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| Identification of Adult Hypophosphatasia |
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# Summary of Thesis

Serum PLP, the circulatory form of vitamin B6 is a substrate of the enzyme alkaline phosphatase (ALP). In adult hypophosphatasia (HPP), mutation of the ALPL gene leads to deficient ALP activity and accumulation of PLP.

Four tested factors (inflammation, reduced kidney function, low ALP, and vitamin B6 supplementation) are associated with significant differences in PLP. PLP is lower in cases of inflammation and reduced kidney function, and higher in cases of low ALP and vitamin B6 supplementation.

In a representative U.S. population sample, PLP levels are significantly different based on age and gender, and race. Reference intervals have been reported that are stratified to reflect these differences, following the exclusion of factors known to confound PLP.

In a U.K. population PLP levels are gender dependent and different in lean compared to obese individuals. 95% reference intervals are reported for PLP, BAP and a novel formation marker ratio (BAP:PINP).

Biochemical indicators of HPP including low BAP, elevated PLP, and low BAP:PINP ratio were present in 23 of 679 subjects from a metabolic bone clinic cohort. These subjects were younger than subjects with biochemical indicators within the normal range.

Mutations associated with HPP were observed in 7 of these subjects (1% of the study population), including 3 novel mutations. Subjects with a mutation display a wide range of clinical symptoms including: foot fractures, femoral fractures, vertebral fractures, stress fractures, chronic pain, and dental abnormalities.

There is no observed genotype-phenotype correlation in adult HPP; clinical symptoms of adult HPP are more severe in women.

The symptomatic variability of HPP is high. 100% displayed musculoskeletal pain or weakness, 50% had dental problems and 50% had mental health problems. The fracture prevalence (71.4%) was higher than the general population. 86% of patients had a gene mutation and the average time to diagnosis was 13.4 years.

# Acknowledgements

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I wish to say a huge thank you to all the staff, fellow students and volunteers within the Academic Unit of Bone Metabolism who have made this work not only possible, but enjoyable.

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Finally thank you to my family, friends and Amy, without whom I wouldn’t be writing these acknowledgements.

# Contributions

Professor Eastell wrote the original protocol and funding application for the HPP study and acted as supervisor throughout the project.

I was responsible for all subsequent applications for ethical approvals for the usage of patient samples stored in the South Yorkshire and North Derbyshire Musculoskeletal Biobank

I was responsible for establishing new laboratory techniques within the Bone Biochemistry Laboratory and for carrying out all novel analyte measurements used in this project. Alongside this I was responsible for the evaluation laboratory methods against internal and external quality assurance standards.

I carried out all statistical analyses and methodologies described in this thesis.

Recruitment to the study, consent of patients, sample processing, and data recording was carried out by myself, alongside a number of research staff including Selina Bratherton, Simon Bowles, Amelia Edmonson, Charlotte Humphries, and Amy Lawson.

Clinical interview of patients with suspected HPP was carried out by Dr Robert Desborough. Professor Eastell reviewed the clinical history to determine which health problems were likely to be related to HPP. Pharmatelligence was commissioned to analyse the interview transcripts.

Genetic screening was carried out at Sheffield Children’s NHS Foundation Trust by Antonio Milano, Richard Kirk, Peter Winship and Sara Morosini. Further clinical genetics advice was offered by Meena Balasubramanian.

Some subject characteristics reported in chapter 4 were recorded by the respective research staff involved during the original studies.

# Presented Abstracts

**Oral Communications:**

Nicklin P, R Eastell, K E Naylor. Establishing reference intervals for pyridoxal 5’-phosphate: the National Health and Nutrition Examination Survey 2007-2008 data. Bone Research Society / British Society for Matrix Biology Joint Meeting 2015; Edinburgh, UK.

Nicklin P, R Eastell, K E Naylor. [Snap poster presentation] Establishing 95% reference intervals for PLP in a representative U.S. population (NHANES Study), to better identify hypophosphatasia in adults. Mellanby Centre for Bone Research, Research Day 2015; Sheffield, UK.

Nicklin P, R Eastell, K E Naylor. Establishing race and gender specific reference intervals for pyridoxal 5’-phosphate to better identify adult hypophosphatasia. Mellanby Centre for Bone Research, Research Day 2016; Sheffield, UK.

Nicklin P, R Eastell, K E Naylor. Establishing race- and gender-specific reference intervals for pyridoxal 5-phosphate to better identify adult hypophosphatasia using data from the NHANES programme. European Calcified Tissue Society Meeting 2017; Salzburg, Austria.

Nicklin P, R Eastell. [Snap poster presentation] Biochemical indicators of

hypophosphatasia in a clinical population. Mellanby Centre for Bone Research, Research Day 2017; Sheffield, UK.

**Poster Presentations:**

Nicklin P, R Eastell, K E Naylor. Establishing reference intervals for pyridoxal 5’-phosphate: the National Health and Nutrition Examination Survey 2007-2008 data. American Society for Bone and Mineral Research Annual Meeting 2015; Seattle, US.

Nicklin P, R Eastell, K E Naylor. Establishing reference intervals for pyridoxal 5’-phosphate: the National Health and Nutrition Examination Survey 2007-2008 data. Bone Research Society / British Society for Matrix Biology Joint Meeting 2015; Edinburgh, UK.

Nicklin P, R Eastell, K E Naylor. Establishing 95% reference intervals for PLP in a representative U.S. population (NHANES Study), to better identify hypophosphatasia in adults. Mellanby Centre for Bone Research, Research Day 2015; Sheffield, UK.

Nicklin P, R Eastell, K E Naylor. Establishing reference intervals for pyridoxal 5’-phosphate to identify adult hypophosphatasia. University of Sheffield, Medical School Research Day, 2016; Sheffield, UK.

# Abbreviations

4PA - 4-Pyridoxic acid

ALP - Alkaline phosphatase

AA - Asfotase alfa

ASFF - Atypical subtrochanteric femoral fractures

BAP - Bone alkaline phosphatase

BMD - Bone mineral density

BMI - Body mass index

BP - Bisphosphonate

CI - Confidence interval

CLIA - Chemiluminescence Immunoassay

CTX - C-telopeptide of type I collagen

CRP - C-reactive protein

dsDNA - Double-stranded DNA

DNA - Deoxyribonucleic acid

DXA - Dual-energy X-ray absorptiometry

EDTA - Ethylenediaminetetraacetic acid

EIA - Enzyme immunoassay

FAB - Fat and bone

GPI-AP - Glycosylphosphatidylinositol-anchored protein

HA - Hydroxyapatite

HPLC - High performance liquid chromatography

HPP - Hypophosphatasia

LC-MS/MS - Liquid chromatography tandem mass spectrometry

NGS - Next-generation sequencing

NDNS - National Diet and Nutrition Survey

NHANES - National Health and Nutrition Examination Survey

OC - Osteocalcin

OMIM - Online Mendelian Inheritance in Man

OPN - Osteopontin

PCR - Polymerase chain reaction

PEA - Phosphoethanolamine

PEA-P-lyase - O-phosphorylethanolamine phospholyase

PINP - Procollagen I intact N-terminal

PL - Pyridoxal

PLP - Pyridoxal 5’-phosphate

PM - Pyridoxamine

PN - Pyridoxine

PPi - Inorganic pyrophosphate

ROC Receiver operating characteristic

SYNDMB - South Yorkshire and North Derbyshire musculoskeletal biobank

TNSALP - Tissue non-specific alkaline phosphatase

XCT - Xtreme CT

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# Chapter 1: Introduction

## Background

Hypophosphatasia (HPP) is a rare inherited condition that results in defective bone and teeth mineralisation as a result of reduced alkaline phosphatase (ALP) activity (Mornet 2008). HPP is a result of loss-of-function mutations of the bone/liver/kidney alkaline phosphatase gene on chromosome 1, *ALPL* (OMIM #171760), responsible for encoding tissue non-specific alkaline phosphatase (TNSALP) (Hessle et al. 2002, Henthorn and Whyte 1992). Currently >340 defects in *ALPL* are associated with HPP (Mornet 2015).

Six forms of HPP have been identified with high symptomatic variability (Table 1), ranging from fatal perinatal forms to the less severe adult-onset form. The severe forms were initially estimated to have an incidence of 1/100,000 live births (Fraser 1957) a number which Fraser himself treated as “speculation”. Other estimates of severe HPP in a larger, multi-national population over 9 years (2000-09) suggest an incidence of approximately 1/300,000 (Mornet et al. 2011).

|  |  |  |
| --- | --- | --- |
| **Subtype** | **Inheritance** | **Clinical Presentation** |
| Perinatal (lethal) (OMIM #241500) | Autosomal recessive | Marked impaired bone mineralisation in utero, blue sclerae, osteochondral projections affecting limbs (often diagnostic), respiratory complications. Often lethal. |
| Prenatal (benign) | Autosomal recessive or autosomal dominant | Limb deformities (may improve during third trimester of pregnancy). Spontaneous improvement of skeletal defects reported. |
| Infantile (OMIM #241500) | Autosomal recessive | Onset before 6 months of age. Respiratory complications, premature craniosynostosis, demineralisation, rachitic changes in the metaphyses, hypercalcaemia, short stature, premature loss of primary teeth. Mortality is often related to respiratory complications. |
| Childhood (OMIM #241510) | Autosomal recessive or autosomal dominant | Onset after 6 months. Skeletal deformities and fractures, short stature, premature loss of primary teeth. Often self-limiting but may re-appear in adulthood. |
| Adult (OMIM #146300) | Autosomal recessive or autosomal dominant | Presents in middle age with metatarsal stress fractures, thigh pain, atypical femur fractures, chondrocalcinosis and osteoarthropathy. Patients may report premature loss of primary +/or permanent teeth. |
| Odontohypophosphatasia (OMIM #146300) | Autosomal recessive or autosomal dominant | Premature exfoliation of primary teeth (most commonly the incisors). Often no skeletal manifestations. |

Table 1: Forms of hypophosphatasia; adapted from Hollis et al. (2013).

* 1. *Adult Hypophosphatasia*

Adult-onset HPP is more common than the severe forms with an estimated prevalence of 1/6370 of the general population (Mornet et al. 2011). Recent investigations into a clinical population of 800,000 adults found that 1/1544 had persistently low ALP and symptoms consistent with HPP (McKiernan, Berg, and Fuehrer 2014), suggesting that actual prevalence may be much higher for the adult form. Inconsistent reporting of prevalence emphasises the need for reliable and cost effective biochemical testing for adult HPP.

The adult form of the disease is typically less symptomatic, and presents with greater variability than the more severe infant forms. Adult HPP is categorised as HPP that becomes symptomatic after 18 years of age, and often presents in middle-age. Dental abnormalities from childhood are often retrospectively identified.

Following assessment of clinical, radiographic and laboratory characteristics of 38 adults with HPP (diagnosed >18 years of age), Schmidt et al., (2017) observed a broad spectrum of clinical symptoms. The median diagnosis age was 43 years for women and 51 years for men. The most common symptoms were muscle pain (61% of patients), headaches (55%). 15 patients (39%) reported a previous fracture and 6 patients (16%) reported multiple fractures, most commonly low-impact fractures of the lower extremities. The observed clinical characteristics of this cohort are summarised in Table 2.

|  |  |  |  |
| --- | --- | --- | --- |
|  | All patients (*N* = 38) | Female patients (*N* = 30) | Male patients (*N* = 8) |
| *N* (%) | *N* (%) | *N* (%) |
| History of fractures -  | 15 (39) | 11 (37) | 4 (50) |
|  Patients with multiple fractures | 6 (16) | 6 (20) | - |
|  Metatarsal stress fractures | 8 (21) | 6 (20) | 2 (25) |
|  Metacarpal stress fractures | 2 (5) | 2 (7) | - |
|  Distal radius fractures | 2 (5) | 1 (3) | 1 (12.5) |
|  Tibial plateau fractures | 1 (3) | 1 (3) | - |
|  Vertebral compression fractures (low-energy fracture) | 3 (8) | 2 (7) | 1 (12.5) |
|  Proximal femur fracture (low-energy fracture) | 1 (3) | 1 (3) | - |
|  Persistent bone marrow oedema | 2 (5) | 2 (7) | - |
|  Fracture healing complication | 3 (8) | 3 (10) | 1 (12.5) |
| Dental abnormalities | 18 (47) | 16 (53) | 2 (25) |
|  Early loss of permanent teeth (<50 years of age) | 10 (26) | 9 (30) | 1 (12.5) |
|  Enamel thinning | 5 (13) | 4 (13) | 1 (12.5) |
|  Severe caries | 4 (11) | 4 (13) |  |
| Joint pain | 13 (38) | 13 (43) | 1 (12.5) |
| Musculoskeletal pain | 23 (61) | 19 (63) | 4 (50) |
| Severe muscle weakness | 4 (11) | 3 (10) | 1 (12.5) |
| Headaches | 21 (55) | 18 (60) | 3 (37.5) |
| Chondrocalcinosis | 8 (21) | 8 (27) | - |
| Patients diagnosed with depressive disorder | 7 (18) | 6 (20) | 1 (12.5) |

Table 2: Clinical characteristics of adult HPP patients, adapted from Schmidt et al., (2017).

Schmidt et al. (2017) also assessed bone mineral density (BMD) by dual-energy X-ray absorptiometry (DXA). Z-scores were slightly lower in the HPP patients, while Z-scores and T-scores were not significantly different between HPP patients with a history of fracture compared to those without. Subnormal BMD in adult HPP may lead to misdiagnosis of HPP as osteoporosis (Sutton et al. 2012). However areal BMD may also be normal or elevated in some instances of adult HPP (Whyte 2017).

There is no obvious genotype-phenotype correlation in adult HPP. Following clinical and genetic investigation Hofmann et al. (2014) noted intra-, and interfamilial variability of phenotypes in patients with identical genotypes. Similarly, no genotype-phenotype correlation was observed in 32 adult patients with confirmed HPP mutations (Schmidt et al. 2017). Clinical characteristics and serum ALP levels were found to vary greatly among patients with the same mutation.

* 1. *TNSALP and Hypophosphatasia*

Deficiency of TNSALP activity is the key biochemical trademark of HPP, leading to an accumulation of three presumed substrates of the enzyme (Whyte et al. 1995). Serum levels of pyridoxal 5’-phosphate (PLP) are increased; urinary levels of phosphoethanolamine (PEA) are increased, whilst inorganic pyrophosphate (PPi) levels are increased in both serum and urine. All three of these analytes may potentially be utilised as diagnostic markers.

ALPL knockout mice have been shown to emulate the biochemical changes typical of HPP described above, which indicates the relevance of defective TNSALP in the pathogenesis of HPP (Fedde et al. 1999).

* 1. *Adult Hypophosphatasia and Bisphosphonates*

Bisphosphonates (BPs) are derivatives of PPi (Figure 1) and similarly inhibit calcification and bind to hydroxyapatite crystals in bone mineral, where inhibition of hydroxyapatite breakdown effectively suppresses bone resorption (Drake, Clarke, and Khosla 2008).

[Image Redacted]

Figure 1: Structural similarities of PPi, synthetic BPs, and phosphocitrate, another naturally occurring mineralisation inhibitor (Terkeltaub 2001).

BPs also suppress bone resorption through direct inhibition of osteoclasts. Internalisation of BPs within osteoclasts cause structural changes that disrupt the cells resorptive processes (Yu et al. 2016). For this reason, they are commonly prescribed in diseases of excess osteoclast mediated bone resorption, primarily osteoporosis. There is no evidence to show that BPs exhibit an effect on PLP levels.

Based on this pharmacological action and evidential case reports, Sutton et al. (2012) have suggested: “patients diagnosed with osteoporosis but carrying a TNSALP mutation are prone to develop atypical subtrochanteric femoral fractures (ASFFs) from BPs”. This hypothesis is further supported by the method of action of BPs (such as alendronate and zoledronate) that act to inhibit TNSALP by binding of Zn2+ and Mg2+ ions - required for catalytic function of the enzyme (Vaisman, McCarthy, and Cortizo 2005). This potential risk highlights the importance of clear diagnostic testing for HPP to differentiate diagnosis from osteoporosis, in order for a more suitable treatment to be prescribed.

## Biochemical Markers of Adult Hypophosphatasia

* 1. *Vitamin B6: Pyridoxal 5’-Phosphate*

Pyridoxal 5’-Phosphate (PLP) is the biologically active coenzyme form of vitamin B6. The term vitamin B6 comprises three vitamers, the pyridine derivatives pyridoxine (PN), pyridoxamine (PM) and pyridoxal (PL), and their 5’-phosphate esters including PLP (Figure 2). PLP is the major component, and adequate indicator of, vitamin B6 status.

In circulation PLP is largely albumin bound and must be dephosphorylated to PL by TNSALP in order for it to enter cells and cross the blood-brain barrier (Whyte et al. 1985). Therefore deficiencies in TNSALP lead to an increased level of PLP and decreased extracellular PL (Cellini et al. 2014). PLP has been shown to be consistently elevated across multiple forms of HPP (Whyte et al. 1985). This study also shows that plasma PL levels are only low in extremely severe HPP, and milder forms of the disease may have normal or even elevated levels. PL therefore cannot be used as a marker for HPP, but does explain the presence of seizures in severe forms of HPP (Waymire et al. 1995). It is suggested that increased PLP however is a reliable biochemical indicator of the disease, in the presence of persistently decreased ALP.

[Image Redacted]

Figure 2: Formation of pyridoxal 5'-phosphate and other phosphate esters from pyridoxine, pyridoxal, and pyridoxamine. Adapted from Do et al. (2012).

Evidence has been mixed regarding the correlation between PLP levels and adult HPP disease severity. In a cohort of 8 adults with HPP, all had elevated PLP, although those who were symptomatic had lower values than the asymptomatic patients (Berkseth et al. 2013). Conversely, using more robust data from a larger cohort of 38 adult patients Schmidt et al. (2017) observed that higher PLP levels correlated with a greater disease severity. PLP was higher in patients with fractures, and also in patients with more symptomatic HPP (>2 symptoms, compared to those with 1-2 symptoms.) (Figure 3a). These mixed observations are reliant on small population samples from which it is difficult to draw conclusions. Furthermore asymptomatic cases are possibly underdiagnosed and therefore underrepresented in clinical evaluations.

[Image Redacted]

Figure 3: Comparison of serum PLP (a) and ALP (b) levels between patients with and without fractures, and between patients with >2 symptoms and those with 1-2 symptoms, in 38 cases of adult HPP (Schmidt et al. 2017). Statistical significance was defined as *p* < 0.05.

* + 1. *Pyridoxal 5’-Phosphate Measurement: High Performance Liquid Chromatography*

Liquid chromatography tandem mass spectrometry (LC-MS/MS) methodology exists that allows simultaneous measurement of all six vitamers of vitamin B6 alongside 4-pyridoxic acid (4PA) (Footitt et al. 2013). The clinical relevance of measuring all six vitamers in HPP has not been established and so these methods are often cost and time ineffective in comparison to more accessible and specific HPLC methods.

Reversed phase high-performance liquid chromatography (HPLC) with fluorometric detection (Figure 4) is the most widely used method for PLP measurement in serum and plasma (Rybak, Jain, and Pfeiffer 2005). Low fluorescence of PLP means that derivatization is required to enhance sample fluorescence. Derivatization is the main variable in PLP measurement techniques by HPLC and may occur pre- or post-column, using bisulfite (Coburn and Mahuren 1983), semicarbazide (Ubbink, Serfontein, and de Villiers 1985), cyanide (Bates et al. 1999), and more recently, chlorite (Rybak and Pfeiffer 2004, 2009). Derivatization also acts to separate PLP from albumin to which it is bound in the circulation.

Figure 4: An example chromatogram of PLP quantification using pre-column cyanide derivatization of a patient plasma sample. Peak is identifiable by comparison of the retention time to aqueous PLP standards after derivatization. Peaks are not detectable after treatment of samples with acid phosphatase – confirming that peaks are representative of a phosphorylated compound.

[Image Redacted]

For ease of use and reproducibility, a commercial kit is available for HPLC measurement of PLP in serum, plasma and whole blood that utilises a simple pre-column sample derivatization method using cyanide (Chromsystems, Germany). Cyanide derivatization converts PLP to the more fluorescent 4-pyridoxic acid 5’-phosphate (Bates et al. 1999) through saturation of the double bond at the 4- position of the pyridine ring of PLP (Figure 5) (Bonavita 1960).

[Image Redacted]

Figure 5: Derivatization of PLP by cyanide. The R region denotes the pyridine ring of the PLP molecule (Bonavita 1960).

Rybak and Pfeiffer (2004) showed that using chlorite post-column derivatization offered a fourfold increase of sensitivity in comparison to previous methods using bisulfite post-column derivatization. However post-column derivatization requires additional equipment and sample analysis time, making it unsuitable for large sample numbers. More recently Cabo et al. (2014) developed a gradient flow method using bisulfite derivatization that is suitable for analysis of low sample volumes (25μl sample) in clinical trials and shows a low coefficient of variance (6%).

* + 1. *Pyridoxal 5’-Phosphate Measurement: Enzymatic Method*

Plasma PLP has also shown to be quantifiable enzymatically using radioactive tyrosine and the enzyme tyrosine decarboxylase (Chabner and Livingston 1970). Numerous adaptations have been made to the assay over time (Shin-Buehring, Rasshofer, and Endres 1981, Camp, Chipponi, and Faraj 1983, Lequeu, Guilland, and Klepping 1985). More recently a homogeneous non-radioactive enzymatic method was established (Han et al. 2002), and is commercially available for the measurement of PLP in plasma (A/C Diagnostics, San Diego, USA).

* + 1. *Pyridoxal 5’-Phosphate Measurement: Method Comparison*

A laboratory comparison study (10 laboratories included) found that laboratories using both techniques reported higher imprecision in the enzymatic method in comparison to HPLC (Rybak, Jain, and Pfeiffer 2005). Additionally, although HPLC sample preparation and derivatization methods differed across labs (bisulfite, chlorite, and semicarbazide derivatization) all were similarly effective for PLP measurement. An unpublished crossover study (*n*=236) highlights differences between the commercially available enzymatic assay and HPLC methods (NHANES 2014a). The HPLC method has a lower limit of detection than the enzymatic method (0.3nmol/L compared to 10nmol/L) and a greater detection rate (100% compared to 71%). HPLC values were also higher than the enzymatic assay (mean, 82.1; median, 45.9nmol/L; compared to mean, 49.0; median, 23.3nmol/L). This study suggests that data from different methods should not be combined, and that HPLC methods for PLP determination are most suitable for research and clinical applications.

* + 1. *Preanalytical Handling Considerations for Pyridoxal 5’-Phosphate Analysis*

PLP is light sensitive and so it is recommended that serum/plasma sample handling is carried out under non-UV lighting, using amber vials, or a similar technique to preserve sample PLP (Bailey, Wright, and Southon 1999, NHANES 2014a). The effect of photochemical degradation was examined by Talwar et al. (2003) who demonstrated (Figure 6a) that after 2 hours exposure to natural light within the lab PLP was significantly reduced (17%), and after 24 hours the majority of PLP was lost in underivatised plasma samples. Derivatised plasma samples had no loss of PLP for 48 hours, when in natural light at room temperature. Underivatised plasma was however stable for at least 12 hours in the dark at room temperature (Figure 6b). Finally, it was determined that PLP in plasma was stable for at least 12 months when stored at -70°C.

In addition it has been demonstrated that under normal lighting at room temperature there is no significant loss of PLP from serum or plasma samples within six hours of sample collection (Reynolds and Brain 1992) – suggesting that although precautions should be taken during sample preparation, no deviation from typical sample collection procedures is required, so long as samples reach the laboratory or are stored in darkness within 6 hours of collection.

Vitamin B6 supplementation is known to elevate circulatory PLP, with values returning to a pre-supplement baseline after 2-3 weeks (Morris et al. 2008) (Ye, 2010) (McKiernan, 2017). Therefore, vitamin supplement intake should be ceased three weeks prior to sample collection.

Serum PLP has been shown to decrease following glucose or high carbohydrate intake (Leklem and Hollenbeck 1990), therefore fasting blood samples should be collected to accurately measure PLP. Circulatory PLP levels are not known to be influenced by circadian rhythm (Ueland, 2015). It is therefore not necessary to maintain a consistent time of sample collection for PLP measurement.

[Image Redacted]

Figure 6: Stability of PLP in derivatised (■) and underivatised (□) plasma samples, when exposed to natural light (a) and in darkness at ambient temperature (b); each plot represents a mean value determined from three aliquots - adapted from Talwar et al. (2003).

Rybak and Pfeiffer (2004) noted that serum and heparinised plasma PLP values obtained through HPLC (with post-column derivatization) correlated with strong significance (*r*=0.98-1.00, *P*<0.0001), while EDTA plasma samples showed “noticeable difference from their respective serum reference counterparts”. This was due to chromatographic interference whereby EDTA is detected as a wide-based peak on the chromatogram at a similar retention time to PLP. This peak trails over the PLP peak and thus increases the area of the PLP peak, confounding PLP quantification.

Talwar et al. (2003) reported that the use of different anti-coagulants (heparin and EDTA) had no effect on PLP values when measured by HPLC with pre-column derivatization, which is the method used in this study.

Due to the possibility of interference, it would be best practice to consider appropriate sample collection protocols depending on the method/kit used.

PLP measurement is often requested by the clinician investigating suspected adult HPP. Typical clinical indicators of HPP that may prompt PLP measurement include: osteomalacia, pseudofractures, joint pain associated with chondrocalcinosis, and dental anomalies. Low total ALP, typically measured in the clinical setting as part of a bone profile test, may also prompt a clinician to measure PLP where present alongside clinical symptoms. Other bone biomarkers and bone mineral density may be within the normal range.

* + 1. *Establishing Reference Ranges for Pyridoxal 5’-Phosphate*

As elevated circulating PLP levels are indicative of HPP, reliable reference intervals, particularly regarding the upper limit, need to be established. Bates et al. (1999) established plasma (heparinised) reference intervals for elderly (>65 years) males (*n*=136, 17.9-98.8 nmol/L) and females (*n*=161, 14.4-98.7) who were not receiving vitamin B6 supplementation. It was noted that these ranges were comparable at the 2.5 and 50 percentiles with a larger cross-sectional group for whom PLP was measured in The National Dietary and Nutrition Survey. However it should be noted that not all reported reference intervals are similarly comparable.

One study explored inter-laboratory variation between reported reference intervals for PLP measured by HPLC with fluorescence detection (Hoad et al. 2013). PLP measurements in plasma, rather than in whole blood, showed the greatest variation in ranges (Figure 7). Laboratories 4 to 6 showed a relative consensus of ranges and all utilised a commercially available kit (Chromsystems, Munich, Germany) to measure PLP in whole blood. Labs 1 to 3 measured PLP in plasma; labs 1 and 2 utilised in-house assays and reported ranges with considerably higher upper limits, while laboratory 3 used the commercial kit and displayed a range more similar to the other commercial kit using labs, 4 to 6. The previously discussed lab comparison study (Rybak, Jain, and Pfeiffer 2005) suggests that calibration and quality control are particular problem areas in relation to PLP measurement, a viewpoint that Hoad et al. (2013) support in this study.

[Image Redacted]

Figure 7: An inter-laboratory comparison of PLP Reference intervals in whole blood (black bars) and plasma (spotted bars) samples (Hoad et al. 2013).

The National Health and Nutrition Examination Survey (NHANES) has recently been used to establish serum reference ranges for PLP in a representative US population sample, aged 1 year and older (*n*=8311). The range was wider in comparison to the ranges reported by Hoad et al., (2013) (Figure 7) with a significantly higher upper limit: 11.3-302 nmol/L (2.5th-97.5th percentile) (NHANES 2014b). This is a robust estimation of PLP reference intervals available due to the large sample size, diversity and representation of the sample. However there were no exclusions made based on factors known to affect serum/plasma PLP.

Haynes et al. (2013) studied the association between physiological variables and PLP in the NHANES 2003-2006 dataset (aged ≥20 years), and showed that serum PLP was associated with a number of physiological factors.

Inflammation (C-reactive protein ≥5.0 mg/L) was significantly associated with a reduced PLP (Table 3); PLP may have a protective effect against inflammation (Morris et al. 2010). Reduced renal function was also associated with limited changes in serum PLP (Table 3). PLP measurements in chronic kidney disease (CKD) stages 3-5 were comparable to normal levels, but stage 1 or 2 CKD with the presence of albuminuria had reduced PLP. The effect of renal dysfunction is unclear; Morris et al. (2008) found that in measurements of PLP “excluding people with signs of renal dysfunction and those with heart attack or stroke history did not alter results”.

|  |  |
| --- | --- |
| **Inflammation** |  |
| Overall | No | Yes | *P* Value |  |
| 50.1 | 55.2 | 36.5 | <0.001 |  |
| **Renal Function** |  |
| Overall | Normal | CKD stage 1 or 2 | CKD stage 3-5 | *P* Value |
| 50.3 | 51.5 | 41.8 | 51.6 | 0.001 |

Table 3: Mean PLP concentration (nmol/L) by inflammation and renal function category. Normal renal function was defined as eGFR ≥60 mL/(min . 1.73m2) and the absence of albuminuria; stage 1 or 2 CKD was defined as eGFR ≥60 mL/(min . 1.73m2) and the presence of albuminuria; stage 3-5 was defined as eGFR <60 mL/(min . 1.73m2). Adapted from Haynes et al. (2013).

Pregnancy was associated with a higher prevalence of abnormal PLP, which is consistent with a previous study (Trumbo and Wang 1993) that showed plasma PLP to be significantly lowered in pregnant compared to non-pregnant women.

Vitamin B6 supplementation has been shown to increase plasma PLP significantly (Figure 8). Dietary sources rich in vitamin B6 such as ready-to-eat cereals are also associated with an increase in plasma PLP (Ye et al. 2010). False positive PLP results as a response to vitamin B6 supplementation occur when PLP is quantified within a week of supplementation (Mornet and Nunes 1993). Blood samples for measurement of PLP should ideally be taken 2 weeks after cessation of supplementary vitamin B6 intake (McKiernan, Berg, and Fuehrer 2014).

Figure 8: Increase in PLP correlates with increasing Vitamin B6 supplementation. Data is shown as least squared geometric means - subjects (*n*=6165) aged ≥1 year were categorised into 32 groups (*n*=approx.200) (Morris et al. 2008).

[Image Redacted]

As a variety of physiological factors can affect plasma and serum PLP measurements future work should aim to establish reference intervals for PLP that investigate confounding factors further and include exclusion criteria to reduce interference. Dietary and supplementation status should also be considered in elevated PLP measurements. Furthermore reference intervals that are race and gender specific are required as PLP has been shown to be higher in males than females, and lower in non-Hispanic blacks than other ethnicity groups, within U.S. subjects (CDC 2012) (Nicklin, Eastell, and Naylor 2015). Similarly, in a large (n=523, adults aged 18-63 y) European cohort PLP concentrations were significantly lower in women (Albersen et al. 2014).

* 1. *Alkaline Phosphatase*

ALP has long been used as a marker of bone formation, as the enzyme is involved in bone mineralisation (Harris 1990); circulating levels reflect osteoblast activity and are measured clinically as an indicator of bone formation.

ALP is also present on the cell surface as a glycosylphosphatidylinositol-anchored protein (GPI-AP). The GPI anchor is manufactured in the endoplasmic reticulum and added to ALP during post-translational modification. The secretory pathway transports the GPI-AP unit to the cell membrane, mediated by GPI. Enzymatic cleavage of the GPI backbone by phosphatidylinositol-specific phospholipase C (PI-PLC) promotes shedding of GPI-APs such as ALP. This is thought to contribute to circulatory levels of ALP (Murakami et al. 2012) (Fedde, 1990).

ALP isoenzymes are encoded for by 4 genes related to their origin tissue, placental, germ cell, intestinal, and tissue non-specific (TNSALP). TNSALP (consisting of liver/bone/kidney ALP) is responsible for 95% of total circulating ALP and is highly expressed in bone and liver tissues (Halling Linder et al. 2009); “total ALP” is a measurement of all isoforms. Measurement of specific isoenzymes of TNSALP such as the bone ALP isoform (BAP) alone offers increased sensitivity, for example a total ALP measurement within expected normal ranges may not represent subtle changes in specific isoenzyme activity (Rosalki and Foo 1984).

There is an inverse relationship between total serum ALP and PLP, in HPP ALP is significantly reduced alongside an increased PLP (Anderson et al. 1980). Low ALP, hypophosphatasaemia, is a biochemical indicator of HPP but cannot be used to diagnose HPP in isolation (Mornet 2007). In HPP serum ALP is persistently low due to a genetic deficiency, but ALP may also be reduced, either persistently or transiently in a number of other conditions (Table 4).

|  |  |  |
| --- | --- | --- |
| Persistently low | Transiently low |  |
| * Hypophosphatasia
* Cleidocranial dysplasia
* Mseleni joint disease
* Benign familial hypophosphatasaemia
 | * Profound hypothyroidism
* Cushing disease
* Bisphosphonate therapy
* Adynamic renal osteodystrophy
* Milk-alkali syndrome
* Vitamin D intoxication
* Wilson disease
* Nutritional deficiencies
* Hypozincaemia
* Coeliac disease
* Pernicious anaemia
* Radioactive heavy metal intoxication
 | * Cardiac bypass surgery
* Major trauma
* Major surgery
* Cancer chemotherapy
* Multiple myeloma
* Massive transfusion
* Starvation
* Severe sepsis
* Multi-organ failure
* Analytical error
* Improper specimen collection
 |

Table 4: Potential causes of low serum ALP; adapted from McKiernan et al. (2017).

Serum ALP and BAP levels reflect disease severity in the childhood forms of the disease (Whyte et al. 1996, Whyte 2010). Despite persistently low ALP in adult HPP, the same correlation between ALP and disease severity is not observed. No differences were observed in serum ALP in patients with fractures compared to those without, and also in patients with more symptomatic forms of the disease compared to those with a less symptomatic presentation (Schmidt et al. 2017) (Figure 3b).

* + 1. *Measurement of Alkaline Phosphatase*

Automated measurement of total serum ALP is possible with the use of an enzymatic substrate and colourimetric absorbance (Morgenstern et al. 1965, Rosalki and Foo 1984). A number of techniques are available to quantify specific ALP isoenzymes such as bone ALP alone. Quantification of bone ALP specifically was first available using a range of techniques such as heat inactivation (Moss and Whitby 1975), whereby bone and liver isoenzymes are separated based on differing heat stabilities; wheat-germ lectin precipitation (Behr and Barnert 1986) that separates bone ALP from the liver form via precipitation, followed by a quantification of the mostly bone ALP precipitate; and electrophoresis (Van Hoof et al. 1988). Electrophoresis based techniques can achieve limited determination of specific isoenzymes but are limited due to incomplete separation of bone and liver ALP; Magnusson (1992) states that when using adult serum samples “often only one bone and two liver ALP isoenzymes can be identified” by electrophoresis.

Immunoassays are now the primary method of clinical evaluation of bone ALP (BAP) (Woitge, Seibel, and Ziegler 1996), and can accurately distinguish between ALP isoenzymes. A comparison of immunoassay methods for determining bone ALP found good correlation between total and bone ALP in disorders of bone metabolism (Figure 9).

The methods for measurement of ALP and BAP used in this study are outlined in chapter 2.

[Image Redacted]

Figure 9: Comparison of bone ALP levels against total ALP (TAP), in patients with non-skeletal disorders and metabolic bone disease. L-BAP, lectin precipitation assay; I-BAP, immunoradiometric assay; E-BAP, enzyme linked immunosorbent assay (ELISA) (Woitge, Seibel, and Ziegler 1996).

The main limitation of immunoassay methods is that cross reactivity of up to 20% exists between bone and liver isoenzymes of the TNSALP gene (Seibel 2005) which may confound measurements or provide falsely “normal” results in certain circumstances. Conditions of elevated liver ALP (such as biliary obstruction) should be considered during total ALP measurement; in the absence of liver disease, total ALP is sufficient to provide a measure of bone formation and assessment of HPP (Mornet 2007), however bone ALP is increasingly preferred due to greater specificity (Farley, Chesnut, and Baylink 1981).

* + 1. *Measurement of Specific Alkaline Phosphatase Isoforms*

Variations in the TNSALP isoforms are a result of posttranslational modification in carbohydrate composition following expression of the gene locus in different cells.

Perhaps the gold standard measurement of specific bone isoforms is achieved by HPLC (Magnusson, Lofman, and Larsson 1992); this method specifically detects two bone specific ALP isoforms (B1 and B2), and a third bone/intestinal isoform (B/I); a fourth isoform (B1x) has also been identified in patients with chronic kidney disease (Magnusson et al. 2001).

Magnusson et al. (1992) demonstrated that the chromatographic profile of a patient with HPP (total ALP 30 U/L) shows a decrease in all ALP isoforms, compared to normal serum (total ALP 162 U/L). Bone ALP isoforms are reduced the most in HPP; the B2 isoform was undetectable (Figure 10).

[Image Redacted]

Figure 10: The left chromatogram is that of a normal serum sample: peaks at 4.78, 6.10, and 9.42 minutes correspond to the intestinal/bone, bone 1, and bone 2 isoforms respectively. The right chromatogram is from serum of a patient with HPP: All isoform activities were decreased and the bone 2 isoform is undetected (Magnusson, Lofman, and Larsson 1992).

## Other Biomarkers in Hypophosphatasia

* 1. *Procollagen I intact N-terminal pro-peptide*

In the early stages of bone formation osteoid (the unmineralised organic component of bone) is formed. Type I collagen constitutes around 90% of osteoid content and is formed from cleavage of type I procollagen. This cleavage process leads to the release of procollagen I intact N-terminal pro-peptide (PINP) into circulation where it is a measurable marker of bone formation (Naylor and Eastell 2012).

Formation markers such as PINP appear unremarkable in adult HPP (Schalin-Jäntti et al. 2010, Iqbal et al. 2017, Schmidt et al. 2017).

* 1. *BAP:PINP Ratio*

A formation marker ratio is proposed to identify the ratio of HPP-sensitive BAP to insensitive PINP. In adult HPP Schalin-Jäntti et al (2010) observed BAP to be low, alongside a PINP concentration within the normal range. We will validate this observation by calculating a BAP:PINP ratio as a potential diagnostic indicator of HPP in cases where the ratio is low.

* 1. *Phosphoethanolamine*

Although the metabolic pathway and origin of PEA is uncertain (Whyte 2010), it had been presumed that PEA is a natural substrate of TNSALP (Whyte et al. 1995). Urinary PEA negatively correlates to serum ALP (Millan et al. 1980) and as such is increased in HPP; however this increase is not pathognomonic and is displayed in other metabolic bone disorders (Mornet 2007). Urinary PEA may be positively correlated with disease severity as Berkseth et al. (2013) found PEA levels to be consistently higher in HPP patients who were symptomatic or had experienced a fracture. Recently it has been shown that activity between all ALP isoforms and PEA is extremely low and much lower than activity with PLP (800-5,000 times lower) and PPi (10,000 times lower); suggesting that PEA is actually unlikely to be a natural substrate of TNSALP as once thought (Halling Linder et al. 2009). Although the mechanism of increased levels of urinary PEA is unclear, Millan and Plotkin (2012) postulate that the role of PLP as a cofactor in the action of O-phosphorylethanolamine phospholyase (PEA-P-lyase) may be an indirect cause of the elevated PEA. Deficient TNSALP activity in HPP leads to reduced cellular levels of PLP and therefore PEA-P-lyase activity is reduced, as a result PEA excretion is elevated.

* 1. *Inorganic Pyrophosphate*

PPi is comprised of two inorganic phosphate (Pi) molecules, joined by an ester bond (Figure 1), that is hydrolysed by TNSALP (Whyte et al. 1995). Deficiency of TNSALP in HPP leads to elevated levels of PPi (Russell et al. 1971). This explains the reduced level of bone mineralisation in HPP, as PPi accumulatesextracellularly and potentlyinhibits calcification (Meyer 1984) leading to osteomalacia. Measurements of elevated PPi are not pathognomonic to HPP as other enzymes act as PPi regulators, including inorganic pyrophosphatases and certain acid phosphatases (Terkeltaub 2001).

* 1. *4-Pyridoxic Acid*

The catabolic end product of vitamin B6 is 4-Pyridoxic acid (4PA), which can be quantified in urine or serum/plasma via HPLC; 4PA has also been investigated as a potential marker of HPP. 4PA is a degradative product of PLP but despite increased PLP in HPP, 4PA is unchanged in urinary measurements of HPP patients (Whyte et al. 1985) and is therefore unsuitable for use as a biomarker. Measurement of 4PA can however be utilised as an indicator of short-term vitamin B6 status, it has been suggested that PLP alone may not be a sufficient status indicator (Leklem 1990).

* 1. *Osteopontin*

Osteopontin (OPN) is a glycoprotein that is highly phosphorylated. The role of OPN has not been fully elucidated, but it has been demonstrated to inhibit bone mineralisation by inhibition of hydroxyapatite (HA) formation; experimentally OPN has been shown inhibit HA formation by 50% (Hunter, Kyle, and Goldberg 1994). The phosphorylation of OPN is related to its ability to inhibit mineralisation; it has been demonstrated that removal of bound phosphates from OPN by ALP treatment reduced its inhibitory effect on bone mineralisation by “more than 40-fold” (Hunter, Kyle, and Goldberg 1994). Millan and Plotkin (2012) have suggested that OPN may be a natural substrate of TNSALP.

Animal studies have found elevated serum levels of OPN in ALPL knockout mice (Harmey et al. 2006) as well as a correlation between elevated OPN and PPi, whereby PPi is thought to control expression of OPN. There is potential for OPN as a biomarker of HPP but further work is needed before robust conclusions can be drawn.

* 1. *Calcium*

Although hypercalcaemia is observed in the infantile form of the disease (Hollis et al. 2013), there is little evidence to suggest calcium levels are high in the adult form of the disease. Schmidt et al. (2017) measured serum calcium levels in 38 cases of adult HPP with varying disease severity and all were within the normal reported reference range of 2.13-2.63 nmol/L.

## Treatment of Hypophosphatasia

### *Asfotase Alfa*

A human recombinant enzyme replacement therapy, asfotase alfa (AA), has recently been approved for treatment of HPP in several countries, including the UK. Administration of AA replaces defective TNASLP with the active recombinant enzyme. AA is administered as a subcutaneous injection and can be administered three times a week at a dosage of 2mg/kg of bodyweight or six times a week at a dosage of 1mg/kg of bodyweight.

Clinical trial data shows that AA treatment markedly improves skeletal development and respiratory function in perinatal, infantile and childhood HPP when compared to historical age matched controls (Hofmann, Seefried, and Jakob 2016). Bone mineralisation defects are resolved and mortality rates in perinatal and infantile HPP are shown to be reduced from around 97% and 60% respectively to ~10% (Bishop, Munns, and Ozono 2016).

Treatment of adult HPP with AA is less well documented. A phase 2 clinical study observed improved mobility and proximal muscle strength in a cohort of adults with adult- and childhood-onset HPP when treated with AA (Kishnani et al. 2018). AA treatment was also associated with a dose-dependent decrease in PLP and PPi to within the normal range. In 2 case study reports (Klidaras et al. 2018), AA treatment was shown to stimulate fracture healing, reduce pain and increase mobility in an adult patient living with infantile-onset HPP. The second case involved an individual with adult HPP who suffered a fragility fracture of the femur and subsequent non-union and chronic pain for 8 years. Complete fracture healing, reduced pain and improved quality of life were reported within 4 months.

Due to the novelty of the treatment, safety and efficacy data is not available for 7+ years, although Kishnani et al. (2018) note that “asfotase alfa was generally well tolerated over 5 years of treatment”. The most common adverse events observed during AA treatment are injection site reactions, observed in upwards of 70% of patients treated with AA (Kishnani et al. 2017). There is potential for long-term treatment with AA to be associated with ectopic calcification as a result of reduced inhibition of hydroxyapatite crystal deposition. Clinical evidence has linked AA treatment to ectopic calcification that is not reported as clinically important or symptomatic (Whyte et al. 2016) (Gospe et al. 2019). Longer treatment periods may be associated with clinically significant ectopic calcifications.

### *Teriparatide*

Other treatments such as teriparatide, a human recombinant form of parathyroid hormone (PTH 1-34) have shown effectiveness in reported case study treatment of adult HPP. Teriparatide is commonly prescribed to treat postmenopausal and glucocorticoid-induced osteoporosis patients at high risk of fracture. Teriparatide stimulates osteoblast precursor cells, increasing the rate of bone formation and mineralisation; which is associated with an increase in BMD and increased circulatory markers of bone formation including PINP and BAP (Eastell and Walsh 2017, Panico et al. 2011, Neer et al. 2001). Current clinical recommendations suggest a continuous treatment period of 24 months only.

Case reports of teriparatide treatment in adult HPP show: a decrease in HPP associated pain and functional difficulty, alongside normalised ALP levels (Righetti et al. 2018), reduced pain in stress fracture sites and increased ALP levels (Whyte, Mumm, and Deal 2007), improved pain, mobility and fracture healing alongside significantly increased ALP levels (Schalin-Jäntti et al. 2010).

Teriparatide treatment of adult HPP is not always effective. In a 61 year old male initial teriparatide treatment failed to stimulate fracture healing; subsequent treatment with AA was successful in stimulating fracture healing (Klidaras et al. 2018). In an adult with childhood-onset HPP, teriparatide treatment had no effect on fracture healing or circulatory levels of ALP or BAP (Laroche 2012). The effectiveness of teriparatide as a treatment of HPP in adults may be related to the severity of the *ALPL* mutation, whereby complete absence of the BAP isoform in severe cases cannot be remedied by teriparatide treatment.

### *Anti-Sclerostin Antibody*

Anti-sclerostin antibody is another treatment that has been investigated for the treatment of adult HPP. Sclerostin is a protein expressed by bone cells that has been shown to down-regulate osteoblast activity (Lewiecki 2014). Anti-sclerostin treatments such as romosozumab have been shown to increase osteoblast activity; improving fracture healing and significantly increasing BMD.

One small scale study in adult HPP patients found short-term increases in ALP and BAP enzyme activity, as well as increases in bone formation markers (PINP and OC) and BMD (Seefried et al. 2017). This suggests that anti-sclerostin antibody treatment may be a viable therapy for adult HPP, although further investigation is needed.

## Conclusion

The literature suggests that the best biochemical markers of adult-onset HPP are a combination of PLP and ALP. Raised PLP alone is not entirely pathognomonic of adult-onset HPP and so measurement of PLP should be used as an identifier of HPP alongside persistently reduced ALP. Additionally, the effect of HPP on bone-specific isoforms of ALP should be further investigated. The role of OPN in HPP and its specificity as a marker of disease also needs elucidating further.

The increased detection rate, precision, and lower limit of quantification of HPLC methods compared to enzymatic methods, along with the availability of commercially available kits make HPLC a suitable and relatively simple method to assess PLP. Analysis of bone ALP isoforms is only currently possible through HPLC; total and bone ALP can be effectively quantified using enzymatic methods.

Reference intervals that are established from a representative population, such as those drawn from the NHANES study and similar UK populations are of most value in the assessment of PLP in HPP. The suggestion is made that further work is needed to establish gender and race specific PLP reference intervals that account for confounders of PLP.

## Thesis Aims

**To investigate biological and demographic factors associated with significant with significant differences in PLP and report 95% reference intervals that reflect these differences (Chapter 3)**

1. To examine whether inflammation, reduced kidney function, vitamin B6 supplementation, and low ALP are associated with significant differences in PLP.
2. To investigate age, race and gender associated differences in PLP.
3. To calculate 95% reference intervals for PLP from a large, representative sample population that reflect these differences.

**To investigate factors associated with significant differences in markers of bone health relevant to adult hypophosphatasia in a local population, and report 95% reference intervals that reflect these differences (Chapter 4)**

1. To determine whether population differences such as gender, age, and BMI are associated with significant differences in biochemical markers relevant to HPP.
2. To calculate 95% reference intervals for these markers that account for these differences in a Sheffield based population.
3. To calculate a novel 95% reference interval of BAP:PINP ratio for identification of HPP in adults.

**To determine the prevalence, biochemical profile and genetic profile of adults with hypophosphatasia in a clinical population (Chapter 5)**

1. To determine the prevalence of biochemical changes suggestive of HPP in a clinical population through application of previously calculated 95% reference intervals of PLP, BAP and BAP:PINP ratio.
2. To report the clinical profile of individuals with abnormal biochemistry.
3. To establish the prevalence of HPP-positive mutations in cases of abnormal biochemistry.

**To report the clinical profile of adults known to have hypophosphatasia who attend the Sheffield Metabolic Bone Centre (Chapter 6)**

1. To report the clinical, biochemical and genetic profile of adults with HPP attending the Sheffield Metabolic Bone Centre.

# Chapter 2: Methods

## Study Procedures

### *Recruitment*

Study participants were recruited from the clinical population of the Metabolic Bone Centre (MBC), Northern General Hospital in Sheffield.

All patient visitors were made aware prior to their visit that they would be able to volunteer to take part in the study. Due to the high numbers of patients attending the MBC, potential participants were typically approached following their initial routine in cases where “work-up” blood samples were needed as part of their clinical evaluation. This was to minimise both the invasiveness of the study and the burden placed on departments staff. All potential participants received a participant information sheet and gave written informed consent prior to undergoing any study procedures. Informed consent was obtained in accordance with Good Clinical Practice guidelines to ensure patient confidentiality and privacy.

### *Questionnaire*

All participants completed the skeletal health questionnaire as part of their visit to the MBC. This includes a range of questions designed to assess bone and general health including questions related to fracture history, and measurements of height and weight. Current medication including any vitamin supplements was also recorded; where this was unknown patients were re-contacted at a later date.

## Sample Collection

Blood samples were collected where possible immediately after consent was obtained during the recruitment phase of the study.

### *Serum, Plasma and Whole Blood Collection*

Blood samples were collected for biochemical and genetic assessment. Samples obtained for serum preparation were collected into 2x 8.5 mL tubes containing a polymer gel and spray-coated with silica. Blood tubes stood at room temperature in darkness to allow clotting, followed by centrifugation at 3000rpm for 10 minutes prior to sample aliquoting. Samples obtained for plasma preparation and whole blood were collected into 4.0 mL tubes containing the anti-coagulant EDTA (Ethylenediaminetetraacetic acid). Plasma was obtained through centrifugation at 3000rpm for 15 minutes within 30 minutes of sample collection. All sample aliquots were stored at -80°C until analysis.

## Biochemical Methods

### *Pyridoxal 5’-Phosphate (PLP)*

Serum PLP was measured by reversed-phase high performance liquid chromatography (HPLC) with fluorometric detection using a commercially available reagent kit and column (Chromsystems, Gräfelfing, Germany). The manufacturers report an inter-assay CV of between 2.5 - 3.2%. The limit of quantification is 1.0 μg/l.

### *Bone Alkaline Phosphatase (BAP)*

Serum BAP was measured by automated enzyme-linked immunosorbent assay (ELISA) using the IDS-iSYS multi-discipline automated system (Immunodiagnostic Systems, Boldon, UK). The manufacturers report an inter-assay CV of 7.3%. The reportable range of the assay is 1 - 75 μg/l.

### *Total Alkaline Phosphatase (ALP)*

Serum total ALP was measured by colourimetric assay (CA) using a Roche/Hitatchi Cobas C auto-analyser (Roche Diagnostics, Indianapolis, US). The manufacturers report an inter-assay CV of 1.8%. The reportable range of the assay is 5 - 1200 U/l.

The lower boundary of reported ALP reference intervals is typically between 30-40 U/L and the upper boundary of normal between 115-129 U/L (Lum 1995, McKiernan et al. 2014, Riancho-Zarrabeitia et al. 2016). The criteria for low ALP in this study was defined as <36 U/L as per the NHANES laboratory methods guidelines (Collaborative Laboratory Services 2009).

### *Procollagen I intact N-terminal pro-peptide (PINP)*

The serum biomarker of bone formation PINP was measured by chemiluminescence immunoassay (CLIA) using the IDS-iSYS multi-discipline automated system (Immunodiagnostic Systems, Boldon, UK). The manufacturers report an inter-assay CV of 4.6%. The reportable range of the assay is 2 - 230 ng/ml.

## Assay Principles

### *High Performance Liquid Chromatography (HPLC)*

Prior to analysis, samples undergo a preparation and derivatisation process. This procedure includes protein precipitation which liberates 88% of PLP from its bound status. Derivatisation then produces a fluorescent PLP derivative suitable for detection with fluorescence detector.

Reversed-phase HPLC produces a separation of analytes based on polarity. The assay principle involves the injection of pre-derivatised sample volume into a polar aqueous mobile phase which is circulating through the system. Hydrophilic molecules within the sample have a greater affinity for the mobile phase than more hydrophobic molecules and therefore pass through the non-polar stationary phase (in the column packing) more quickly and elute sooner. This leads to separation of the sample components based on the retention times of the molecules passing through the column. Once the sample has passed through the column it reaches a fluorescence detector, here the aqueous solution enters a flow cell and the optical emission of light by molecules in the solution is measured following excitation by a xenon lamp at a specific wavelength. A schematic of the HPLC system is shown in figure 11.

A calibration standard of known PLP concentration is analysed prior to patient samples to allow calculation of PLP concentration from peak height. The following formula is used:

$$Sample \left(μg/l\right)= \frac{ASample}{ACalibrator}×CCalibrator$$

Where:

* ASample = Peak height of PLP in the chromatogram of the sample.
* ACalibrator = Peak height of PLP in the chromatogram of the calibrator.
* CCalibrator = The Concentration of PLP in the calibrator.

A conversion factor of 4.04 was applied to convert results to nmol/l.



Figure 11: Schematic of a HPLC system.

### *Enzyme Immunoassay (EIA)*

The automated EIA method utilised by the IDS iSYS analyser quantifies BAP concentration through addition of biotin labelled, BAP-specific monoclonal antibodies to the patient sample. This is followed by an incubation step and addition of streptavidin labelled magnetic particles. Following further incubation a magnet captures magnetic particles and a wash step removes any unbound BAP. Final incubation with an enzyme substrate allows spectrophotometric quantification of substrate turnover, with signal proportional to the amount of BAP present in the sample.

### *Chemiluminescence Immunoassay (CLIA)*

The automated CLIA method utilised by the IDS iSYS analyser quantifies intact PINP. Patient samples are diluted, followed by incubation with an anti-PINP antibody, an acridinium labelled antibody, and streptavidin labelled magnetic particles. A magnet captures the magnetic particles and a wash step removes the unbound particles. Adding trigger reagents stimulates light to be emitted by the acridinium label which is directly proportional to the concentration of intact PINP in the sample.

### *Colourimetric Assay (CA)*

Total ALP is measured by CA. Patient samples are incubated in the presence of magnesium and zinc ions; p-nitrophenyl phosphate is cleaved by phosphatases present in the patient sample into phosphate and p-nitrophenol:

$$p ̵nitrophenyl phosphate +H\_{2}O → phosphate+p ̵nitrophenol$$

ALP activity is directly proportional to the release of p-nitrophenol which is measured through spectrophotometric changes in absorbance.

## Biochemistry Quality Control

For all biochemical measurements completed internally, the possibility of inter-operator variability was removed as all measurements were completed by the same experienced technician.

As standard procedure in the laboratory, prior to sample measurement all analysers were calibrated and maintained as per the manufacturers recommendation for each analyte respectively. Analyte-specific quality control samples were measured at the beginning and end of each sample batch, with results validated against the manufacturers known concentrations to ensure the accuracy of all measurements. Internal quality control serum samples were used to monitor the long term accuracy and stability of all laboratory measurements. Where possible, laboratory methods such as the determination of PLP by HPLC were quality assured against external quality assurance standards such as the INSTAND e.V. service.

To minimise inaccuracy all analytes were measured in fresh aliquots of sample wherever possible; no samples underwent more than two freeze thaw cycles.

## Genetic Testing

Next-generation sequencing (NGS) was utilised to identify mutations of the *ALPL* gene coding region in patients with suspected HPP. All identified mutations were confirmed by Sanger methodology. A summary of the *ALPL* gene sequencing process follows:

1. DNA Extraction

DNA is isolated from patient whole blood samples using a QIAsymphony SP system in conjunction with the QIAsymphony DSP Midi kit (Qiagen, Venlo, Netherlands). An overview of the DNA extraction and purification process is shown in figure 12.

[Image Redacted]

Figure 12: DNA extraction process.

1. DNA Quantification

DNA is quantified using a DNA Qubit assay kit and Qubit Fluorometer (Thermo Fisher Scientific, Mass., US). Dyes selective for dsDNA are added to the DNA extract which fluoresce at a level directly proportional to the concentration of the bound sample. This fluorescence is detected and quantified.

1. NGS AmpliSeq Library Preparation

DNA targets are amplified using AmpliSeq primer pools (Thermo Fisher Scientific, Mass., US); following amplification primer sequences are partially digested before ligation of patient-specific DNA barcodes and generic adapters. DNA libraries can then be quantified.

1. Library Quantification

DNA libraries are quantified using TapeStation 2200 (Agilent, Santa Clara, US)

1. Chip Loading

The Ion Chef system is an automated system that carries out emulsion PCR to amplify AmpliSeq libraries and attach them to ion sphere particles. Multiple barcoded patient DNA samples are then loaded onto a semi-conductor chip which can be sequenced.

1. Sequencing

The Ion S5 (Thermo Fisher Scientific, Mass., US) is a NGS platform that converts chemical data into a digital data output. The DNA sample chip is loaded into the apparatus where it is sequentially flooded with solutions containing a single nucleotide. This causes the release of an H+ ion as a by-product, in turn decreasing pH which is detectable by ion sensors on the apparatus. The detected changes in pH are associated with a specific nucleotide. This data is converted to into a digital binary data output.

1. Run NGS Pipeline

Data generated by NGS is processed by in-house scripts to produce useable sequencing results.

1. Sanger Sequencing Confirmation

All pathogenic variants identified by NGS are confirmed by Sanger sequencing. Gaps identified via NGS are PCR amplified and Sanger sequenced on a Core Robotics System (Thermo Fisher Scientific, Mass., US).

1. Sequencing Analysis – Mutation Surveyor

Mutation Surveyor software (SoftGenetics, State College, US) is used for analysis of Sanger sequencing data files.

# Chapter 3: Establishing 95% Reference Intervals for PLP in the NHANES Population

## Research Aims

1. To produce 95% reference intervals for PLP from a large, representative sample population.
2. To examine factors (including inflammation, reduced kidney function, vitamin B6 supplementation, and low ALP) that may be associated with significant differences in PLP.
3. To investigate potential age, race and gender differences in PLP.

## Materials and Methods

### *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 U.S. population. Data from 20,015 subjects collected during two years of the survey were collated for this investigation (2007-2008, *n* = 9762; 2009-2010, *n* = 10,253). This investigation included data from adults (ages 20-80) from three pre-defined race/ethnicity groups, Hispanic-Mexican American, Non-Hispanic Black and Non-Hispanic White. 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.

Cases with ambiguous race/ethnicity identification (Hispanic-Other Hispanic, Other) were excluded based on the difficulty of applying the MDRD equation to calculate eGFR. Cases were omitted where data was incomplete for any variable of interest. Following omissions the study population was 9069 (50.6% female; *n* = 4458 from 2007-2008; *n* = 4611 from 2009-2010). Further exclusions were made based on the presence of one or more known confounders of PLP (*inflammation, reduced kidney function, low ALP, vitamin B6 supplementation*), providing a final reference interval population of *n* = 4463, 44.4% female.

### *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 (Rybak and Pfeiffer 2004, 2009).

Estimated glomerular filtration rate (eGFR) was calculated from serum creatinine using the Modification of Diet in Renal Disease Study (MDRD) equation (Levey et al. 1999). Reduced kidney function was defined in individuals with an eGFR <60 mL/(min • 1.732), typically described as chronic kidney disease (CKD) stage 3-5.

Inflammation was defined as C-reactive protein (CRP) ≥5.0 mg/L (Haynes et al. 2013).

### *Statistical analyses*

SAS Universal Viewer version 1.4 (SAS Institute, North Carolina, USA) was used for data assembly. Statistical analyses were performed using MedCalc version 16.8.4 (MedCalc Software, Ostend, Belgium).

PLP values 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, as well as identifying gender differences in PLP. Differences in PLP associated with race were assessed using the Kruskal-Wallis test. For all tests significance was defined as *p* < 0.05.

The remaining PLP measurements had a log normal distribution so 95% RIs were calculated from data back-transformed after logarithmic transformation.

## Results

General characteristics of the study population before (total) and following exclusions (RI population) are presented in Table 5. Both years of the NHANES survey included in this analysis are remarkably similar. Levels of the biochemical markers of interest are similar in both collections, prior to and following exclusions. The numbers of excluded subjects for each confounding variable is also similar across both collections, as is the gender and race stratification of the population.

|  |  |  |
| --- | --- | --- |
|  | **2007-08** | **2009-10** |
|  | Total | RI population | Total | RI population |
| n | 4458 | 2188 | 4611 | 2275 |
| % Female | 50.4 | 44.5 | 50.8 | 44.2 |
| Age | 51 (50 – 51) | 44 (43 – 45) | 49 (49 – 50) | 45 (44 – 46) |
| PLP | 44.1(43.0 –45.4) | 39.70(38.1 – 40.8) | 41.0(39.9 – 42.6) | 37.8(36.6 – 39.2) |
| CRP | 2.0 (2.0 – 2.1) | 1.4 (1.4 – 1.5) | 1.9 (1.8 – 2.0) | 1.3 (1.2 – 1.4) |
| eGFR | 93.44(92.64 – 94.56) | 99.55(98.19 – 100.71) | 91.95(91.12 – 92.93) | 96.26(95.12 – 97.51) |
| ALP | 67 (66 – 68) | 66 (66 – 67) | 66 (65 – 67) | 65 (64 – 66) |
| Race categories; n (%): |  |  |  |  |
| Mexican American | 937 (21.0) | 563 (25.7) | 955 (20.7) | 566 (24.9) |
| Non-Hispanic White | 2514 (56.4) | 1118 (51.1) | 2706 (58.7) | 1227 (53.9) |
| Non-Hispanic Black | 1007 (22.6) | 507 (23.2) | 950 (20.6) | 482 (21.2) |
| Excluded subjects; n: |  |  |  |  |
| Inflammation | 1051 | 1057 |
| Reduced kidney function | 366 | 375 |
| Low ALP | 88 | 111 |
| Vitamin B6 supplementation | 1312 | 1321 |

Table 5: Population characteristics, median (95% CI for the median)

### *Confounder analysis*

All four independently tested variables were associated with significant differences in PLP (Table 6). PLP was significantly lower in individuals with inflammation (*p* < 0.0001), and those with reduced kidney function (*p* = 0.0005). Conversely PLP was shown to be significantly higher in subjects with low ALP (*p* < 0.0001), and those receiving a vitamin B6 supplement (*p* < 0.0001).

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Variable | Present | Median | IQR | Sig. | *N* |
| Inflammation, CRP ≥5.0 mg/L | No | 47.4 | 28.7 – 82.8 | *p* < 0.0001 | 6961 |
| Yes | 29.3 | 18.3 – 52.6 |  | 2108 |
| Reduced Kidney Function, eGFR <60 mL/(min • 1.732) | No | 43.2 | 25.8 – 75.0 | *p* = 0.0005 | 8328 |
| Yes | 37.5 | 21.4 – 74.2 |  | 741 |
| Low ALP, <36 U/L | No | 42.3 | 25.1 – 73.5 | *p* < 0.0001 | 8870 |
| Yes | 83.3 | 49.4 – 137.8 |  | 199 |
| Vitamin B6 Supplement | No | 34.5 | 22.2 – 55.1 | *p* < 0.0001 | 6436 |
| Yes | 81.3 | 48.2 – 143.0 |  | 2633 |

Table 6: PLP (nmol/L) in subjects according to the presence and absence of tested confounders

### *Gender, race and age differences in PLP*

Gender, race and age differences in PLP were found in the reference interval population (*n* = 4463) (Table 7). Changes in PLP associated with ageing were gradual (Figure 13).

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  |  | Median | IQR | Sig. | *N* |
| Gender1 | Male | 43.3 | 28.7 – 64.9 | *p* < 0.0001 | 2483 |
|  | Female | 33.2 | 22.4 – 51.6 |  | 1980 |
| Race/ethnicity2 | Mexican Americana | 44.6 | 30.0 – 63.9 | *p* < 0.0001 | 1129 |
|  | Non-Hispanic Whiteb | 37.8 | 24.6 – 58.1 |  | 2345 |
|  | Non-Hispanic Blackc | 34.3 | 22.8 – 55.0 |  | 989 |
| Age3 |  | -0.204† | -0.232 – -0.176ǂ | *p* < 0.0001 | 4463 |

Table 7: A comparison of gender, race and age differences in PLP (nmol/L).

1Mann-Whitney U Test was used for gender comparisons. 2Kruskal Wallis Test was used for race comparisons. abcPost-hoc analysis showed each race was significantly different from the two other races (p < 0.05). 3Spearman’s rho. †Coefficient of rank correlation (rho) between age and PLP. ǂ95% confidence interval for rho.



Figure 13: Scatterplot of PLP (nmol/L) vs. Age with LOESS line (5% smoothing)

### *95% reference intervals*

Due to the differences in PLP relative to gender, race and age, the 95% RIs were stratified to reflect these differences (Table 8 and 9). Young adults were defined as 20-49 years old, and older adults ages 50+. A 95% RI for PLP prior to any exclusions or race/gender/age stratification is shown for comparison in table 10.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
|  |  | GM | 95% RI | 95% CI of lower limit | 95% CI of upper limit | *N* |
| Mexican American | Male | 57.6 | 21.0 – 157.7 | 19.5 – 22.6 | 146.4 – 169.9 | 389 |
|  | Female | 40.1 | 12.6 – 128.0 | 11.5 – 13.8 | 116.5 – 140.6 | 323 |
| Non-Hispanic White | Male | 49.0 | 14.8 – 162.3 | 13.9 – 15.8 | 152.2 – 173.1 | 737 |
|  | Female | 34.8 | 9.3 – 129.9 | 8.6 – 10.1 | 120.2 – 140.4 | 605 |
| Non-Hispanic Black | Male | 49.6 | 14.4 – 170.5 | 13.0 – 16.0 | 154.0 – 188.7 | 313 |
|  | Female | 30.7 | 9.3 – 101.0 | 8.4 – 10.3 | 90.9 – 112.1 | 274 |

Table 8: Race- and gender-specific 95% RIs for PLP (nmol/L) in young adults (ages 20-49).

*GM* geometric mean

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
|  |  | GM | 95% RI | 95% CI of lower limit | 95% CI of upper limit | *N* |
| Mexican American | Male | 40.1 | 13.0 – 123.3 | 11.7 – 14.6 | 110.3 – 137.8 | 216 |
|  | Female | 36.8 | 9.6 – 141.2 | 8.4 – 11.0 | 123.0 – 162.1 | 201 |
| Non-Hispanic White | Male | 35.5 | 9.5 – 132.5 | 8.8 – 10.3 | 122.5 – 143.3 | 595 |
|  | Female | 34.3 | 9.2 – 127.4 | 8.4 – 10.1 | 115.9 – 140.1 | 408 |
| Non-Hispanic Black | Male | 32.0 | 8.3 – 124.2 | 7.3 – 9.4 | 109.1 – 141.3 | 233 |
|  | Female | 29.4 | 8.7 – 99.6 | 7.6 – 9.9 | 86.8 – 114.1 | 169 |

Table 9: Race- and gender-specific 95% RIs for PLP (nmol/L) in older adults (ages 50+).

*GM* geometric mean

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| GM | 95% RI | 95% CI of lower limit | 95% CI of upper limit | *N* |
| 45.7 | 8.9 – 234.9 | 8.7 – 9.1 | 229.1 -240.8 | 9069 |

Table 10: 95% RI for PLP (nmol/L) in the total study population.

*GM* geometric mean

## Discussion

The standardised protocol of NHANES allowed us to combine data from two years of the programme, providing an increased sample representative of the noninstitutionalised U.S. population. Due to this large sample we have been able to calculate robust 95% RIs for PLP that are specific to gender, race and age as required by the significant differences displayed based on these factors. These reference intervals show much variation around the upper limit of the ranges; old black women have the lowest upper limit (99.6 nmol/L) compared to young black men who have the highest (170.5 nmol/L). The variation around the upper limit of the RIs shows the importance of gender/race/age stratification for the diagnosis of HPP.

Likewise, in comparison to a 95% RI calculated within the total study population without exclusion of confounding variables (Table 10), the upper limit of all stratified RIs are lower than the upper limit of the total population (234.9 nmol/L). The geometric mean of the total population (45.7) was also higher than most stratified RIs, except young Mexican American, non-Hispanic white, and non-Hispanic black males (57.6, 49.0 and 49.6 respectively).

PLP was found to be higher in males than in females, different across all race/ethnicity groups, highest in Mexican Americans, and higher in young adults compared to adults aged 50 and over. Differences in PLP associated with ageing were consistent and gradual. 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.

Of the four tested physiological variables, 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 (Gray et al. 2004, Duncan et al. 2012), including an investigation of data from the 2005-5006 NHANES collection (Haynes et al. 2013). Upwards of 90% of serum PLP is albumin bound (Whyte et al. 1985); 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 (Duncan et al. 2012).

Reduced kidney function was also associated with lower PLP. 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 m2) and absence of albuminuria) and those with stage 3-5 CKD (eGFR <60 ml/(min • 1.732)). However a third stratification was investigated in that study (stage 1 or 2 CKD, eGFR ≥60 mL/(min • 1.73 m2) and presence of albuminuria) which was not represented in this study, as our investigation examined only those with stage 3-5 CKD, compared to those with normal kidney function, defined as eGFR ≥60 mL/(min • 1.73 m2).

PLP was higher in individuals with low ALP (<36 U/L), than those with normal or high values. This negative correlation has been previously documented (Anderson et al. 1980). Cases with high PLP and low ALP may represent undiagnosed instances of HPP within the population, thus PLP is higher in these individuals. It is not known whether higher PLP has an effect on supressing or increasing ALP activity.

PLP was also higher in individuals taking vitamin B6 supplementation, compared with those who weren’t. As PLP levels were higher in subjects receiving vitamin B6 supplements, blood tests in clinical investigation of PLP should be taken preferably at least 2 weeks after cessation of supplementary vitamin B6 intake (McKiernan, Berg, and Fuehrer 2014).

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 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.

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, seven factors have been shown to be associated with differences in PLP and should be considered in the interpretation of clinical PLP measurements, along with gender/race/age differences.

# Chapter 4: Establishing 95% Reference Intervals for PLP in a Sheffield Population

## Research Aims

1. To determine whether population differences such as gender, age, and BMI are associated with significant differences in biochemical markers of HPP such as serum PLP and BAP.
2. To calculate 95% reference intervals for these markers that account for these differences in a Sheffield based population.
3. To calculate a 95% reference interval for serum BAP:PINP ratio for identification of HPP.

## Materials and Methods

### *Study population*

Serum samples from previous investigations held at the South Yorkshire and North Derbyshire Musculoskeletal Biobank (SYNDMB) were accessed in order to produce a Sheffield-based reference interval population. This population consisted of study cohorts from 3 prior investigations:

1. Reference Range Study (Glover et al. 2008):

200 healthy women were recruited between March and July 2005. Subjects were aged 30 to 45 years and were recruited from the Northern General and Royal Hallamshire hospitals (Sheffield, UK) via poster and email.

No subjects were diagnosed with conditions believed to affect bone metabolism, nor were they receiving medication known to affect bone metabolism.

Subjects were excluded from the study population who had experienced a fracture within the previous 12 months, or had undergone a surgical intervention in the previous 3 months. Pregnant women and those receiving contraceptives were also excluded.

1. Xtreme CT Study (XCT) (Walsh et al. 2017):

180 healthy subjects were recruited between May 2009 and May 2010. Subjects were recruited from posters and emails to departments within the Northern General and Royal Hallamshire hospitals (Sheffield, UK), University of Sheffield staff, and mailing from Sheffield general practices.

The study population included 30 men and 30 women stratified across three age groups; 16 to 18 years, 30 to 32 years, and 70+ years.

No subjects were diagnosed with conditions believed to affect bone metabolism, nor were they receiving medication known to affect bone metabolism.

Subjects were excluded if they had any conditions that may affect reliability of study measurements (including previous fractures of both radii or both tibiae, or they did not have 2 evaluable vertebrae by DXA). Pregnant women and those receiving oral or hormonal contraceptive were also excluded.

1. Fat and Bone Study (FAB) (Evans et al. 2015):

200 adults were recruited during 2012 to 2013. Subjects were recruited from posters and emails to departments within the Northern General and Royal Hallamshire hospitals, University of Sheffield staff, and mailing from Sheffield general practices.

The study population included 104 women and 96 men across two age groups (25 to 40 years and 55 to 75 years), and two classifications based on BMI (lean 18.5 to 24.9 kg/m2 or obese >30 kg/m2). Obese cases were matched to lean individuals based on age (±3 years), height (±5 cm), the first part of their postcode (e.g. S6), and whether or not they were a current smoker.

No subjects were diagnosed with conditions believed to affect bone metabolism, nor were they receiving medication known to affect bone metabolism.

Subjects were excluded from the study population who had experienced a fracture or undergone orthopaedic surgery within the previous 12 months, had a history of immobilisation, cancer, eating disorders, consumed more than 21 units of alcohol per week, were actively losing weight or a competitive athlete.

Approval for sample analysis was granted by the SYNDMB prior to analysis.

These combined cohorts would allow investigation of population differences in biomarker levels dependent on age, gender and BMI, in order to calculate stratified 95% RIs within a healthy local population.

Limited sample volumes meant that biomarkers could not be measured in all of the original study samples. Depending on volumes certain markers were measured in more samples than others, e.g. PLP was measured prior to other markers and so was measured in a greater number of subjects in studies with limited volumes.

### *Biochemical measurements*

All biochemical analyses were completed at the Sheffield Bone Biochemistry Laboratory within the University of Sheffield. PLP was measured by HPLC, BAP and PINP were measured using the IDS iSYS auto-analyser methods.

Biochemical analyses of samples from the Reference Range study were completed between November 2015 and September 2016. Biochemical analyses of samples from the Extreme CT study were completed between December 2017 and January 2018. Biochemical analyses of samples from the PRP study were completed between December 2017 and January 2018.

### *Statistical analyses*

Statistical analysis was carried out using SPSS Statistics version 22.0.0.1 (IBM, Armonk, NY, U.S.). 95% reference intervals were calculated using MedCalc version 16.8.4 (MedCalc Software, Ostend, Belgium).

95% References intervals were calculated as mean ±1.96 standard deviation (SD). Confidence intervals around the upper and lower limits of the reference interval were calculated as ±1.96 standard error (SE) where:

$$SE= √(SD^{2} / N+1.96^{2}×SD^{2} / 2N)$$

PLP and BAP data was not normally distributed in any of the study populations (Table 11) and so statistical analyses were carried out on log10 transformed data. 95% Reference intervals were established using log10 transformed data and then back transformed. BAP/PINP ratio was calculated from the untransformed measurements and was log10 transformed prior to analysis.

|  |  |  |
| --- | --- | --- |
| Study Population | Analyte | Sig. |
| Reference Range | PLP | *p* > 0.001 |
|  | BAPBAP:PINP ratio | *p* > 0.001*p* > 0.001 |
| Xtreme CT | PLP | *p* > 0.001 |
|  | BAPBAP:PINP ratio | *p* > 0.001*p* > 0.001 |
| FAB | PLP | *p* > 0.001 |
|  | BAPBAP:PINP ratio | *p* > 0.001*p* > 0.001 |

Table 11: Shapiro-Wilk normality test.

## Results

Tables 12-15 show the study subject characteristics for each of the 3 study populations as well as the combined study populations.

|  |  |  |  |
| --- | --- | --- | --- |
| Variable | *n* | Mean (SD) | Median (minimum; maximum) |
| Age (years) | 200 | 39.2 (4.28) | 39.5 (30.2; 45.7) |
| Height (m) | 188 | 1.65 (0.06) | 1.64 (1.45; 1.79) |
| Weight (kg) | 187 | 69.8 (14.8) | 67.1 (44.0; 134.0) |
| BMI (kg/m2) | 186 | 25.7 (5.42) | 24.8 (17.5; 50.9) |
| PLP (nmol/l) | 200 | 64.8 (60.4) | 47.5 (12.2; 433.8) |
| BAP (μg/l) | 199 | 11.1 (5.1) | 10.2 (0.99; 40.1) |
| PINP (ng/ml) | 198 | 38.1 (18.6) | 35.1 (10.1; 177.4) |
| BAP:PINP ratio | 197 | 0.306 (0.11) | 0.286 (0.047; 0.865) |

Table 12: Subject characteristics in the Reference Range study population.

|  |  |  |
| --- | --- | --- |
|  | Women | Men |
|  | 16 to 18n = 22 | 30 to 32n = 22 | 70+n = 24 | 16 to 18n = 19 | 30 to 32n = 14 | 70+n = 11 |
| PLP (nmol/l) | 63.3 (40.8) | 84.5 (82.7) | 57.8 (43.7) | 94.7 (56.1) | 94.3 (36.1) | 88.3 (71.1) |
| BAP (μg/l) | 21.0 (7.7) | 12.6 (3.2) | 20.4 (5.8) | 27.8 (12.9) | 14.0 (3.8) | 13.3 (5.7) |
| PINP (ng/ml) | 95.7 (35.9) | 39.9 (15.8) | 53.8 (18.2) | 137.8 (57.3) | 62.7 (25.5) | 39.8 (20.4) |
| BAP:PINP ratio | 0.250 (.147) | 0.343 (.104) | 0.408 (.139) | 0.206 (.069) | 0.242 (.065) | 0.363 (.116) |

Table 13: Subject characteristics in the XCT study by age group; mean (SD).

|  |  |  |
| --- | --- | --- |
|  | Women | Men |
|  | 25 to 40 yearsn = 42 | 55 to 75 yearsn = 53 | 25 to 40 yearsn = 35 | 55 to 75 yearsn = 53 |
|  | Norm. BMI | Obese | Norm. BMI | Obese | Norm. BMI | Obese | Norm. BMI | Obese |
| PLP (nmol/l) | 64.8 (39.7) | 48.0 (57.3) | 97.8 (147.3) | 38.2 (20.1) | 89.1 (46.7) | 78.2 (41.6) | 67.5 (49.2) | 47.5 (28.1) |
| BAP (μg/l) | 14.6 (4.8) | 17.4 (3.9) | 20.0 (6.3) | 18.9 (4.4) | 19.7 (8.0) | 18.1 (6.2) | 17.9 (6.6) | 15.9 (5.5) |
| PINP (ng/ml) | 48.4 (12.2) | 39.5 (12.7) | 54.95 (21.6) | 41.7 (13.3) | 72.1 (46.0) | 50.6 (22.3) | 45.6 (18.2) | 33.0 (11.5) |
| BAP:PINP ratio | 0.716 (1.76) | 0.458 (.111) | 0.393 (.131) | 0.472 (.103) | 0.320 (.119) | 0.373 (.088) | 0.398 (.118) | 0.505 (.168) |

Table 14: Subject characteristics in the FAB study by age and BMI group; mean (SD).

|  |  |  |  |
| --- | --- | --- | --- |
| Variable | n | Mean (SD) | Median (minimum, maximum) |
| PLP (nmol/l) | 495 | 68.3 (64.1) | 50.8 (4.6; 827.9) |
| BAP (μg/l) | 495 | 15.3 (7.3) | 13.7 (1.0; 63.7) |
| PINP (ng/ml) | 495 | 49.2 (31.5) | 39.8 (10.1; 229.6) |
| BAP:PINP ratio | 495 | 0.343 (.133) | 0.319 (0.047; 0.963) |

Table 15: Subject characteristics of the combined study populations.

### Reference Range Study

Glover’s original analysis of measurements of bone turnover markers in this study (Glover et al. 2008) population found a difference in marker levels in subjects depending on age, whereby marker concentrations were higher in subjects aged 30 to 35 compared to those aged 35 to 45. The relationship between age and biomarker levels have been visualised (Figure 14). LOWESS curves have been used to observe the trend of the data; no clear trend was observed. This potential effect was further investigated for the three markers measured in this study. Non-parametric independent-sample testing did not find any differences in marker concentration in subjects aged 30 to 35 compared to those aged 35 to 45 (PLP, *p* = 0.080; Bone ALP, *p* = 0.971; PINP, *p* = 0.293).

95% reference intervals were calculated for each marker and Bone ALP:PINP ratio using all available measurements; data was back transformed following log transformation (Table 16).

|  |  |
| --- | --- |
|  |  |
|  |  |

Figure 14: Scatter plots of age against PLP, BAP and PINP with LOWESS curves.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  | *n* | GM | 95% RI | 95% CI of lower limit | 95% CI of upper limit |
| PLP (nmol/l) | 200 | 51.0 | 14.6 – 177.9 | 12.9 – 16.6 | 156.4 – 202.3 |
| Bone ALP (μg/l) | 199 | 10.1 | 4.18 – 24.3 | 3.82 – 4.58 | 22.2 – 26.6 |
| PINP (ng/ml) | 198 | 34.9 | 15.5 – 78.6 | 14.2 – 16.8 | 72.2 – 85.5 |
| Bone ALP:PINP ratio | 197 | 0.289 | 0.147 – 0.570 | 0.137 – 0.158 | 0.531 – 0.611 |

Table 16: 95% Reference intervals in the reference range study population.

### Xtreme CT Study

A two-way ANOVA was performed in order to determine the effect of gender and age on log10 transformed serum PLP and BAP values; data was back transformed following analysis.

*PLP*

Gender had a statistically significant effect on log10 transformed PLP (*p* = 0.002), whereas age does not (*p* = 0.244). The interaction age\*gender did not have a significant effect on PLP (*p* = 0.939). Back-transformed PLP values were higher in men (mean: 79.4; 95% CI: 64.9 – 97.3) compared to women (mean: 52.5; 95% CI: 44.8 – 61.5) (Table 17).

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | *n* | Mean | 95% Confidence Interval | Sig. |
| Lower Bound | Upper Bound |
| Men | 44 | 79.4 | 64.9 | 97.3 | *p* = 0.002 |
| Women | 68 | 52.5 | 44.8 | 61.5 |  |
| 16 to 18 years | 41 | 66.1 | 53.7 | 81.1 | *p* = 0.244 |
| 30 to 32 years | 36 | 73.3 | 58.6 | 91.8 |  |
| 70+ years | 35 | 55.6 | 43.8 | 70.6 |  |

Table 17: PLP (nmol/l) differences in the XCT Study; values were back transformed after logarithmic transformation.

*BAP*

Gender did not have a statistically significant effect on log10 transformed BAP (*p* = 0.527). BAP was different depending on age (*p* < 0.001), and the interaction age\*gender was also significant (*p* < 0.001). BAP was different depending on age group, with the youngest subjects having the highest values (mean: 22.5; 95% CI: 1.1 – 20.2), followed by the oldest subjects (mean: 15.5; 95% CI: 1.1 – 13.7). BAP was lowest in the 30 to 32 year old subjects (mean: 12.8; 95% CI: 1.1 – 11.4). Post-hoc Tukey testing (Tukey) showed BAP to be significantly different in each age group (Table 18).

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | *n* | Mean | 95% Confidence Interval | Sig. |
| Lower Bound | Upper Bound |
| Men | 43 | 16.1 | 14.5 | 17.9 | *p* = 0.527 |
| Women | 68 | 16.8 | 15.5 | 18.3 |  |
| 16 to 18 yearsa | 40 | 22.5 | 20.2 | 25.0 | *p* < 0.001 |
| 30 to 32 yearsb | 36 | 12.8 | 11.4 | 14.4 |  |
| 70+ yearsc | 35 | 15.5 | 13.7 | 17.6 |  |

Table 18: BAP (μg/l) differences in the XCT Study; values were back transformed after logarithmic transformation.

abcTukey post-hoc test differences.

*PINP*

Gender did not have a statistically significant effect on log10 transformed PINP (*p* = 0.081). PINP was different depending on age (*p* < 0.001), with 16 to 18 years olds having higher PINP (mean: 106.2; 95% CI: 93.3 – 121.1) than the older age groups, who had statistically similar PINP (30 to 32 year olds means: 46.3; 95% CI: 40.4 – 53.2; and 70+ year olds means: 42.5; 95% CI: 36.6 – 49.2) (Table 19).

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | *n* | Mean | 95% Confidence Interval | Sig. |
| Lower Bound | Upper Bound |
| Men | 42 | 63.8 | 56.2 | 72.3 | *p* = 0.081 |
| Women | 68 | 55.3 | 50.1 | 61.0 |  |
| 16 to 18 yearsa | 39 | 106.2 | 93.3 | 121.1 | *p* < 0.001 |
| 30 to 32 yearsb | 36 | 46.3 | 40.4 | 53.2 |  |
| 70+ yearsb | 35 | 42.5 | 36.6 | 49.2 |  |

Table 19: PINP (ng/ml) differences in the XCT Study; values were back transformed after logarithmic transformation.

abTukey post-hoc test differences.

*BAP:PINP Ratio*

Gender and age were both associated with significant differences in the formation marker ratio (*p* = 0.006 and *p* < 0.001 respectively). The ratio was higher in women (mean: 0.305; 95% CI: 0.280 – 0.331) than men (mean: 0.251; 95% CI: 0.225 – 0.279). Post-hoc testing showed the ratio was higher in older age groups; all three groups were significantly different from one another (Table 20).

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | *n* | Mean | 95% Confidence Interval | Sig. |
| Lower Bound | Upper Bound |
| Men | 42 | 0.251 | 0.225 | 0.279 | *p* = 0.006 |
| Women | 68 | 0.305 | 0.280 | 0.331 |  |
| 16 to 18 yearsa | 39 | 0.208 | 0.187 | 0.233 | *p* < 0.001 |
| 30 to 32 yearsb | 36 | 0.277 | 0.245 | 0.311 |  |
| 70+ yearsc | 35 | 0.366 | 0.322 | 0.415 |  |

Table 20: BAP:PINP ratio differences in the XCT Study; values were back transformed after logarithmic transformation.

abcTukey post-hoc test differences.

### Fat and Bone Study

A three-way ANOVA was performed in order to determine the effect of gender, age, and BMI on log10 transformed serum PLP and BAP values; data was back transformed following analysis.

*PLP*

Gender and BMI were both associated with significant differences in PLP (*p* = 0.025, and *p* > 0.001 respectively). PLP was higher in men (mean: 57.4; 95% CI: 49.7 – 66.2) than women (mean: 45.8; 95% CI: 40.0 – 52.4). PLP was also higher in adults with a lean BMI (mean: 63.0; 95% CI: 55.1 – 72.1) compared to obese adults (mean: 41.7; 95% CI: 36.1 – 48.1). There was no significant difference in PLP between the two age groups investigated (*p* = 0.055) (Table 21).

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | *n* | Mean | 95% Confidence Interval | Sig. |
| Lower Bound | Upper Bound |
| Men | 88 | 57.4 | 49.7 | 66.2 | *p* = 0.025 |
| Women | 95 | 45.8 | 40.0 | 52.4 |  |
| 25 to 40 years | 77 | 56.4 | 48.9 | 65.5 | *p* = 0.055 |
| 55 to 75 years | 106 | 46.5 | 41.0 | 52.9 |  |
| Lean BMI (18.5 – 24.9) | 98 | 63.0 | 55.1 | 72.1 | *p* < 0.001 |
| Obese BMI (>30) | 85 | 41.7 | 36.1 | 48.1 |  |

Table 21: PLP (nmol/l) differences in the FAB Study; values were back transformed after logarithmic transformation.

*BAP*

There were no significant observed differences in BAP dependent on gender, age or BMI (*p* = 0.860, *p* = 0.334, and *p* = 0.983 respectively) (Table 22).

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | *n* | Mean | 95% Confidence Interval | Sig. |
| Lower Bound | Upper Bound |
| Men | 83 | 16.8 | 15.7 | 18.1 | *p* = 0.860 |
| Women | 94 | 17.0 | 15.9 | 18.1 |  |
| 25 to 40 years | 72 | 16.5 | 15.3 | 17.8 | *p* = 0.334 |
| 55 to 75 years | 105 | 17.3 | 16.3 | 18.4 |  |
| Lean BMI (18.5 – 24.9) | 95 | 16.9 | 15.8 | 18.1 | *p* = 0.983 |
| Obese BMI (>30) | 82 | 16.9 | 15.7 | 18.1 |  |

Table 22: BAP (μg/l) differences in the FAB Study; values were back transformed after logarithmic transformation.

*PINP*

Gender did not have a statistically significant effect on log10 transformed PINP (*p* = 0.760). PINP was different depending on age (*p* = 0.005), with 25 to 40 years olds having higher PINP (mean: 47.6; 95% CI: 43.7 – 52.1) than the 55 to 75 year olds (mean: 40.6; 95% CI: 37.8 – 43.6). Obese individuals had a lower PINP (mean: 38.5; 95% CI: 35.5 – 41.9) than lean individuals (mean: 50.2; 95% CI: 46.5 – 54.2) (Table 23).

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | *n* | Mean | 95% Confidence Interval | Sig. |
| Lower Bound | Upper Bound |
| Men | 79 | 44.4 | 40.8 | 48.2 | *p* = 0.760 |
| Women | 90 | 43.7 | 40.5 | 47.1 |  |
| 25 to 40 years | 67 | 47.6 | 43.7 | 52.1 | *p* = 0.005 |
| 55 to 75 years | 102 | 40.6 | 37.8 | 43.6 |  |
| Lean BMI (18.5 – 24.9) | 91 | 50.2 | 46.5 | 54.2 | *p* < 0.001 |
| Obese BMI (>30) | 78 | 38.5 | 35.5 | 41.9 |  |

Table 23: PINP (ng/ml) differences in the FAB Study; values were back transformed after logarithmic transformation.

*BAP:PINP Ratio*

In the FAB study subjects there was no significant difference in BAP:PINP ratio depending on gender (*p* = 0.491); however age and obesity were both associated with significant differences (both *p* < 0.001). The ratio was higher in 55 to 75 year olds (mean: 0.423; 95% CI: 0.398 – 0.449) than in 25 to 40 year olds (mean: 0.344; 95% CI: 0.319 – 0.371). The ratio was also higher in obese (mean: 0.436; 95% CI: 0.406 – 0.467) compared to lean BMI individuals (mean: 0.334; 95% CI: 0.313 – 0.357) (Table 24).

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | *n* | Mean | 95% Confidence Interval | Sig. |
| Lower Bound | Upper Bound |
| Men | 79 | 0.375 | 0.349 | 0.403 | *p* = 0.491 |
| Women | 90 | 0.388 | 0.364 | 0.414 |  |
| 25 to 40 years | 67 | 0.344 | 0.319 | 0.371 | *p* < 0.001 |
| 55 to 75 years | 102 | 0.423 | 0.398 | 0.449 |  |
| Lean BMI (18.5 – 24.9) | 91 | 0.334 | 0.313 | 0.357 | *p* < 0.001 |
| Obese BMI (>30) | 78 | 0.436 | 0.406 | 0.467 |  |

Table 24: BAP:PINP ratio differences in the FAB Study; values were back transformed after logarithmic transformation.

### Combined Study Populations

95% reference intervals were calculated that best reflected the population differences in the markers and ratio of interest (Table 25).

In both the XCT and FAB studies PLP was higher in men than women, but no age differences were observed. PLP was also higher in obese compared to lean individuals. Therefore gender specific 95% reference intervals for PLP have been calculated using data from women in the reference range study, and a combined population of men from the XCT and FAB studies, with obese individuals (*n* = 40) excluded.

There were no gender or BMI differences in BAP, and age differences were only observed in the XCT study population. As such 95% reference intervals for BAP were calculated using the original reference range study population.

As there were no gender differences in PINP, 95% reference intervals for BAP:PINP ratio were also calculated from the original reference study population of women only.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  | *n* | GM | 95% RI | 95% CI of lower limit | 95% CI of upper limit |
| PLP (nmol/l) | Men | 92 | 69.8 | 19.4 – 250.7 | 16.0 – 23.6 | 206.4 – 304.6 |
| Women | 200 | 51.0 | 14.6 – 177.9 | 12.9 – 16.6 | 156.4 – 202.3 |
| Bone ALP (μg/l) | 199 | 10.1 | 4.18 – 24.3 | 3.82 – 4.58 | 22.2 – 26.6 |
| PINP (ng/ml) | 198 | 34.9 | 15.5 – 78.6 | 14.2 – 16.8 | 72.2 – 85.5 |
| Bone ALP:PINP ratio | 197 | 0.289 | 0.147 – 0.570 | 0.137 – 0.158 | 0.531 – 0.611 |

Table 25: 95% Reference intervals in the combined study populations.

## Discussion

This investigation has shown that selected indicators of bone formation (BAP, PINP) and markers of HPP (PLP) are different according to factors including gender, age and BMI. Based on these differences, 95% reference intervals have been calculated from measurements made in the most suitably representative local populations (Table 25).

Although the pre-existing reference range study provided the most suitable population sample for determining reference ranges in these markers – a sufficiently large sample of healthy subjects at presumed skeletal maturity – inclusion of measurements from the XCT and FAB cohorts allowed investigation of biomarker differences in populations that were stratified by gender, age and BMI.

### *Gender associated differences in biomarkers*

Neither marker of bone formation (BAP, PINP) was found to be different in men and women in either the XCT or FAB study populations. PLP was the only marker that showed gender differences with men having significantly higher levels. This finding mirrors what was previously observed in the larger population of the NHANES investigation (Chapter 3).

Vitamin B6 supplementation is one factor known to influence PLP levels; however during recruitment of these study populations no data on supplement usage was gathered so differences between the male and female subjects based on supplementation cannot be explored. Dietary intake of vitamin B6 may account for gender differences in serum PLP. Previous investigation of gender differences in plasma B6 vitamers has shown that in healthy adults not taking vitamin B6 supplements, men typically have a higher B6 intake than women irrespective of age. In the same study, levels of plasma pyridoxal were also higher in males aged 25 to 50 than women at any age, although PLP levels were “similar” across age and gender groups (Driskell, Giraud, and Mitmesser 2000). Metabolomics investigation has identified that pyridoxate (a degradation product of pyridoxine) is higher in men. Furthermore, pathway analysis found no gender differences in super-pathways of vitamin metabolism; however pathway activity related to “pyridoxal metabolism” was elevated in men (Krumsiek et al. 2015).

### *Age associated differences in biomarkers*

Contrary to findings from the NHANES investigation there were no age based differences in PLP in the reference range, XCT or FAB study cohorts. This may be a reflection of differences between these populations whereby “healthy” individuals were recruited, in comparison to the more representative population sample provided in the NHANES. Therefore age differences in PLP observed in the NHANES study may be associated with diseases of ageing.

Age-associated differences in BAP were observed in the XCT study and reflect the study’s design whereby subjects were observed at three key stages of skeletal development. BAP was highest in 16 to 18 years olds (skeletal growth - high formation), lower in 30 to 32 year olds (peak bone mass – stable bone turnover), and lowest in 70+ year olds (loss of bone mass).

This effect is also seen in PINP concentration as values were significantly higher in 16 to 18 years olds than the older age groups – again reflecting the role of PINP as a marker of bone formation. This effect is not as clearly observed in the FAB study due to the use of wider and less distinct age groupings at 25 to 40 years and 55 to 70 years. Only PINP was significantly different, and was higher in the younger group.

In the original reference range study segmental linear regression showed a higher BAP and PINP in 30 to 35 year olds compared to 35 to 45 year olds (Glover et al. 2008). In this analysis BAP and PINP were both measured by IDS iSYS auto-analyser (compared to Alkphase B ELISA and Roche Elecsys auto-analyser in the Glover study); no differences in either BAP or PINP was found in subjects aged 30 to 35 compared to those aged 35 to 45 and so the entire reference range population was used for the calculation of these reference intervals.

### *BMI associated differences in biomarkers*

The use of samples from the FAB study allowed for the comparison of biomarker levels in lean (BMI 18.5 – 24.9) and obese (BMI >30) individuals. There were no differences in BAP between lean and obese individuals. PLP and PINP were both lower in obese individuals. Similarities in BAP between obese and lean adults has been previously reported (Viljakainen et al. 2014).

Previous investigation within this cohort (Evans et al. 2015) showed lower dietary vitamin D intake in the obese population, and although dietary intake data for vitamin B6 was not collected, similar dietary differences may be contribute to the differences in PLP. Obesity is also associated with increased levels of pro-inflammatory factors and as observed previously (Chapter 3), pro-inflammatory factors are in turn associated with lower serum PLP. Albumin, a carrier protein of PLP was also shown to be lower in in this cohort (Evans et al. 2015) and with potentially more than 90% of circulatory PLP being albumin-bound (Whyte et al. 1985), may be associated with lower PLP values in obesity. The observation of lower PLP values in obese individuals should be considered when interpreting biochemical measurements in cases of suspected HPP.

Previous investigation within this cohort (Evans et al. 2015) also identified that PINP was lower in obesity. Obesity has also been previously associated with lower levels of PINP in young adults (Viljakainen et al. 2014).

### *Reference intervals*

PLP values were unchanged based on age, higher in males, and lower in obesity. 95% reference intervals of PLP were therefore calculated to reflect these differences. The original reference range study population was selected for the female range (*n* = 200) and a combined cohort of males (*n* = 92) was drawn from the XCT and FAB study cohorts following exclusion of obese individuals.

Despite the presence of some age-related differences in BAP and PINP, the limited sample size make calculating age stratified reference intervals unreliable. Therefore these reference intervals were calculated from the original reference range study population. No exclusion of younger women were made from the cohort and all available measurements were used (BAP, *n* = 199; PINP, *n* = 198).

### *Limitations*

Little or no information regarding factors known to affect serum PLP levels such as vitamin B6 supplementation and dietary vitamin B6 was available for the three studies populations in this investigation and so it is impossible to determine how these factors may have influenced the gender differences observed in PLP reference intervals. Limited population stratification only allowed comparison of lean to obese BMI individuals. The study population could be expanded to allow investigation of differences in underweight and overweight individuals.

Despite the gender specific PLP reference intervals calculated, the male population sample is small (*n* = 92). Reed at al. (1971) recommend a sample of at least *n* = 120 for calculation of reference intervals; for sample sizes below 120 the “Robust” method of calculation can be used. In this scenario the robust method was not used in order to ensure all reference intervals reported were calculated through the same method. Finally the reference range study population is also 95% white and so race differences observed in the large NHANES cohort were not possible to examine in this U.K. sample.

# Chapter 5: Biochemical Indicators of HPP in a Clinical Population

## Research Aims

1. To determine the prevalence in biochemical changes suggestive of HPP in a Sheffield Metabolic Bone Centre clinical population.
2. To report the clinical profile of individuals with abnormal biochemistry.
3. To establish the prevalence of HPP-positive mutations in cases of abnormal biochemistry.

## Materials and Methods

### *Study design*

This is a single-centre cross-sectional observational study. Patients attending the Sheffield Metabolic Bone Centre provided serum and whole blood for biochemical and genetic evaluation. Upon identification of abnormal biochemical findings suggestive of HPP, genetic evaluation in the form of NGS and Sanger sequencing was carried out to identify and confirm *ALPL* gene mutations. Figure 15 shows the patient pathway in a CONSORT diagram.



Figure 15: CONSORT diagram to show patient pathway through the study.

### *Study population*

Clinical patients under investigation for osteoporosis were recruited to the study population at the Metabolic Bone Centre, Northern General Hospital Sheffield.

The typical patient pathway at the centre includes a dual energy X-ray absorptiometry (DXA) scan to evaluate bone mineral density, followed by blood and urine sample collection in some patients for evaluation of marker relevant to bone health.

During the initial stages of recruitment patients were only approached when “work up” blood tests were required as part of their clinical evaluation. This allowed research blood samples to be collected alongside clinical bloods, minimising the burden placed on phlebotomists within the department

To increase the recruitment rate of patients into the study population, additional phlebotomists were assigned. This allowed for further recruitment of any consenting patients whether or not they required blood tests as part of their clinical investigation.

During a period of 23 months (February 2016 to December 2017) 704 patients were recruited to the investigation. Of these, 1 patient withdrew from the study prior to sample analysis, 19 were consented where clinical staff were unable to draw a suitable volume of blood, 5 patient samples were insufficient for laboratory analysis.

Potential participants were not recruited to the study when the presence of factors known to affect serum ALP and PLP were identified, including:

* Coeliac disease
* Vitamin B12 deficiency
* Untreated hypothyroidism
* Wilson’s disease
* Pregnancy

Other factors known to affect PLP levels such as vitamin B6 supplement intake were recorded in the study questionnaire at the time of consent.

### *Biochemical measurements*

Biochemical analysis of PLP, BAP and PINP were completed at the Academic Unit of Bone Metabolism Biochemistry Laboratory (University of Sheffield). PLP was measured by HPLC, BAP and PINP were measured using the IDS iSYS auto-analyser methods. Biochemical analyses of samples in this investigation were completed alongside recruitment during in three batches during February 2016 to December 2017.

Total ALP was measured by automated colourimetric assay at the Department of Clinical Chemistry, Northern General Hospital. Total ALP measurements were made during clinical investigations, and were retroactively accessed via patient records with consent of the study participant. All measurements were made between February 2016 and February 2018; for subjects with multiple measurements the most recent result was selected.

### *Screening*

Participants were screened in order to identify individuals with biochemistry which may be suggestive of HPP. Subjects with abnormal biochemistry were identified based on the 95% reference interval values calculated in a healthy local population (Chapter 4, Table 25). Cases were identified based on the presence of one or more of the following characteristics:

1. High PLP (men >250.7 nmol/l; women >177.9 nmol/l)
2. Low BAP (<4.18 μg/l)
3. Low BAP:PINP ratio (<0.147)

### *Genetic assessment*

Participants with biochemical results warranting genetic evaluation underwent next-generation sequencing (NGS) to identify mutations of the *ALPL* gene coding using whole blood samples. Sanger sequencing methodology was used to confirm suspected mutations. Genetic assessment was done by Sheffield Children’s Hospital Clinical Genetics Laboratory.

## Results

Subject characteristics of the entire study population are shown in table 26. The study population included *n* = 529 women (77.9%) and *n* = 150 men (22.1%). Gender stratified subject characteristics are shown in table 27.

|  |  |  |  |
| --- | --- | --- | --- |
| Variable | *n* | Mean (SD) | Median (minimum, maximum) |
| Age (years) | 679 | 65.8 (13.8) | 68.3 (17.5, 97.0) |
| Height (cm) | 596 | 162.8 (8.6) | 161.9 (144.4, 195.4) |
| Weight (kg) | 596 | 70.8 (16.7) | 67.9 (34.3, 148.4) |
| BMI (kg/m2) | 596 | 26.7 (5.6) | 25.8 (12.8, 49.1) |
| PLP (nmol/l) | 640 | 48.3 (72.9) | 30.8 (0.93, 1034.0) |
| BAP (μg/l) | 676 | 18.2 (9.2) | 16.1 (3.24, 59.9) |
| PINP (ng/ml) | 675 | 47.3 (34.3) | 38.6 (3.80, 227.1) |
| BAP:PINP ratio | 673 | 0.493 (0.288) | 0.425 (0.082, 2.810) |
| Total ALP (IU/L) | 620 | 82 (51) | 72 (10, 837) |

Table 26: Subject characteristics in the clinical population.

### *Gender differences*

Non-parametric independent-sample testing (Mann-Whitney U Test) did not find any differences in age (*p* = 0.057) or any measured biomarkers or formation marker ratio between men and women in the study population (PLP, *p* = 0.936; ALP, *p* = 0.782; Bone ALP, *p* = 0.367; PINP, *p* = 0.930; BAP:PINP ratio, *p* = 0.199). Men had a greater height, weight, and BMI (*p* < 0.001, *p* < 0.001, and *p* = 0.028 respectively) than women.

|  |  |  |
| --- | --- | --- |
|  | Men (22.1%) | Women (77.9%) |
| Variable | *n* | Mean(SD) | Median(minimum, maximum) | *n* | Mean(SD) | Median(minimum, maximum) |
| Age (years) | 150 | 63.5 (15.4) | 65.8 (17.5, 91.5) | 529 | 66.5 (13.3) | 68.7 (19.3, 97.0) |
| Height (cm)\* | 134 | 172.5 (8.4) | 172.1 (152.9, 195.4) | 462 | 160.0 (6.3) | 160.0 (144.4, 183.0) |
| Weight (kg)\* | 134 | 81.2 (16.3) | 80.3 (47.0, 148.4) | 462 | 67.8 (15.6) | 66.0 (34.3, 127.0) |
| BMI (kg/m2)\* | 134 | 27.3 (4.9) | 27.0 (15.4, 43.5) | 462 | 26.5 (5.7) | 25.3 (12.8, 49.1) |
| PLP (nmol/l) | 139 | 48.7 (72.3) | 32.5 (3.3, 656.7) | 501 | 48.3 (73.1) | 30.1 (0.93, 1034.0) |
| BAP (μg/l) | 148 | 19.0 (9.9) | 16.1 (3.5, 49.9) | 528 | 17.9 (9.0) | 16.0 (3.2, 59.9) |
| PINP (ng/ml) | 148 | 49.6 (39.4) | 37.4 (6.3, 214.6) | 527 | 46.6 (32.8) | 39.0 (3.8, 227.1) |
| BAP:PINP ratio | 147 | 0.536 (39.4) | 0.463 (0.104, 2.136) | 526 | 0.481 (0.269) | 0.416 (0.082, 2.810) |
| Total ALP (IU/L) | 141 | 88 (77) | 72 (14, 837) | 479 | 82 (41) | 73 (10, 603) |

Table 27: Gender stratified subject characteristics in the clinical population.

\*Significant gender differences, *p* < 0.05.

### *Vitamin B6 supplement intake*

Regular vitamin B6 supplement intake was recorded in *n* = 41 study participants; *n* = 8 of these specifically took vitamin B6 or a vitamin B complex, the remaining *n* = 33 took multivitamins containing vitamin B6. Of the *n* = 41 participants taking a supplement, one had a serum PLP concentration below the measureable range. Of the 41 individuals who recorded taking a vitamin supplement including vitamin B6, over half (*n* = 23) had a PLP value above the normal range.

Non-parametric independent-sample testing (Mann-Whitney U Test) found PLP concentration to be significantly higher in participants taking vitamin B6 supplements, compared to those not taking supplements (*p* < 0.001) (Table 28).

|  |  |  |
| --- | --- | --- |
|  | No supplement (93.75%) | Supplement (6.25%) |
| Variable | *n* | Mean(SD) | Median(minimum, maximum) | *n* | Mean(SD) | Median(minimum, maximum) |
| PLP (nmol/l)\* | 600 | 43.1 (65.3) | 29.3 (0.9, 1034) | 41 | 126.4 (122.2) | 88.4 (7.3, 497.4) |

Table 28: Effect of vitamin B6 supplementation on serum PLP concentration.

\*Significant differences, *p* < 0.001.

### *Abnormal biochemistry*

Screening identified *n* = 23 cases with biochemistry results outside the normal expected range (Table 29). Non-parametric independent-sample testing (Mann-Whitney U Test) identified a number of differences between cases with abnormal biochemistry when compared to normal individuals. PLP was higher in the abnormal cases (*p* < 0.001), whereas BAP, BAP:PINP ratio and total ALP were all lower in cases with abnormal biochemistry (all *p* < 0.001). The abnormal cases were younger than the normal individuals (*p* = 0.048). There were no differences in height, weight, BMI or PINP between the 2 groups. Gender representation was equal in the normal biochemistry (77.9% female) and abnormal biochemistry (78.3% female) groups.

|  |  |  |
| --- | --- | --- |
|  | Normal Biochemistry | Abnormal Biochemistry |
| Variable | *n* | Mean(SD) | Median(minimum, maximum) | *n* | Mean(SD) | Median(minimum, maximum) |
| Age (years)\* | 655 | 66.1 (13.7) | 68.4 (17.5, 97.0) | 23 | 59.2 (16.3) | 63.4 (30.3, 89.2) |
| Height (cm) | 580 | 162.8 (8.7) | 161.9 (144.4, 195.4) | 16 | 161.6 (5.2) | 162.5 (152.6, 168.8) |
| Weight (kg) | 580 | 71.0 (16.8) | 68.2 (34.3, 148.4) | 16 | 64.2 (12.4) | 60.8 (41.3, 82.7) |
| BMI (kg/m2) | 580 | 26.7 (5.6) | 25.8 (12.8, 49.1) | 16 | 24.5 (4.2) | 24.9 (16.3, 29.7) |
| PLP (nmol/l)\* | 617 | 38.8 (31.4) | 29.7 (0.9, 229.1) | 23 | 304.5 (235.6) | 303.4 (9.2, 1034.0) |
| BAP (μg/l)\* | 653 | 18.41 (9.20) | 16.15 (4.80, 59.91) | 23 | 11.40 (7.44) | 8.89 (3.24, 25.77) |
| PINP (ng/ml) | 652 | 46.6 (32.8) | 38.6 (3.8, 214.6) | 23 | 65.1 (62.7) | 46.5 (8.1, 227.1) |
| BAP:PINP ratio\* | 650 | 0.501 (0.287) | 0.432 (0.150, 2.810) | 23 | 0.286 (0.242) | 0.197 (0.082, 1.097) |
| Total ALP (IU/L)\* | 599 | 82.9 (51.6) | 73.0 (26.0, 837.0) | 21 | 53.4 (29.9) | 51.0 (10.0, 109.0) |

Table 29: Population comparison in cases of normal vs. abnormal biochemistry.

\*Significant differences, *p* < 0.05.

Individual profiles of cases with abnormal biochemistry are shown in table 30. Of the subjects with an elevated PLP, 5 individuals were receiving a vitamin B6 supplement and had no other abnormal biochemistry.

The BAP:PINP ratio utilised here is a novel parameter intended to be interpreted as a “formation marker ratio”. Although not directly related to HPP, the ratio will indicate where BAP activity is reduced in relation to overall bone formation, represented by another formation marker, PINP. The ratio is not currently applied as a screening indicator of HPP; we utilise the ratio here to explore whether BAP:PINP is consistently low in cases of reduced ALP activity.

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| ID | Gender | Age | PLP (nmol/l) | BAP (μg/l) | BAP:PINP ratio | PINP (ng/ml) | Total ALP (IU/L) | Vitamin B6Supplement |
| HM20008 | M | 58 | 46.58 | 15.55 | **0.104** | 149.59 | 59 |  |
| HM20053 | F | 73 | **424.20** | 20.72 | 0.253 | 81.94 | 109 | Yes |
| HM20073 | F | 31 | **497.36** | 19.60 | 0.392 | 50.04 | 61 | Yes |
| HM20103 | F | 56 | **351.68** | 4.96 | **0.104** | 47.65 | 42 |  |
| HM20108 | F | 62 | **454.86** | 8.07 | 0.267 | 30.21 | 32 | Yes |
| HM20181 | M | 45 | **376.57** | 4.33 | **0.122** | 35.40 | 19 |  |
| HM20185 | F | 68 | 45.45 | 22.48 | **0.107** | 209.56 | 51 |  |
| HM20258 | F | 44 | **179.01** | **3.24** | 0.197 | 16.45 | 23 |  |
| HM20326 | M | 70 | **301.22** | 8.89 | 1.097 | 8.10 | 59 |  |
| HM20327 | F | 39 | 104.47 | 5.06 | **0.095** | 53.47 | 22 |  |
| HM20370 | F | 71 | **248.34** | 16.07 | 0.502 | 32.04 | 100 | Yes |
| HM20384 | M | 69 | 34.58 | 19.87 | **0.136** | 146.43 | 89 |  |
| HM20456 | F | 71 | **343.04** | 17.05 | 0.545 | 31.32 |  |  |
| HM20492 | F | 44 | 20.20 | 20.76 | **0.091** | 227.07 | 86 |  |
| HM20508 | F | 74 | **210.81** | 10.52 | **0.114** | 92.17 | 50 |  |
| HM20566 | F | 65 | **303.36** | 25.77 | 0.303 | 85.01 | 77 |  |
| HM20568 | F | 30 | **191.13** | 11.52 | **0.140** | 82.21 | 31 |  |
| HM20574 | F | 72 | **337.14** | 6.68 | 0.500 | 13.35 |  |  |
| HM20580 | M | 47 | **656.74** | **3.52** | **0.136** | 25.83 | 14 |  |
| HM20585 | F | 89 | 9.17 | **3.37** | 0.333 | 10.12 | 40 |  |
| HM20606 | F | 36 | **1034.00** | **3.82** | **0.082** | 46.54 | 10 |  |
| HM20623 | F | 63 | **399.35** | **4.04** | 0.335 | 12.07 | 52 |  |
| HM20630 | F | 78 | **433.98** | 6.26 | 0.617 | 10.14 | 96 | Yes |

Table 30: Case profiles of patients with abnormal biochemical findings. Abnormality is defined as high PLP, low BAP, and/or low BAP:PINP where values fall outside of the defined 95% RIs (Chapter 4, Table 25).

We have identified 23 adults (3.4%) with biochemical abnormalities suggestive of HPP in a clinical population of 679. Of the abnormal cases identified the majority (*n* = 17) had an elevated PLP value; of these four also had a low BAP:PINP ratio, two also had low BAP, and two had all three. Five cases with only an elevated PLP were receiving supplements including vitamin B6; these cases constitute all the cases identified with abnormal biochemistry who were also taking a supplement. Four cases were identified with high PLP only, and were not receiving a vitamin B6 supplement. An additional five cases were identified on the basis of a low BAP:PINP ratio only; and one case had only a low BAP.

### *Clinical observations*

Clinical observations were carried out in 10/23 subjects with abnormal biochemistry. It was not possible to complete clinical observations in all. Summary observations are shown in table 31.

|  |  |  |
| --- | --- | --- |
| ID | Clinical Observation | Treatment/Medication |
| HM20008 | N/A | Cream steroids, Alendronate, Adcal-D3, Ramiprie, Bendromflumethzide, Amitriptyline |
| HM20053 | Osteoporosis diagnosed 2016. 3 vertebral fractures. 1 Rib fracture. Multiple ‘greenstick’ wrist fractures in childhood; Left wrist fracture (traumatic), Left ankle fractures. No dental issues in childhood. Dentist extracted all teeth at 21 years. No spontaneous loss of whole teeth. | Prednisolone Esomeprazole, Gabapentin, Ramipril, Simvastin, Quinine Bisulphate, Bisoprolol |
| HM20073 | N/A | Depo-Provera, multi vitamin, vitamin D, Omega 3 |
| HM20103 | Right femur stress fracture. Shaft cortical stress fracture. Thigh pain starting in childhood. No tooth loss. | Meloxicam, Omeprazole, Escitalopram, Colecalciferol, Co-Dydramol, Codamol, Fexofenadine |
| HM20108 | N/A | Alendronate (stopped after 5 days), calcium and vitD supplement |
| HM20181 | Fractures; Ribs, Right ankles (eversion injury 15 years ago). No dental problems. | Betamethasone valerate, Hydrocortisone |
| HM20185 | Osteoporosis. Left hip replacement in 2016. Fractures; Left Wrist - traumatic (2016), Right Shoulder - traumatic (skiing), Scapuloid fracture in RTA. No loss of whole teeth. Right knee pain. | Pamapril |
| HM20258 | Fibromyalgia; Fractured left foot; Anxiety. | Steroid Cream |
| HM20326 | Radial/Ulnar fracture – traumatic; Possible vertebral crush fractures (asymptomatic) picked up on VFA. No dental issues, Bilateral Shoulder Pain; Fibromyalgia. | Alendronate, Prednisolone, Omeprazole, Adcal-D3 |
| HM20327 | No fractures; No dental problems; Pain in wrist, thumbs, knees. | Contraceptive pill, Vitamin D supplement |
| HM20370 | N/A | Ibandronate, Prednisolone, ventolin, Colecaiferol, Budesondie, Amiodrone, wafarin, Nitrazepam, Trazodone, Hydroxocobalamin |
| HM20384 | N/A | Furosemide, salbutamol inhaler, Thiamine, Vitamin B compound, diprobase cream |
| HM20456 | N/A | Steroid Cream, Glucosomide sulphate, Vitamin B complex, Risedronatwe  |
| HM20492 | N/A | Lansoprozole, Sertraline, Doxycline, Diltiazem SR capsules, Amitriptyline |
| HM20508 | N/A | Alendronate, Steroid Cream |
| HM20566 | N/A | Steroid Cream, Fluoxetine, Cetrizine, Clobetasol, Hydromol ointment |
| HM20568 | Bilateral wrist fractures - traumatic; T11 vertebral fractures - non-traumatic. Low BMD; No dental problems; Joint hypermobility; Chronic back pain/tenderness since childhood. | None recorded |
| HM20574 | N/A | None recorded |
| HM20580 | Right clavicular fracture (traumatic); no tooth loss; Hypertension & High Cholesterol; Muscular aches (calves); now resolved; Cervical neck pain (No diagnosis, MRI normal) | None recorded |
| HM20585 | N/A | None recorded |
| HM20606 | Right radial fracture – traumatic; Right elbow – traumatic; Right hand fracture; Multiple toes fracture – stubbing left & right; Left wrist fracture. Dental- 5 teeth lost, ‘crumbled, weak teeth’; joint pain in hips, knees. | None recorded |
| HM20623 | N/A | Alendronate (stopped), Denosumab (current), Levothyroxine |
| HM20630 | N/A | None recorded |

Table 31: Clinical observations of 23 subjects with abnormal biochemistry. The treatment/medication each subject was taking as recorded in the clinical questionnaire is also shown.

Clinical observations varied greatly amongst the cohort of subjects with abnormal biochemistry. Some subjects (e.g. HM20103, HM20568 and HM20606) display symptoms that could be suggestive of a HPP phenotype such as stress fractures, low-trauma fractures, chronic pain and premature tooth loss. 2 patients (HM20053 and HM20185) were diagnosed with osteoporosis and showed few typical manifestations of adult HPP beyond pain and unexplained tooth removal in early adulthood.

### *Genetic evaluation*

Results of genetic screening are shown in table 32.

|  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| ID | M/F | cDNA | Protein | Clinical Form1 | DNE%1 | Comment | PLP nmol/L | BAP µg/l | BAP:PINP | Total ALP IU/L | Supplement |
| HM20008 | M |  |  |  |  | No change detected | 46.58 | 15.55 | **0.104** | 59 |  |
| HM20053 | F |  |  |  |  | No change detected | **424.20** | 20.72 | 0.253 | 109 | Yes |
| HM20073 | F |  |  |  |  | No change detected | **497.36** | 19.60 | 0.392 | 61 | Yes |
| HM20103 | F | c.215T>C | p.Ile172Thr | Odonto (heterozygote) | 40.2 | Likely pathogenic | **351.68** | 4.96 | **0.104** | 42 |  |
| HM20108 | F |  |  |  |  | No change detected | **454.86** | 8.07 | 0.267 | **32** | Yes |
| HM20181 | M | c.1161A>G | p.Gly387Gly | - |  | Unknown sig. | **376.57** | 4.33 | **0.122** | **19** |  |
| HM20185 | F |  |  |  |  | No change detected | 45.45 | 22.48 | **0.107** | 51 |  |
| HM20258 | F | c.1396C>G | p.Pro466Ala | - |  | Unknown sig. | **179.01** | **3.24** | **0.197** | **23** |  |
| HM20326 | M |  |  |  |  | No change detected | **301.22** | 8.89 | 1.097 | 59 |  |
| HM20327 | F | c.400\_401CA:NA | p.Thr134His | Perinatal (compound heterozygote) |  | Likely pathogenic | 104.47 | 5.06 | **0.095** | **22** |  |
| HM20370 | F |  |  |  |  | No change detected | **248.34** | 16.07 | 0.502 | 100 | Yes |
| HM20384 | M |  |  |  |  | No change detected | 34.58 | 19.87 | **0.136** | 89 |  |
| HM20456 | F |  |  |  |  | N/A | **343.04** | 17.05 | 0.545 |  |  |
| HM20492 | F |  |  |  |  | No change detected | 20.20 | 20.76 | **0.091** | 86 |  |
| HM20508 | F |  |  |  |  | N/A | **210.81** | 10.52 | **0.114** | 50 |  |
| HM20566 | F |  |  |  |  | N/A | **303.36** | 25.77 | 0.303 | 77 |  |
| HM20568 | F | c.526G>A | p.Ala176Thr | Childhood (compound heterozygote) | 58.0 | Pathogenic | **191.13** | 11.52 | **0.140** | **31** |  |
| HM20574 | F |  |  |  |  | No change detected | **337.14** | 6.68 | 0.500 |  |  |
| HM20580 | M | c.346G>A | p.Ala116Thr | Adult (heterozygote) | 40.0 | Pathogenic | **656.74** | **3.52** | **0.136** | **14** |  |
| HM20585 | F |  |  |  |  | No change detected | 9.17 | **3.37** | 0.333 | 40 |  |
| HM20606 | F | c.143C>G | p.Thr48Ser | - |  | Unknown sig. | **1034.00** | **3.82** | **0.082** | **10** |  |
| c.1171C>T | p.Arg391Cys | Childhood (compound heterozygote) | 50.5 | Pathogenic |  |
| HM20623 | F |  |  |  |  | No change detected | **399.35** | **4.04** | 0.335 | 52 |  |
| HM20630 | F |  |  |  |  | No change detected | **433.98** | 6.26 | 0.617 | 96 | Yes |

Table 32: *ALPL* gene sequencing variants identified in patients with abnormal biochemistry.

1Classification and (genotype) of the patient where previously reported in the TNSALP Gene Mutations Database (Mornet 2015).

Where available, DNE: Dominant Negative Effect %.

Abnormal biochemistry results are **bolded**; defined as high PLP, low BAP, and/or low BAP:PINP where values fall outside of the defined 95% RIs (Chapter 4, Table 25).

Of the 23 subjects who underwent genetic screening 7 were found to have a mutation of the *ALPL* gene, with one subject (HM20606) having compound mutations.

The clinical form of HPP associated with each mutation was matched from the TNSALP Gene Mutations Database (Mornet 2015), including mutations associated with the odonto, perinatal, childhood and adults forms of the disease. 3 mutations not previously recorded in the database were observed.

It was not possible to draw whole blood samples from 3 subjects (HM20456, HM20508 and HM20566).

## Discussion

### *Genetic and clinical evaluation*

Of the 23 subjects who were selected for genetic screening on the basis of abnormal biochemistry, 7 out of 20 were found to have mutations associated with the *ALPL* gene. It was not possible to screen the remaining 3 subjects as insufficient sample volume was drawn from the subjects and time did not permit a second clinic visit. Incidentally, 3 of the subjects with abnormal biochemistry (HM20103, HM20181 and HM20258) were attending the clinic with suspected HPP at the time of recruitment.

There were no correlations observed between clinical findings and genetic results; chronic pain and multiple/recurrent fractures were the most common clinical symptoms observed but no one symptom was observed in all cases. However the most severe clinical manifestation was observed in the subject with compound mutations (HM20606), including multiple, recurrent fractures, chronic joint pain, and dental abnormalities.

The classification of the clinical forms of HPP associated with the observed mutations did not correlate with the clinical symptoms observed. 3 subjects were observed with mutations which had previously been associated with the childhood or perinatal forms of HPP; these severe disease forms were not manifested phenotypically in the subjects observed likely due to the absence of homozygous or compound heterozygous mutations. The mutation observed in subject HM20103 has previously been associated with the odonto form of HPP, however in our observations there were no dental problems in this subjects. This supports previous clinical observations that found no clear phenotype-genotype correlation in adult HPP (Schmidt et al. 2017). Even among family member with identical mutations, disease phenotype has been reported to vary greatly (Hofmann et al. 2014). However genotype-phenotype correlation has been observed in the odonto form of the disease within a previously studied family (Martins et al. 2013).

3 mutations were identified which did not match the 381 mutations recorded in the TNSALP Gene Mutations Database. In subject HM20181 a synonymous mutations was observed (c.1161A>G p.Gly387Gly) resulting in no structural protein change. Further novel missense mutations were observed in HM20258 (c.1396C>G p.Pro466Ala) and HM20606 (c.143C>G p.Thr48Ser).

In our study population the clinical presentation of adult HPP was more severe in women; vertebral fractures, foot fractures, femoral stress fractures and dental abnormalities were only observed in women. Berkseth et al. (2013) proposed that “gender may influence the clinical expression of HPP” following the observation of foot fractures and subtrochanteric femoral fractures exclusively in women. Unlike our study Berkseth et al. observed vertebral fractures exclusively in men. Additional similar investigations (Riancho-Zarrabeitia et al. 2016) did not report any data on gender differences in either mutation rate or symptoms.

Despite the small cohort, the gender split of HPP cases with a mutation generally reflected the overall study population; 71.4% of the subjects with observed mutations were female and females comprised 77.9% of the study population. Berkseth et al. (2013) observed a similar proportion of women with adult HPP (68% or 15/22 subjects were women), however no comment was made regarding the gender proportion of the total population. Therefore gender differences in mutation rate may reflect the composition of the population investigated. Clinical presentation of HPP in men may be mild or un-symptomatic and therefore may be under-investigated or reported in the general population.

### *Biochemical indicators of adult HPP*

Of the biochemical criteria utilised during screening, low BAP:PINP ratio was observed in all subjects with a mutation (7/7). Elevated PLP was observed in 6/7 of the subjects with a mutation and low BAP was observed in 2/7. A combination of abnormal biochemistry criteria was observed in 6/7 subjects with a mutation. One subject (HM20606) displayed levels that were out of the normal range for all three screening tests.

Due to the small sample size it is difficult to suggest one that one biochemical criterion alone is more reliable in the identification of HPP than the others; in fact a combination approach may be the most reliable method to identify adult HPP. All 7 subjects with a mutation presented with a combination of a low BAP:PINP ratio alongside another biochemical abnormality. However, the low BAP:PINP ratio identified 4 false positives (HM20008, HM20185, HM20384, HM20492) where the only abnormal biochemical indicator was the low formation marker ratio; none of these four cases had a mutation. In the case of subject HM20103 serum BAP and total ALP were both measured at a low-normal concentration but within the normal range; application of the formation marker range ratio allowed identification that in comparison to the level of a HPP-insensitive formation marker, PINP, BAP activity was suppressed. The formation marker ratio provides greater insight to BAP activity in cases of borderline biochemical deficiency.

Within the cohort of patients screened, 5 subjects were identified on the basis of an elevated serum PLP alone and were taking vitamin B6 supplements. These could be classified as false positive cases of abnormal biochemistry as none were found to have genetic abnormalities. The effect of vitamin B6 supplementation to elevate serum PLP is well documented (Morris et al. 2008, Ye et al. 2010, McKiernan et al. 2017). As expected the subjects in this study known to be receiving vitamin B6 supplementation were also found to have higher PLP than those not. Vitamin supplementation should therefore be considered during interpretation of PLP levels when attempting to identify HPP. In order to avert this effect, cessation of supplementary vitamin B6 intake should occur 2-3 weeks prior to serum sample collection, in order to allow circulatory PLP levels to return to baseline. In such cases where this is not possible or PLP measurement is not available it could be suggested to use the formation marker ratio as an alternative indicator of disease.

Although not used a diagnostic marker in this study, total serum ALP results were obtained from patient records where possible for the subjects with abnormal biochemistry. Low ALP (<36 IU/L) was observed in 7/23 subjects, 6 of whom were found to have an *ALPL* mutation. This shows that low ALP as an indicator of adult HPP may have greater specificity than the other biochemical indicators investigated here. However the effectiveness and greater selectivity of low ALP as an indicator of HPP related mutations observed here has not been seen in in larger, general clinical populations; Riancho-Zarrabeitia et al. (2016) observed that only 50% of patients with an unexplained persistently low ALP had a TNSALP associated mutation.

The identification rates of subjects with HPP associated mutations based on abnormal biochemistry is similar to a previous study. In a review of serum ALP measurements from a hospital serving 350,000 patients in Northern Spain it was shown that that of 42 patients with an unexplained, persistently low ALP, 21 (50%) had a mutation of the *ALPL* gene. Approximately 50% of these (10 cases) had a reduced enzyme function associated with an elevated PLP (>180nmol/l) (Riancho-Zarrabeitia et al. 2016). Although the biochemical indicator differed (total ALP as opposed to high PLP, low BAP or a low formation ratio), the mutation rate in the genetic screening cohort was similar to that observed in my study. Likewise Riancho-Zarrabeitia et al did not observe an elevated PLP level in *all* cases with a mutation present, which supports the observation that not all TNSALP associated mutations result in substrate (PLP) accumulation.

Abnormal biochemistry suggestive of HPP was also observed at a higher rate in my study than has previously been shown in a general hospital population. Riancho- Zarrabeitia et al (2016) evaluated ~500,000 ALP test results from a Spanish population and identified only 181 patients with low ALP. The prevalence of HPP-associated mutations observed in this study (7/704) is much higher than previous estimates of adult HPP affecting 1/6370 adults (Mornet et al. 2011). This likely reflects the nature of the population investigated, as participants were drawn specifically from a specialist metabolic bone clinic. Additionally, previous efforts to identify abnormal biochemistry indicative of adult HPP have relied solely on low total ALP as a screening marker. If we treat patients with a mutation as disease positive cases, we can determine the positive predictive value of each biomarker as an indicator of disease:

* Low BAP: **60%** (identified 12 cases, all 7 with mutation)
* Low BAP:PINP: **58%** (identified 5 cases, 3 with mutation)
* High PLP: **50%** (identified 12 cases [excluding 5 taking vitamin B6 supplement], 6 with mutation)

Each biomarker had a comparable positive predictive value (between 50-60%); however, the BAP:PINP ratio identified all 7 disease cases compared to the other markers (3 and 6). Furthermore, the BAP:PINP ratio is not affected by vitamin supplementation. It is impossible to produce a negative predictive value for each biomarker without completing genetic screening in all 679 patients.

*Differences between normal vs abnormal biochemistry*

The observed differences in population characteristics between the normal and abnormal biochemistry groups reflected the criteria used to divide the patients into, whereby differences in biomarkers are observed in the two groups.

Between the two groups there were no differences in height, weight, BMI or gender. The study population was mostly female which reflects the nature of the clinical population observed, as the most common metabolic bone disease, osteoporosis is approximately four times as prevalent in women than men in adults over 50 (Wade et al. 2014), and osteoporosis is the main cause for patient visits to the clinic.

These biochemical differences in the normal vs. abnormal groups would be expected if we were to compare individuals with HPP to individuals without HPP; as high PLP, low BAP and low total ALP are commonly observed in all forms of HPP (Whyte et al. 1985, Magnusson, Lofman, and Larsson 1992, Schmidt et al. 2017).

Age differences between the two groups, whereby abnormal cases were younger, may be explained by the onset age of symptomatic HPP in adults in comparison to more prevalent diseases of bone such as osteoporosis. If we are to assume that biochemical differences in the two groups correctly distinguish between the presence and absence of HPP, it would be reasonable to assume that patients with HPP would be younger than the overall clinical population. Previous assessment of 38 adults with HPP reported a median age of diagnosis as 38, a range of 20 to 72 (Schmidt et al. 2017). Whereas osteoporosis could broadly be defined as a disease of aging (Vondracek and Linnebur 2009).

Vitamin supplements including vitamin B6 were taken by 40 of the 679 participants. As expected, and in-line with previous investigations (Mornet and Nunes 1993, Morris et al. 2008, Ye et al. 2010) PLP was higher in the subjects with a supplement intake.

### *Limitations*

Our study population is a specific cohort of patient visitors to the Sheffield Metabolic Bone Centre. Despite being the largest of its kind in the UK our findings are unlikely to be widely generalisable. Future work is planned to include expansion to another UK research centre.

It was not possible to complete genetic screening in 3/23 subjects and clinical observations in 13/23 subjects with abnormal biochemistry. This was primarily due to patient preference for no further contact beyond the initial visit or time constraints of the study.

Due to resource limitations it was not possible to expand genetic screening beyond the identified cases with abnormal biochemistry. Ongoing work plans to match these cases to control cases for comparison of biochemical, clinical, and genetic identifiers to better characterise this population in against a control cohort. Currently it is not known how the clinical presentation of these identified subjects compare to the wider study population.

In conclusion, this chapter shows approximately 1% of patients (7/679) attending the Sheffield MBC had a mutation associated with HPP. 23/679 subjects had one or more biochemical indicators associated with HPP and were typically younger than the rest of the population. Of these, 7 had an HPP-associated mutation of the *ALPL* gene, including 3 novel mutations. The clinical symptoms observed in these patients varied greatly and were generally more severe in women.

Alongside high PLP, low BAP and low ALP, the use of BAP:PINP as a formation marker ratio has been described as a viable indicator of HPP-associated abnormal biochemistry.

# Chapter 6: The Clinical Profile of Adult Hypophosphatasia

## Research Aim

1. To report the clinical profile of adults with HPP attending the Sheffield Metabolic Bone Centre.

## Materials and Methods

### *Study design*

This is a single-centre observational retrospective study to characterise the clinical symptoms of HPP in adults. Participants attended a single visit to provide informed consent, provide a fasting blood sample and conduct the clinical interview. Patients were asked to stop any vitamin B6 supplement at least two weeks before the visit.

### *Study population*

Participants were recruited from adult patients under investigation for HPP at the Metabolic Bone Centre (Northern General Hospital, Sheffield). Participants all had a clinical diagnosis of HPP (with or without previous genetic test confirmation) and were able and willing to consent to participate.

### *My contribution to the study*

As a PhD student I was unable to carry-out the clinical interviews and evaluations described in this chapter. Clinical interview of patients with suspected HPP was carried out by Dr Robert Desborough. Professor Eastell reviewed the clinical history to determine which health problems were likely to be related to HPP. I observed the clinical interviews and was present during the discussion of patient cases. I was present during the review of genetic results with Dr Meena Balasubramanian and all other project meeting.

I completed the biochemical measurements in patient samples where these had not been previously measured. I also supported the study in an administrative capacity which required involvement in patient recruitment and consenting, and managing patient records and biochemistry results.

### *Biochemical measurements*

A fasting blood sample was collected for the measurement of PLP and total ALP, and to test for *ALPL* mutations if the test had not already been carried out. PLP was measured by HPLC at the Academic Unit of Bone Metabolism Biochemistry Laboratory (University of Sheffield). Total ALP was measured by automated colourimetric assay at the Department of Clinical Chemistry (Northern General Hospital, Sheffield).

### *Genetic assessment*

Genetic evaluation was carried out in the form of next-generation sequencing (NGS); Sanger sequencing methodology was used to confirm suspected mutations. Genetic assessment was done by Sheffield Children’s Hospital Clinical Genetics Laboratory.

### *Clinical interview*

The clinical interview was conducted by a physician with experience in the evaluation of metabolic bone disorders. A standardised case data report form was used throughout the interviews to ensure that all data relevant to the investigation was recorded (appendix A). A review of participant hospital notes was also carried out as required. The obtained clinical history was reviewed to evaluate which health problems and healthcare use are likely to be related to HPP. Each item was assessed as unrelated, possibly related or likely related.

Health problems specifically assessed in the clinical interview included: fractures, dental problems, skeletal complications, mobility problems, mental health problems, seizures, and respiratory complications.

## Results

### *Subject characteristics*

The subject characteristics of adult HPP patients are shown in table 33.

|  |  |  |  |
| --- | --- | --- | --- |
|  | Overall | Male | Female |
| N (%) | 28 (100) | 10 (35.7) | 18 (64.3) |
|  | Age (years), mean, median (LQ-UQ) |
| At disease presentation\* | 31.8, 32.5 (19.3-40.0) | 32.6, 30.0 (24.5-39.0) | 31.5, 34.0 (16.0-40.0) |
| At diagnosis | 43.8, 44.0 (36.5-51.3) | 44.7, 46.5 (40.5-51.5) | 43.2, 41.0 (35.3-51.0) |
| At interview | 46.8, 48.0 (38.8-55.5) | 47.6, 50.0 (45.5-54.3) | 46.3, 43.5 (36.5-56.0) |
| Years between presentation and diagnosis | 13.4, 10.0 (1.0-20.0) | 13.9, 14.0 (1.0-18.0) | 13.2, 8.5 (1.3-20.1) |

Table 33: Subject characteristics and age of adult HPP patients

\*Date of disease could not be established for one patient.

The clinical symptoms of HPP and the frequency thereof reported by patients are shown in table 34. Fracture sites included: clavicle, elbow, femur, foot, metatarsal, ankle, toe, hand, rib, hip, shoulder, tibia, vertebra, and wrist. The most common fracture site was the metatarsal (41.4% of overall patients). Dental problems included enamel anomalies, loose teeth, and tooth loss. Mental health symptoms recorded included depression, anxiety, suicidal thoughts, low mood, and mental fatigue. The most prevalent mental health symptom was depression (35.7% of overall patients).

|  |  |  |  |
| --- | --- | --- | --- |
|  | OverallN (%) | MaleN (%) | FemaleN (%) |
| Patients reporting ≥1 Fracture | 20 (71.4) | 8 (80.0) | 12 (66.7) |
| Stress fracture, any | 8 (28.6) | 2 (20.0) | 6 (33.3) |
| Mobility problems | 7 (25.0) | 2 (20.0) | 5 (27.8) |
| Musculoskeletal pain or weakness | 28 (100.0) | 10 (100.0) | 18 (100.0) |
| Dental problems | 14 (50.0) | 5 (50.0) | 9 (50.0) |
| Family history of HPP | 3 (10.7) | 1 (10.0) | 2 (11.1) |
| Mental health | 14 (50.0) | 3 (30.0) | 11 (61.1) |

Table 34: Clinical symptoms reported by adult HPP patients

The time between the presentation of HPP and diagnosis varied greatly, from 1 year to >30 years (table 35).

|  |  |
| --- | --- |
| Time from presentation to diagnosis (years) | N (%) |
| 0 – 9 | 14 (51.9) |
| 10 – 19 | 6 (22.2) |
| 20 – 39  | 1 (3.7) |
| 30 – 39 | 6 (22.2) |

Table 35: Delay in diagnosis of adult HPP

N = 27; date of disease presentation could not be established for one patient

### *Genetic and biochemical assessment*

Results of biochemical measurements and genetic screening are shown in table 36.

Of the 28 patients with adult HPP, 24 (86%) were found to have a mutation of the *ALPL* gene, with four patients having compound mutations.

The clinical form of HPP associated with each mutation was matched from the TNSALP Gene Mutations Database (Mornet, 2015), including mutations associated with the odonto, perinatal, childhood, infantile and adults forms of the disease; six mutations not previously recorded in the database were observed.

PLP was above the normal range in 16 patients (57%), total ALP was below 36 IU/L in 25 patients (89%).

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| ID | Gender | Age | cDNA | Protein | Clinical Form1 | DNE%2 | Comment | PLP nmol/L | ALP IU/L |
| BHA01 | Female | 57 | c.215T>C | p.Ile172Thr | odonto | 40.2 | Heterozygous | **305** | 38 |
| BHA02 | Female | 60 | c.550C>T | p.Arg184Trp | perinatal | 36.7 | Heterozygous | **275** | **18** |
| BHA03 | Male | 59 | c.891C>A | p.Tyr297\* | infantile |  | Heterozygous | **261** | **33** |
| BHA04 | Male | 55 | c.212G>C | p.Arg71Pro | perinatal |  | Heterozygous | 108 | **16** |
| BHA05 | Female | 74 | c.526G>A | p.Ala176Thr | childhood | 58.0 | Compound heterozygous | **182** | **14** |
| c.571G>A | p.Glu191Lys | infantile | 79.5 |
| BHA06 | Female | 53 |  |  |  |  | No change detected | 68 | **21** |
| BHA07 | Female | 44 | c.1396C>G | p.Pro466Ala | - |  | Unknown sig. | **242** | **35** |
| BHA08 | Female | 41 |  |  |  |  | No change detected | 45 | **33** |
| BHA09 | Male | 52 | c.571G>A | p.Glu191Lys | infantile | 79.5 | Compound heterozygous | **822** | **15** |
| c.1406dupA | p.His469fs | - |  |
| BHA10 | Female | 49 | c.346G>A | p.Ala116Thr | adult | 40.0 | Heterozygous | **267** | **31** |
| BHA11 | Female | 28 |  |  |  |  | No change detected | 30 | 41 |
| BHA12 | Male | 45 | c.346G>A | p.Ala116Thr | adult | 40.0 | Heterozygous | **377** | **19** |
| BHA13 | Female | 39 | c.400\_401CA:NA | p.Thr134His | perinatal |  | Heterozygous | 104 | **22** |
| BHA14 | Female | 51 | c.1161A>G | p.Gly387Gly | - |  | Unknown sig. | **462** | **17** |
| BHA15 | Female | 35 | c.1551\_1566del | p.517\_522del | - |  | Heterozygous | **1119** | **18** |
| BHA16 | Male | 20 | c.1161A>G | p.Gly387Gly | - |  | Unknown sig. | **784** | **27** |
| BHA17 | Female | 38 | c.575T>C | p.Met192Thr | infantile |  | Heterozygous | 95 | **31** |
| BHA18 | Male | 49 | c.526G>A | p.Ala176Thr | childhood | 58.0 | Compound heterozygous | **1853** | **17** |
| c.658G>A | p.Gly220Ar | perinatal |  |
| BHA19 | Female | 57 | c.1250A>G | p.Asn417Ser | perinatal | 26.5 | Heterozygous | **206** | **25** |
| BHA20 | Female | 43 | c.1044\_1055del | p.Leu349\_Ala352del | perinatal |  | Heterozygous | N/A | **14** |
| BHA21 | Female | 65 | c.346G>A | p.Ala116Thr | adult | 40.0 | Heterozygous | **261** | **22** |
| BHA22 | Female | 33 |  |  |  |  | No change detected | 47 | **27** |
| BHA23 | Male | 47 | c.346G>A | p.Ala116Thr | adult | 40.0 | Heterozygous | **789** | **14** |
| BHA24 | Male | 39 | c.472+1\_472+3het\_delGTG | p.? | - |  | Heterozygous | 148 | **29** |
| BHA25 | Female | 36 | c.143C>G | p.Thr48Ser | - |  | Unknown sig. | **1178** | **10** |
| c.1171C>T | p.Arg391Cys | childhood | 50.5 | Heterozygous |
| BHA26 | Male | 59 | c.431G>A | p.Gly144Glu | adult |  | Heterozygous | 138 | **28** |
| BHA27 | Female | 30 | c.526G>A | p.Ala176Thr | childhood | 58.0 | Heterozygous | <LoD | **31** |
| BHA28 | Male | 51 | c.526G>A | p.Ala176Thr | childhood | 58.0 | Heterozygous | 179 | 43 |

Table 36: Biochemical and genetic screening results in adult HPP patients. PINP and BAP measurements had not been completed in these subjects at the time of writing.

1Previous clinical classification of the disease form registered in the TNSALP Gene Mutations Database (Mornet, 2015). Abnormal biochemistry results are bolded.

2Where available, DNE: Dominant Negative Effect %.

## Discussion

### *Age at presentation and diagnosis*

The mean age of presentation was 31.8 years and the mean age at diagnosis was 43.8 years. The mean time to diagnosis was 13.4 years. This long delay between presentation and diagnosis potentially relates to the unfamiliarity of physicians with HPP. It could also relate to the non-specific and wide-ranging nature of the symptoms displayed in adult HPP. For example, only 3 of the 28 patients presented with femoral stress fracture and 7 of 28 with metatarsal stress fractures, both fractures typically associated with adult HPP. One patient waited >30 years between the first presentation of the disease and diagnosis. The true prevalence of adult HPP is therefore likely to be underestimated in the wider population.

Our findings are similar to previous case series of adults presenting with HPP. In a series of 22 adults presenting with HPP (Berkseth et al. 2013) the mean age at diagnosis was 44 years, the same as in our study. In the HIPS/HOST survey of 125 adults with HPP (Weber et al. 2016), the mean age was 45 years, but the age at presentation was age 12. Their population was more severely affected than ours, with a majority (67%) reporting paediatric-onset of disease.

### *Clinical features of HPP*

All patients in our cohort reported musculoskeletal pain, compared to the 41% and 95% reported in other case series (Berkseth et al. 2013, Weber et al. 2016). Prior fractures were reported in 71% of our patients compared to 54% and 85% in previous case series (Berkseth et al. 2013, Weber et al. 2016). Many of the reported fractures were classical of adult HPP, namely metatarsal stress fractures in 7 patients and femoral shaft stress fractures in 3 patients in our study. The overall fracture rate was higher than the lifetime prevalence for fractures in the UK, 38.2% (Donaldson et al. 2008).

Dental problems are another hallmark of adult HPP; we reported dental problems in 50% of our patients, 4 of these reported tooth loss. This is higher than similar investigations which report dental problems in 13% of adult HPP patients (Berkseth et al. 2013).

We also report a high prevalence of problems related to mental health. We found that 50% had some problem, and the commonest of these was depression (36%) and some patients had suicidal thoughts. This may be related to the high prevalence of chronic musculoskeletal pain.

Despite the genetic origin of HPP we only reported a family history of HPP in four patients (14%). This is similar to the 9% reported by Berkseth et al. (2013), but is surprisingly low considering autosomal dominant inheritance is often reported in HPP (Kishnani et al. 2017).

### *Genetic and biochemical evaluation*

HPP related mutations were observed in 24/28 (86%) of our patients. Four gene negative cases (14%) were reported; this is similar to the 16% reported in another study (McKiernan et al. 2017), but lower than the 50% reported by Riancho-Zarrabeitia et al. (2016). The 4 gene-negative cases shared a similar biochemical profile, whereby total ALP was low and PLP was normal. PLP was elevated in 18/24 patients with mutation – this shows that neither low ALP nor high PLP alone is pathognomic of HPP. As discussed in previous chapters a combination of biochemical indicators are perhaps more suitable in the identification of adult HPP.

PLP was elevated, and ALP was depressed in all 4/28 (14%) patients with mutations of unknown significance, suggesting these are true cases. A number of mutations were observed in multiple patients (571G>A, 526G>A, 346G>A); none of these patients were known to be related.

It was not possible to assess the efficacy of the previously described formation marker ratio in identifying biochemical indicators of HPP in these patients. Further measurements of BAP and PINP would allow us to establish the prevalence low BAP:PINP ratio.

In conclusion adult HPP is associated with a varied clinical presentation, including musculoskeletal, dental and mental health problems. This may explain the long delay between presentation and diagnosis typical in adult HPP. The biochemical indicators of disease are also varied, and elevated PLP alone is not pathognomic of HPP. A combination of biochemical criteria should be used when identifying HPP.

# Chapter 7: Summary

## Main Findings

The main aim of this thesis was to describe the prevalence of biochemical and genetic indicators of adult HPP in a clinical population. 95% reference intervals for potential biomarkers of HPP were reported following the investigation and exclusion of a range of confounding factors of PLP, including low ALP activity, inflammation, reduced kidney function, and vitamin B6 supplement intake. Biomarker differences related to gender, age and race were investigated. Stratified reference intervals were reported from a large representative U.S. sample, and a healthy U.K. based population that reflected the observed differences in biomarker levels.

Abnormal biochemistry associated with HPP was observed in 3.4% of the study population - a much higher prevalence than has been previously reported. Genetic screening in these subjects revealed *ALPL* mutations in 1% of the study population, including 3 novel mutations. Clinical examination revealed the symptoms associated with HPP mutations to be highly variable, but comparable to previously reported presentations. There was no genotype-phenotype correlation observed, and HPP was more symptomatic in women. Likewise, in our cohort of adult HPP patients the clinical and biochemical symptoms of the disease varied greatly between individuals where chronic pain was the only common factor. The varied clinical profile of adult HPP means that the average time between presentation and diagnosis of the disease is over 13 years.

## How the Study was Unique

This is the first study to report 95% reference intervals for PLP using a large representative U.S. population sample. Potential confounders of PLP including low ALP, inflammation, vitamin B6 supplementation and reduced kidney function were all investigated and shown to be associated with significant differences in serum PLP. The proven confounders of PLP were excluded from the reference interval population. These reference intervals have been stratified to reflect the proven differences in PLP based on age, gender and race which have not previously been reported.

Further reference intervals have been calculated using a local population where gender differences in PLP were also observed and reference intervals reported appropriately. A 95% reference interval for a formation marker ratio (BAP:PINP) has been reported for the first time. It was also the first time a reference range for a formation marker ratio has been applied as a diagnostic criterion in the identification of HPP. The ratio successfully identified individuals with suppressed BAP activity in relation to the formation marker PINP in cases of HPP related mutations.

This is the first study of this scale to determine the prevalence of biochemical changes suggestive of HPP in an adult clinical population in the UK. It was shown for the first time that *ALPL* mutations are present in as many as 1% of the clinical population. This study adds further insight to the clinical and biochemical profile of adult HPP, including the differing severity of adult HHP dependent on gender, and the absence of genotype/phenotype correlations. Novel *ALPL* mutations have been described, along with the clinical and biochemical profile of the subjects.

## Limitations

A main limitation of this study is the use of a cross-sectional observation based study. Reduced ALP activity in adult HPP is thought to be associated with biochemical markers that are persistently outside the normal range; it was not possible in this study to determine whether biochemical abnormalities observed in our study population were transient of persistent.

Limitations are present due to the use of a study population recruited from a specific clinic. Despite providing new insights into the prevalence and clinical presentation of adult HPP, findings may not be generalisable to the wider population. There is a potential bias in attempting to identify diseased individuals in a clinical population compared to the general public; other factors such as the average age and general state of health of participants are not representative of the wider population.

Patient recruitment and sample collection, was carried out in an ad-hoc, non-standardised manner. Therefore no attempt was made to control potential pre-analytical variability associated with controllable factors such as diet, lifestyle or circadian effects. Markers of bone turnover have been shown to be sensitive to circadian effects, lifestyle choices such as smoking may be associated with elevated BAP (Glover et al. 2008), and dietary vitamin B6 affects serum PLP (Morris et al. 2008). Samples collected in the reference interval populations were collected at standardised times from subjects in a fasting state to minimise variability.

Finally, time and resource constraints prevented clinical and genetic evaluation in all subjects to provide case matched control comparisons, and identify the prevalence of *ALPL* mutations in subjects with biochemical measurements within the normal range.

## Future Work

Further studies are required to better classify the clinical symptoms observed in our cohort. Comparison to age- and gender-matched controls from the same population without HPP related mutations will provide better distinction into the clinical symptoms of adult HPP. Further investigation is required to characterise the intra-family genotypic and phenotypic variation that may exist in our subjects with known HPP related mutations. This will provide further understanding of genotype-phenotype correlation in relatives with the same mutation.

The use of a 95% reference interval of a formation marker ratio has proven effective in identification of adult HPP; further work should be carried out to examine the effectiveness of this ratio in other cohorts. The effectiveness of other formation markers, such as osteocalcin could also be elucidated in order to better understand the relevance of formation marker levels in adult HPP.

Further biochemical analysis should be undertaken to elucidate the role of other bone turnover markers in adult HPP. It has been suggested that osteopontin (OPN) may be a natural substrate of ALP (Millan and Plotkin 2012), therefore OPN may accumulate in response to TNSALP associated mutations.

Expansion of the study to a less specific population will allow a better understanding of the prevalence of HPP related biochemical indicators in the general population. Previous studies have examined the prevalence of persistently low total ALP to determine the prevalence of adult HPP (Mornet et al. 2011, McKiernan, Berg, and Fuehrer 2014, Riancho-Zarrabeitia et al. 2016, McKiernan et al. 2017). Future efforts should explore the use of the biochemical indicators of adult HPP described in this study (low BAP, high PLP, and low formation marker ratio) to better identify adult HPP in the wider population.

## Conclusions

In conclusion, adult HPP-related mutations are present in approximately 1% of the clinical population examined. The symptomatic and biochemical profile of adult HPP is highly variable, and shows no genotype-phenotype correlation. Adults with HPP are typically younger than other patients with metabolic disorder of bone and there is no observed difference in the prevalence of HPP between men and women.

Low BAP, high PLP and low BAP:PINP ratio are all suitable biochemical indicators of adult HPP, although for more reliable indication a combination of criteria should be utilised. Serum PLP is shown to be confounded by a range of factors, including low ALP activity, inflammation, reduced kidney function and vitamin B6 supplement intake. Serum PLP is also shown to be different based on gender, age and race – these factors should be considered during interpretation of PLP measurements.

# Appendix A





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