Bone mass, density and geometry in phenylketonuria, reviewing the evidence

Introduction

There has been contradictory published evidence as to whether adults and children with phenylketonuria (PKU) have worse bone geometry (please refer to section 1) compared to their peers. There is a lack of consensus on the extent and cause of any bone abnormalities within the PKU population.

Literature reporting bone mass and density have included cross sectional and cohort studies. Different methodologies have been used to investigate different parameters of bone and the correlation of variables such as blood phenylalanine (Phe) control and protein substitute adherence. It is known that bone shape, size, mass and density could be influenced by several factors such as nutritional status and intake, physical activity, body composition or genetic factors. In addition, for children, pre and post pubertal developmental age and growth status and for adults, lifestyle choices such as smoking or alcohol intake need to be taken into account. Many of the studies failed to assess all of these important parameters. Some studies have used mouse models, the human studies have recruited both children and adults in small numbers, and subjects have followed different dietary regimens with different nutrient compositions. This makes it difficult to interpret the evidence in order to give the best counsel to patients on achieving optimal bone density.

Bone and its relationship with PKU has had renewed interest since the introduction of glycomacropeptide (GMP)-based protein substitutes as an alternative to amino acid (AA)-based protein substitutes for the dietary management of PKU. It has been hypothesised (via animal models) that protein substitutes based on GMP could provide benefit for bone mass and density in PKU.

The objective of this summary is to assess the available evidence relating to PKU and bone investigating the hypothesis that GMP-based protein substitutes are beneficial for bone health.

The aims of this review are to examine:

1. how bone mass and density are defined and measured
2. the current evidence
3. factors affecting bone in PKU including dietary management
4. the effect of protein substitutes, including GMP.

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Optimal bone mass, density and geometry are key to preventing fractures as we get older. Various factors influence bone mass and density such as genetics, physical activity, body composition and diet [1, 2]. The term bone density in this summary specifically refers to the amount of mineral content in the bones, such as calcium and phosphorus. It is a measure of how compact and dense the bones are. Higher bone density generally indicates stronger bones [1, 3].

Glossary of terms:

- **Bone Density or Bone Mineral Density (BMD):** The average concentration of mineral in a 2- or 3-dimensional image or defined section of bone. This term is also used to refer to results of all types of bone densitometry.
- **Bone Mass:** The amount of bone tissue as the total of protein and mineral in the whole skeleton or in a particular segment of bone.
- **Bone Mineral Content (BMC):** The amount of mineral measured in a defined section of bone. Total bone mineral content refers to the amount of mineral in the whole skeleton or in a particular segment of bone.
- **Bone formation and resorption:** Both are a part of the remodelling process of bones. During resorption specific cells will break down bones, while during formation other cells will form new bones and/or grow and heal existing bones.
- **Bone Turnover Markers (BTM):** Enzymes or substances that can be measured in the blood or urine to assess the rate of bone remodelling. They provide information about the balance between bone formation and bone resorption, which is important for evaluating bone health and diagnosing conditions such as osteoporosis.
- **Dual-energy X-ray absorptiometry (DXA):** An instrument that allows bone density to be calculated. This gives specific measurements of the lumbar spine, areal bone mineral density, bone mineral content and total body mineral content. A measure of the amount of calcium and other minerals per square centimetre of bone and used to assess fracture risk.
- **Osteopenia:** A term originating from the Working Group of the World Health Organisation to refer to T-scores between -1.0 and -2.5.
- **Osteoporosis:** Defined by the Working Group of the World Health Organisation as a bone density T-score at or below -2.5. A diagnosis of osteoporosis is also made based on a vertebral fracture confirmed by radiograph.
- **Total bone less head (TBLH):** DXA measurement which has excluded the head, used in assessment of BMD in children.
- **Potential Renal Acid Load (PRAL):** PRAL is a measure of the acid-base load of foods and estimates renal net acid excretion.

Collaborators

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Contents

1 Bone geometry essentials

The human skeleton is a mechanical structure, designed to provide protection, structure and support. It is made up from two types of bone as shown in **figure 1**:

Figure 1: diagram of human bone

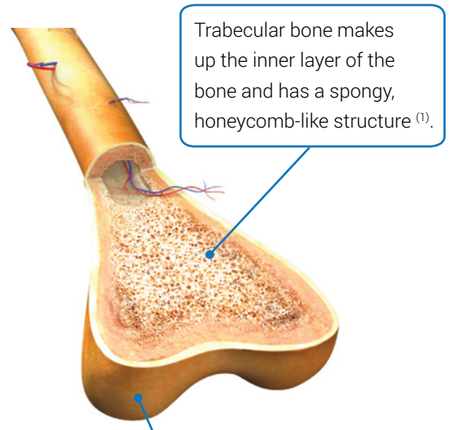
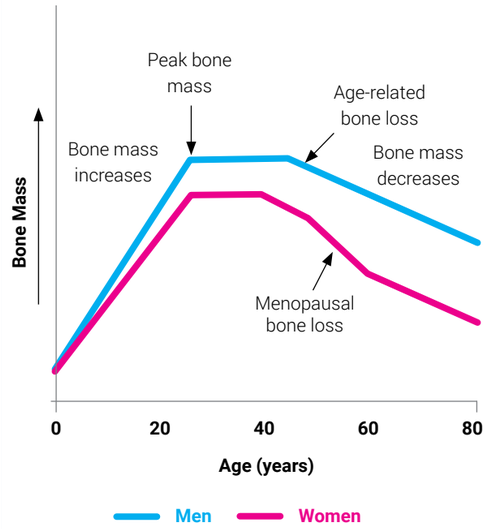


Figure 2: accumulation of bone mass



A child's skeleton is constantly changing in both size and composition. As the skeleton grows the bones are constantly being built and broken down; a process known as modelling and remodelling. Bone growth occurs in two ways; by increasing size and the accruing of bone minerals. In children these processes occur at different rates and times, however, by age 20-30 years the skeleton has reached its maximum bone mineral density, known as peak bone mass. Age-related bone loss then starts to occur where bone is removed faster than being replaced [4]. This process is shown in **figure 2**.

A person's bone mass depends on the peak bone mass achieved and on the rate of loss later in life as they grow older. Bone mineral density (BMD) is a measure of bone mass. Women experience an acceleration of bone loss around the time of menopause which lasts approximately 5-10 years [4, 5]. In children and adolescents who have not reached their peak bone mass, it is important to be able to assess if either bone growth or the build-up of bone minerals are altered, which would increase the risk of fragility fractures in childhood or later in life.

Low bone mass is associated with increased risk of osteoporosis and fracture [4]. Risk factors for low bone mass include:

- Unmodifiable factors: certain ethnicities, female gender, increasing age, family history of fracture.
- Modifiable factors: low body mass index (BMI), smoking, weight-bearing exercise, excess alcohol, vitamin D deficiency, low calcium intake, hormonal disorders and certain medications [4].

1 Bone geometry essentials

Measuring bone mass and density

Bone mass, density and geometry can be measured in different ways including:

- **Bone blood parameters:** total plasma calcium, plasma phosphate, parathyroid hormone (PTH), 25-hydroxyvitamin D, urinary calcium.
- **Serum bone blood turnover markers (BTM):** bone alkaline phosphatase (bone ALP), osteocalcin (OC), type I collagen β crosslinked C-telopeptide (β-CTX), procollagen type 1 N terminal propeptide (P1NP).
- **Urine turnover markers:** deoxypyridinoline (DPD), pyridinoline (PYD), urine calcium/ creatinine ratio
- **Bone Mineral Density (BMD):** dual-energy X-ray absorptiometry (DXA), quantitative computed tomography.
- **Bone geometry:** peripheral quantitative computed tomography (pQCT).
- **Diagnostic imaging:** radiographs (X-ray), radionuclide scans, quantitative ultrasound (QUS), magnetic resonance imaging (MRI).

Bone blood turnover markers

BTM involved in formation, resorption (breakdown) and regulation are released into the blood during bone remodelling in adults. It has been advocated to use BTM in combination with the measurement of BMD to provide a more comprehensive clinical assessment of fracture and osteoporosis risk [6].

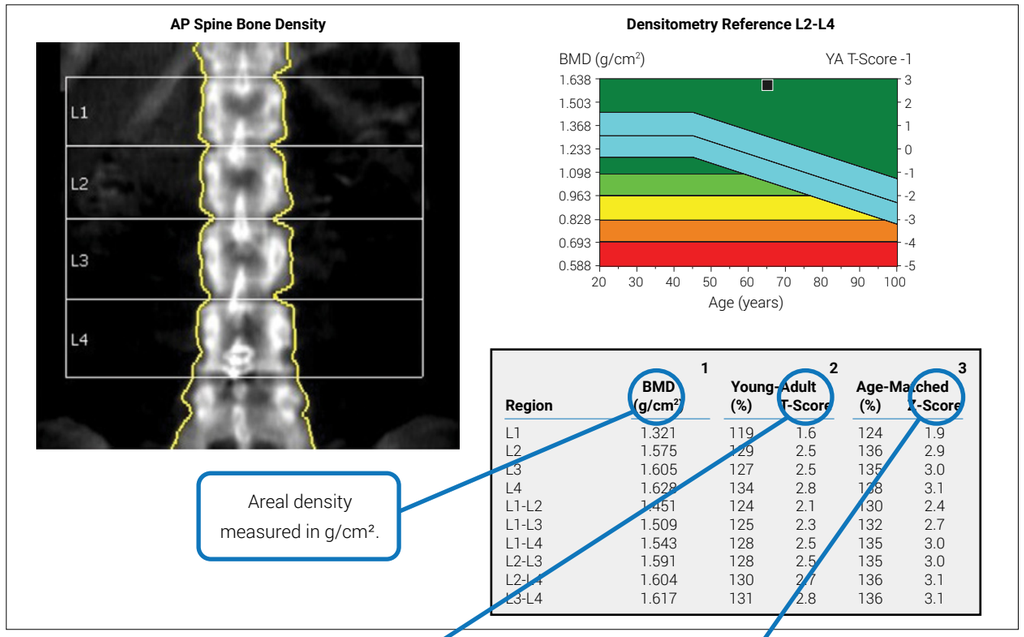
Bone mineral density

BMD is a measure of the amount of calcium and other minerals (the bone mineral content) per square centimetre of bone. DXA is the most commonly used method to measure BMD. DXA measures a specific bone or bones, usually the spine L2-L4 (lumbar) and hip (femur) but can also be wrist (radius) and total (whole body). Reporting at different sites can alter the findings [1].

Spinal BMD is significant for trabecular bone, and femoral BMD for cortical bone. The density of measured bone is compared with an average index based on age, sex, and size to determine risk for fractures and the stage of osteoporosis (if any) in an individual [1, 7]. See **figure 3** for an example of a DXA scan report with explanation of the measurements which identify low BMD/osteopenia and osteoporosis. A specific image of the whole spine called the Lateral Vertebral Assessment (LVA) is particularly important and regarded as a standard measurement assessing skeletal fragility and undiagnosed spinal fractures.

1 Bone health essentials

Figure 3: Example DXA scan report with measurements explained.



T-score

The number of standard deviations above or below the mean when the patient is compared to healthy 30-year-old adults of the same sex and, in some cases, ethnicity [5].

The World Health Organization (WHO) diagnostic descriptors of T-score results are:

- Normal: -1.0 or higher
- Osteopenia/Low BMD: -1.0 to -2.5
- Osteoporosis: -2.5 or less
- Severe osteoporosis: -2.5 and below + fragility fracture

This is illustrated in the top right hand example graph with the black result box within the normal range for a 65 year old.

Because BMD declines with age, T-scores are consistently lower than Z-scores after about the age of 40 years, and the difference increases with age [5, 8].

Z-score

The number of standard deviations above or below the mean compared to patients of the same age and sex, and in some cases, ethnicity [5].

The International Society for Clinical Densitometry (ISCD) state: Z-score of -2.0 or lower is defined as "below the expected range for age", and a Z-score above -2.0 is "within the expected range for age" [9].

Z-scores are preferred when reporting BMD in females prior to menopause and in males younger than 50 years. The incidence of low BMD in the general population is 2%. Low BMD in children is defined as a BMD Z-score of less than -2.0. Osteoporosis cannot be diagnosed in children or men under 50 years based on BMD Z-score alone but must be coupled with a significant fracture history [5].

NOTE: DXA is the preferred method of measuring bone density, although it provides only an areal view instead of true bone volume. In children this method has limitations, as children with reduced height compared to peers have an appropriate reduced bone mass. DXA overestimates bone density in a tall child while underestimating bone density in a short child; this may lead to misinterpretation of results. In children, the head constitutes a large portion of the total bone mass and is larger compared to body size. Therefore, it is important to exclude the head from DXA scans measuring total BMD in children, this adapted measurement is known as total bone less head (TBLH).

2 Bone mass, density and geometry in PKU

Bone mass, density and geometry in PKU are affected by the same factors as the general population. However, in PKU there may be additional factors to consider. It has been suggested that genetics, and the necessity to follow a restrictive diet, could play significant roles in altering bone density. One of the first reports on abnormal bone density was by Feinberg and Fisch in 1962 [10], which reported striations on long bones in neonates with PKU. In recent years there have been considerable advances in the dietary management for PKU and so it should be expected that outcomes for bone density should follow.

There have been four systematic reviews published on bone health in PKU: Enns et al., 2010 [11], Hansen and Ney, 2014 [12], Demirdas et al., 2015 [13] and de Castro *et al.*, 2020 [14]. Since Demirdas *et al.*, 2015 [13] systematic review and meta-analysis was published there have been several more publications regarding bone health in PKU [15-23]. Findings from these reviews and the subsequent studies have been tabulated in appendix 1 to allow comparison. It is evident from these publications that low BMD in PKU is not universal.

BMD has been the most investigated measure of bone in PKU. BMD Z-scores are most commonly reported in studies of bone density in PKU because the population is relatively young and results from paediatric and adult subjects are combined [13]. However, different ways of measurements (e. g. DXA Z-scores measured at different bone sites or measurements in children as TBLH) make it difficult to compare studies. In 2019, a review provided more detail of the techniques available to measure bone density and their potential limitations, it illustrated assessing bone density in children is challenging and measurements in isolation cannot provide a definitive diagnosis or conclusion [24].

All the publications in appendix 1 that reported mean DXA Z-scores showed PKU patients to be within the expected range for age (above -2.0) according to ISCD official positions [9]. Five publications compared DXA Z-scores with controls or reference populations. One conference report found no difference between patients and controls [17]. Four publications reported BMD Z-scores as being lower in their PKU cohort [12, 13, 19, 22]. One publication compared adults only to the reference population and found mean BMD Z-scores were significantly lower for all skeletal sites except the radius [22]. Frequency of low BMD was observed in 1.6-5.5% with the maximum being observed at the spinal level, however, this is lower than described previously.

Statistical significance was not provided in the other three publications and authors were conflicted on the clinical significant of their findings [12, 13, 19].

Few studies have reported on bone mineral content (BMC) or bone turnover markers (BTM) in PKU. In the Demirdas *et al.*, 2015 [13] review authors reported the clinical implications of BMC in PKU were unknown. It stated that the results on BTM were ambiguous and consensus on the utility of BTM, including reliable methods of collection and reference ranges, should be established for further investigation. Geiger *et al.*, 2016 [16] found no abnormalities in BTM and Choukair *et al.*, 2017 [18] found normal BTM in the majority of their cohort. A three-year longitudinal study from Daly *et al.*, 2021 found that BTM followed the expected age-dependant variations [23].

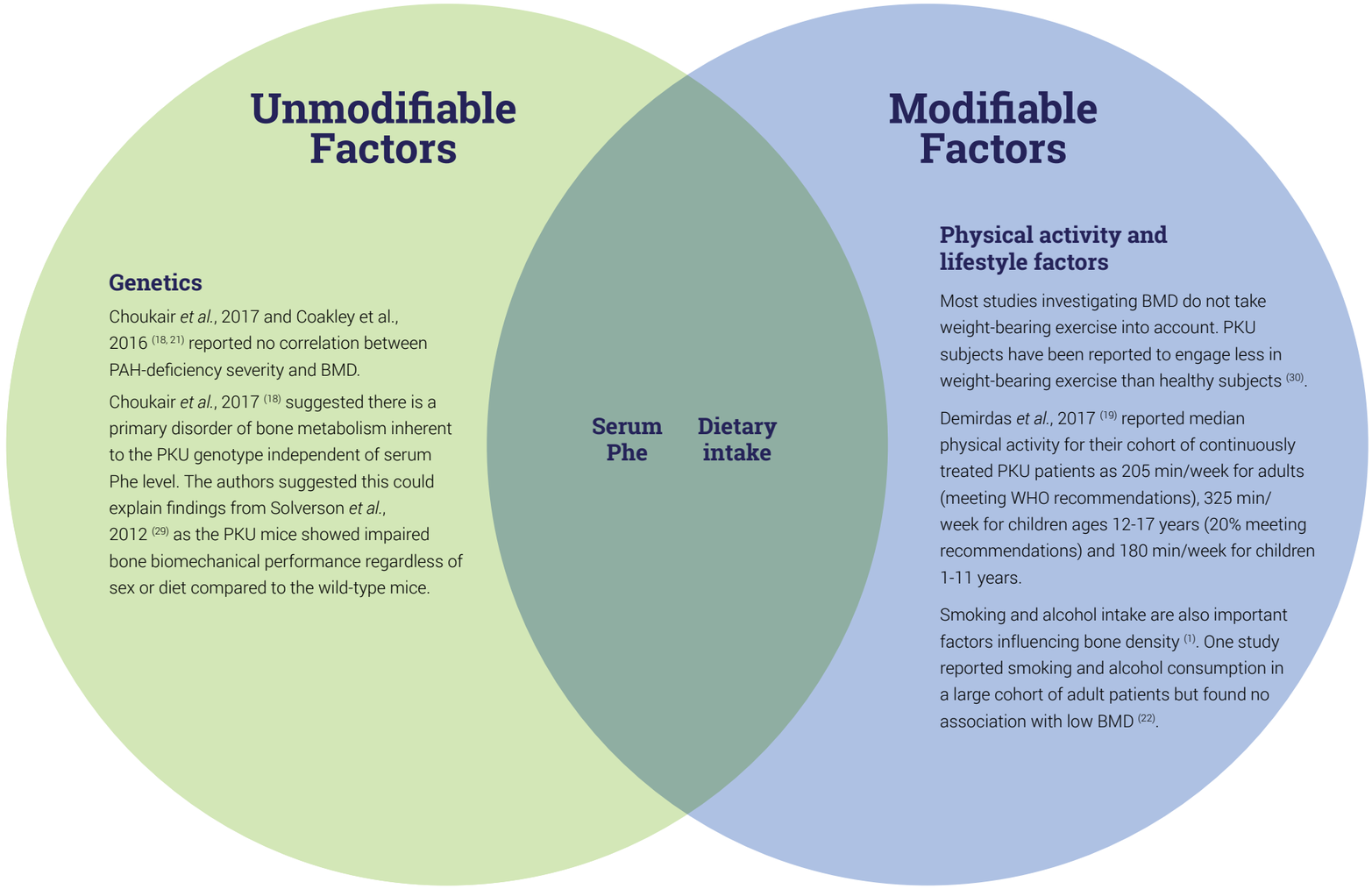
Considerations

Recent studies on bone density measurements in PKU, summarised in **appendix 1**, varied in study participants and methodologies including; BMD measured at different body sites, wide age range, and use of sapropterin dihydrochloride (Kuvan®). Details on physical activity, body composition and fracture history are lacking in most of the available publications. Many factors related to growth and development have been shown to influence BMD and peak bone mass in healthy children [25] with height particularly influencing BMD and BMC. While height of PKU children is statistically comparable to peers based on Z-scores [26, 27] other factors that need to be considered in PKU are pubertal status and age at menarche, which is often unreported in study findings. Few studies reported time spent exercising.

Nutritional intakes, genetic and lifestyle factors are known to influence bone development and health in the general population [4]. **Section 3** explores factors which have been identified to affect bone health in PKU. **Appendix 2** tabulates findings reported by studies included in **appendix 1** which investigated dietary associations.

3 Factors affecting bone mass and density PKU

Section 2 summarised the evidence on bone density in PKU and demonstrated that the presence of impairment is contested. The likely cause of any bone impairment has been debated whether it could be inherent to PKU or related to the dietary management [13, 18, 28]. Many factors affect bone density and skeletal development, some of which have been investigated in the human PKU population.



Serum Phe

Reviews by Hansen and Ney, 2014 [12] and Demirdas *et al.*, 2015 [13] concluded there were no correlations between serum Phe and bone density in PKU. This finding has also been consistently reported in subsequent studies [16, 18, 21, 22].

Dietary intake

Appendix 2 tabulates the findings from the review and any subsequent studies which have investigated associations between dietary intake and outcomes of bone density. These primarily focused protein, vitamin D, calcium, phosphate and magnesium.

Six out of ten studies assessed for a correlation between bone density and nutrient intakes [13, 16, 20-22, 31]. One review reported no correlation between dietary intake and outcomes [13]. One study reported no correlation between calcium and vitamin D supplementation and BMD [22]. Two out of the six studies reported BMD was significantly positively correlated with protein substitute intake in cohorts containing a combination of patients who were meeting minimum protein requirements (either synthetic or natural) [16, 21]. Two studies reported a non-significant negative correlation or no correlation between BMD and protein substitute intake, participants reported lifelong adherence to the PKU diet [20, 31]. The impact of overall protein status, including biological value of intact versus protein substitute and percent of total protein derived from protein substitute on bone were not considered by any of the studies.

In PKU protein substitutes provide essential nutrients for normal health. Improvements in dietary management in recent years include stricter blood Phe target ranges, increased monitoring, improved access and continuing the diet for life [32-35]. Improvements in technologies to optimise taste and convenience of protein substitutes are linked to improved adherence and nutritional status [16, 18, 36, 37]. Therefore, assessment of bone density in individuals have not received optimal nutritional management could explain an inaccurate representation of bone density in the PKU population.

4 GMP and bone in PKU

Glycomacropeptide (GMP) is a macropeptide derived from natural protein. Un-modified it is an incomplete protein source. GMP is supplemented with the limiting, indispensable amino acids (apart from phenylalanine) in order to provide a viable alternative to AA-based protein substitutes for the dietary management of PKU. GMP-based protein substitutes contain some residual Phe and must be used with caution in individuals with a low Phe-tolerance. GMP-based protein substitute have been shown to provide a suitable alternative to AA-based protein substitutes for growth, micronutrient status and metabolic control when used as the sole protein substitute or combined with other AA-based protein substitutes in adults and children with PKU [38-40] with careful monitoring of the Phe levels. It has been recommended that GMP-based protein substitutes should be introduced carefully and systematically in children [38].

Evidence available involving GMP-based protein substitutes and BMD in PKU:

(Solverson *et al.*, 2012) [29]

Subjects:

Wildtype (WT) and PKU mouse models (n=217).

Investigations:

Mice consumed either a casein, AA, or GMP diet from weaning. DXA, 3-point bending testing and diaphyseal structure of femur.

Findings:

BMD was significantly lower in PKU mice compared to WT regardless of diet. No difference in BMD found between the diets in PKU mice. In WT mice femur size and strength reduced in AA group compared to GMP and casein group.

Considerations:

Disease specific mouse models are produced with intensive brother-sister mating to produce mice with practically identical genomes in order to knock out specific genes more easily [48]. Differences seen in bone fragility between the inbred PKU and wildtype mice could be affected by genetic factors, this was supported by subsequent findings [18]. Activity levels were not assessed. It was acknowledged that neurological damage from Phe toxicity in PKU mice would likely have reduced physical activity levels and increased their risk of skeletal fragility. The differences in skeletal structure and development in mice compared to humans limit direct conclusion [16, 49].

(Stroup *et al.*, 2017) [31]

Subjects:

8 early-treated PKU patients aged 16-35 years.

Investigations:

Two staged, crossover pilot study. Potential renal acid load (PRAL)* of protein substitute calculated. Food records and 24-hour urine collection after consuming low-Phe diet in combination with high-PRAL* AA -based, or low-PRAL* GMP-AA-based, protein substitutes for 1-3 weeks. Patients taking a low-PRAL* AA-based protein substitute were excluded. DXA completed at baseline when taking usual AA-based protein substitute.

Findings:

9 out of 10 AA-based protein substitutes had a 1.5–2.5 - fold higher PRAL* than a GMP-AA-based protein substitute. A statistically significant increase in renal net acid excretion (RNAE) and calcium and magnesium urine losses were found in participants taking high-PRAL* AA-based protein substitutes, compared to those taking low-PRAL* GMP-AA based protein substitutes. Suggested that the cause of the increased skeletal fragility is associated with PRAL*.

Considerations:

Small cohort and short duration of GMP-based protein substitute exposure. DXA was taken at baseline when participants were taking AA-based protein substitutes and not repeated after GMP intake. High-sodium, low-PRAL* AA-based protein substitutes excluded. Correlation between PRAL and BMD was not published. PRAL* statistically significantly affected by sodium content of product.

Positive health benefits of GMP have been proposed including improvement of bone and gut health, prebiotic and anti-inflammatory properties and nitrogen retention [38, 41-47].

Most of the evidence on bone geometry in PKU summarised in **section 2** is based on research conducted on PKU patients taking AA-based protein substitutes. Only one study examined the effect of GMP-based protein substitutes on bone health over a three-year period in children with PKU [23].

(Stroup *et al.*, 2018) [20]

Subjects:

15 PKU patients aged 15-50 years.

Investigations:

DXA completed reflective of usual AA-based protein substitute. 3-day food record diaries. PRAL* of protein substitute calculated. 24-hour urine collection after 1-3 weeks of taking either high-PRAL* AA, or low-PRAL* GMP-based, protein substitutes.

Findings:

Males (6) had statistically significantly lower total body and femur BMD compared to females (no other BMD measurement reached statistical significance). Mean total femur DXA Z-score was negatively correlated with intakes of AA-based protein substitutes (p=0.048) but not spine or total body. No significant difference was found between male and female PRAL*, RNAE, or AA-based protein substitute intake (g PE/kg/day).

Considerations:

Small cohort and DXA not repeated after intake of GMP-based protein substitute. It was concluded that higher intakes of AA-based protein substitute with a higher PRAL* value results in low BMD in males, however, no significant difference between male and female PRAL* intake found. The correlation between mean PRAL* and BMD was not reported.

(Daly *et al.*, 2021) [23]

Subjects:

48 children with PKU (aged 5-16 years).

Investigations:

3 groups (13 children taking GMP-based protein substitutes, 16 taking a combination of GMP- and AA-based protein substitutes, 19 taking AA-based protein substitutes) were monitored for 36 months. Serum blood, urine BTM and blood bone biochemistry were measured at enrolment, 6, 12, and 36 months, DXA was measured at enrolment and 36 months, pQCT was measured at 36 months only.

Findings:

The study found that long-term consumption of GMP-based protein substitutes supports normal bone growth consistent with AA-based protein substitute consumption in patients having good metabolic control. All biochemistry markers (calcium, phosphate, magnesium, vitamin D, and parathyroid hormone) were within the normal reference range for all 3 groups. As the bone density was lower than the population mean (expressed as median Z-score), but in the known range, no increased osteoporotic risk in PKU patients could be found. Additionally, BTM showed an active bone turnover profile demonstrating that bone remodelling processes were active in children taking either GMP- or amino acid-based protein substitutes suggesting normal bone growth in all 3 participating groups.

Considerations:

Each group had small patient numbers with no healthy control group. The GMP concentrations differed between the participating groups. As children from 5 to 16 years were observed, some of them already reached puberty which could have had an influence on growth, bone marker, etc. An extended follow up period will be needed to overcome those obstacles.

*See **appendix 3** "What is potential renal acid load (PRAL) and how does it relate to PKU?"

Considerations

When reviewing the literature available on bone density and GMP-based protein substitutes it is important to consider that all published evidence relating to BMD measurements in PKU are based on dietary management with AA-based protein substitutes and that poor bone density reported in early, continuously and adequately treated patients with PKU is contested.

One study from Daly *et al.* [23] reported on BMD and further markers of bone health in patients taking GMP-based protein substitutes. Although it is known in PKU that the median Z-scores are below the general population mean, in the study bone density was normal, blood biochemistry and bone turnover markers were within the reference range. The authors concluded that GMP-based protein substitutes do not have any advantage for these parameters compared to AA-based protein substitutes [23]. This supports that GMP- and AA-based protein substitutes both support normal bone growth. Studies which suggest that GMP-based protein substitutes benefit bone in PKU attribute this to providing a lower PRAL* value [20, 31]. A causal association between dietary acid load, measured with PRAL*, and osteoporotic bone disease is not supported by evidence in the general population [50, 51]. PRAL* calculation used to investigate protein substitutes in PKU is significantly influenced by mineral and electrolyte content of the product, particularly sodium content. Patients taking a high-sodium, low-PRAL* AA-based protein substitute were not included in the investigation. The correlation between BMD and PRAL* value of the protein substitutes, which would support the hypothesis that high-PRAL* reduces BMD, was not reported in either publication. It was reported that the correlation between intake of high-PRAL* protein substitutes and BMD measures did not reach statistical significance [20, 31].

* See **appendix 3** "What is potential renal acid load (PRAL) and how does it relate to PKU?"

5 Discussion

Bone health in PKU is complex and recent studies have shown mean BMD Z-scores are within the normal range according to ISCD definitions [9, 12, 13, 15-21]. However, BMD is often lower compared to controls or reference populations, and the clinical significance of this is unknown [12, 13, 19]. All research providing evidence of BMD, BMC and fractures have been based on patients who have taken AA-based protein substitutes. Few studies have investigated correlations between dietary intake and bone density. Those that have, linked improvement in bone density with adequate intakes of calcium and vitamin D, adherence to the Phe-restricted diet and adherence to prescribed amounts of protein substitutes [16, 19, 21].

It has been suggested that GMP-based protein substitutes could be beneficial for bone density. Although the prospect of clinical benefit of GMP is appealing, more research is needed. Considering the complexity of genetic, clinical, nutritional and lifestyle factors which influence bone health, it is unlikely that changes in bone health could be attributed to a single dietary component such as GMP. The evidence available from Solverson et al. 2012 [29], conducted on a mouse model, has limited application on informing clinical decisions for patients, while Daly et al. 2021 [23] concluded that the bone density was clinically normal for patients taking GMP-based or AA-based protein substitutes.

The studies by Daly et al. 2021 [23, 52] represents data of a three-year clinical trial which compare bone health, including TBLH, in children with PKU after consuming AA or GMP-based protein substitutes (PKU sphere™) for three years. The results show no statistically significant difference between those taking GMP-based protein substitutes compared to AA-based protein substitutes for height, weight, BMI, percentage total fat lean mass BMC, bone mineral apparent density and TBLH [23, 52]. However, a trend on improved body composition could be observed in the group taking GMP-based protein substitutes as sole source of protein source [52].

Current practical recommendations to optimise bone density for individuals with PKU include [21, 34]:

- **promote adherence to prescribed amount of protein substitute,**
- **ensuring adequate intakes of calcium and vitamin D,**
- **optimisation of natural protein intake,**
- **regular weight-bearing exercise.**

6 Conclusion

The objective of this evidence summary was to review the data available to investigate whether GMP-based protein substitutes are beneficial for bone health for individuals with PKU. More research is needed to conclusively determine whether GMP-based protein substitutes affect bone health in PKU. As stated previously, bone health is multifactorial and confounding factors need to be controlled for in future research. It is important to ensure future research is conducted with early treated individuals with PKU who have adequate nutritional intakes.

GMP-based protein substitutes offer an alternative choice for clinicians and patients, providing a different taste and mouthfeel which many patients find preferable to AA-based protein substitutes [37, 38, 53-55]. As a result, GMP-based protein substitutes may help to promote dietary adherence for individuals with PKU [37, 54, 56].

Adherence to any protein substitute is likely to promote more optimal clinical outcomes for PKU patients, especially when the protein substitute is fortified with a comprehensive nutrient profile beneficial for bone health [37, 57-59]. Particularly, GMP-based protein substitutes seem to lead to a better adherence to the diet due to the favourable taste and mouth feel compared to AA-based protein substitutes. It has been suggested that introducing GMP as early as possible into the diet may help implant its flavour profile by repeated exposure in the early years [60].

7 Appendix 1: Table 1: summary of publications investigating bone health in PKU

Author, year, country	Study type	Outcomes investigated	Number of PKU patients	Mean/median BMD Z-score (g/cm²)	Fractures assessed	Physical activity assessed	Nutrient intake assessed	Author's Conclusions	Limitations
Enns *et al.*, 2010 [11], USA	Systematic review.	Not reported.	Not reported.	Not reported.	✗	✗	✗	Osteopenia and osteoporosis has been detected in the adult PKU population. The decrease in peak BMD in adult patients may be explained by long-standing dietary deficiencies or a primary defect in bone turnover inherent to the disease itself.	Misinterpretation of DXA Z-scores to diagnose osteoporosis and osteopenia. Limited studies included which reviewed BMD. Limited information reported. Mean BMD z-scores not provided to allow comparison.
Hansen and Ney [12], 2014, USA	Systematic review.	BMD Z-score, fractures.	67 Mean age in studies on BMD: 9 ± 2 years, 11 ± 4 years, 9 ± 4 years.	Spine: - 0.10.	20% fracture rate of 263 subjects.	✗	✗	Spine BMD is lower in PKU than control subjects, statistical significance not reported. Studies inconsistently controlled for reported smaller body size of PKU subject. Data lacking to show if lower spine BMD results in a higher fracture rate. The cause of low BMD in PKU is unknown. Suggestion of a clinical fracture rate of 20% among PKU subjects, fracture rates in controls are lacking.	Young cohorts. Inclusion of late diagnosed PKU patients and patients who liberalised their diet after the age of 8 years. These patients would likely have had reduced mobility associated with cognitive impairment and/or nutritional deficits which would increase their risk of lowered BMD.
Demirdas *et al.*, 2015 [13], USA and Netherlands	Systematic review and meta-analysis.	BMD Z-score, BTM, BMC, fractures.	360 Age range: 11-57 years.	Total: - 0.45. Lumbar: -0.70. Femur: -0.96.	✗	✗	✗	Mean BMD is within the normal range in PKU subjects, although mean BMD is lower in PKU patients compared to reference groups, statistical significance not reported. 90% of early treated patient with PKU are not at risk of low BMD. Clinical significance of a slightly lower BMD Z-scores is unknown.	Adherence to dietary treatment has not been assessed in the systematic review. Studies provided insufficient evidence to establish conclusions on BTM and other factors that may influence BMD including blood Phe concentrations, and nutrient intake. Fractures were not included in the search terms when reviewing the papers.
Geiger *et al.*, 2016 [16], USA	Single centre, cross-sectional retrospective record review and prospective cross-sectional study in PKU patients.	Hip and lumbar BMD, dietary intakes, 25 hydroxyvitamin D2 and D3, iPTH, plasma calcium, ALP.	88 IEM retrospective review. 20 PKU prospective study. Age range 8-20 years.	16 had normal BMD at both hip and lumbar. 2 had reduced BMD in the hip (-2.4 and -3.6). 1 had reduced lumbar BMD (-2.1). None had reduced BMD at both hip and lumbar. Mean Z-scores not reported.	✗	✗	✓	No evidence found for reduced BMD in children with PKU on specialised diets. Higher BMD was associated with calcium intake. In 19 participants, 3 had low BMD for chronological age (Z-score ≤ -2) measurement at either the hip or the spine and none had a low BMD at both the hip and the spine.	Mean BMD z-scores not provided to allow comparison. Control group had other IMD and were not 'healthy' controls.
Leiva *et al.*, 2016 [17], Chile	Single centre, cross-sectional study. (conference abstract).	Lumbar, femur and total BMD.	16 Age range: 6-23 years.	Control: lumbar: -0.4, femur: 0.2, total: 0.5. PKU: lumbar: -0.3, femur: -0.3, total: 0.2. mHPA: lumbar: -0.05, femur: 0.65, total: 1.25.	✗	✗	✗	No significant difference in BMD between groups ($p<0.05$). This could be because the cohort has maintained an adequate follow-up that includes sustained contributions of calcium and vitamin D provided by protein substitutes from diagnosis at NBS onwards.	Small cohort. Conference report therefore limited information provided.
Demirdas *et al.*, 2017 [19], Netherlands	Multi-centre, prospective, cross-sectional study.	Dietary intake, blood micronutrient concentrations, fatty acid status, physical activity, fracture history, BMD Z-score.	60 Age range: 1-39 years.	Lumbar: -0.1. Femur: -0.45. Hip: -0.3.	41.7% PKU participants had fracture(s), 38.2% in general population.	✓	✓	BMD Z-scores are within the normal range but lowered compared to the general population, statistical significance not reported. The clinical implications may be limited as none of the patients have osteoporosis as defined by ISCD. Lifetime fracture prevalence was normal.	The authors were unable to investigate association between dietary intake, blood concentrations of nutrients and BMD or fracture history.
Coakley *et al.*, 2016 [21], USA	Single centre, prospective, cross-sectional study.	Anthropometry, BMD Z-score, BMC, dietary intake, blood amino acid concentrations, micronutrient status.	88 Mean age: 18.8 ± 11 years.	Total: -0.326.	✗	✗	✓	No subject had low BMD for chronological age (Z-score ≤ -2) which represents a lower prevalence of low BMD compared to previous reports. Compliance with medical food (protein substitute) prescription was the strongest predictor of total BMD Z-score.	Fracture history and physical activity not assessed.
Choukair *et al.*, 2017 [18], Germany	Single centre, cross-sectional study.	Total BMD of distal and proximal radius Z-score, cortical and trabecular BMD Z-score, grip force, anthropometry, fractures, blood phenylalanine, PTH, 25-(OH-) vitamin D_3, serum calcium, serum phosphate, ALP, osteocalcin, TRP, urinary calcium/creatinine ratio, DPD crosslinks.	56 Age range: 11.8-41.5 years.	Distal radius total: -1.05. Proximal radius total: -0.11. Cortical: 0.12. Trabecular: -0.18.	63% of female PKU participants had fracture(s) compared to 71% in female study population.	✗	✗	The radial bone is characterised by inadequately reduced bone strength in relation to muscular force, reduced cortical thickness, and reduced total BMD at the metaphyseal site. These alterations indicate a mixed bone defect in PKU, both of which are due to primary alterations of bone metabolism and to secondary alterations in response to neuromuscular abnormalities.	Conclusions drawn on radial bone BMD only. Proximal radius, trabecular or cortical BMD were not significantly altered. 64% of study population took any protein substitute, no sub analysis of correlation of protein substitute intake and outcomes reported.
Stroup *et al.*, 2017 [31], USA	Baseline results of cross-over trial.	BMD Z-score for total body, lumbar, femur, and radius, body composition, potential renal acid load (PRAL) from protein substitute, dietary intake, 24-hour urine samples.	8 Age range: 16-35 years.	Mean Z-scores for the cohort not reported.	✗	✗	✗	2 of 8 participants had low BMD-for-age (Z-score ≤ -2) and evidence of bone microarchitectural degradation.	Small cohort. Mean BMD Z-score of the cohort for any BMD measurement not provided to allow comparison. No participant had low BMD at both femur and lumbar.
Stroup *et al.*, 2018 [20], USA	Baseline results of randomised cross-over trial.	Total body, lumbar and femur BMD Z-score, body composition, PRAL from protein substitute.	15 Age range: 15-50 years.	Males: total body: -0.9, lumbar: -1.3, femur: -0.7. Females: total body: 0.2, lumbar: -0.4, femur: 0.4.	✗	✗	✗	Males with PKU have lower total body and femur BMD compared with females with PKU which may be related to higher intake of AA-based protein substitutes and greater calcium excretion.	Small cohort. No significant differences found between male and female PRAL, g PE AA-based protein substitute, RNAE, magnesium or sulphate to support hypothesis that higher intake of AA-based based protein substitutes result in lower BMD.
Lubout *et al.*, 2019 [22]. Netherlands	Multicentre retrospective survey study.	BMD Z-score for lumbar, femoral neck, total proximal femur, radius and total body. Natural protein intake, calcium and vitamin D supplements, use of sapropterin dichloride, mean Phe the year before the recent DXA scan, smoking and alcohol consumption.	183 early treated PKU (ETPKU) adult patients.	Mean Z-score (+/- 1SD) Lumbar: -0.527 Femoral neck: -0.324 Total proximal femur: -0.262 Radius: -0.298 Median Z-score Total body: -0.400	Fractures described in 30 patients (16.4%), which is significantly lower than the estimated age-standardized fracture prevalence of 38.2% for England.	✗	✓	Most ETPKU patients have a BMD within normal range, with only a maximum of 5.5% having a low BMD. A DXA scan should potentially be requested in PKU patients aged >35-40 years and in those PKU patients considered to be at increased risk for fractures.	Most participating centres routinely performed a DXA scan in all patients, some centres only carried out a DXA scan in a selection, this may cause bias in under or over estimation of low BMD. Additional analysis on risk factors unable to be carried out due to low prevalence of low BMD. Described fracture prevalence may be underreported as based on chart studies which are less reliable.

7 Appendix 1: Table 1: summary of publications investigating bone health in PKU

Author, year, country	Study type	Outcomes investigated	Number of PKU patients	Mean/median BMD Z-score (g/cm²)	Fractures assessed	Physical activity assessed	Nutrient intake assessed	Author's Conclusions	Limitations
De Castro *et al.*, 2020 [14] **Spain**	Systematic review of cross-sectional and cohort studies.	Bone mineral status, levels of bone turnover markers, and levels of minerals and elements related to bone health.	327 PKU patients and 10 HPA patients, 54 participated in cross-sectional studies (in total: 14 studies). Age range: up to 40 years.	Several.	✗	✗	✗	Compared to healthy population, BMD is reduced in PKU patients, which does not correlate with an increased prevalence of bone mass below the expected range. Trend towards imbalance between bone formation and resorption was found. Inconclusive data of involved minerals and hormones.	Risk of bias as several different studies and study types were compared.
Daly *et al.*, 2021 [23] **UK**	Longitudinal three years study.	Blood chemistry (calcium, magnesium, phosphate, vitamin D, parathyroid), BTM (Serum and urine), median DXA Z-score and median pQCT Z-scores.	48 Age range: 5-16 years	At enrolment: GMP100: -0.2, GMP50: -0.1, L-AA: -0.1. After 36 months: GMP100: -0.6, GMP50: -0.1, L-AA:-0.5.	✗	✗	✓	All measured blood biochemistry markers were within the reference range, with no statistical differences in the groups of children taking either L-AA or CGMP-AA protein substitutes.	Small cohort and no healthy control group present. Age of participants differs a lot within each group, additionally puberty had an influence on several bio markers. Extended follow-up period needed to evaluate long-term effects.

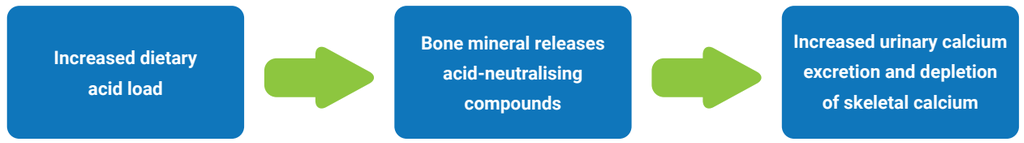
7 Appendix 2: Table 2: summary of publications investigating dietary factors in patients taking AA-based protein substitutes

Author and year	Protein	Vitamin D	Calcium, phosphate and magnesium	Other findings	Author's Conclusions	Limitations
Demirdas *et al.*, 2015 [13]	Total protein intake did not correlate with BMD. Correlation with protein substitute intake was inconsistent, one study reported a positive correlation and one study reported no correlation.	Vitamin D (25(OH)D) status did not correlate to BMD. Vitamin D intake was not assessed.	Lower plasma calcium concentrations reported in children with PKU but impact on bone ambiguous. Blood phosphorus and magnesium concentrations not linked to bone status.	n/a	Dietary compliance and dietary intake assessed as reported protein substitute intake, total protein or phenylalanine intake were not correlated to BMD or BTM. Vitamin D status does not seem to influence BMD.	The impact of overall protein status, including biological value of intact versus protein substitute and percent of total protein derived from protein substitute on bone were not considered by any study included in the review. Micronutrient intake and correlation to BMD was not investigated.
Geiger *et al.*, 2016 [16]	Significant positive correlation between protein substitute intake and spine BMD.	No significant difference in 25(OH)D levels between IEM patients and the control children. No correlation between serum 25(OH)D and BMD.	Significant, positive correlation between calcium intake and spine BMD.	n/a	BMD was a significantly correlated with dietary calcium and protein substitute intake, suggesting that consumption of protein substitute, which provide most key nutrients important for bone health, play a crucial role in developing peak bone mass among patients with PKU. No evidence of low serum vitamin D in their population of children with IEMs compared to control children.	Small cohort of 12 PKU patients completed 3-day food diaries which lowered statistical power to investigate relationships between dietary intakes and BMD.
Demirdas *et al.*, 2017 [19]	All patients had protein intakes above minimum safe recommendations.	Vitamin D intake was below minimum requirements in 20% of participants. Vitamin D status was low in 14% of participants.	Not reported.	58% of participant's total fat intake was below minimal recommended 20% of energy.	Dutch patients with PKU on long-term dietary treatment have a near normal nutrient status, however, supplementation of micronutrients of which deficiency may be deleterious (e.g. vitamin D and selenium) should be considered.	The authors were unable to investigate association between dietary intake, blood concentrations of nutrients and BMD or fracture history.
Coakley *et al.*, 2016 [21]	Positive correlation found between compliance with protein substitute and actual protein substitute intake (gPE) and BMD. Total BMD Z-score was significantly negatively correlated with protein substitute prescription. Dietary phenylalanine and total protein intake were not correlated to BMD.	Normal vitamin D status found in 84% of their participants. Serum vitamin D status was not correlated with BMD. BMD (adjusted for BMI) was positively correlated to vitamin D intake.	BMD (adjusted for BMI) was positively correlated to calcium intake.	BMD was significantly negatively correlated with dietary carbohydrate intake, dietary sugar intake, total glycaemic load and caffeine intake. BMD was negatively correlated with DEXA scans being taking in winter months.	BMD Z-score was most positively associated with compliance with protein substitute prescription and dietary vitamin D intake and most strongly negatively correlated with caffeine intake and total glycaemic load. Promoting optimal protein substitute compliance may be a feasible strategy to improve BMD Z-score.	3-day food diaries do not represent long-term food intake patterns.
Choukair *et al.*, 2017 [18]	Did not report correlation between BMD and protein substitute intake. 64% of participants took any protein substitute.	Vitamin D deficiency or insufficiency reported in 83% of the cohort. No correlation between serum vitamin D concentration and BMD.	Not reported.	Not reported.	No BMD parameter was related to serum 25(OH)D concentrations. Hence, to what extent the high prevalence (83%) of vitamin D deficiency or insufficiency in this PKU cohort contributes to the altered macroscopic bone architecture cannot be assessed.	Nutritional intakes and association with markers of bone health were not assessed. Large proportion of the cohort not taking any protein substitute which was not included in sub analysis of results.
Stroup *et al.*, 2017 [31]	Intake of PE from AA-based protein substitute was negatively correlated with total body BMD (p=0.04) but not lumbar BMD.	Serum concentrations of 25(OH)D were within normal limits.	Serum concentrations of calcium were within normal limits.	AA-based protein substitutes provided 1.5-2.5-fold higher potential renal acid load (PRAL)* compared to GMP-based protein substitutes.	The authors established that AA-based protein substitutes provided a high-PRAL and the high-PRAL* was associated with higher urinary excretion of RNAE, calcium, magnesium and sulphate, which was considered likely to contribute to skeletal fragility in PKU.	Small cohort. High-sodium, low-PRAL* AA-based protein substitutes not included. Correlation between PRAL* and mean total BMD for total cohort not reported.
Stroup *et al.*, 2018 [20]	Mean total femur DXA Z-score was negatively correlated with intakes of AA-based protein substitutes (p=0.048) but not spine or total body.	Not reported.	Not reported.	PRAL determined for protein substitutes. Correlation between PRAL* and BMD not reported.	Males with PKU have lower total body BMD Z-scores and may be at greater risk for osteoporosis than females with PKU. The authors hypothesised that low-normal BMD Z-scores found in male participants may be related to low-normal lean mass and/or higher intakes of AA-based protein substitutes with a correspondingly greater loss of urinary calcium.	Small cohort. Correlation between PRAL* and mean total BMD for total cohort not reported. No significant difference between male and female PRAL intake to support hypothesis that decreased BMD in males related to increased intake of high-PRAL protein substitute.
Lubout *et al.*, 2019 [22]	It was not possible to draw conclusions on the exact amount of natural protein intake and medical food protein intake. It was not clear what type of medical foods were being consumed.	Low vitamin D concentration reported in 32% of 173 patients. Vitamin D supplementation documented in 26% of 162 patients. An association with BMD was not found for either.	Calcium supplementation reported in 19% of 161 patients but no association with BMD found.	Of 106 patients, 22% smoked and 26% consumed on average > 2 units alcohol per day. No association found with BMD.	No statistically significant differences were found in possible risk factors between patients with low BMD and patients with a BMD within normal range.	Dietary factors were derived from patient charts, therefore micronutrient intakes not assessed and not complete enough to draw conclusions on total protein and medical food protein intakes.
De Castro *et al.*, 2020 [14]	Not possible to determine the precise intake of natural protein, protein coming from medical foods or Phe-levels in patients.	Due to high variability of the included studies the data are not conclusive. It should be noted that protein substitutes are often fortified with micronutrients. If patients are compliant to the diet it, they are more likely to have normal levels of micronutrients, e. g. Vit D, despite a low BMD. Decreased natural protein is insufficient for normal bone development although adequate micronutrient intake (Ca, P, Vit D) is ensured.	Due to high variability of the included studies the data are not conclusive. It should be noted that protein substitutes are often fortified with micronutrients. If patients are compliant to the diet it, they are more likely to have normal levels of micronutrients, e. g. Ca, despite a low BMD. Decreased natural protein is insufficient for normal bone development although adequate micronutrient intake (Ca, P, Vit D) is ensured.	Quality and diversity of protein substitutes improved significantly together with a more universal recognition of the need to keep patients under follow-up with better metabolic control could be seen in the last decade, leading to improved nutritional and clinical management practices.	Compared to the healthy population the BMD in PKU patients is reduced, which is not directly connected with an increased prevalence of bone mass below the expected range for age. An imbalance between bone formation and resorption with a trend to bone removal was observed. Serum levels of micronutrients were inconclusive.	Protein substitute and dietary intake was not assessed. Risk of bias as several different studies and study types were compared. Studies from the last decade included, which had a poorer quality in several points, than newer ones. Inconclusive data.
Daly *et al.*, 2021 [23]	All patients had safe protein intake, taking only GMP-based protein substitutes, a mix of GMP-based and AA-based protein substitutes, or taking only AA-based protein substitutes. No clinically significant difference between the three groups could be measured.	Median concentration for vitamin D was within normal reference ranges for all the groups over the 36-month study period.	No correlation or statistically significant differences were found between the three groups for lean body mass, %body fat, fat mass and height. However, a trend towards improved body composition was observed with CGMP-AA when it provided the entire protein substitute requirement.	No statistical differences between the groups of children taking either L-AA or CGMP-AA protein substitutes could be found.	Small cohort and no healthy control group present. Age of participants differs a lot within each group, additionally puberty had an influence on several bio markers. Extended follow-up period needed to evaluate long-term effects.	Dietary factors were derived from patient charts, therefore micronutrient intakes not assessed and not complete enough to draw conclusions on total protein and medical food protein intakes.

* See **appendix 3** "What is potential renal acid load (PRAL) and how does it relate to PKU?"

7 Appendix 3: What is potential renal acid load (PRAL) and how does it relate to PKU?

PRAL is a measure of the acid-base load of foods and estimates renal net acid excretion (RNAE) [51]. It is suggested that increased acid load has a negative impact on bone health by the following process [31]:



PRAL is a controversial theory and is not widely accepted [50, 51]. It is reported that a causal association between dietary acid load and osteoporotic bone disease is not supported by evidence [50, 51].

Stroup et al. in 2017 and 2018 [20, 31] applied the PRAL theory to the PKU diet and implicated the PRAL value of AA-based protein substitutes as a cause of poor bone health in PKU. The calculation* that was used to determine PRAL of protein substitutes in these publications is heavily influenced by the minerals and electrolyte content of the products, the calculation only includes two amino acids. A high PRAL calculation for the AA-based protein substitutes examined in these studies were significantly influenced (p=0.006) by the higher sodium content of the GMP-based compared to amino acid-based protein substitutes.

The correlation between BMD and PRAL value of the protein substitutes, which would support the hypothesis that high PRAL reduces BMD, was not reported in either publication. It was reported that the correlation intake of high PRAL protein substitutes and BMD measures did not reach statistical significance [20, 31].

*The calculation (61) reference. PRAL = (2 × (0.00503 × mg Met/d)) + (2 × (0.0062 × mg Cys/d)) + (0.037 × mg phosphorus/d) + (0.0268 × mg chloride/d) − (0.021 × mg potassium/d) − (0.026 × mg magnesium/d) − (0.013 × mg calcium/d) − (0.0413 × mg sodium/d).

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