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Creatine energy substrate increases bone density in the Pah^{enu2} classical PKU mouse in the context of phenylalanine restriction

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ABSTRACT

Pathophysiology of osteopenia in phenylalanine hydroxylase (PAH) deficient phenylketonuria (PKU) is poorly characterized. The Pah^{enu2} mouse is universally osteopenic where dietary phenylalanine (Phe) management with amino acid defined chow does not improve bone density. We previously demonstrated Pah^{enu2} osteopenia owes to a skeletal stem cell (SSC) developmental deficit mediated by energy dysregulation and oxidative stress. This investigation demonstrates complexity of Pah^{enu2} SSC energy dysregulation. Creatine use by bone tissue is recognized. *In vitro* Pah^{enu2} SSCs in osteoblast differentiation respond to creatine with increased *in situ* alkaline phosphatase activity and increased intracellular ATP content. Animal studies applied a 60-day creatine regimen to Pah^{enu2} and control cohorts. Control cohorts include unaffected littermates (wt/wt), Pah^{enu2} receiving no intervention, and dietary Phe restricted Pah^{enu2}. Experimental cohorts (Phe unrestricted Pah^{enu2}, Phe restricted Pah^{enu2}) were provided 1% creatine *ad libitum* in water. After 60 days, microcomputed tomography assessed bone metrics. Equivalent osteopenia occurs in Phe-restricted and untreated Pah^{enu2} control cohorts. In Phe unrestricted Pah^{enu2}, creatine was without effect as bone density remained equivalent to Pah^{enu2} control cohorts. Alternatively, Phe-restricted Pah^{enu2} receiving creatine present increased bone density. We hypothesize small molecule dysregulation in untreated Pah^{enu2} disallows creatine utilization; therefore, osteopenia persisted. Dietary Phe restriction enables creatine utilization to enhance SSC osteoblast differentiation and improve *in vivo* bone density. PKU intervention singularly focused on Phe reduction enables residual disease including osteopenia and neurologic elements. Intervention concurrently addressing Phe homeostasis and energy dysregulation will improve disease elements refractory to standard of care Phe reduction mono-therapy.

1. Introduction

Dietary Phe restriction is the most broadly applied intervention to treat PAH deficient PKU [1–7]. Newborn screening enables early intervention, disallowing neurologic devastation; however, cognitive deficit, executive function deficit, neuropsychiatric phenotypes, and osteopenia persist among treated patients. Osteopenia is not fully penetrant among PKU patients; however, clinical description is extensive [8–11]. Among early-identified, continuously treated patients, lumbar spine bone mineral density Z scores of −2.0 are observed [12] as is similarly reduced total body bone mineral density [13]. Unlike other PKU phenotypes, the degree of hyperphenylalaninemia is not a correlative metric as osteopenia occurs in therapy noncompliant and therapy compliant patients [12].

Osteopenia was originally considered secondary to diet therapy; however, osteopenia occurs in patients that never received diet therapy and a plethora of studies disprove this argument [12–14]. Several studies find no correlation [8,11,14–19] between hyperphenylalaninemia and bone disease; others show a negative correlation between hyperphenylalaninemia and bone disease [20,21]. Among biochemical elements associated with osteopenia, neither bone formation markers [20,21], bone resorption markers [22,23], nor other osteopenia risk factors [20,22,24] provide insight in the PKU system.

The emerging pathophysiological mechanisms of PKU osteopenia cites an SSC (skeletal stem cell formerly called mesenchymal stem cells [25]) developmental deficit in the osteoblast pathway precipitated by

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energy dysregulation and oxidative stress [26–28]. Osteopenia is universal in the Pah^{enu2} mouse model of classical PKU where Phe restriction with amino acid defined chow neither improves nor worsens bone density [26–28]. We previously demonstrated Pah^{enu2} SSCs provided glutamine energy substrate during *in vitro* osteoblast differentiation displayed augmented alkaline phosphatase activity and mitochondrial oxygen consumption [29]. Providing a 60-day glutamine regimen to Phe unrestricted Pah^{enu2} increased *in vivo* bone density [29]. Pah^{enu2} receiving a creatine regimen have increased bone density; however, eliciting increased bone density requires concurrent Phe management. Energy repletion addresses a PKU pathophysiologic element underappreciated by intervention singularly focused on systemic Phe reduction providing means to address disease elements refractory to standard of care.

2. Methods

2.1. Pah^{enu2} and control animals

Pah^{enu2} and C57bl/6 background strain (unaffected littermates) are propagated in the Rangos Research Center at Children's Hospital of Pittsburgh with an appropriate IACUC protocol and oversight. Animal breeding (Pah^{enu2}, controls) and genotyping is as previously described [30]. Animals were weaned day 21 of life. Cohorts for both *in vitro* SSC studies and *in vivo* bone density studies utilized homozygous genotypes (enu2/enu2, wt/wt). For *in vitro* SSC studies, animals (Pah^{enu2}, unaffected littermates C57bl/6 background strain) were maintained on standard lab diet and sacrificed at 2–3 months of age. Immediately after weaning, Pah^{enu2} animals used for *in vivo* bone density studies receive one of the following diets **1.** standard mouse chow (hyperphenylalaninemic control cohort); **2.** Phe-free amino acid defined chow with Phe (0.35 g/L) supplemented drinking water (Phe restricted control cohort); **3.** standard mouse chow with creatine supplemented water (1% creatine) provided *ad libitum* (experimental cohort 1); **4**. Phe-free amino acid defined chow with Phe and creatine-supplemented in drinking water (0.35 g/L Phe, 1% creatine) provided *ad libitum* (experimental cohort 2). Among Pah^{enu2} animals, standard laboratory chow (with or without supplemental creatine) maintains plasma Phe of ~2000 μM in males and ~ 2200 μM in females. Phe-free amino acid defined chow (with or without supplemental creatine) maintains Pah^{enu2} plasma Phe of ~200 μM in male and female animals [30]. Water formulations (creatine; Phe and creatine) were freshly provided each Monday, Wednesday, and Friday throughout the 60-day regimen. The normal control cohort consisting of unaffected littermate animals (wt/wt) were provided standard mouse chow. Experiments used animals in the fed state. Animals were sacrificed (control cohorts, experimental cohorts) by $C0_2$ asphyxiation.

2.2. Skeletal stem cell culture and osteoblast differentiation

Each animal cohort (experimental, control) contained at least 4 animals with equivalent or near equivalent male/female representation. SSCs were prepared from 2 to 3 month animals (control, Pah^{enu2}) as described [26–29]. Briefly, bone marrow was aspirated with culture media (RPMI-1640 10% fetal calf serum) from the femur and tibia. Cells are plated for ~18 h at 37 °C to remove rapidly adhering fibroblast-like cells. Non-adherent cells were quantified and re-plated at a density of $2*10^6$ cells/cm^2. After 72 h non-adherent cells were discarded and adherent cells provided SSC proliferation medium (MesenCult media, Stemcell Technologies). Osteoblast differentiation studies use passage 4-7cells. Osteogenic differentiation is as described applying supplemented MesenCult media (35 μg/mL L-ascorbic acid, 10 mM β-glycerol phosphate, 10pM adrenocorticotropic hormone, 10 nM 1α,25-dihydroxyvitamin D3, 0.5 mM $CaCl_2$) for 14 or 21 days [31]. SSC osteoblast differentiation media in Pah^{enu2} cultures is supplemented with Phe to 1200 μM. Pah^{enu2} SSC cultures receiving creatine supplementations includes 0.5 mM creatine and Phe to 1200 μM. Throughout differentiation, culture media was replaced every other day. SSC osteoblast differentiation by *in situ* alkaline phosphatase activity and mineralization were as described [26–29]. Briefly *in situ* alkaline phosphatase activity used 0.01% napthol AS-MX substrate and fast blue to visualize product [26–29]. Visualizing mineralization applied von Kossa silver staining [26–28]. Densitometry analysis was performed after grayscale image conversion, inverted, and the mean white area was measured with ImageJ software. Data analysis was performed as described [26–29].

2.3. Cellular ATP determination

SSCs were cultured, induced to osteoblast differentiation, and supplemented (Phe, creatine) as described above (Mesenchymal Stem Cell Culture and Osteoblast Differentiation) generating equivalent cohorts. After 21 days of osteoblast differentiation, cellular ATP quantification was performed. The ATPlite Luminescence Assay system (Perkin Elmer) was applied according to manufacturer's instructions. Data analysis applied student *t*-test with GraphPad software.

2.4. Micro-computed tomography

Experimental cohorts (Pah^{enu2} Phe restricted diet plus creatine, Pah^{enu2} unrestricted diet plus creatine) and control cohorts (C57bl/6, unmanaged Pah^{enu2}, Phe restricted Pah^{enu2}) each contained 6 animals with equal male *vs* female representation. All animals were sacrificed 2 months post-weaning. Trabecular bone analysis applied blinded micro-computed tomography as described [26–29]. Briefly, fixed lumbar vertebrae were scanned in 70% ethanol at 6 μm resolution with a 0.25 mm aluminum filter; voltage and current were set at 69 kV and 100 μA. Image reconstruction utilized NRecon and InstaRecon software. Cross sectional images used Dataviewer software. Quantitative analysis assessed a 1.2 mm region at the midpoint of the fourth lumbar vertebrae. Determined were bone volume/total volume, bone surface density, trabecular number, and total porosity. Data represents that of the entire cohort (experimental, control) not parsed by sex. Data analysis is as described [26–29]. Briefly, individual group comparisons (*e.g.* 1:1 comparisons) are applied comparing experimental cohorts to the appropriate control cohort (*e.g.* Pah^{enu2} untreated *vs.* Pah^{enu2} with Phe-restriction plus creatine) with data assessed with GraphPad software and student *t*-test.

3. Results

3.1. Creatine energy substrate increased Pah^{enu2} in situ alkaline phosphatase activity

Fig. 1 provides *in situ* alkaline phosphatase activity. We reported that Pah^{enu2} SSCs in osteoblast differentiation have a statistically significant decrease in alkaline phosphatase activity compared to unaffected controls [26–29]. Pah^{enu2} SSCs provided creatine energy substrate demonstrate a statistically significant increase in *in situ* alkaline phosphatase activity. Similar to our study with glutamine energy substrate [29], mineralization did not increase with creatine energy substrate (data not shown).

3.2. Creatine energy substrate increases Pah^{enu2} SSC cellular ATP content in osteoblast differentiation

Fig. 2 applied the ATPlite assays to quantify ATP in SSCs following 14 days of osteoblast differentiation. Creatine energy substrate induces a statistically significant increase in cellular ATP content of Pah^{enu2} SSC in osteoblast differentiation.

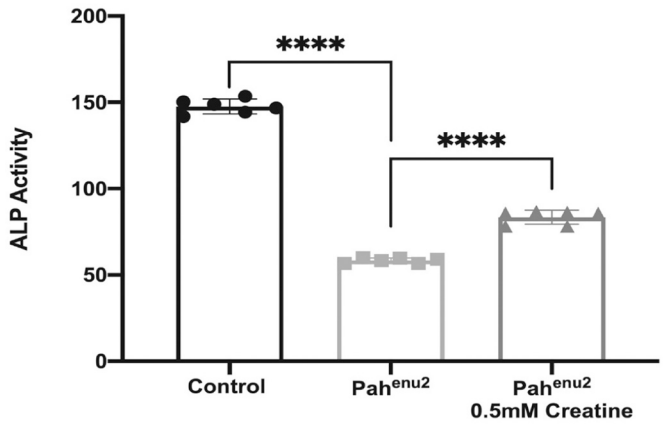

Fig. 1. Creatine energy substrate anaplerosis increases alkaline phosphatase activity in Pah^{enu2} SSCs in osteoblast differentiation.
SSCs from Pah^{enu2} (2 male, 3 female) and C57bl/6 (2 male, 2 female) were differentiated in standard media or media supplemented with 0.5 mM creatine. **** $p = 0.0001$. SSCs derived from male and female animals responded similarly.

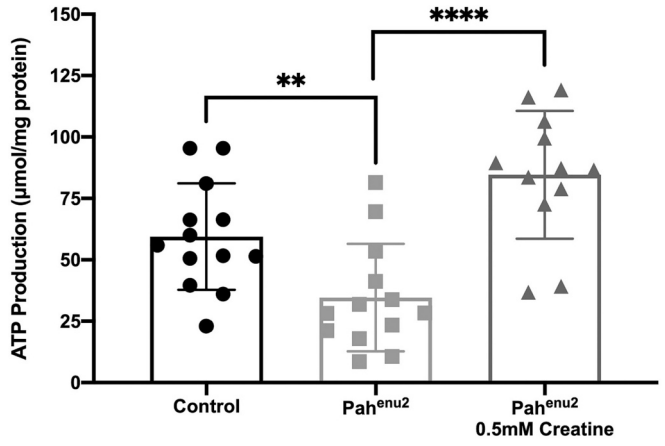

Fig. 2. Creatine Energy Substrate Anaplerosis Increases Cellular ATP in Pah^{enu2} SSCs in Osteoblast Differentiation.
SSCs from Pah^{enu2} (3 male, 4 female) and C57bl/6 (2 male, 2 female) were differentiated in standard media or media supplemented with 0.5 mM creatine. ** $p = 0.01$ **** p = 0.0001. SSCs derived from male and female animals responded similarly.

3.3. Creatine energy substrate increases in vivo bone density among Phe restricted Pah^{enu2} animals

In vivo bone density assessment by microcomputed tomography was applied to Pah^{enu2} treatment cohorts (**1.** Phe unrestricted plus creatine, **2.** Dietary Phe restriction plus creatine) and control cohorts (1. C57bl/6 control, 2. Pah^{enu2} hyperphenylalaninemic control; 3. Pah^{enu2} dietary Phe restricted control). At sacrifice, the weight of Pah^{enu2} animals was determined with no significant difference between cohorts (data not shown). Microcomputed tomography, assayed the fourth and fifth lumbar vertebrae as previously reported [26–29]. Fig. 3 provides data sets for each metric assessed in control and experimental cohorts. Statistical comparisons are 1:1 with appropriate controls. All bone metrics (bone volume/total volume, bone surface density, trabecular number, total porosity) indicate a common degree of osteopenia among Pah^{enu2} hyperphenylalaninemic controls, dietary Phe restricted Pah^{enu2} controls, and the Pah^{enu2} experimental hyperphenylalaninemic cohort receiving creatine supplementation. Similar statistically significant differences (Figs. 3A-D) are realized between the dietary Phe restricted

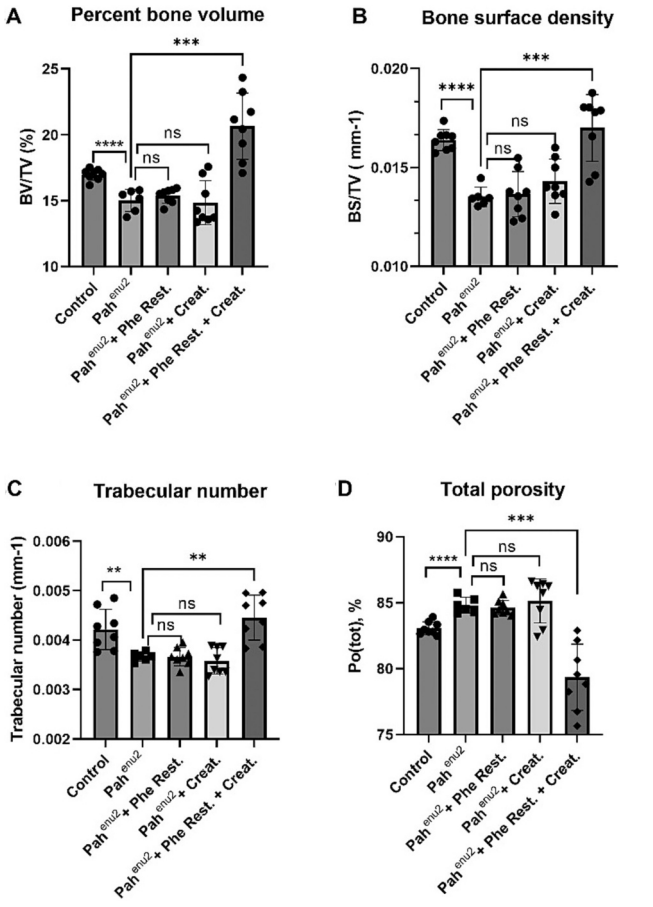

Fig. 3. Creatine energy Substrate Increases Pah^{enu2} Bone Density in Phe Restricted Animals.
Static histomorphometry of control wt/wt animals, unmanaged Pah^{enu2}, Phe-restricted Pah^{enu2}, unmanaged Pah^{enu2} provided creatine energy substrate, and, Phe-restricted Pah^{enu2} provided creatine energy substrate. Control animals are background strain C57bl/6 littermates. All cohorts have a minimum of five animals. Histomorphometry metrics determined in 3rd or 4th lumbar vertebrae. 3A. Percent bone volume, 3B. Bone surface density, 3C. Trabecular number, 3D. Total porosity. Rest = restricted, Creat = creatinine, ** = $p \leq 0.01$; *** $p \leq 0.001$; **** = $p \leq 0.0001$, ns = no significant difference. Male and female animals responded similarly.

Pah^{enu2} cohort and hyperphenylalaninemic Pah^{enu2} plus creatine cohort when compared to C57bl/6 background strain littermates (data not shown). Pah^{enu2} animals concurrently receiving dietary Phe restriction and creatine supplementation display improvement of every bone metric with bone density rivaling that of the C57bl/6 unaffected littermate control cohort.

4. Discussion

PKU clinical phenotypes occur in brain and bone, neither tissue express the PAH gene nor hydroxylate Phe. There is evidence of an emerging, incompletely penetrant PKU ocular phenotype being an additional clinical phenotype in a tissue where neither PAH is expressed nor Phe hydroxylated [32,33] PKU clinical phenotypes are secondary to biochemical dysregulation impacting susceptible tissues and cells. The universally osteopenic Pah^{enu2} mouse is an ideal osteopenia model. We determine Pah^{enu2} SSCs have an osteoblast developmental deficit where energy dysregulation and oxidative stress contribute [26–29]. In the context of hyperphenylalaninemia, Pah^{enu2} SSCs provided Gln energy substrate present increased *in vitro* alkaline phosphatase activity (a measure of osteoblast differentiation) and mitochondria oxygen

consumption (a measure of oxidative energy production) [29]. *In vivo* Gln energy substrate improved Pah^{enu2} bone density concurrent to unregulated hyperphenylalaninemia [29]. This study reports creatine energy substrate increased *in vitro* osteoblast differentiation, *in vitro* SSC cellular ATP content, and *in vivo* bone density. However, recovery of bone density required concurrent Phe-restriction demonstrating complexity of PKU energy dysregulation relating to osteopenia resolution.

SSC osteoblast differentiation and bone formation is energy intensive. We demonstrated energy dysregulation in PKU brain and bone, postulating energy deficit is an under-appreciated disease driver not addressed by current interventions [34]. As dietary Phe restriction with amino acid defined diet does not increase Pah^{enu2} bone density, causation by asymmetric LAT1 (SLC7A5 gene product) amino acid transport is refuted. Phe restriction with glycomacropeptide, while not reducing plasma Phe to the degree of amino acid defined diet (~200 μM Phe amino acid diet *vs.* ~700 μM Phe glycomacropeptide), improves bone density [35]. We identified superoxide oxidative stress in Pah^{enu2} SSCs [28]. Glycomacropeptide reduces inflammatory processes which may facilitate bone mass recovery [36].

Applying creatine to treat bone density has principally been done in geriatric patients. Conflicting reports create controversy regarding creatine efficacy to improve density of normal bone [37–40]. Our study applies creatine in the context of PKU disease pathology that elicits an SSC energy deficit mediating osteopenia. Alternative pathway creatine energy repletion improves Pah^{enu2} SSC *in vitro* function, SSC *in vitro* ATP content, and *in vivo* bone density. Providing creatine to differentiating SSCs from young adult Pah^{enu2} present increased alkaline phosphatase activity (Fig. 1) indicating augmented osteoblast differentiation. Increased *in vitro* osteoblast differentiation secondary to creatine supplements was previously reported [41]. Similar to glutamine substrate anaplerosis [29], creatine did not improve SSC mineralization. During osteoblast differentiation, Pah^{enu2} SSCs have lower cellular ATP content than SSCs from C57bl/6 background strain littermates. Creatine increased Pah^{enu2} SSC cellular ATP content (Fig. 2) that we suggest enables energy intensive osteoblast differentiation (*e.g.* collagen synthesis, alkaline phosphatase activity). Bone marrow expresses X-linked SLC6A8 high affinity creatine transporter. The creatine kinase/phosphocreatine system creates a cytosolic phosphocreatine pool buffering energy and maintaining the ATP/ADP ratio during energy intensive osteoblast differentiation. Biochemical dysregulation is causal to all PKU clinical phenotypes. Creatine essentially normalized bone density in Phe-restricted Pah^{enu2}; however, no effect is realized in hyperphenylalaninemic Pah^{enu2} (Fig. 3). Our Pah^{enu2} metabolomic assessments show high-level over-representation of Phe, Phe catabolites, Phe conjugates, oxidative stress response analytes, energy related analytes, and other analytes [42]. We hypothesize a creatine pathway element (transport, creatine kinase, other) is inhibited by an over-represented analyte(s) intrinsic to untreated PKU. Presently there is no insight regarding the offending analyte or stage wherein creatine utilization is disallowed; additional investigation is required to riposte these outstanding elements. Notable is creatine increases *in vitro* alkaline phosphatase activity and cellular ATP content in the context of 1200 μM Phe, while in hyperphenylalaninemic Pah^{enu2} animals *in vivo* bone density did not increase. Two elements likely contribute. *In vitro* cell culture studies applied 0.5 mM creatine, a concentration unlikely to occur within the *in vivo* bone compartment. The high creatine concentration in cell culture may over-come inhibitory elements. PKU cell culture studies apply a Phe concentration of 1200 μM the metric defining classical PKU. *In vivo* Pah^{enu2} Phe homeostasis is typically 2000–2200 μM and spikes higher postprandial. High *in vivo* Phe concentration and the plethora of other secondary over-represented analytes [42] may induce greater inhibitory effect.

5. Conclusion

Creatine and glutamine energy substrates increase *in vivo* Pah^{enu2} bone density; however, effect occurs at opposite ends of the Phe homeostasis spectrum highlighting complexities of energy anaplerosis. It is tempting to suggest Gln anaplerosis may be utilitarian in therapy non-compliant osteopenic PKU patients while creatine may be better suited to address osteopenia in therapy compliant patients. Additional investigation is required to characterize efficacy and precise mechanisms of alternative anaplerosis regimens. The role of creatine in cerebral energy metabolism is unequivocal; an outstanding question is how creatine may improve PKU neurologic disease. Even among adult/adolescent PKU patients retaining therapy compliance, treatment refractory neurologic disease (cognitive decline, executive function deficit, neuropsychiatric phenotypes) is common. We previously demonstrated PKU cerebral energy deficit [40], posit energy deficit contributes to neurologic phenotypes, and suggest creatine energy support may provide intervention opportunity for both osteopenia and neurologic phenotypes.

Author contributions

Dobrowolski & Blair: study design, data interpretation, manuscript composition.

Tourkova & Larrouture: histomorphometry, SSC cellular studies.

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Declaration of Competing Interest

None of the authors have competing interests relating to these studies.

Data availability

Data will be made available on request.

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