Enzyme replacement therapy prevents loss of bone and fat mass in murine homocystinuria

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Abstract

Skeletal and connective tissue defects are the most striking symptoms in patients suffering from classical homocystinuria (HCU). Here, we determined body composition and bone mass in three mouse models of HCU and assessed whether a long-term administration of enzyme replacement therapy (ERT) corrected the phenotype. The mouse models of HCU were analyzed using dual-energy X-ray absorptiometry and the data were complemented by plasma biochemical profiles. Both the mouse model lacking CBS (KO) and the one expressing human CBS mutant transgene on a mouse CBS null background (I278T) showed marked bone loss and decreased weight mostly due to a lower fat content compared with negative controls. In contrast, the HO mouse expressing the human CBS WT transgene on a mouse CBS null background showed no such phenotype despite similar plasma biochemical profile to the KO and I278T mice. More importantly, administration of ERT rescued bone mass and changes in body composition in the KO mice treated since birth and reversed bone loss and improved fat content in the I278T mice injected after the development of clinical symptoms. Our study suggests that ERT for HCU may represent an effective way of preventing the skeletal problems in patients without a restricted dietary regime.

KEYWORDS
bone mineralization, bone–fat interactions, DXA, osteoporosis, preclinical studies

1 | INTRODUCTION

Classical homocystinuria (HCU, MIM \#236200) is a monogenic inborn error of sulfur amino acid metabolism caused chiefly by missense mutations in the cystathionine beta-synthase (CBS) gene. The CBS enzyme catalyzes the conversion of homocysteine, a toxic intermediate of the methionine cycle, to cystathionine and thus diverts the sulfur from methionine recycling to cysteine synthesis through the transsulfuration pathway (Finkelstein, 1990). Human CBS is a homotetrameric enzyme containing two cofactors (heme and pyridoxal-5'-phosphate: PLP) and whose activity is regulated by S-adenosylmethionine binding to the C-terminal region of a 63 kDa-long subunit (Majtan, Pey, Ereno-Orbea, Martinez-Cruz, & Kraus, 2016). The lack of CBS activity in HCU patients results in accumulation of the metabolites above the block, such as homocysteine (Hcy), S-adenosylhomocysteine, and methionine (Met), and depletion of metabolites below the block, such as cystathionine (Cth) and cysteine (Cys). This metabolic imbalance is accompanied by a variety of pathological abnormalities in four major areas: the eye (myopia, ectopia lentis), the skeletal and connective tissues (osteoporosis, scoliosis, brittle and thin skin, fine fair hair), the vascular system (thromboembolism, stroke), and the central nervous system (mental retardation, seizures) (Mudd, Levy, & Kraus, 2001).

The severity of complications primarily depends on two main factors: the age, when the patient was initially diagnosed, and the amount of his/her residual CBS activity. Roughly half of the HCU patient population clinically responds to high doses of pyridoxine (vitamin B\textsubscript{6}), a precursor of PLP, which results in an increase in residual CBS activity leading, in limited number of cases, to normalization of plasma metabolites. In the remaining patients, however, metabolic control can be achieved only by methionine (protein)-restricted diet with cysteine supplementation. The diet of HCU patients is often combined with betaine, which serves as a methyl donor for alternative, liver-dependent conversion of Hcy back to Met via betaine homocysteine methyltransferase (Finkelstein, 1990). Unfortunately, many patients and their families cannot follow the strict dietary regime, which results in a poor compliance and exacerbation of complications, mainly thromboembolic events (Walter, Wraith, White, Bridge, & Till, 1998). Thus, an alternative treatment would lessen or ideally eliminate the restricted diet and restore the normal metabolic balance.

To understand the pathogenic mechanism of CBS deficiency and to enable development of novel therapeutics, several mouse models of HCU were made. The mouse entirely lacking CBS activity (the CBS KO mouse) suffered from a neonatal lethality due to liver failure thus severely restricting the utility of this model (Watanabe et al.,...
In order to circumvent the survival problem, the transgenic mice expressing human CBS p.Ile278Thr mutant cDNA from a zinc-inducible promoter was engineered (the I278T mouse) (Wang et al., 2005). The I278T alteration (c.833T > C) is the most common pyridoxine-responsive mutation observed in HCU patients. However, no significant change in I278T mice plasma metabolite profiles was detected when given pyridoxine via drinking water despite an 8-fold increase in the plasma PLP levels (Chen, Wang, Fazlileva, & Kruger, 2006; Wang et al., 2005). Although the transgene conferred the mice with only 2%–3% of CBS WT activity, it prevented neonatal lethality, but did not rescue HCU manifesting biochemical and clinical sequelae similar to human patients (Gupta & Kruger, 2011; Gupta et al., 2009). In addition to pyridoxine supplementation, various dietary interventions commonly used in HCU patients have been studied to impact disease phenotype in I278T mouse (reviewed in Gupta, Wang, & Kruger, 2016). Only a decreased methionine intake was able to significantly affect the I278T mouse phenotype, while betaine and cysteine supplementation resulted in a mixed or essentially no improvement of the disease. Another potentially valuable HCU mouse model was generated by inserting the whole human CBS WT gene on a mouse CBS null background (so called “human only” HO mouse) (Maclean et al., 2010). The expression of human WT enzyme was limited in HO mice and varied in different organs from 2% of CBS WT activity in the kidney to 16% in the brain. The HO model reproduces the biochemical profile of human HCU, is not neonatal lethal, and does not incur any liver damage. Thus, there are enough animal models of HCU for long-term proof of concept studies.

We have recently introduced an enzyme replacement therapy (ERT) for HCU and showed that the administration of polyethylene glycol (PEG)-modified truncated human CBS lacking the C-terminal regulatory domain (PEG–CBS) to HO mice resulted in ∼75% decrease of Hcy and normalization of Cys in plasma (Bublil et al., 2016). In addition, this improved plasma metabolite profile was accompanied in KO mice by increased survival and decreased liver disease. Engineering of PEG–CBS molecule by testing different PEG chemistries resulted in identification of conjugates, which retained biological activity for a long-term period (Majtan, Park, Carrillo, Bublil, & Kraus, 2017). Here, we present the impact of PEG–CBS on bone mass and body composition in three different mouse models of HCU.

2 METHODS

2.1 Chemicals

Unless stated otherwise, all materials were purchased from Sigma-Aldrich (St. Louis, MO, USA) or Thermo Fisher Scientific (Waltham, MA, USA).

2.2 Protein purification and PEGylation

Human truncated CBS carrying the p.Cys15Ser mutation (htCBS C15S) was expressed and purified as described elsewhere (Bublil et al., 2016). The purified enzyme was formulated and concentrated using Labscale TFF system (Merck Millipore, Burlington, MA, USA) equipped with Pellicon XL 50 Ultracel 30 cartridge (regenerated cellulose 30 kDa MWCO membrane) into 100 mM sodium phosphate pH 7.2. The PEG (ME-200GS; linear 20 kDa NHS-activated PEG) was purchased from NOF (Tokio, Japan). PEGylation of htCBS C15S was carried out in 50 mM sodium phosphate pH 7.2 by adding PEG dissolved in milliQ water in a 10-fold molar excess of PEG over htCBS C15S subunit to a final protein concentration of 5 mg/ml. The reaction was carried out at 4°C overnight. Subsequently, the reaction mixture was diluted twice with milliQ water, buffer exchanged into Gibco 1× PBS (Thermo Fisher Scientific) and concentrated using Labscale TFF system with Pellicon XL 50 Biomax 100 cartridge (polyethersulfone 100 kDa MWCO membrane; Millipore). The final PEG–CBS conjugate was filter sterilized (Millipore’s 0.2 μm PVDF membrane filter), aliquoted, flash frozen in liquid nitrogen and stored at −80°C.

2.3 Animals

All animal procedures were approved under animal protocol# B-49414(03)1E by the University of Colorado Denver (UCD) Institutional Animal Care and Use Committee. The UCD animal facility is an AAALAC-accredited (#00235), Public Health Service-assured (#A 3269-01), and USDA-licensed (#B-4-R-0059) institution. Three models of murine HCU (HCU mice) were used in the studies summarized in this report. A breeding pair of heterozygous CBS KO mice of the C57BL6 strain background was originally purchased from the Jackson Laboratory (Bar Harbor, ME, USA) and subsequently propagated and genotyped at UCD as described previously (Bublil et al., 2016; Watanabe et al., 1995). The breeding pair of heterozygous transgenic I278T mice of the C57BL6 background was generously gifted by Dr. Warren Kruger (Fox Chase Cancer Center, Philadelphia, PA) and subsequently propagated and genotyped at UCD as described previously (Wang et al., 2005). The HO mice expressing low levels of human CBS WT were previously generated in our lab (Maclean et al., 2010) and propagated and genotyped at UCD as described previously (Bublil et al., 2016). Animals were maintained on extruded standard diet 2920X (Envigo, Madison, WI, USA) containing 18 mg/kg pyridoxine. To produce homozygous KO /+−, I278T /−−, and HO /−− mice, homozygous −/+ males were bred with heterozygous females to produce litters consisting of equal numbers of homozygous /−− and heterozygous /+/− pups. At 7–10 days of age, mice were foot pad tattooed and collected tail snips were used for extraction of genomic DNA and identification of mouse genotype using real-time QPCR as described previously (Bublil et al., 2016). Weaning and ear tagging was done at 21 days of age, when male and female siblings were gang housed separately with no more than 5 mice per cage. A single-use lancet for submandibular bleeding was used for blood collection into Capiject T-MLHG lithium heparin (12.5 IU) tubes with gel (Terumo Medical Corporation, Somerset, NJ, USA). Tubes were then centrifuged at 1,200×g for 10 min, followed by transfer of plasma to 1.5 ml tubes and storage at −80°C.

2.4 Study design

Since the KO mouse phenotype is neonatal lethal, we treated entire litters from birth with three subcutaneous (SC) injections of PEG–CBS
(7.5 mg/kg) given per week to rescue them and allow them to survive into adulthood (Bublil et al., 2016). At 35 days of age, the mice were randomized into three groups. The first group consisted of heterozygous KO +/− mice (n = 8M+6F), which were left untreated from day 35 on and used as negative controls. The second group contained KO −/− mice (n = 6M+10F), which were left untreated from day 35 on, thus serving as positive controls. The third group of KO −/− mice (n = 8M+9F) continued receiving treatment as during the neonatal period. The mice were terminated at the age of ~5 months.

As I278T transgenic mice readily survive into adulthood, it allowed us to study the effect of ERT on substantially older mice. Average age of the 1278T mice at the beginning of the study was 6.5 months and these mice had developed a full spectrum of the phenotypical changes including osteopenia, decreased body weight, low fat content or facial alopecia (Gupta, Melnyk, & Kruger, 2014). Mice were randomized into three groups. The first group consisted of untreated heterozygous I278T +/− mice (n = 10M+11F) used as negative controls. The positive control group contained untreated I278T −/− mice (n = 8M+8F). The third group involved I278T −/− mice (n = 7M+9F) injected thrice weekly with PEG–CBS (SC, 7.5 mg/kg) for a period of ~6 months. The study concluded when the mice reached age of 12–13 months.

The study on HO transgenic mice included a group of untreated HO −/− mice (n = 8M+11F) of the average age 5.5 months, which were compared with age-matched, untreated healthy heterozygous HO +/− controls (n = 9M+10F).

2.5 Dual-energy X-ray absorptiometry measurement

Bone density and body composition was measured in anesthetized mice (combination of 60 mg/kg ketamine and 15 mg/kg xylazine in PBS injected intraperitoneally) using a GE Lunar PIXImus scanner (Lunar Corp., Madison, WI, USA; software version 2.10). Mice were placed on a tray in the prone position and scanned. The analyzed region represented whole body area excluding head and neck area as well as tail. After the scan, mice were placed back to their cage to recover.

2.6 Determination of metabolite concentrations

Plasma metabolites were determined by stable-isotope-dilution gas chromatography mass spectrometry as previously described (Allen, Stabler, & Lindenbaum, 1993). The analysis was performed in a blinded fashion without knowledge of the animal genotype and/or treatment regimen. The plasma samples (~40 µl) were collected a week prior to the DEXA scanning and 24 hr after the injection of treated mice.

2.7 Statistical analysis

All data are presented as means ± standard errors (SEMs). Statistical comparisons of two groups were conducted using an unpaired, two-tailed Students t-test. Statistical analysis of three or more factor levels was conducted by ANOVA followed by Tukey’s multiple comparison test to determine significance. For all the tests, a value of P < 0.05 was considered significant. Significance in figures is shown by letters above the error bars with no letter indicating nonsignificance.

3 RESULTS

3.1 ERT prevented osteopenia and changes in body composition of KO mice

Figure 1 shows results of dual-energy X-ray absorptiometry (DXA) analysis of body composition and bone mass in negative controls compared with both untreated and PEG–CBS-treated KO −/− mice. The DXA scans were performed when the mice reached age of ~5 months. Weight and fat content but not the amount of lean mass of the KO −/− mice was significantly decreased compared with negative controls (Figure 1A–C). This effect was more pronounced in males than females. Treatment of KO −/− mice since birth normalized weight and fat content in males, but fat content in females remained similar compared with negative controls. Compared with negative controls, the KO −/− mice developed a significant osteopenia characterized by decreased bone mineral density (BMD; Figure 1D) and bone mineral content (BMC; Figure 1E). The long-term treatment of the KO −/− mice yielded normalized BMD and BMC to the levels seen in negative controls. Interestingly, changes in bone density and efficacy of PEG–CBS on BMD and BMC were similar between males and females. Taken together, continuous ERT administration from birth prevented loss of bone tissue and negative changes in body composition in the KO −/− mice.

3.2 ERT rescued bone loss and improved body composition in I278T mice

Further, we determined body composition and bone mineralization in ~12-month-old untreated I278T −/− mice and compared them with age-matched negative controls as well as I278T −/− mice treated with PEG–CBS for a period of ~6 months prior to the measurement (Figure 2). Similarly to significantly younger KO mice, weight and fat content and in females also the amount of lean mass of the untreated I278T −/− mice was significantly reduced compared with negative controls (Figure 2A–C). Treatment of I278T −/− mice for the past 6-month period rescued fat content in both sexes and weight in males, but failed to improve weight and amount of lean mass in females. Untreated I278T −/− mice suffered from significant loss of bone mass as evidenced by decreased BMD and BMC compared with negative controls (Figure 2D and E). On the other hand, the treated I278T −/− mice showed no signs of osteopenia with BMD and BMC values similar to those for negative controls. Taken together, ERT administration to fully symptomatic I278T −/− mice prevented loss of bone mass and improved body composition in the treated mice.

3.3 HO mice did not suffer from decreased muscle and fat content or bone loss

Finally, we determined body composition and bone density in the third studied HCU mouse model, the HO mice (Figure 3). Surprisingly, unlike the KO −/− and I278T −/− mice, the homocystinuric HO −/− mice of ~5–6 months of age did not suffer from impaired body composition or a reduced bone mass compared with age-matched negative
FIGURE 1  Body composition and bone density of KO mice. The treated KO −/− mice (black; n = 8M+9F) received PEG-CBS (7.5 mg/kg, SC, thrice a week) since birth until they reach age of ∼5 months, when they were scanned using DXA and compared with age-matched untreated KO +/− negative controls (white; n = 8M+6F) and KO −/− positive controls (gray; n = 6M+10F). Individual panels show total weight (A), lean mass (B), fat content (C), bone mineral density (D), and bone mineral content (E). Significance was determined by ANOVA followed by Tukey’s post-hoc test and is designated by letters above the error bars. No letter indicates nonsignificance.

FIGURE 2  Body composition and bone density of I278T mice. The I278T −/− mice (black; n = 7M+9F), showing full spectrum of HCU clinical symptoms at the age of ∼6.5 months, received PEG-CBS (7.5 mg/kg, SC, thrice a week) for the following ∼6 months. At the age of 12–13 months, the mice were scanned using DXA and compared with age-matched untreated I278T +/− negative controls (white; n = 10M+11F) and I278T −/− positive controls (gray; n = 8M+8F). Individual panels show total weight (A), lean mass (B), fat content (C), bone mineral density (D), and bone mineral content (E). Significance was determined by ANOVA followed by Tukey’s post-hoc test and is designated by letters above the error bars. No letter indicates nonsignificance.
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FIGURE 3 Body composition and bone density of HO mice. The HO +/− negative controls (white; n = 9M+10F) were compared with HO −/− positive controls (gray; n = 8M+11F). The DXA scans were performed when all the mice reached the age of ∼6 months. Individual panels show total weight (A), lean mass (B), fat content (C), bone mineral density (D), and bone mineral content (E). Significance was determined by an unpaired, two-tailed Students t-test and is designated by letters above the error bars. No letter indicates nonsignificance.

3.4 Is there a threshold for hyperhomocysteinemia resulting in osteopenia and impaired body composition?

The surprising result with the HO −/− mice prompted us to compare plasma levels of sulfur amino acids in the studied mice (Figure 4). As expected, the more severe phenotype of the HCU mouse model (i.e., KO (Figure 4A) > I278T (Figure 4B) > HO (Figure 4C)) and the smaller amount of residual CBS activity (i.e., KO < I278T < HO), the higher plasma Hcy levels were found compared with negative controls. Although all three HCU mouse models showed severely elevated, but quite variable Hcy plasma levels, their concentrations of plasma Cys were similarly and significantly decreased to about 50% of those found in negative controls. On the other hand, plasma Met was slightly, but significantly increased in the HCU mice compared with negative controls. Treatment of KO −/− and I278T −/− with PEG–CBS resulted in substantial decrease of Hcy levels, gross elevation of Cth levels, normalization or improvement of plasma Cys levels and normalization of plasma Met concentrations (Figure 4A and B). Taken together, there seems to be a correlation between impaired body composition and bone density and Hcy levels in murine HCU (Table 1).

4 DISCUSSION

Although not evident at birth and typically not in infants and very young children (Smith, 1967), skeletal abnormalities are among the most striking phenotypical features in HCU patients. Notably, a thinning and lengthening of the long bones result in tall and thin individuals with an appearance similar to that of Marfan syndrome (Mudd et al., 2001). However, osteoporosis, particularly of the spine, which is associated with scoliosis, is one of the distinguishing features of the disease and the most consistent skeletal change in HCU patients (Mudd et al., 2001). It has been described as early as at the age of one year with a frequency often as high as 90%–100% among homocystinuric individuals thus allowing initial diagnosis solely on the basis of a radiological examination (Schedewie, Willich, Grobe, Schmidt, & Muller, 1973). A natural history study showed that a half of untreated HCU patients have radiological evidence of spinal osteoporosis by 15 years of age (Mudd et al., 2001). DXA measurement in late diagnosed HCU patients showed similar findings of osteoporosis in spine and femur for both younger and older patients regardless of a severity of the disease (Parrot, Redonnet-Vernhet, Lacombe, & Gin, 2000).

Here we found that the KO and the I278T, but not the HO mouse model reproduced the skeletal and connective tissue phenotype typical for HCU patients. Our results suggest there is a threshold level of HCU above which an osteopenia, decreased weight and decrease fat controls. On the contrary, all the followed parameters, including weight, lean mass, fat content, BMD, and BMC, were slightly increased in both HO −/− males and females compared with the negative controls. As HO −/− mice failed to reproduce the phenotype shown by the KO and I278T, the treatment with PEG–CBS was not performed.
content develop in mice. Similar threshold effect was observed previously comparing phenotypes of transgenic KO mice expressing either human CBS WT (Tg-WT) or the I278T mutant (Gupta et al., 2009). The Tg-WT mice failed to exhibit any of the phenotypic traits found in the I278T mice, such as facial alopecia, low bone mass, ER stress in the liver and kidney or reduced mean survival, despite plasma Hcy levels greatly elevated (30-fold, ∼170 μM) above normal compared to a more severe elevation in I278T mice (54-fold, ∼300 μM). Thus, the threshold for significant effects on the phenotype of the HCU model was estimated to be in the range of 170–300 μM Hcy in plasma (Gupta et al., 2009). In that respect, the HO model resembles the plasma metabolite profile and phenotypic features of the Tg-WT mouse. In addition, here we showed that the HO mice do not suffer from either reduced bone mass and fat loss suggesting that Hcy levels are the primary driving force of osteopenia and impaired lipid metabolism in homocystinuric mice. More importantly, we showed that this phenotype can be entirely prevented or reversed by ERT administered from birth (KO mice) or later in life once the phenotype is fully expressed (I278T mice). Interestingly, both Tg-WT and HO mouse models have increased plasma Cth levels compared with healthy controls and other HCU mouse models, such as the I278T or KO mice. Previously, it was shown that hepatic steatosis and renal tubule damage is greatly attenuated in HO

**FIGURE 4** Plasma sulfur amino acid profiles in three mouse models of HCU. Panels A, B, and C show plasma levels of total Hcy, Cth, total Cys, and Met in the KO, I278T, and HO mice studied by DXA, respectively. Note that Cth concentrations are shown in a different scale (on the right) compared with other metabolites in all three panels. Significance was determined by ANOVA followed by Tukey’s post-hoc test (for KO and I278T mice shown in panels A and B) or an unpaired, two-tailed Students t-test (for HO mice shown in panel C) and is designated by letters above the error bars.
mice after treatment with tunicamycin compared with the WT controls (Maclean et al., 2012). In addition, short-term elevation of Cth levels, induced by the cystathionine gamma-lyase inhibitor propargylglycine, protected WT mice against tunicamycin-induced steatotic hepatopathy, renal tubular injury and apoptotic cell death (Maclean et al., 2012). Thus, it is plausible that increased plasma Cth levels might be protecting the HO mouse from the HCU clinical phenotype.

Another phenotypic trait commonly shared by HCU patients is a thin build, low body fat content, and lower body mass index (BMI) than the age-matched general population (Brenton, Dow, James, Hay, & Wayne-Davies, 1972; Gibson, Carson, & Neill, 1964; Mudd et al., 2001; Poloni et al., 2014). Here we show that the KO and the I278T but not the HO mouse model replicate the body composition typical for HCU patients by overall lower mass and markedly decreased body fat content. The ERT was less effective correcting this phenotype compared with normalization of bone density, but often resulted in significant improvement of weight and fat content, particularly in males, with no effect on lean mass.

Although SC injected PEG–CBS was unable to fully normalize the plasma Hcy levels, the concentration of Hcy was markedly reduced compared with the untreated controls. The balance between plasma Hcy and Cys seems to be critical for proper bone mineralization as well as body composition as supported by cross-sectional observational studies of healthy population. For example, plasma Hcy levels were significantly inversely associated with both bone ultrasound parameters and BMD (Elsborhaggy et al., 2009; Enneman et al., 2014), while plasma Cys concentrations showed a strong positive association with BMI mediated through fat mass (Elsborhaggy et al., 2008). A pathogenic mechanism responsible for skeletal and connective tissue abnormalities as well as reduced BMI in HCU is not fully understood. It has been shown that hyperhomocysteinemia promoted bone resorption (Herrmann et al., 2009; Herrmann, Widmann, & Herrmann, 2005; Ozdem et al., 2007), increased oxidative stress and apoptosis of osteoblasts (Park, Kim, Kim, Oh, & Cho, 2012; Tyagi et al., 2011) and decreased bone blood flow, remodeling, mineralization by affecting bone extracellular matrix (Khan et al., 2001; Tyagi et al., 2011). Collagen is a major extracellular matrix component of bone providing structural support coming from specific cross-links involving lysine residues within and between collagen chains (Boskey, 2013). N-homocysteinylation of these lysines was hypothesized to impair cross-linking (Jackson, 1973; Lubec, Fang-Kircher, Lubec, Blom, & Boers, 1996), but only recently proven in I278T mice (Perla-Kajan et al., 2016), thus at least partially explaining skeletal and connective tissue defects in HCU. The study comparing Tg-WT and I278T mice showed that, while the amount of bound Hcy to plasma proteins is similar, the amount of total free Hcy (i.e., sum of free reduced Hcy and Hcy from Hcy–Hcy and Hcy–Cys disulfides) was significantly higher in I278T mice suggesting that plasma proteins have a maximum binding capacity for Hcy (Gupta et al., 2009). Thus, the elevated total free Hcy was correlated with the severity of the mice phenotype, which is also consistent with the data from HCU patients. Relationship between plasma total Hcy and free Hcy in a group of 46 Irish patients showed that free Hcy is undetectable below a total Hcy of 55 μM recommending the treatment target limit of 100–120 μM total Hcy, which would correspond to 8–11 μM in free Hcy (Morris et al., 2017; Yap et al., 2001). Indeed, treatment of the KO and I278T mice with PEG–CBS resulted in plasma total Hcy levels of around 100 μM and showed prevention or reversal of osteopenia and modestly improved body composition. Furthermore, concomitant normalization of plasma Cys levels with treatment in both KO and I278T mice improved the balance between plasma Hcy and Cys concentrations. It was found in HCU patients and confirmed in mouse models of the disease that plasma Cys in HCU is largely in the form of free Cys compared with healthy controls, where majority of Cys is bound to plasma proteins (Gupta et al., 2009; Malloy, Rassin, & Gaull, 1981). Although we have not determined it, it is reasonable to anticipate that normalization of total plasma Cys would result in normalized balance between free and bound Cys. This factor alone may play a significant role in rescuing HCU phenotype by ERT. On the contrary, when long-term betaine supplementation was used as a mean to decrease Hcy in HCU

### TABLE 1 Correlation of bone mineral density (BMD) and plasma total homocysteine levels (Hcy) in HCU mice

| HCU mouse strain | Genotype | Sex | untreated PEG–CBS injected | Hcy (μM ± SEM) |
|------------------|----------|-----|---------------------------|---------------|
| KO               | +/-      | M   | 0.049 ± 0.001             | 5 ± 0.1       |
|                  | F        |     | 0.051 ± 0.001             | n/a           |
|                 | +/-      | M   | 0.044 ± 0.001             | 521.2 ± 87.3  |
|                 | F        |     | 0.045 ± 0.001             | 101.7 ± 12.5  |
| I278T            | +/-      | M   | 0.052 ± 0.001             | 8.5 ± 0.5     |
|                 | F        |     | 0.053 ± 0.001             | n/a           |
|                 | +/-      | M   | 0.046 ± 0.001             | 406.7 ± 15.8  |
|                 | F        |     | 0.048 ± 0.001             | 88.4 ± 5.7    |
| HO               | +/-      | M   | 0.053 ± 0.002             | 5.4 ± 0.4     |
|                 | F        |     | 0.053 ± 0.001             | n/a           |
|                 | +/-      | M   | 0.055 ± 0.001             | 243.2 ± 9.6   |
|                 | F        |     | 0.052 ± 0.001             | n/a           |

n/a, not available.
patients and I278T mice (Gahl, Bernardini, Chen, Kurtz, & Horvath, 1988; Gupta et al., 2016), no improvement in bone density was found supposedly due to lack of plasma Cys normalization. In addition, such normalization may positively impact plasma redox environment and signaling and regulation via sulfhydryl cysteinyllation (Paulsen & Carroll, 2013).

Despite clear data, there are several weaknesses of our study. Both negative and positive controls were untreated instead of being vehicle-injected. The question remains what represents the ideal placebo: buffer, PEG in buffer or PEGylated carrier protein, such as BSA, in formulation buffer. Assessment of bone health using DXA is not a reliable tool to measure bone strength, and structural bone analysis should be performed to evaluate bone health.

Another study limitation is comparing the effects of ERT across the KO and I278T mice using a single dose level of PEG–CBS. The HCU models may have different sensitivities rather than overall effects and, at higher dose, both may respond similarly.

Taken together, our study showed a threshold effect of murine HCU on osteopenia and changes in body composition, particularly fat content. While severe elevation of plasma Hcy above 350 μM resulted in observed phenotype in KO and I278T mice, a lower plasma Hcy of ~240 μM in HO mice showed no changes compared with healthy controls. More importantly, long-term treatment of the KO and I278T mice with PEG–CBS rescued the phenotype despite the imperfect metabolic control. Notably, this effect was achieved solely by the ERT on the background of regular Met intake and without betaine supplementation. Study of Parrot et al. (2000) emphasized that early treatment can prevent the development of osteoporosis in HCU patients and argued that even badly managed HCU patients seemed to be protected from thromboembolic events thus considering osteoporosis as the major complication in late-treated, young or aging patients. Thus, our study demonstrates a strong preclinical indication that our ERT represents a promising treatment option for HCU patients with potential to allowing for a better quality of life on an unrestricted diet.

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DISCLOSURE STATEMENT

E.M.B. is employed in Orphan Technologies Ltd. that is engaged in the development of innovative therapies for orphan diseases. T.M., E.M.B., and J.P.K. are inventors on patents related to the processes and products referred here (US patents 9,034,318 and 9,243,239).

AUTHOR CONTRIBUTION

T.M. designed and performed all the studies, prepared, and analyzed PEG–CBS conjugates, analyzed data, prepared figures, wrote the initial draft, revised manuscript, and approved its final version. I.P. took care of the animal colonies, assisted with animal studies, and approved the final version of the manuscript. E.M.B. co-designed the studies, analyzed the data, revised the manuscript, and approved its final form. J.P.K. conceived the idea, designed the studies, revised the manuscript, and approved its final form.

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