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Review

Classical homocystinuria: From cystathionine beta-synthase deficiency to novel enzyme therapies

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ABSTRACT

Genetic defects in cystathionine beta-synthase (CBS), a key enzyme of organic sulfur metabolism, result in deficiency of CBS activity and a rare inborn error of metabolism called classical homocystinuria (HCU). HCU is characterized by massive accumulation of homocysteine, an intermediate of methionine metabolism, and multisystemic clinical symptoms. Current treatment options for HCU are very limited and often inefficient, partially due to a low patient compliance with very strict dietary regimen. Novel therapeutic approaches are needed to cope with the toxic accumulation of homocysteine and restoration of a healthy metabolic balance. Human CBS is a complex intracellular multimeric enzyme that relies on three cofactors (heme, pyridoxal-5′-phosphate and S-adenosylmethionine) for proper function. Engineering and chemical modification of human CBS yielded OT-58, a first-in-class enzyme therapy candidate for HCU. Pre-clinical testing of OT-58 showed its substantial efficacy in lowering plasma and tissue concentrations of homocysteine, improving metabolic balance and correcting clinical symptoms of HCU. In addition, OT-58 showed great safety and toxicity profile when administered to non-human primates. Overwhelmingly positive and extensive pre-clinical package propelled OT-58 into a first-in-human clinical trial, which started on January 2019. In a meantime, other enzyme therapies based on modified human cystathionine gamma-lyase or erythrocyte-encapsulated bacterial methionine gamma-lyase have shown efficacy in decreasing plasma homocysteine in HCU mice. In addition, gene therapy approaches using adenovirus or minicircle DNA have been evaluated in HCU. In this review, we summarize the current efforts developing novel therapies for HCU to address a high unmet medical need among HCU patients.

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Abbreviations

BHMT	betaine-homocysteine methyltransferase
CBS	cystathionine beta-synthase
CGL	cystathionine gamma-lyase
Cth	cystathionine
Cys	cysteine
EC	Enzyme Commission
H_2S	hydrogen sulfide
HCU	homocystinuria
Hcy	homocysteine
hCBS	human CBS
htCBS	human truncated CBS
KO	CBS knock-out mouse model
MAT	methionine adenosyltransferase
Met	methionine
MGL	methionine gamma-lyase
MRD	methionine-restricted diet
MS	methionine synthase
MT	methyltransferase
MTHFR	methyl-tetrahydrofolate-reductase
OMIM	Online Mendelian Inheritance in Man
OT-58	htCBS C15S modified with linear 20 kDa N-hydroxysuccinimide-PEG
PEG	polyethyleneglycol
PLP	pyridoxal-5′-phosphate
REG	regular diet
SAM	S-adenosylmethionine
SAH	S-adenosylhomocysteine
SAHH	SAH hydrolase
Ser	serine
SHMT	serine-hydroxymethyltransferase
THF	tetrahydrofolate
WT	wild type

1. Sulfur amino acid metabolism and classical homocystinuria

The essential amino acid methionine (Met) is one of the four common sulfur-containing amino acids, together with cysteine (Cys), homocysteine (Hcy) and taurine. However, only Met and Cys are incorporated into proteins, while Hcy and taurine are not and, together with Cys, are products of Met metabolism [1]. Met is a source of organic sulfur for eukaryotic cells (Fig. 1). When dietary proteins are ingested and Met transferred into the cells, it is activated by condensation with ATP catalyzed by Met adenosyltransferase (MAT) to form S-adenosylmethionine (SAM) [1]. SAM is the most important cellular methyl donor for a varied repertoire of methylation reactions catalyzed by methyltransferases (MT), such as DNA/RNA methylation, biosynthesis of hormones, neurotransmitters, carnitines, creatine or phospholipids. Transfer of a methyl group from SAM to various substrates generates S-adenosylhomocysteine (SAH), which is rapidly hydrolyzed by SAH hydrolase (SAHH) to adenosine and Hcy. Hcy is a toxic intermediary amino acid residing at the intersection of transmethylation cycle and transsulfuration pathway. In order to conserve Met, Hcy can be remethylated by the action of ubiquitous methionine synthase (MS) or a liver-specific betaine-Hcy methyltransferase (BHMT) using methyl-tetrahydrofolate (methyl-THF) and betaine as methyl donors, respectively. To generate other important cellular sulfur compounds such as Cys, glutathione, taurine and hydrogen sulfide (H_2S), Hcy is irreversibly diverted from transmethylation cycle into the transsulfuration pathway by cystathionine beta-synthase (CBS)-catalyzed condensation with serine (Ser) forming cystathionine (Cth). Cth is subsequently hydrolyzed by Cth gamma-lyase (CGL) into Cys. Of note, SAM regulates Hcy flux through the competing transsulfuration and remethylation by allosteric activation of CBS and inhibition of methyl-THF-reductase (MTHFR). Interestingly, enzymes of both competing pathways require assistance of a particular vitamin B family member: B_2 (riboflavin) in MTHFR, B_6 (pyridoxine) in serine-hydroxymethyltransferase (SHMT), CBS and CGL, B_9 (folic acid) as a one-carbon carrier and B_{12} (cobalamin) in MS. Taken together, CBS governs the flow of organic sulfur from Met to biosynthesis of all other physiologically important sulfur-containing compounds and its proper function is central to the regulation of sulfur amino acid metabolism. Therefore, it is not surprising that the most common inborn error of sulfur amino acid metabolism, referred to as classical homocystinuria (HCU), is caused by deficiency in CBS activity.

In classical HCU (OMIM# 236200), insufficient CBS activity is chiefly caused by the presence of missense pathogenic mutations in the CBS gene [2]. Lack of CBS disrupts entire sulfur amino acid metabolism yielding typical biochemical presentation of the disease, which includes massive accumulation of toxic Hcy in tissues, plasma and urine and highly elevated levels of Met, SAM and SAH accompanied by decreased concentrations of Cys and Cth in circulation (Fig. 1). 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) [3]. Major causes of premature death in HCU patients are thromboembolism and stroke, which are likely caused by pathologically and chronically increased concentrations of prothrombogenic Hcy, dysfunctional H_2S metabolism and decreased antioxidant capacity [4].

Despite these detrimental consequences for patients, treatment options for HCU have been very limited and are often very hard to comply with [3–5] (Table 1). They are mostly focused on decreasing and preventing the buildup of Hcy (Fig. 1). Therefore, the mainstay therapy includes low-Met/protein diet supplemented with a Met-free amino acid formula to deliver adequate levels of other necessary amino acids. Betaine is often used as supplemental therapy to dietary management in patients who cannot achieve target levels of Hcy by other means. Patients with certain mild CBS mutations also benefit from treatment with pyridoxine, which provides essential cofactor for CBS and thus stimulates its residual catalytic activity. In addition, patients are often prescribed multi vitamin B supplements to promote activity of enzymes involved in sulfur amino acid metabolism (Fig. 1) and Cys supplementation is also common.

HCU is a rare disease. According to literature, prevalence varies greatly estimated at 1 in 100,000–200,000 in the USA and 1 in 200,000–335,000 worldwide. In countries like Ireland and Qatar, HCU is more common with a prevalence of 1:65,000 and 1:1,800, respectively [6,7]. Expanded newborn screening suggests that the true rate of occurrence is greatly underestimated [4,8]. Furthermore, recent real-world healthcare data shows that HCU prevalence in the USA is 1 in 10,000, which is substantially higher than previously estimated (Posters# PSY31 and PSY32 presented at ISPOR Europe 2018 meeting). Understanding that HCU is underdiagnosed together with limited efficacy and difficulties in coping with the current treatments, a high unmet need exists in the

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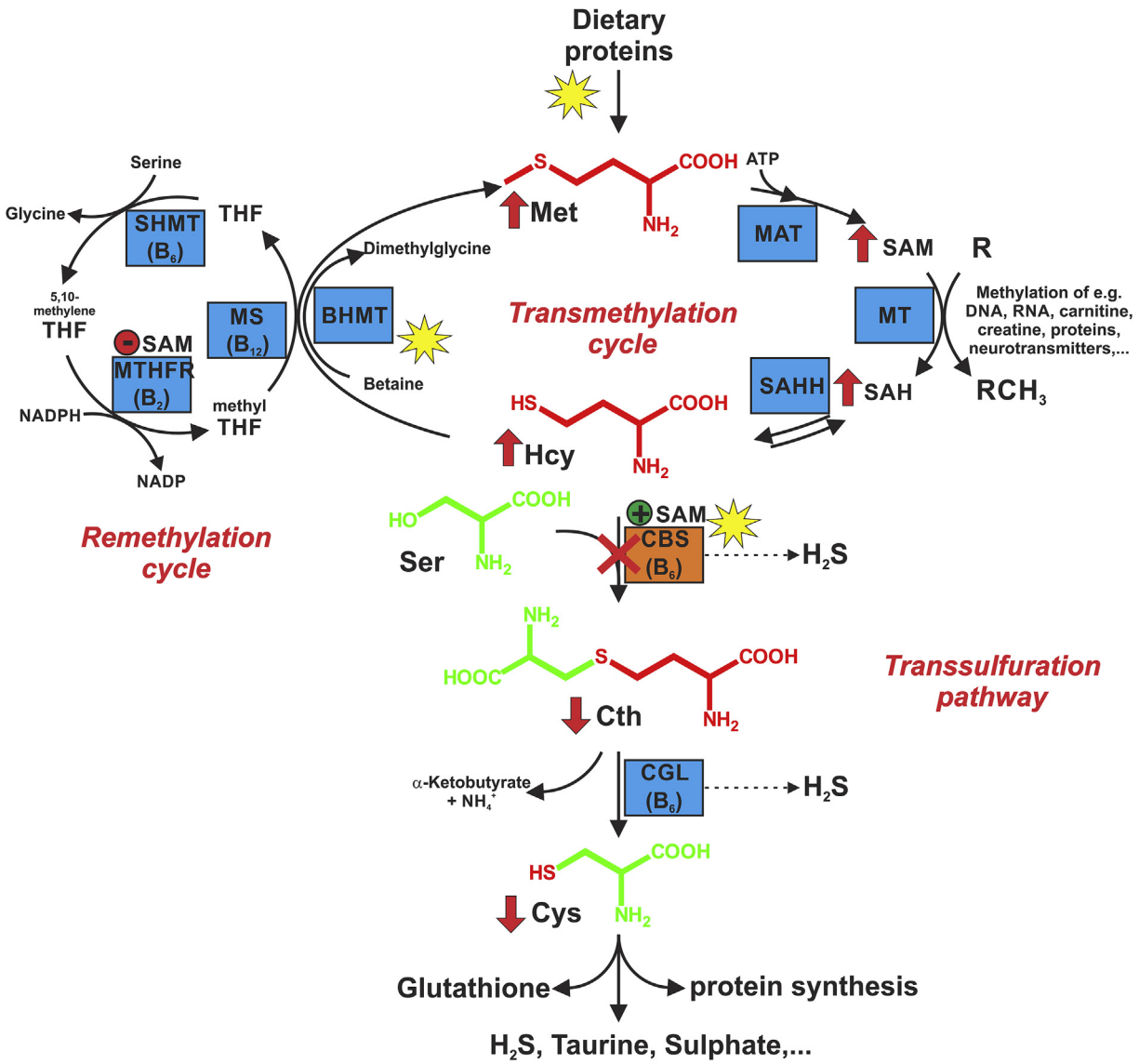

Fig. 1. Sulfur amino acid metabolism and HCU. For details regarding metabolism of Met including pathways, enzymes and compounds, please see the main text. In HCU, sulfur amino acid metabolism is disrupted due to deficient CBS activity (red cross), which leads to elevation of upstream metabolites Hcy, Met, SAM and SAH and decrease of downstream metabolites Cth and Cys as indicated by red arrows. Yellow stars designate locations, where current treatments for HCU works: restriction of dietary Met and protein intake to decrease production of Hcy, pyridoxine supplementation to stimulate residual CBS activity and betaine supplementation to promote Hcy re-methylation back to Met.

community for better diagnosis and, more importantly, novel efficient treatments for HCU. Recently, a growing number of enzyme therapies have been in development for HCU, which we will review in this study. Enzyme therapy is a better suited designation than enzyme replacement therapy, as none of the publicly disclosed and reviewed on-going projects seeks to replace an intracellular dysfunctional CBS in HCU, but rather targets metabolite buildup in blood. We will focus on the first-in-class drug candidate of enzyme therapies for HCU and the most advanced program, which is based on CBS and have been developed by the group of Professor Jan. P. Kraus, who recently passed away [9].

2. Human CBS and OT-58

Human CBS (hCBS, EC# 4.2.1.22) is a unique pyridoxal-5′-phosphate (PLP)-dependent multimeric hemoprotein with a multidomain architecture and an intricate regulation [10]. The 63 kDa hCBS subunits each containing 551 amino acid residues assemble into native homotetramers. Each subunit is comprised of three modules (Fig. 2A). The N-terminal module encompassing the first ~70 residues binds heme cofactor, which is axially coordinated by residues C52 and H65 [11,12]. Function of heme in CBS remains unknown, but is thought to play a role in redox sensing [13] or enzyme folding [14]. The central module spanning the residues 70 to 386 represents the catalytic segment, where the PLP cofactor binds via a Schiff bond to the ε-amino group of the K119 residue [15]. PLP is catalytically versatile cofactor as illustrated by more than 120 distinct activities associated with known PLP-dependent enzymes, which translates into catalytic promiscuity of hCBS [16,17]. In addition to the canonical condensation of Ser and Hcy to form Cth, hCBS can utilize Cys alone or with Hcy to form H_2S. However, it is unclear what directs hCBS towards alternative reactions and how this process is regulated. The C-terminal module houses a tandem of CBS domains CBS1 and CBS2, a conserved structural motif named after CBS, which forms two major cavities designated as sites S1 and S2. This motif is found in diverse and

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Table 1
Overview of current and potential future therapies for HCU.

Therapy	Description	Type	Stage	Mechanism of action
Current therapies				
Met-restriction combined with Met-free amino acid formula	Low protein diet and Met-free amino acid formula to supply the missing amino acids	Diet	In clinical use	Reduces build-up of Hcy by limiting the consumption of its precursor Met. Most patients often find it hard to adhere to the diet.
Pyridoxine	Vitamin B_6	Diet supplement	In clinical use	CBS catalytic co-factor. Increases enzymatic activity of certain CBS mutants. Most HCU patients are either non-responsive or partially responsive.
Betaine	Trimethylglycine	Diet supplement	In clinical use	Promotes Hcy re-methylation to Met via liver-dependent BHMT. Results in build-up of Met. Decreased efficacy over time.
Therapies in development				
OT-58 (Orphan Technologies)	Recombinant PEGylated truncated human CBS C15S variant	Enzyme Therapy	Phase I/II	Converts free Hcy into Cth and indirectly reduces all bound Hcy adducts thus resulting in substantial decrease of total Hcy in plasma as well as in tissues.
AEB4104 (Aeglea)	Recombinant PEGylated human CGL variant engineered to process Hcy	Enzyme Therapy	Pre-clinical	Acts on both the free Hcy and Hcy-Hcy disulfide in plasma.
Erymethionase (Erytech Pharma)	Recombinant *P. putida* methionine gamma-lyase encapsulated in RBCs	Enzyme Therapy	Pre-clinical	Degrades Met and free Hcy that enter into RBCs. Enzyme produces methanethiol or H2S and ammonia, which may be toxic.
RTX-CBS (Rubius Therapeutics)	Recombinant human CBS expressed $CD34^+$ hematopoietic precursor cells by lentiviral gene delivery that mature into RBCs	Enzyme Therapy	Pre-clinical	Acts on free Hcy that enters into RBCs.
rAAV CBS	Human CBS cDNA transduction by recombinant adeno-associated virus to host cells	Gene Therapy	Proof of concept	Expresses human CBS (truncated or full-length) in target cells to replace the defective CBS variants thus allowing for intracellular Hcy processing. The rAAV does not integrate into the host genome and persist in episomal form.
Minicircle CBS DNA	Human CBS cDNA in minicircle-based naked DNA vector	Gene Therapy	Proof of concept	Expression of human CBS WT enzyme in target cells to replace the defective enzyme and to process intracellular Hcy. Does not incorporate into the host genome.

functionally unrelated proteins, where it usually fulfills a regulatory role and/or sensing function upon binding adenosine analogs [18,19]. Indeed, the C-terminal domain of hCBS represents a regulatory domain, which inhibits catalytic activity of the enzyme. Due to steric interference from bulky hydrophobic residues, the site S1 is blocked and remains empty. On contrary, site S2 is exposed and represents the primary binding site for SAM. Binding of SAM kinetically stabilizes and activates the enzyme 3-8-fold by inducing a substantial conformational change in association of the C-terminal region (Fig. 2B–C) [20–22]. Crystal structures of an engineered hCBS mutant hCBSΔ516-525 lacking a loop of 10 amino acid residues from CBS2 domain uncovered molecular mechanism of hCBS regulation by SAM [21–23]. The hCBSΔ516-525 biochemically behaves like native hCBS WT; however, it forms well-defined stable dimers, instead of native tetramers and higher order oligomers of hCBS WT. In a basal conformation, the regulatory domain of one hCBSΔ516-525 polypeptide interacts with the catalytic core of the other subunit and thus blocks the entrance to the PLP-containing catalytic center (Fig. 2B). In contrast, SAM binding to site S2 causes conformational shift of the regulatory domain away from the other subunit catalytic core thus relieving the block and activating the enzyme (Fig. 2C). SAM-bound regulatory domains from two hCBSΔ516-525 subunits associate together to form a so-called CBS module, which kinetically stabilizes the enzyme [20,24]. Complete removal of the C-terminal regulatory domain yields highly active, stable human truncated CBS (htCBS) dimers (Fig. 2D). The htCBS lacking the regulatory domain no longer binds SAM and has similar specific activity as the SAM-activated hCBS or hCBSΔ516-525 [25,26]. Pathogenic mutations kinetically destabilize regulatory domain and thus protein stabilization by SAM or its structural analog may play a key role in modulating steady-state levels of hCBS *in vivo* [20,27].

Complex structural and functional properties of hCBS made it challenging, but not impossible, to develop an enzyme therapy for HCU based on CBS. The first and most advanced project for HCU therapy is OT-58, which has been developed by Kraus and Majtan group at the University of Colorado Anschutz Medical Campus with Orphan Technologies. OT-58 employs engineered, recombinantly produced and chemically modified hCBS (Fig. 2E). To overcome low hCBS catalytic activity and complex regulation by SAM, we used highly active dimeric htCBS lacking residues 414–551 as our starting candidate (Fig. 2D). When injected into HCU mice, htCBS showed very short half-life of 2.7 h after intravenous administration [28]. In addition, despite htCBS being less prone to aggregation than native hCBS WT, it still formed small inconsistent amount of soluble multimers. These forms are not desired as they may stimulate immune response and represent a technological challenge for process development. We found that the process of htCBS multimerization is driven by the formation of inter-dimeric disulfide bridges involving previously identified solvent-exposed C15 residue [29]. The htCBS C15S variant (Fig. 2E) showed discrete dimers essentially free of higher order multimers [28]. In order to increase retention of htCBS in circulation, we explored conjugation of htCBS with inert polyethyleneglycol (PEG) moieties, commonly referred to as PEGylation, a well-recognized and accepted strategy to improve pharmacokinetics and to decrease immunogenicity of drugs [30]. Indeed, PEGylation of htCBS increased its half-life 5-10-fold and resulted in sustainable and substantial decrease of plasma Hcy in HCU mice [28]. Among various chemistries and sizes of PEGs, conjugation of htCBS C15S with linear 20 kDa N-hydroxysuccinimide-PEG (20NHS PEG) resulted in the most reproducible pattern and the highest potency after repeated administration [31].

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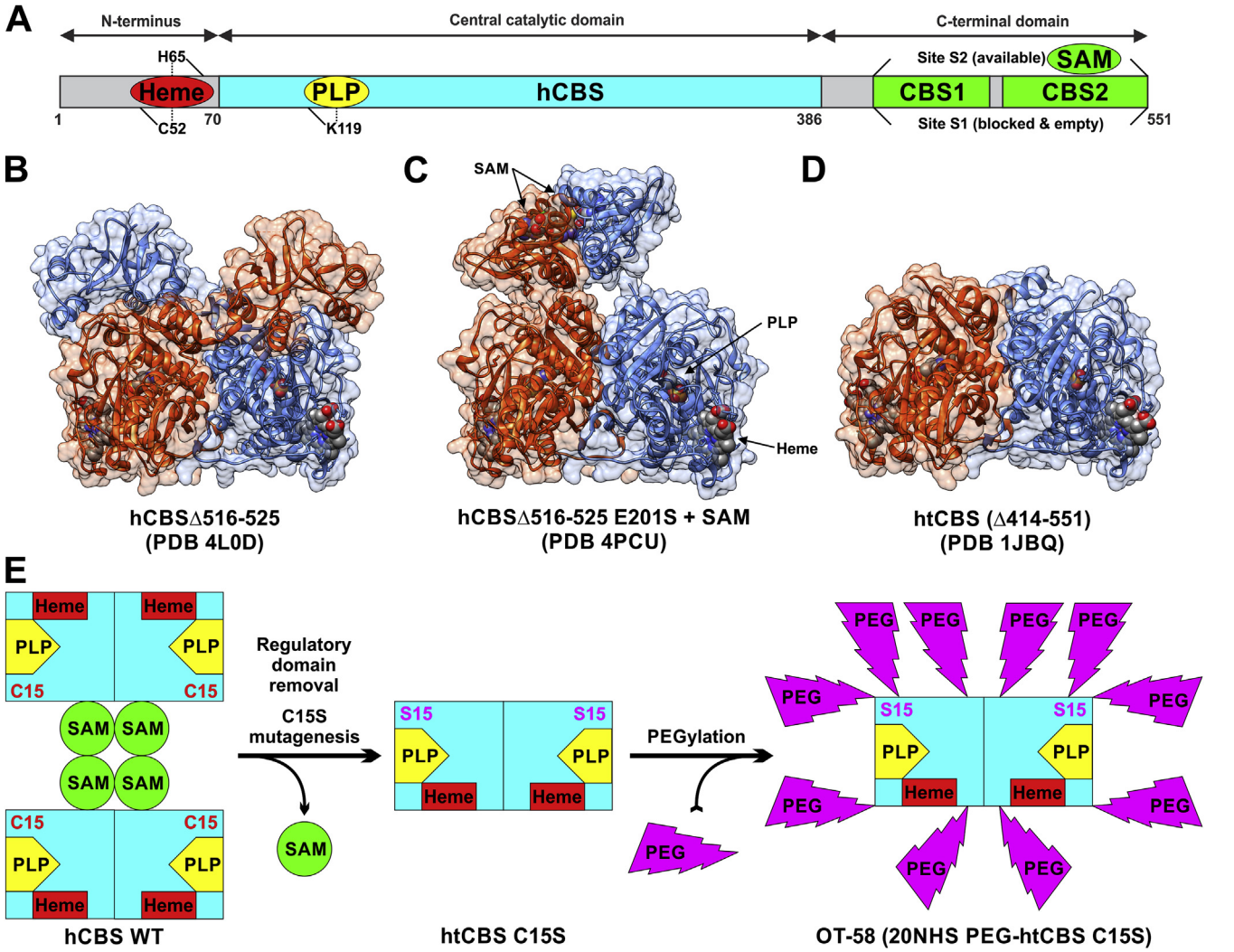

Fig. 2. Structure of hCBS and OT-58 generation. A – Domain architecture of hCBS polypeptide. **B** – Crystal structure of hCBSΔ516-525 in SAM-free basal conformation. Two CBS subunits in each dimer are shown in orange and blue. Cofactors (heme, PLP and SAM) are displayed in spheres. **C** – Crystal structure of hCBSΔ516-525 E201S mutant in SAM-bound activated conformation. **D** – Crystal structure of highly active htCBS lacking the C-terminal regulatory domain (Δ414-551). **E** – Schematics of OT-58 generation. Native tetrameric hCBS WT was subjected to the removal of its regulatory domain and mutagenesis of solvent-exposed C15 residue to generate highly active dimeric htCBS C15S. Purified htCBS C15S was subsequently chemically modified with linear 20 kDa NHS PEG moieties to produce OT-58.

Detailed characterization revealed that each htCBS C15S subunit of 20NHS PEG-htCBS C15S conjugate is modified on average with 5.0 ± 0.5 20NHS PEG moieties (Fig. 2E). When injected to HCU mice, 20NHS PEG-htCBC C15S showed elimination half-life of 2 days after intravenous administration, which decreased to 17.5 h after subcutaneous dosing with bioavailability of 64.6% [32]. Taken together, consistency in production of 20NHS PEG-htCBS C15S and its ability to markedly and sustainably reduce plasma Hcy concentrations in HCU mice resulted in its designation as OT-58 and its progress into an expanded pre-clinical testing.

3. Pre-clinical testing of OT-58

For extensive pre-clinical testing of OT-58, we used three mouse models of HCU, which differ in severity of the disease and associated clinical symptoms. The KO mice are the most severely affected suffering from neonatal lethality due to a complete knock-out of CBS gene [33]. To overcome the survival issue of KO mice, the transgenic I278T mice express the most common pathogenic hCBS I278T variant from zinc-inducible promoter [34]. Expression of the hCBS I278T transgene confers mice with only 2–3% of CBS WT activity, which prevented neonatal lethality, while still manifesting biochemical and clinical phenotype of HCU. Similarly to I278T mice, transgenic HO mice variably express hCBS WT giving the mice 2–16% of CBS WT activity [35]. The HO mice still reproduce the biochemical profile of HCU, but clinical symptoms of the disease are less prominent or missing entirely.

Bulletproof pre-clinical proof-of-concept that enzyme therapies targeting extracellularly accumulated Hcy, such as OT-58, can represent a novel, viable avenue to treat HCU came from our study using KO mice [36]. When injected 3 times a week with a dose of 7.5 mg/kg administered subcutaneously from 2 days after birth, 93% of KO mice survived beyond 5 weeks compared to none injected with vehicle (phosphate-buffered saline). The cohort, which continued with the treatment up to 17 weeks of age, showed 100% survival compared to 86% survival rate of those, which were treated with OT-58 only during the initial 5 weeks of life (Fig. 3A). Presence of OT-58 in circulation of 18-days-old KO pups resulted in 80% decrease of plasma Hcy compared to age-matched vehicle-treated KO mice (Fig. 3B). More importantly, Hcy levels in liver, kidney and

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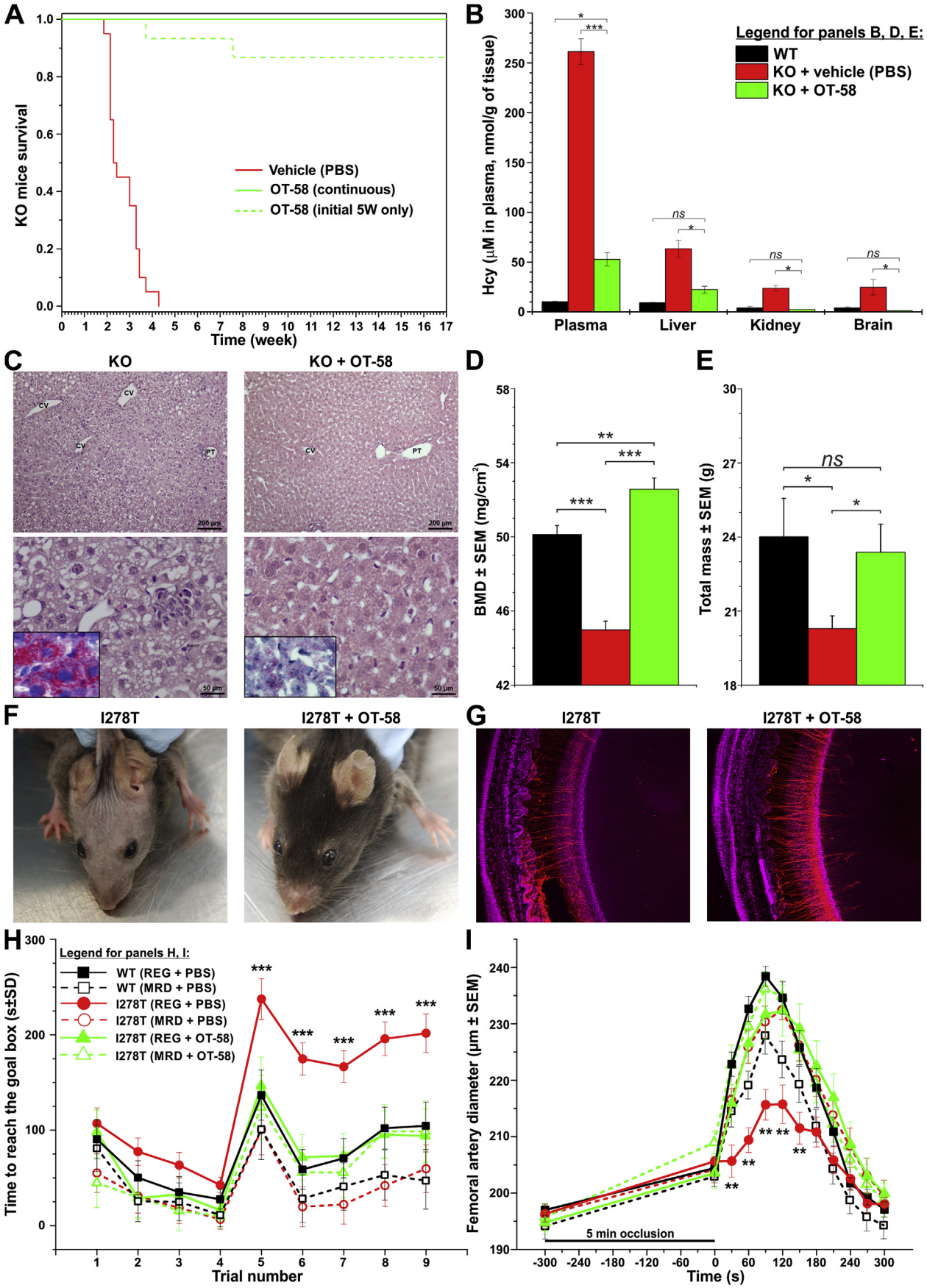

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brain of OT-58-treated KO mice were normalized (Fig. 3B). This result suggests that OT-58 in circulation functions as a metabolic sink creating a concentration gradient for Hcy secreted from tissues and processed in plasma. In addition, plasma and tissue concentrations of Cys and Met were fully normalized [36]. The KO mice suffer from severe liver damage characterized by steatosis, hepatocellular necrosis, inflammation, swollen mitochondria and disorganized endoplasmic reticulum (Fig. 3C). Administration of OT-58 completely rescued KO mice livers from these pathological changes (Fig. 3C). Furthermore, long-term administration of OT-58 to KO mice completely rescued or even improved their bone mineralization (Fig. 3D) and body weight (Fig. 3E).

Skeletal abnormalities and connective tissue defects are among the most striking features in HCU patients with osteoporosis associated with scoliosis as one of the distinguishing symptoms of the disease [3]. We found that the KO and the I278T, but not the HO mice reproduced this phenotype characterized by low bone mineral content, low body fat and low total weight compared to WT controls [37]. Long-term administration of OT-58 completely rescued bone mineralization and substantially improved body composition of the treated KO and I278T mice. These results showed efficacy of OT-58 to correct this phenotype, but also point to a threshold effect of murine HCU, where less severe elevation of plasma Hcy in HO mice compared to KO or I278T mice resulted in the absence of the phenotype.

Our study testing efficacy of OT-58 in I278T mice suggests that initiation of treatment before onset of the clinical symptoms can entirely prevent disease phenotypes. Facial alopecia in I278T mice has an onset of 105–120 days characterized by a complete loss of hair including whiskers (Fig. 3F) [38]. Administration of OT-58 (3x a week, 7.5 mg/kg SC) from day 21 of age completely prevented loss of facial hair in treated I278T mice resulting in an image indistinguishable from WT mice (Fig. 3F). Myopia and dislocated optic lenses are characteristic clinical symptoms of HCU [3]. Untreated 4-months-old I278T mice showed markedly disrupted ciliary zonules characterized by reduced density and length of zonular fibers, which hold the lens in axis of sight (Fig. 3G). Treatment with OT-58 from day 2 after birth fully rescued the zonular structure in the eyes of treated I278T mice (Fig. 3G). In addition to corrected sulfur amino acid metabolism, treatment of I278T mice with OT-58 resulted in an improvement of disturbed lipid and glucose metabolism and systemic decrease of oxidative stress and inflammation [39].

Another study evaluated efficacy and benefits of a long-term treatment with OT-58 on the background of normal Met intake in I278T mice and compared it with Met restriction, the current standard of care for HCU patients [40]. At 24 days of age, mice were put on amino acid-defined diets containing 4% and 0.5% Met designated as regular (REG) and Met-restricted diet (MRD), respectively. At the same time, subcutaneous administration of vehicle or OT-58 (3x a week, 10 mg/kg) was initiated. We found that, compared with untreated I278T mice, OT-58 treatment of I278T mice fed with the REG diet resulted in a 90% decrease of plasma Hcy concentrations and correction of learning/cognition deficit (Fig. 3H), endothelial dysfunction (Fig. 3I), hemostasis, bone mineralization, and body composition [40]. On the background of the MRD, OT-58 performed equally well with plasma Hcy entirely normalized. The MRD alone decreased plasma Hcy by 67% and corrected the HCU phenotype in I278T mice. However, the MRD increased anxiety and reduced bone mineral content in both I278T mice and WT controls. This study illustrates that OT-58 is a highly efficacious novel treatment for HCU on the background of either normal or restricted methionine intake. The data also argue that OT-58 should allow for elimination of Met/protein restriction and thus, in turn, substantially improve quality of life of HCU patients and their families.

In addition to HCU mice, OT-58 appeared to be well-tolerated in non-human primates with >80% bioavailability of OT-58 after subcutaneous administration [32]. Based on pharmacokinetics/pharmacodynamics relationship studies in mice and monkeys and using allometric scaling, we estimated that one weekly subcutaneous injection of 0.66 mg/kg OT-58 should result in a significant correction of biochemical imbalance in HCU patients [32].

Backed by these extensive, convincing and overwhelmingly positive results from pre-clinical studies, OT-58 is currently being evaluated in the first-in-human clinical trial evaluating safety and efficacy (phase 1/2) in HCU patients (ClinicalTrials.gov Identifier NCT03406611).

4. Other enzyme therapies for HCU in development

In addition to OT-58, there are several other enzyme therapies in development for HCU (Table 1). To date, however, there is very little information available with no studies published in peer-reviewed journals. AEB4104, developed by Aeglea Biotherapeutics, is a novel recombinant PEGylated engineered human CGL designed to change its native substrate specificity from Cth towards Hcy. AEB4104 has been optimized to act on both the reduced Hcy or its oxidized disulfide form, homocystine. AEB4104 significantly reduced plasma Hcy concentrations by at least 90% after intraperitoneal administration of 50 mg/kg to a diet-induced hyperhomocysteinemia mouse model (Oral presentation# O61 presented at ICHOCM 2017 meeting). Furthermore, intraperitoneal administration of 25 mg/kg AEB4104 from day 10 until day 50 resulted in a full survival of the treated KO mice with improved liver abnormalities including resolution of steatosis (Poster# 980F presented at ASHG 2018 meeting). AEB4104 has >80% bioavailability from subcutaneous compartment in monkeys, which is similar to that of OT-58 [32].

Another approach to enzyme therapy for HCU, developed by Erytech Pharma, utilizes Met gamma-lyase (MGL) to degrade Met in circulation. By limiting Met supply, this therapy was primarily developed to treat cancer, but it can act as a substrate reduction therapy in HCU and decrease buildup of Hcy, a downstream product of Met metabolism. In fact, MGL displays enzymatic activity with both Met and Hcy with similar K_m constants, but a higher V_{max} for Hcy [41]. By degrading Met and Hcy, MGL generates toxic by-products: methanethiol and H_2S, respectively, and ammonia. Erymethionase is MGL from *Pseudomonas putida* entrapped in red blood cells using company's proprietary ERYCAPS technology platform [42]. Encapsulation of MGL into red blood cells provides several advantages. First, erythrocytes are a natural pool of PLP cofactor, which MGL has a low affinity for and needs to stay active. Second, bacterial MGL is likely highly immunogenic and being hidden in erythrocytes, MGL is protected from the host's immune system. Third, entrapment of MGL in red blood cells greatly extends

Fig. 3. Pre-clinical testing of OT-58 using various mouse models of HCU. A – OT-58 rescues neonatal lethality of KO mice [36]. **B** – OT-58 substantially reduced plasma Hcy and normalized Hcy concentrations in liver, kidney and brain of rescued KO mice [36]. **C** – OT-58 prevented severe liver damage in KO mice characterized by steatosis, hepatocellular necroses and inflammation [36]. **D, E** – Long-term administration of OT-58 rescued bone mineral density and total mass of treated KO mice [36,37]. **F** – Long-term administration of OT-58 before onset of clinical symptoms completely prevented facial alopecia characteristic for untreated I278T mice [39]. **G** – Start of OT-58 treatment from day 2 after birth rescued structure of ciliary zonules in the eyes of I278T mice [39]. **H** – I278T mice on the REG diet showed significant cognitive impairment and learning deficit in a puzzle box test, which was fully corrected by administration of OT-58 or the MRD diet [40]. **I** – I278T mice fed with the REG diet suffer from significant endothelial dysfunction as assessed by determining flow-mediated vasodilation, which was completely rescued by administration of OT-58 or the MRD diet [40].

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drug's half-life in circulation. When injected to I278T mice, Erymethionase reduced plasma Hcy by more than 40% 24 h after intravenous administration, which remained significantly lower compared to baseline six days after dosing (Poster# 314 presented at ICIEM 2017 meeting).

Another form of an erythrocyte-encapsulated drug is RTX-CBS developed by Rubius Therapeutics. Company's proprietary RED PLATFORM utilizes CD34 $^{+}$ hematopoietic precursor cells from healthy type O negative donors, that are transduced with lentiviral vector or gene cassette to express CBS. The cells are then expanded and differentiated into mature erythrocytes, which eject their nucleus and thus no longer contain foreign DNA, yielding red blood cells loaded with expressed CBS. RTX-CBS is expected to rapidly drop plasma Hcy to clinically meaningful target levels, but no further details are available.

5. Outlook for treatment of HCU

In addition to the enzyme therapies mentioned above, gene therapy approaches have been studied to correct CBS deficiency in HCU as well (Table 1). The first study employed a recombinant adeno-associated virus vector carrying hCBS cDNA [43]. When administered to KO mice by intramuscular or intraperitoneal injection, their plasma Hcy levels dropped from 400 μM to 240–300 μM and their life span was 3–7 days longer than for untreated KO mice. Although not overwhelming, particularly compared to OT-58 or AEB4104, the results of this study established a proof of concept for potential benefits of CBS gene delivery. In another study, E1E3E4-deleted adenoviral vectors for hepatocyte-restricted overexpression of CBS were administered to diet-induced hyperhomocysteinemic *Ldlr*$^{-/-}$ *Cbs*$^{+/-}$ mice to evaluate hyperhomocysteinemia as a risk factor for atherosclerosis [44]. Gene transfer resulted in >80% decrease of plasma Hcy, which remained stable for the entire duration of the experiment. This selective Hcy lowering corrected endothelial dysfunction in the mice, but did not have any effect on atherogenesis. It would be interesting to see how this CBS adenovirus would perform when administered to mouse models of HCU, such as KO or I278T, instead of using a diet-induced mouse model of hyperhomocysteinemia. More recently, I278T mice were used to evaluate potential of minicircle-based naked DNA gene therapy to treat HCU [45]. Liver-specific expression of hCBS resulted in 34-fold increase of liver CBS activity compared to untreated I278T mice (12.8 versus 432 units), which translated into 63% reduction of plasma Hcy 21 days after the dosing (131 versus 351 μM). The Hcy lowering effect persisted up to 42 days and Hcy concentration returned to time 0 levels 70 days after the administration. Interestingly, such treatment of young I278T mice, subsequently followed for 202 days, greatly reduced facial alopecia characteristic for the I278T mouse model.

It is noteworthy that novel therapies for HCU, particularly the enzyme therapies processing Hcy, may be of benefit to other causes of hyperhomocysteinemia. These may include genetic disorders in other enzymes of sulfur amino acid metabolism and related pathways, such as the cobalamin-related remethylation disorders (cblC/D/E/F/G/J types), MTHFR, MAT I/III and MS reductase (MSR) deficiencies, as well as hyperhomocysteinemias of non-genetic origin, particularly in chronic kidney disease and in several cardiovascular, neuropsychiatric, and skeletal diseases. Therapeutic application of enzyme therapy, such as OT-58, in these indications would very likely result in substantial decrease or normalization of plasma Hcy. However, it is premature to speculate what clinical benefits could be achieved in these indications solely by correcting elevated plasma Hcy concentrations.

Taken together, recent years have seen an increased interest in addressing critical unmet need of HCU community for novel and better treatment options than the current decade-old and often insufficient standard of care. Enzyme therapies, and potentially gene-based therapies, can revolutionize treatment and management of HCU and substantially improve the quality of life of HCU patients. The availability of more treatment options for this rare disease will also generate higher awareness about HCU, which may result in an improved diagnosis. With OT-58 being in clinical phase 1/2 trial and several novel treatments in development, HCU patients and their caregivers may be optimistic that novel effective treatments could be available in a near future.

Authorship contribution statement

TM conceived the manuscript outline and prepared figures. EMB and TM finalized the concept of the manuscript, wrote it, revised it and approved its final version.

Declaration of competing interest

EMB is an employee of Orphan Technologies, a private pharmaceutical company developing OT-58 enzyme therapy for classical HCU. TM and EMB are inventors on patents related to OT-58 (US patents 9,034,318 and 9,243,239). TM provides *ad hoc* consulting services to Orphan Technologies Ltd.

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