

## Novel erythropoiesis stimulating agents in thalassemia

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## A B S T R A C T

Anemia in thalassemias results from globin chain imbalance leading to ineffective erythropoiesis and hemolysis. In non-transfusion-dependent thalassemias, (NTDT) progressive loss of erythropoietin response exacerbates anemia, particularly in older adults. Erythropoiesis-stimulating agents, such as erythropoietin, are plausible but risk exacerbating extra-medullary erythropoiesis and iron hyperabsorption. A better strategy is to increase the effectiveness of erythropoiesis. This can be improved by correcting globin chain imbalance through promoting HbF synthesis, such as with hydroxyurea or butyrates. This has generally achieved variable and only modest hemoglobin increments clinically, although new approaches to HbF promotion are under pre-clinical evaluation. Combination strategies, e.g. erythropoietin with hydroxyurea, have shown encouraging hemoglobin increments and significant improvements in Quality of Life (QoL). Restriction of transferrin iron delivery to the erythron by Tmprss6 inhibition, hepcidin manipulation or apotransferrin infusion, although not proven clinically, may increase effective erythropoiesis by decreasing oxidative damage in the erythron. Jak2 inhibition corrects the proliferation/differentiation imbalance, decreases splenomegaly, and is under clinical trials in thalassemias. The most advanced novel agents in clinical trials (sotatercept and luspatercept) are activin receptor IIa/b traps for molecules involved in TGF- $\beta$  signaling, mainly inhibiting GDF11. Preliminary phase II findings show hemoglobin increase of more than 1.5 g in NTDT, transfusion requirement reduction in transfusion-dependent thalassemia (TDT), and an acceptable tolerability profile.

### Learning goals

At the conclusion of this activity, participants should:

- understand the pathophysiological mechanisms underlying anemia in thalassemias;
- understand the mechanisms and consequences of ineffective erythropoiesis (IE);
- understand the contrasting approaches to correcting IE in thalassemias;
- appreciate the degree of correction of anemia required for clinical benefit;
- be aware of emerging novel approaches to correcting anemia in thalassemias.

## Erythropoiesis in thalassemia and its consequences

In thalassemia syndromes, the degree of globin chain imbalance, measured as the  $\alpha/non-\alpha$ ratio, correlates with the disease severity.<sup>1,2</sup> In βthalassemia syndromes, this results in precipitation of electron-dense  $\alpha$ -globin inclusions, beginning at early polychromatophilic stages, and increasing during maturation.3 These precipitates, which may contain both heme-free iron and hemichromes, generate harmful reactive oxygen species (ROS) leading to accelerated membrane oxidation.4 This pathophysiological process leads to the hallmark feature of thalassemia syndromes, namely ineffective erythropoiesis (IE).5 Globin imbalance may be ameliorated by molecular chaperones such as AHSP6 and the heat shock protein 70 (HSP70),7 or by the ubiquitin-proteasome system and by autophagy.<sup>2</sup> IE also occurs in healthy individuals (where up to 25% of erythropoiesis may be ineffective) but this is much increased in thalassemias where IE redirects 85%-95% of the erythroid iron flux back to plasma.8 A secondary effect of IE and hypoxia is expansion of immature erythroblasts that are unable to bypass apoptotic crisis at the polychromatophilic stage,<sup>5</sup> and which can expand to 5-6 times that of healthy controls. A large fraction of these precursors fails to mature into erythrocytes and their survival and proliferation is erythropoietin (EPO)-dependent on persistent phosphorylation of Jak2 kinase.9,10 The balance between proliferation and differentiation is also modulated by other factors such as GDF11.11,12 A tertiary effect of IE is inhibition of hepcidin synthesis, by factors derived from the expanded erythron,9 leading to increased iron absorption. Growth differentiation factor 15 (GDF15) and TWSG1 have been proposed in humans,13 and erythroferrone in mice.14 This results in preferential portal and hepatocyte iron loading in NTDT.

# What hemoglobin increase is required to improve outcomes?

The increase in Hb required in NTDT for improvement of QoL is a key question when deciding the utility of novel erythroid-stimulating agents (ESA) but, until recently, relatively little prospective randomized data were available. In an interesting study<sup>15</sup> using the SF-36 tool, changes in QoL were compared in patients randomized to two treatments aimed at improving Hb in transfused thalassemia intermedia (TI) patients. Treatment with EPO plus hydroxyurea (HU) led to a rise in Hb of 1.6 g, associated with a significant improvement in QoL. A more modest Hb increase of 0.7 g/dL with HU monotherapy still showed a smaller but significant improvement in QoL. This suggests that other agents that may increase Hb by more than 1.5 g may also have a significant QoL benefit. In TDT, the objective of ESAs is to decrease the transfusion requirement. This in turn will lead to decreased blood utilization, which could be important, particularly in regions where safe blood is scarce. Other potential advantages are decreased chelation requirement and a reduction in the number of hospital visits for transfusion. In clinical trials, it is more challenging to demonstrate a decrease in transfusion requirement in TDT than an increase in Hb in NTDT patients. Since clinically TDT and NTDT are a continuum, some carefully selected patients on regular transfusion may be weaned off transfusion without other interventions, as shown with EB-thalassemia.16 This suggests that with novel interventions, relatively small changes in erythropoiesis may result in transfusion independence in some cases of TDT, depending on the study population and local clinical practices.

## Approaches improving erythropoiesis in thalassemia

The disparate mechanisms by which erythropoiesis may be improved are described below.

#### **Erythropoiesis-stimulating agents**

Mechanisms and rationale for use: erythropoietin has been the most extensively evaluated ESA in thalassemia.<sup>17</sup> EPO stimulates the proliferation, differentiation, and maturation of erythroid progenitors by binding to the EPO receptor (EpoR), a class-1 cytokine on the surface of earliest erythroid progenitors, BFU-E. The EPO/EpoR triggers a complex signal transduction cascade with phosphorylation of the tyrosine kinase Jak2, and the downstream signaling molecule Stat5.<sup>18</sup> EPO also acts on death receptor Fas and FasL expression in erythroblasts and increases the erythroblasts that escape apoptosis.<sup>19,20</sup> Exogenous EPO may also have net anti-oxidant effects by improving glutathione, reducing ROS and membrane lipid peroxides.<sup>21</sup> Hypoxia-mediated EPO synthesis may also augment y-globin in healthy individuals<sup>22</sup> and in some thalassemia patients,23 although this has not been consistently shown.24 The net effect is to increase Hb in conditions of relative or absolute EPO deficiency.

*Clinical trials:* the use of EPO in thalassemia came from the realization that EPO levels may not be sufficiently increased for the degree of anemia. In NTDT, EPO levels decrease with age,<sup>25</sup> as does erythropoietic response to EPO,<sup>16</sup> hence the notion of relative EPO insufficiency that may be corrected by exogenous administration. Clinical trials with EPO are summarized in Table 1.

Hb increments with EPO in NTDT have been highly variable but increases of up to 3 g/dL and transfusion independence of TDT patients have been reported with typical doses of 1000 u/kg from weekly to twice weekly. Some trials used iron supplementation to maximize the response,<sup>35</sup> which seems paradoxical in the light of recent reports of the benefits of iron restriction (see below). There are reports of EPO combined with HU, which, in addition to promoting HbF, may mitigate the effects of excessive erythron expansion (see below). A cheaper ESA, darbopoietin alpha, which has a longer half-life, was used at 4.5 ug/kg/week for eight weeks with dose escalation rules for suboptimal response, and showed increases in Hb of between 1-1.5 g/dL in one study.<sup>24</sup> Trials with ESAs have generally not been long term so that the risks of expansion of extra-medullary erythropoiesis or splenomegaly are unclear.

#### **Corrections of ineffective erythropoiesis**

#### Fetal hemoglobin (HbF) induction

Patients with higher HbF percentage levels have a milder clinical course, are often transfusion-independent,<sup>36,37</sup> with a less likely range of morbidities.<sup>38</sup> Fetal Hb-inducing agents increase the production of  $\gamma$ -globin, which binds to  $\alpha$ -chains to produce HbF, thereby decreasing globin chain imbalance. This reduces ineffective erythropoiesis and hemolysis. Several classes of drugs induce HbF through mechanisms that result in transcriptional reactivation of  $\gamma$ -globin gene expression.

Chemotherapeutic agents: responses to hydroxyurea have been reviewed,<sup>39,40</sup> and will be described here to provide a reference point for responses with newer agents. HU has several effects but acts primarily to inhibit ribonucleotide reductase, causing cell-cycle arrest in S phase, thus encouraging differentiation at the expense of proliferation with a consequent reduction in switching from fetal to adult hemoglobin. Unfortunately, most clinical information with HU is from small-scale single-arm trials or retrospective analyses. Results differ widely, e.g. mean Hb responses of 1 g,<sup>41</sup> (41),  $2 g^{42}$  (42) or >2 g/dL<sup>43</sup> have been reported. Similar variability has been reported in Eβ-thalassemia; an increase of only 0.6 g/dL in the largest study44 but larger increases of 1-3 g in smaller series.45 Significant improvement in QoL has been demonstrated in Egyptian β-TI patients.<sup>46</sup> In a large retrospective multivariate analysis of 500 patients, HU treatment was protective for EMH, PHT, leg ulcers, hypothyroidism, and osteoporosis.47 In TDT, reduced transfusion requirements and even transfusion independence have been reported, for example, with a decrease in annual transfusion requirements of more than 70% in 45% of Algerian TDT patients.48 In Iranian patients with 'intermedia' defined as beginning transfusion after two years of age, 83 of 106 became transfusion independent.<sup>49</sup> The highly variable response between studies is likely to reflect differences in the thalassemia genotype, variable previous transfusion histories, and treatment policies in different regions. 5-azacytidine<sup>50</sup> and decitabine<sup>51</sup> have anti-proliferative, hypo-methylating and cytotoxic effects, and have been shown to increase Hb by more than 1.5 g in small studies (maximum 4 g in one patients on 5-azacytidine). The long-term oncogenic and fertility risks of such chemotherapeutic agents have not been reported systematically in prospective studies.

*Short-chain fatty acid derivatives (SCFADs):* SCFADs act to transcriptionally activate the Aγ gene promoter and possibly also inhibit histone deacetylase (HDAC). Intravenous arginine butyrate (high dose IV continuous for 2-3 weeks;

500 mg/kg/day) was reported to increase %F and the total Hb by 0.6-5 g/dL (mean 2.7).<sup>52</sup> In the search for an oral preparation, oral sodium phenylbutyrate (SPB) at high doses of 20 g/day was shown to increase mean Hb by 2.1 g/dL (range 1.2-2.8 g/dL) in 8 patients.<sup>53</sup> The very large doses required (>300 mg/kg), together with its strong musty odor, led to the search for an alternative. Sodium 2,2 dimethylbutyrate (HQK-1001), an orally bioavailable short-chain fatty acid, was recently evaluated in a randomized placebo controlled study of 21 patients with Eβ-thalassemia or TI over eight weeks at a more manageable dose of 20 mg/kg; a median increase of 6% in HbF and mean Hb increase of 1.1 g/dL was seen in only 4 of 9 subjects.<sup>54</sup> This compound has not been selected for further development in thalassemia thus far.

Other developments in gamma globin induction: retroviral transfer of EPO gene in hematopoietic cells improves the erythrocyte phenotype in murine thalassemia,<sup>55</sup> corrects  $\alpha$ /non- $\alpha$  globin ratio and increases hematocrit. Continuous delivery of EPO from muscle AAV-mediated gene transfer in mice with TI led to stable correction of anemia, RBC morphology, less hemichromes and more effective erythropoiesis.<sup>56</sup> Electrotransfer of EPO cDNA in a mouse  $\beta$ -thalassemia model yielded long-lasting improvement of hematocrit, increase in RBC lifespan and re-establishment of globin balance.<sup>57</sup> Novel approaches in preclinical development are summarized in Table 2.

#### Jak2 inhibition

Unrelenting anemia in thalassemia skews erythropoietic response so that proliferation outpaces differentiation. Relative paucity of differentiating cells and later complications of differentiation in hemoglobinizing normoblasts are the hallmarks of IE in thalassemia. Improving the balance between proliferation and differentiation using inhibitors of Jak2 kinase may improve IE.<sup>66</sup> Murine studies have shown improvement in IE and decrease in spleen size. In humans with myelofibrosis, INCB018424 inhibitor in phase I/II studies reduced spleen size.<sup>67</sup> The Jak2 inhibitor AG490 is able to lower hepcidin and was investigated pre-clinically in some cancers.<sup>68</sup> A phase IIa study with ruxolitinib (INC424, Jakavi) is ongoing in thalassemia syndromes (NCT02049450). A reduction of spleen size in TDT may decrease transfusion requirement if negative effects on residual endogenous erythropoiesis do not outweigh these.

## Modulation of GDF11 (activin receptor traps ACE-011 and ACE-536)

Background and mechanisms of action: ACE-011 (sotatercept) is a novel and first-in-class recombinant human homodimeric activin type IIa receptor (ActRIIA) fusion protein. ACE-536 (luspatercept) is a similar fusion protein for activin receptor type IIb. These act as traps for a range of ligands in the TGF- $\beta$  superfamily. Ligand traps are molecules that inhibit signaling by binding ligands and sequestering them away from their receptors. ACE-011 consists of an extracellular domain (ECD) of ActRIIA linked to the human IgG1-Fc domain. The molecule is comprised of 2 disulfide-linked chains, dimerized through the Fc region and the Fc region has 2 additional intra-molecular disulfide bonds. Domains of this molecule (ECD and Fc) contain N-linked oligosaccharides.

The concept of an activin receptor trap was originally developed to improve bone mineral density (BMD) in menopausal women, where inhibition of activin signaling is

Authors	HbF modulation	Comments
		Genetic approaches
de Vasconcellos <i>et al.</i> <sup>58</sup>	let-7a miRNAs	Downregulation of let-7a, one of the gene family involved in developmental timing events, in <i>ex vivo</i> erythroblasts increases $\gamma$ globin expression and HbF up to 40%.
Li et al. <sup>59</sup>	miR-144 inhibitor	1.8-fold increase in HbF positive cells mediated by 300 nM miR-144 antagomir
Breda <i>et al</i> . <sup>60</sup>	ZF-Ldb1	Lentiviral-mediated zinc finger (ZF) ZF-Ldb1 gene transfer leads to HbF reactivation; appears superior to pharmacological compounds.
Brendel <i>et al.</i> <sup>61</sup>	Repression of BCL11A	Targeted expression of shRNAmiRs to the erythroid compartment driven by a $\beta$ -globin promoter/LCR element circumvented the detrimental effect on HSC engraftment, while still mediating efficient BCL11A knockdown, leading to high $\gamma$ -globin induction and HbF formation in human CD34s <i>in vitro</i> .
	Phar	macological approaches
Rivers et al. <sup>62</sup>	Inhibition of LSD1 demethylating enzyme	RN-1, a more potent LSD1 inhibitor than tranylcypromine, is a powerful HbF-inducing drug with activity similar to decitabine.
Shearstone <i>et al.</i> <sup>63</sup>	ACY-957, a selective inhibitor of HDAC1/2	CD34 cells undergoing erythroid differentiation were treated with ACY-957, which indirectly activated HbF through GATA2 induction and HDAC1/2 inhibition dependent Bcl11a and Sox6 reduction.
Krivega et al. <sup>64</sup>	G9a methyltransferase inhibitor	Inhibition of G9a methyltransferase by the synthetic compound UNC0638 activates (5-fold) $\gamma$ - and represses (3-fold) $\beta$ -gene expression in adult human CD34*
Ruiz <i>et al.</i> <sup>65</sup>	Ascorbic acid	Ascorbate, a co-factor of TET dioxygenases, is a DNA-hypomethylating agent that increases γ-globin gene expression; vitamin C deficiency may limit HbF induction by drugs targeting epigenetic silencing mechanisms.

miRNA: microRNA; Ldb1: LIM domain binding 1 protein gene; shRNA: microRNA-adapted short hairpin RNA; LCR: locus control region; HSC: hematopoietic stem cell; BCL11A: B-cell lymphoma/leukemia 11A protein gene; CD34: cluster of differentiation 34, a hematopoietic progenitor cell antigen; LSD1: lysine-specific histone demethylase 1A; GATA2: GATA sequence binding protein 2; Sox6: a transcription factor belonging to sex determining region Y-related (SRY) transcription factor family; G9a methyltransferase: methyltransferase methylating lysine 9 of histone H3; TET: ten-eleven translocation mythylcytosine dioxygenase.

### Table 2. Examples of novel HbF modulators potentially applicable for thalassemias.

known to prevent osteoclast-dependent bone resorption. Clinical trials with ACE-011 in 31 healthy post-menopausal women showed increased BMD and biomarkers of bone formation, but surprisingly Hb values increased markedly.69 This Hb increase has subsequently been shown with ACE-356.70 Unlike ACE-011, ACE-356 does not bind with high affinity to activin A, and this may explain why significant increases in BMD were not seen. RAP-011 and RAP-356, murine versions of ACE-011 and ACE-356, respectively, where the human IgG-Fc was exchanged for a murine IgG-Fc, were consequently evaluated in mouse models. An Hb increase was seen in both healthy animals and in non-clinical models of anemia including: chronic renal disease, myelodysplastic syndromes, Diamond Blackfan anemia<sup>71</sup> and iron-restricted anemia.<sup>72</sup> In a murine model of  $\beta$ -TI, Hbb(th1/th1), RAP-536 reduced overactivation of Smad2/3 in splenic erythroid precursors. In addition, treatment of Hbb(th1/th1) mice with RAP-536 or RAP-011 reduced anemia,  $\alpha$ -globin aggregates in peripheral red cells, and ROS elevation in erythroid precursors and peripheral red cells.<sup>11,73</sup> Iron overload, splenomegaly and bone pathology also improved, while reducing EPO levels, improving erythrocyte morphology, and extending erythrocyte lifespan in this model.

Investigation of the mechanisms of action has provided fundamental insights into the regulation of hematopoiesis, both in healthy bone marrow and in thalassemias.<sup>11,12,71</sup> Effects with both drugs occur at later stages of erythropoiesis than with EPO,<sup>71</sup> with more rapid stimulation of Hb and, unlike EPO effects, appearing to require accessory cells.74 Also unlike EPO, which induced iron-restricted erythropoiesis in C57BL/6 and in hepcidin over-expressing mice, RAP-11 had no such effect.<sup>72</sup> Evidence now points to GDF11 regulation in erythroid progenitors as key to the hematopoietic effects. Overexpression of GDF11 may be responsible for the ineffective erythropoiesis associated with β-thalassemia as GDF11 is strongly up-regulated in spleen and erythroid cells of thalassemic animals (and in serum of thalassemia patients). GDF11 itself also inhibited erythroid maturation in mice in vivo and ex vivo, while expression of GDF11 and ActRIIB in erythroid precursors decreased progressively with maturation, suggesting an inhibitory role for GDF11 in late-stage erythroid differentiation.<sup>71</sup> Furthermore, inactivation of GDF11 decreased oxidative stress, α-globin membrane precipitates in RBCs, and also corrected the abnormal ratio of immature/mature erythroblasts by inducing apoptosis of immature erythroblasts through the Fas-Fas ligand pathway.11 Therefore, as GDF11 is a ligand for activin II receptors, RAP-011 and RAP-536 may act by blocking effects of GDF11 on erythropoiesis via the Smad2/3 pathway. By inhibiting GDF11 signaling, ACE-536 or ACE-011 may induce apoptosis of GDF11-expressing erythroid progenitors, restoring their ability to differentiate and, hence, alleviating the anemia.12

*Clinical trials:* a phase-IIa dose-escalation of ACE-011 (sotatercept) in TDT and NTDT study is ongoing and preliminary results have been reported.<sup>75</sup> Because of the long plasma half-life of approximately 23 days, dosing can be given 3-weekly. Patients initially received ACE-011 subcutaneously at 0.1 mg/kg once every three weeks. Dose escalation to 1.0 mg/kg is ongoing. Efficacy is assessed by Hb increase from baseline for NTDT patients and RBC transfusion burden reduction for TDT patients. Of 25 patients in the 0.1, 0.3, and 0.5 mg/kg dose groups, 5 (20%) had  $\beta$ -thalassemia major (TDT) and 20 (80%) had NTDT. A dosedependent Hb increase of 1 g/dL or more from baseline was seen during the first three cycles: in 0, 5 (84%), and 5 (84%) at doses of 0.1, 0.3, and 0.5 mg/kg, respectively, in NTDT. An increase of 2 g/dL or more was seen in 0, 1 (16%), and 2 (33%) of the respective dosing groups. Increased drug exposure was associated with higher Hb increases in the first three cycles for NTDT patients. A significant improvement in RBC morphology was also noted in NTDT. Some patients with TDT have reduction in transfusion burden and these results, together with those at 0.75 mg/kg in NTDT, will be presented at the EHA 2015 congress. Tolerability has been generally good. Adverse events have included: grade 3 bone pain in one TDT patient with a history of osteoporosis and one grade 2 phlebitis in one NTDT patient.75 A similar phase Ha dose finding trial is also ongoing with ACE-536 (luspatercept)<sup>76</sup> in TDT (defined as  $\geq$ 4 units RBCs/8 weeks prior to baseline) and NTDT (defined as <4 units RBCs/8 weeks prior to baseline). Preliminary data in 30 patients (23 NTDT/7 TDT) have been reported.<sup>76</sup> In TDT, the transfusion burden for any 12 weeks on treatment was compared with the 12 weeks prior to treatment with 3-weekly subcutaneous injections for up to 5 doses. Sequential cohorts (n=6 patients/cohort) received doses of 0.2, 0.4, 0.6, 0.8, 1.0, 1.25 and 1.5 mg/kg. In NTDT patients in the 0.6 to 1.0 mg/kg cohorts, a maximum Hb increase from baseline of 1.5 g/dL or more was seen in 7 of 11 (64%) patients. In 7 TDT patients treated in the 0.6 to 1.0 mg/kg cohorts, a more than 50% reduction in transfusion burden was seen for all 7 patients. Two patients with long-standing leg ulcers (one NTDT, one TDT) healed within three months. The most frequent related adverse events included bone pain, headache and myalgia. An expansion cohort (n=30) is planned. These preliminary clinical data for ACE-011 and ACE-536 suggest both improve anemia in NTDT and may reduce transfusion requirements in TDT without significant short-term tolerability issues. These compounds appear to increase Hb values by amounts that have been shown to increase QoL using other approaches in NTDT.15 They also show promise for decreasing the transfusion burden in TDT, although the most appropriate way to apply treatment in TDT needs further evaluation. It is likely that one of these two similar agents will be selected for phase III studies.

### Molecular chaperones for alpha globin

Manipulation of molecular chaperones provides a novel target for decreasing IE. The chaperone heat shock protein 70 (HSP70) is constitutively expressed in erythroblasts and, at later stages of maturation, translocates into the nucleus, thereby protecting GATA-1 from caspase-3 cleavage. This chaperone participates in the refolding of proteins denatured by cytoplasmic stress. During the maturation of human TDT erythroblasts, HSP70 interacts directly with free  $\alpha$ -globin chains. As a consequence, HSP70 is sequestrated in the cytoplasm and GATA-1 is no longer protected, resulting in end-stage maturation arrest and apoptosis. Transduction of a nuclear-targeted HSP70 mutant or a caspase-3-uncleavable GATA-1 mutant restores terminal maturation of TDT erythroblasts, which may provide a rationale for new targeted therapies.<sup>7</sup>

#### Iron restriction to modulate ineffective erythropoiesis

An increasing body of work suggests that iron restriction may improve the effectiveness of erythropoiesis in thalassemias. This is superficially at odds with early work that suggested better improvements in Hb in NTDT patients when EPO treatment was supplemented with iron. However, the site and extent of iron restriction may be critical to any benefits that may ensue.

Iron restriction by transferrin treatment: iron restriction to the erythron in mice may be achieved by intra-peritoneal human apo/holotransferrin;<sup>77,78</sup> in th1/th1 mice, increased Hb and increased hepcidin levels were also seen. This effect was attributed to less iron delivery to normoblasts and therefore less iron for heme synthesis, lower heme, hemichromes, ROS-mediated oxidative stress and apoptosis. In thalassemic mice, apotransferrin decreased serum iron, transferrin saturation, cytosolic iron, MCV, reticulocytes and splenomegaly while increasing Hb.<sup>79</sup> A possible explanation for decreased delivery to the erythron is that additional apotransferrin causes redistribution of iron from diferric only to diferric and monoferric species, the latter having a much slower rate of uptake in the erythron.<sup>80</sup> There is some clinical experience with large doses of purified human apotransferrin to patients with restricted erythropoiesis and raised free plasma iron (NTBI), showing effective binding of this fraction by apotransferrin,<sup>81</sup> as well as prevention of full transferrin saturation.<sup>82</sup> However, this approach may be difficult from a practical point of view in thalassemias and is more of interest as a proof of principle than as a therapeutic modality.

*Iron restriction by increasing hepcidin:* hepcidin insufficiency is a known consequence of IE through mechanisms described above. In principle, by increasing hepcidin, iron exit from macrophages or enterocytes through ferroportin channels is reduced, thereby decreasing transferrin saturation. This principle is supported by observations that an iron-restricting diet in th3/+ mice improves IE and prevents iron overload, and from crossbreeding the same mice with a mouse strain expressing moderate hepcidin levels; this abolishes iron overload and improves Hb and RBC indices.<sup>83</sup> In principle, upregulation of hepcidin can be achieved either by

targeting proteins that exert negative regulatory pressure or by stimulating main positive pressors. Examples of these approaches are listed in Table 3.

Tmprss6 modulation: One way of modulating hepcidin synthesis is through Tmprss6, which is a membrane bound serine protease (matriptase-2) that cleaves hemojuvelin. The latter is a co-receptor with BMP6 receptor for BMP6 and necessary for full activation of the SMAD pathway, and hence hepcidin expression. Tmprss6 deactivates hemojuvelin by cleavage forming soluble hemojuvelin, thereby decreasing hepcidin synthesis. Thus a constitutive increase in hepcidin levels is observed in Tmprss6 knockout mice. Tmprss6 is, therefore, a target for correcting hepcidin insufficiency. An antisense oligonucleotide approach, using RNAse H mechanism which degrades Tmprss6 RNA species, has shown reduction in Tmprss6 protein with corrected hepcidin insufficiency in murine thalassemia and hemochromatosis models, leading to reduction in liver iron content and improvement of anemia in the former.91 A different approach is to inject siRNA formulated in lipid nanoparticles for preferential hepatotropism.92 Using this approach in thalassemic and HFE mice, iron overload was reduced and anemia was improved with correction of IE and extension of RBC survival.

*Iron restriction using hepcidin agonists:* hepcidin itself has a poor pharmacokinetic profile so that synthetic hepcidin agonists have been investigated<sup>83,93</sup> based on the concepts of mimicry or of stimulating production. Mimics are oligopeptides that repeat the necessary amino acid sequence that binds with ferroportin, but have been modified to prevent easy digestion or to improve bioavailability. These mini-hepcidins (e.g. PR65, PR73 and PR73SH) have been tested in hemochromatotic mice, where they prevented iron overload and even caused iron restriction anemia. Iron redistribution was seen in already-overloaded animals (Table 3).

*Iron restriction using iron chelators:* in humans with NTDT, the effects of chelation on Hb levels are somewhat inconsistent, but this may provide mechanistic insight into the principles of iron deprivation as a means of improving

Mechanism of action	Examples	Comments
Mimicry	Minihepcidins	Oligopeptides which repeat the necessary amino acid sequence that binds with feroportin but have been modified to prevent easy digestion or to improve bioavailability, e.g. PR65, PR73 and PR73SH.
Stimulation of production	Tmprss6 silencing	Tmprss6 RNAseH mechanism, siRNA, Tmprss6-ASO, approaches that reduce Tmprss6 protein level and so increase mHJV/sHJV ratio.
	Furin inhibitors	Increase mHJC/sHJV ratio that increases BMPR signaling, possible off-target effects.
	BMP-6	Increases BMPR signaling, known off-target effetcts. 84
	Genistein	An isoflavone, enhancer of hepcidin transcripiton. <sup>84</sup>
	Quercetin	Modulatory effect on hepcidin through BMP6/SMAD pathway in conjunction with other factors and not on its own.85
	Estrogen membrane receptor agonists	Increases hepatic hepcidin but not serum hepcidin in ovariectomized mice. <sup>86</sup>
	IL-22	Agonist fusion protein IL22:IgG1Fc used in mice. <sup>87</sup>
	Interferon- $\alpha$	Up-regulates hepcidin. <sup>88</sup>
	Ezitimibe	Increases hepcidin mRNA in mice.89
	Glucose	75 g in vivo increases hepcidin in humans, potentially from $\beta$ islet cells in pancreas and not the liver. <sup>90</sup>

### Table 3. Potential hepcidin agonists for thalassemias.

Triprss6: transmembrane protease, serine 6; siRNA: short interfering RNA; mHJV/sHJV: membrane and soluble hemojuvelin; BMPR: bone morphogenic protein receptor; IL-22: interleukin-22; IgG1Fc: immunoglobulin G heavy chain.

| 316 | Hematology Education: the education program for the annual congress of the European Hematology Association | 2015; 9(1)

IE. The idea of improving Hb with chelation was suggested by findings that RBC membranes from thalassemia patients carry abnormal deposits of iron, presumed to mediate a variety of oxidative-induced membrane damage and that membrane iron was reduced ex vivo by DFP but not with DFO.94 In patients, after two weeks of deferiprone (DFP) treatment, membrane iron also decreased by 50%. In β-thalassemic mice, DFP decreased membrane free iron by 50% and improved oxidation and survival of biotinylated RBC.95 In wild-type mice, DFP can actually decrease Hb relative to controls by nearly 2 g/dL at 200 mg/kg for 60 days,96 so net effects on Hb in animal models may depend on a balance of such effects. Clinical studies in TDT with DFP showed decreased membrane iron, which was followed by reduced KCI co-transport activity.97 In Eβ-thalassemia patients treated with DFP, Hb rose in 3 patients and transfusion requirements reduced in 4 patients.<sup>98</sup> In a subsequent study of 17 patients, a decrease in LPI and ferritin was found in E\beta-thalassemia after 13-17 months of DFP at 50 mg/kg but there was a non-significant change in Hb, and paradoxically, EPO levels increased significantly.99 No Hb increase was found in

NTDT patients treated with deferasirox, some of whom achieved near normalization of body iron.<sup>100</sup> Together, these findings suggest general iron removal from body stores is insufficient to improve IE in thalassemia and that iron restriction must be targeted.

Recently, DFP chelation has been evaluated together with other approaches to iron restriction in animal models. A 2ndgeneration Tmprss6-ASO<sup>101</sup> has been evaluated in combination with DFP using th3/+ mice. However, whereas DFP alone reduced organ iron content without improvement in erythropoiesis, Tmprss6-ASO alone improved LIC (comparably to DFP), IE, RBC and Hb (by 2 g/dL). The authors concluded that chelation alone was insufficient to improve erythropoiesis, despite de-ironing of organs. Similarly, the use of DFP with mini-hepcidins in a thalassemia mouse model<sup>102</sup> showed, in contrast to monotherapy with DFP, correction of inefficient erythropoiesis with an Hb increase of 1.6 g/dL, reduction of apoptosis, ROS, and improvement in RBC indices. Thus the value of chelation therapy alone or in combination with other interventions for improving IE in thalassemias remains an open question.



Figure 1. Approaches to targeting erythropoiesis in thalassemia. Erythropoiesis requires a well-tuned balance of proliferation and differentiation. While this is perturbed in thalassemia, different mechanisms have been utilized in treatments that stimulate erythropoiesis or correct ineffective erythropoiesis. EPO acts on the proliferating compartments; hydroxyurea has anti-proliferative effects by inhibiting ribonuceotide reductase, but allows differentiation; activin receptor traps (e.g. sotatercept) correct GDF11 overabundance and trigger apoptosis of an expanded proliferating compartment and thus promote differentiation; hepcidin agonists and transferrin, by reducing transferrin saturation (and increasing ratio of monoferric to differric species) act predominantly on differentiating compartment where hemoglobinization peaks; HSP70 mutants prevent maturation arrest by protecting cells from excess alpha chain precipitates; butyrates act on early maturation by transcriptionally activating the A $\gamma$  gene promoter and possibly also inhibit histone deacetylase.

#### Conclusions

Anemia in thalassemia can be corrected by globally increasing erythropoiesis, such as with EPO, or by rendering ineffective erythropoiesis more effective. The latter can be achieved pharmacologically by correcting globin chain imbalance through promoting HbF synthesis, such as with HU or butyrates. These agents have given highly variable but typically modest clinical improvements. Diverse novel and exciting approaches to y-globin activation are in preclinical development. Examples of novel pharmacological agents include inhibitors of histone demethylase, G9a methyltransferase or histone deacetylase HDAC. Genetic manipulation is being investigated by targeted repression of BCL11a through RNA inhibition, gene therapy using lentiviral gene transfer to reactivate y-globin synthesis, and forced chromatin looping with zinc finger motifs. A further novel approach is to modify signaling pathways that affect the balance between proliferation and differentiation. Two examples currently undergoing clinical trials (sotatercept and luspatercept), activin receptor traps that affect GDF11 signaling pathways, appear highly effective at increasing Hb by 1.5 to 2.0 g/dL in phase II trials, and one of these has been selected for phase III development. Another approach is to inhibit Jak2 EPO signaling pathways, such as with ruxolitinib, and this may be particularly useful for patients with hypersplenism. Iron restriction, using apotransferrin, minihepcidin or manipulation of hepcidin synthesis through Tmprsss6, improves IE in murine thalassemia models and is a promising novel approach in pre-clinical development.

#### Abbreviations

AAV: adeno-associated virus AHSP: alpha hemoglobin stabilizing protein; ASO: antisense oligonucleotide; BFU-E: burst forming unit-erythroid; BMD: bone mineral density; BMP6: bone morphogenic protein 6; DFO: desferroxamine; DFP: deferiprone; EMH: extra-medullary haematopoiesis; EPO: erythropoietin; EpoR: erythropoietin receptor; ESA: erythropoiesis stimulating agent(s); GATA-1: GATA sequence binding erythroid transcription factor 1 GDF11: growth differentiation factor 11; GDF15: growth differentiation factor 15; HDAC:, histone deacetylase; HFE: human hemochromatosis gene; HSP70: heat shock protein 70; HU: hydroxyurea; IE: ineffective erythropoiesis; Jak2: Janus kinase 2 LIC: liver iron content. LPI: labile plasma iron; MCV:mean cell volume; NTBI: non-transferrin-bound iron; NTDT: non-transfusion-dependent thalassemia; PHT: pulmonary hypertension; Pts: patients; QoL: Quality of Life; RNAse: ribonuclease; ROS: reactive oxygen species; SCFAD: short chain fatty acid derivatives; SMAD: small mothers against decapentaplegic SPB: sodium phenylbutyrate; Stat5: Signal transducer and activator of trancription 5 TDT: transfusion-dependent thalassemia; TGF-b: transforming growth factor beta; TI: thalassemia intermedia; TM: thalassemia major TWSG1: twisted gastrulation factor 1.

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| 318 | Hematology Education: the education program for the annual congress of the European Hematology Association | 2015; 9(1)

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