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REVIEW

TMPRSS6 as a Therapeutic Target for Disorders of Erythropoiesis and Iron Homeostasis

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ABSTRACT

TMPRSS6 is a serine protease highly expressed in the liver. Its role in iron regulation was first reported in 2008 when mutations in TMPRSS6 were shown to be the cause of iron-refractory iron deficiency anemia (IRIDA) in humans and in mouse models. TMPRSS6 functions as a negative regulator of the expression of the systemic iron-regulatory hormone hepcidin. Over the last decade and a half, growing understanding of TMPRSS6 biology and mechanism of action

has enabled development of new therapeutic approaches for patients with diseases of erythropoiesis and iron homeostasis.

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Keywords: Iron restriction; Iron overload; Hemochromatosis; Ineffective erythropoiesis; Thalassemia

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Key Summary Points

Iron is essential for erythropoiesis, and the restriction of iron supply inhibits erythropoiesis.

Hepcidin, the principal hormone of iron homeostasis, controls dietary iron absorption and plasma iron concentration.

Hepcidin is negatively regulated by the membrane protease TMPRSS6.

TMPRSS6 is an attractive target for the treatment of iron and erythrocyte disorders.

The membrane-associated serine protease TMPRSS6 (also called matriptase 2 or MT2) is predominantly expressed in the liver. Its essential role in iron regulation was established when mutations in TMPRSS6 were shown to be the cause of iron-refractory iron deficiency anemia (IRIDA) in humans and in mouse models [1, 2]. In hepatocytes, TMPRSS6 negatively regulates the expression of the systemic iron-regulatory hormone hepcidin. Increasing understanding of TMPRSS6's biology and mechanism of action has enabled development of new therapeutic approaches for patients with diseases of erythropoiesis and iron homeostasis.

ERYTHROPOIESIS

Erythroid Maturation

Erythropoiesis [3] is the process of cell division and differentiation that produces red blood cells (erythrocytes). Erythrocytes are small, specialized end-stage cells which in humans and other mammals are biconcave and full of hemoglobin but lacking a nucleus or other organelles. In the bone marrow, hematopoietic stem cells sequentially divide and differentiate to ultimately generate, among a variety of hematopoietic progenitor cells, the first

erythroid lineage-committed progenitors named erythroid burst-forming unit-erythroid (BFU-E) and colony-forming unit-erythroid (CFU-E). These progenitor cells undergo terminal erythroid differentiation, starting with the first morphologically recognizable proerythroblast stage, which sequentially divide to produce basophilic, polychromatic, and orthochromatic erythroblasts. Eventually, orthochromatic erythroblasts expel their nuclei and other organelles to generate reticulocytes that enter blood circulation and rapidly mature to erythrocytes.

Erythropoiesis Is Regulated by the Concentration of Erythropoietin

An essential driver and regulator of erythropoiesis is the cytokine/hematopoietic hormone erythropoietin (EPO). Circulating EPO binds its cognate receptor (EPOR) on erythroid progenitors, triggering multiple signaling pathways that control the survival and maturation of erythroid progenitors. EPO is secreted by specialized hypoxia-sensing interstitial cells in the kidney. Changes in hemoglobin concentration affect oxygen delivery to the kidney, which modulates stability of Hypoxia Inducible Factor-2 α (HIF2 α), the transcription factor that controls the synthesis and secretion of EPO. This pathway has been targeted with drugs that inhibit prolyl hydroxylases and therefore increase stability of HIFs for treatment of anemia in chronic kidney disease and other inflammatory conditions [4].

EPO-Independent Hormonal Regulation of Erythropoiesis

Although essential for erythropoiesis, EPO is not the only erythropoietic hormone regulating this process. Another unanticipated pathway controlling erythropoiesis has been identified by the use of activin receptor trap ligands (namely sotatercept and luspatercept) that were initially designed to target TGF- β ligands and improve osteoporosis [5]. These drugs can increase RBC production in normal individuals and patients affected by β -thalassemia and

myelodysplastic syndromes (MDS) by a pathway distinct from that of EPO and affecting predominantly the erythroblasts [6]. The molecular mechanisms that underlie the activity of activin traps are not clear. While GDF11-SMAD-mediated signaling was initially hypothesized as the pathway inhibited by activin receptor ligand traps, more recently it has been shown that lack of GDF11 does not improve anemia in WT animals or mice affected by β -thalassemia. Nevertheless, published data suggest strongly that the drugs are likely to target one or more members of the TGF β superfamily.

Erythropoiesis Is Regulated by the Concentration of Iron

The other principal regulator of erythropoiesis is the amount of iron available to erythroid precursors in the form of diferric or monoferric transferrin (collectively referred to as iron-transferrin or Fe-Tf). Tf is an 80-kDa glycoprotein predominantly synthesized by the liver. Under normal circumstances, 20–45% of the iron-binding sites on transferrin are occupied by ferric iron. The concentration of iron in blood plasma and the storage and distribution of iron in the organism are subject to homeostatic regulation. The main function of Tf is to deliver iron to cells by receptor-mediated endocytosis, carried out by the transferrin receptor TfR1. Although all cells require iron to maintain vital processes, most Fe-Tf is utilized for erythropoiesis, and most TfR1 receptors are located in erythroid precursors. Tf also plays an important role in modulating the sensitivity of the erythroid precursors to EPO, likely by interaction with the second transferrin receptor, TfR2 [7, 8]. Compared to the production of white cells and platelets, the rate of erythrocyte production appears to be preferentially inhibited by decreases in Fe-Tf concentration. The coupling of erythropoiesis to iron supply is achieved through multiple mechanisms that prominently include iron modulation of lineage commitment in bipotent megakaryocytic/erythroid progenitors (MEP) [9] and the modulation by Fe-Tf of EPOR delivery to (pro)erythroblast membranes [10].

Additionally, the production of hemoglobin, by far the predominant protein of erythrocytes, is also sensitive to the concentration of Fe-Tf, which is the sole source of intracellular iron for heme synthesis in erythroblasts. Intracellular iron controls heme synthesis via the iron-regulatory proteins that inhibit translation by binding to the 5'-untranslated region of the mRNA encoding the rate-limiting enzyme of heme synthesis, erythroid-specific 5-aminolevulinic synthase (eALAS, a.k.a., ALAS2) [11]. The intracellular concentration of heme in turn translationally regulates the synthesis of globin chains, coordinating the stoichiometric requirements for normal Hb production [12]. Accordingly, erythrocytes produced under iron-deficient conditions contain less hemoglobin per cell than those produced under iron-sufficient conditions.

IRON REGULATION

Normal Systemic Iron Metabolism

The concentration of circulating iron in blood plasma is maintained at 10–30 μ M by balancing dietary absorption, storage, and recycling of iron by specialized cells against iron utilization for essential metabolic processes by all cells. The hormone hepcidin is the principal regulator of iron homeostasis [13]. Hepcidin (encoded by the *HAMP* gene) controls dietary iron absorption and iron recycling by binding, occlusion, and internalization of the sole known cellular iron exporter ferroportin (FPN1); this prevents iron egress from cells into blood plasma. Hepcidin is a small peptide secreted primarily by hepatocytes. The production of hepcidin is induced by iron loading and inflammation and suppressed by iron deficiency and ineffective erythropoiesis. At the cellular level, the regulation of hepcidin by iron is transcriptional and involves multiple interacting proteins, whose role in iron regulation was identified and verified by the disruptive effects of their mutation on iron homeostasis in humans and laboratory animals. These include the transferrin receptors TfR1 and TfR2, hemochromatosis-associated protein (HFE), hemojuvelin (HJV), bone

morphogenetic proteins (BMPs), bone morphogenetic protein receptors (BMPR), neogenin and matrilysin-2 (TMPRSS6) [14]. These components interact to regulate *HAMP* gene transcription through BMPR and its SMAD signaling pathway.

Hepcidin Regulation by Iron

Hepcidin regulation by extracellular iron involves Fe-Tf binding to hepatocyte TfR2 that, in part, potentiates the BMP/SMAD signaling pathway and hepcidin transcription in response to iron. The interaction of TfR1 and HFE on hepatocytes is disrupted by Fe-Tf, freeing HFE to enhance signaling through the BMP/SMAD signaling pathway. The specific interactions of the various components with each other and with BMPR are not yet understood in detail. Liver sinusoidal endothelial cells are the primary source of BMP2 and BMP6 and are stimulated to increase the production of BMPs as iron stores increase through mechanisms that are not yet understood. Thus, under high iron conditions BMP2 and BMP6 in the liver initiate heterodimerization between BMP receptors type I (ALK2/ALK3) and type II (BMPRII/ActRIIA).

The Role of TMPRSS6 in Iron Homeostasis

TMPRSS6 is a transmembrane serine protease which is primarily expressed in hepatocytes. TMPRSS6 is a critical negative regulator of the receptor complex that controls hepcidin transcription [15]. The exact mechanism of action of TMPRSS6 is still not understood, but TMPRSS6 interacts with HJV and possibly other components of the BMP receptor complex and acts as both an allosteric inhibitor and a protease that degrades components of the receptor complex [16–18]. As a result, TMPRSS6 decreases SMAD1/5/8 signaling and suppresses hepcidin transcription. This facilitates iron absorption and iron release from stores and increases systemic iron availability. Confirming the essential role of TMPRSS6 in iron homeostasis, genome-wide association study (GWAS) analyses have demonstrated that TMPRSS6 variants are associated with differences in red

blood cell indices as well as biomarkers of iron homeostasis [19–22]. TMPRSS6 protein levels increase in response to chronic iron deficiency or erythropoietin treatment in rodents via a posttranscriptional mechanism [23], and this mechanism ensures appropriate hepcidin suppression in conditions of iron deficiency or stimulated erythropoiesis.

Role of the Intestine in Systemic Iron Homeostasis

The intestine is also a critical regulator of systemic iron homeostasis. In addition to renal EPO regulation, HIF2 α also plays a major role in iron absorption in the duodenum, in the settings of iron deficiency, erythropoiesis, and in hepcidin deficiency [24–26]. Therefore, intestinal oxygen sensing is essential for adaptive increases in iron absorption. In fact, HIF2 α , under condition of hypoxia, can upregulate transcription of key enzymes and transporters responsible for duodenal iron absorption, such as the duodenal cytochrome B (DcytB), divalent metal transporter 1 (DMT1), and FPN1 [27].

Iron Overload Disorders

Homozygous or compound heterozygous mutations in *HFE*, *HJV*, *TFR2*, and the *HAMP* cause the iron overload disease hemochromatosis [28], whose analysis has informed our understanding of iron homeostasis. The common feature of these disorders is inadequate or absent production of hepcidin, causing excessive iron absorption from the diet and dysregulated release of iron from macrophages that recycle iron from senescent erythrocytes. Transferrin may become saturated with iron, resulting in the appearance of non-transferrin-bound iron in circulation, a reactive form of iron wherein iron is bound to citrate or other polyanionic substances. This form of iron is taken up by various organs through alternative iron uptake mechanisms that are not subject to physiologic regulation, causing tissue iron overload and injury. Heterozygous mutations in ferroportin that cause resistance to hepcidin

phenocopy the forms of hemochromatosis caused by hepcidin deficiency.

Iron-Refractory Iron Deficiency Anemia (IRIDA)

Mutations in both copies of *TMPRSS6* cause a phenotype opposite to hemochromatosis [29]. In IRIDA, hepcidin concentrations in blood are inappropriately high when considered in the context of serum iron concentrations or iron stores. Patients with this genetic disorder are severely iron deficient as indicated by very low serum iron and transferrin saturation. Their erythrocytes are very small (microcytosis) and their hemoglobin concentration is abnormally low. Attempts to treat their iron deficiency and anemia with oral or intravenous iron are at most partially effective as iron is poorly absorbed in the duodenum, becomes trapped in macrophages and is unavailable for erythropoiesis. Although the reported number of cases is relatively small, it appears that erythropoiesis is disproportionately affected, and, despite the severity of iron deficiency in many cases, to our knowledge there have not been any reports of nonhematologic developmental abnormalities in children with IRIDA.

ERYTHROPOIESIS PROMOTES IRON ABSORPTION AND MOBILIZATION

Erythropoiesis Modulates Iron Homeostasis

Studies of humans and mice subjected to erythropoietic stimuli such as blood loss or the administration of EPO show that the erythropoietic stimulus results in a sustained decrease of serum hepcidin concentration that begins within hours [30, 31]. By contrast, the decrease in serum iron concentration is relatively minor and transient, indicating that the increased demand for iron induced by the erythropoietic stimulus is met by increased iron mobilization from stores and increased iron absorption, promoted by the drop in hepcidin concentration. The response requires both EPO and a marrow

that is capable of erythropoietic response [32], suggesting that the substance that suppresses hepcidin in this setting originates in the marrow. An unbiased search for this substance led to the discovery of erythroferrone (ERFE) [31], a member of the C1q-TNF superfamily of glycoproteins, as an erythroid suppressor of hepcidin. ERFE is secreted by EPO-stimulated erythroblasts and acts on the liver to suppress hepcidin transcription. ERFE appears to act by trapping BMPs secreted by sinusoidal endothelium in the liver [33], and the consequent loss of BMP signaling then decreases hepcidin transcription in hepatocytes. Increase in *TMPRSS6* levels after stimulation of erythropoietic activity is likely important for the full ERFE effect considering that loss of *TMPRSS6* [34] severely blunts the suppressive effect of EPO and ERFE on hepcidin expression.

Ineffective Erythropoiesis as a Cause of Pathological Hepcidin Suppression and Iron Overload

Ineffective erythropoiesis is defined as decreased production of erythrocytes caused by cell death of differentiating erythroblasts in the marrow so that relatively few erythroblasts reach maturity. The resulting anemia stimulates EPO production, which causes immature erythroblast populations to expand and secrete excessive amounts of ERFE and other erythrokinins [35]. High ERFE concentrations suppress hepcidin and cause the hyperabsorption of dietary iron and eventual systemic iron overload. Anemias with prominent ineffective erythropoiesis include β -thalassemia, congenital dyserythropoietic anemias, sideroblastic anemias, and pyruvate kinase deficiency. In these diseases, iron overload develops even in patients who do not receive blood transfusions. Blood transfusions are a rich source of iron (200–250 mg/unit) and become the predominant cause of iron overload in those patients who receive them. By lowering EPO, blood transfusions also transiently decrease ineffective erythropoiesis.

IRON DEFICIENCY RESTRICTS ERYTHROPOIESIS

Inhibition of Erythropoiesis by Hypoferremia

Compared to other tissues with proliferating cell populations, erythropoiesis is uniquely sensitive to the supply of transferrin-bound iron [36]. During iron deficiency, the rate of production of erythrocytes is decreased, and the erythrocytes become smaller and contain less hemoglobin. When iron sufficiency is restored by the administration of parenteral iron, the number of erythroblasts in the marrow increases within days followed by the release of reticulocytes into the circulation [37]. The mechanisms that underlie the tight coupling of erythropoiesis to iron availability are still incompletely understood.

Iron Regulates Erythroid Lineage Commitment

Many patients with iron deficiency anemia have an increased number of circulating platelets. Recent studies in mouse models and human hematopoietic progenitors revealed that MEPs exposed to an iron-deficient environment develop metabolic changes that result in MEP bias towards differentiating to megakaryocytes rather than erythrocytes [9, 38]. The receptor that senses iron deficiency in this setting is Tfr2, also involved in iron sensing by hepatocytes.

Iron Regulates Erythroid Precursor Sensitivity to EPO

Restriction of iron supply to the erythroid marrow reduces the erythropoietic response to EPO. In this setting, iron deficiency again appears to be sensed by Tfr2 [39], which in turn interacts with the receptor control element Scribble [10] to reduce EPO sensitivity by limiting cell surface display of EPOR, impairing erythroid proliferation and survival.

Iron Regulates the Synthesis of Hemoglobin and Other Erythrocyte Proteins

The production of both heme and globin chains is decreased during iron deficiency so that the normal stoichiometry of hemoglobin composition is maintained without excess (potentially cytotoxic) production of any of the components. The production of heme is regulated by iron availability at the level of the first component of the heme synthetic pathway in erythroblasts, the erythroid form of aminolevulinic acid synthase, ALAS2, whose translation is inhibited during cellular iron deficiency by iron-regulatory proteins IRP1 and IRP2 [40]. Under the same conditions, the translation of globin chains and some other erythrocyte proteins is inhibited by heme-regulated inhibitor (HRI, heme-regulated eIF2 α -kinase) [41]. Here HRI also cross-talks with the mammalian target of rapamycin (mTOR) pathway to inhibit erythroblast protein synthesis during iron deficiency [12].

TARGETING TMPRSS6 FOR IRON-RESTRICTIVE THERAPY IN HEMATOLOGIC DISORDERS

Rationale for Iron-Restrictive Therapy

Iron-restrictive therapy is defined as an intervention that inhibits the absorption of dietary iron and the release of stored iron from macrophages and hepatocytes. This can be achieved by therapeutically raising hepcidin concentrations or by inhibiting ferroportin on the cell membranes in tissues that deliver iron to blood plasma. The expected effect of these interventions is to lower serum iron and transferrin saturation, with secondary effects on erythropoiesis depending on the degree of iron restriction. These pharmacologic effects can be achieved by the administration of hepcidin or hepcidin analogs, or other inhibitors of ferroportin function or expression, or the administration of compounds that stimulate the

endogenous production of hepcidin. Approaches taken by different groups (Table 1) differ in mode of action, route, and frequency of administration. In the past years, hepcidin mimetics, RNA and DNA therapeutics against *TMPRSS6* including antisense oligonucleotide (ASO) and siRNA, and small molecule ferroportin inhibitor have been tested in various preclinical models of iron overload and hematologic disorders. Several programs have announced promising data in human clinical trials.

Hepcidin Agonists

Hepcidin, or compounds that mimic its action on ferroportin, have been developed for the treatment of diseases characterized by hepcidin deficiency and for diseases where iron restriction is expected to offer therapeutic benefits (Table 1). As a class they validate the targeting of the *TMPRSS6* pathway at the level of its sole known nonredundant effector. Rusfertide, a hepcidin-like peptide administered by subcutaneous injection, has shown efficacy in

preclinical models of β -thalassemia [42] and polycythemia vera (PV). In Phase 2 trials in human PV, rusfertide was well tolerated and effectively replaced phlebotomy for the control of hematocrit [43]. Patients reported improved symptoms, and adverse events consisted mainly of nonserious injection site reactions. Vamifeport is an orally administered small molecule drug which binds to ferroportin, blocks cellular iron export, and causes ferroportin internalization [44]. Vamifeport induced iron restriction and ameliorated β -thalassemia in preclinical models [45]. In healthy human volunteers, the drug was well tolerated and induced the expected hypoferremic response. LJPC-401 is a peptide drug chemically identical to human hepcidin but formulated for rapid absorption after subcutaneous administration. Treatment of hemochromatosis patients with LJPC-401 significantly decreased the need for phlebotomy, but in another trial in patients with transfusion-dependent β -thalassemia, LJPC-401 did not reduce cardiac iron (primary endpoint) or decrease serum iron concentrations (one of several secondary endpoints). It is possible that

Table 1 Pharmacologic modulators of the hepcidin-ferroportin axis

Agent	Sponsor	Chemistry	Mechanism of action	Route and frequency of administration
Hepcidin LJPC-401	La Jolla Pharmaceutical Company	Peptide	Binds to, causes internalization and destruction of ferroportin	SC Daily or weekly
Rusfertide (PTG-300)	Protagonist Therapeutics	Peptide	Binds to, causes internalization and destruction of ferroportin	SC weekly
Vamifeport (VIT-2763)	Vifor Pharma	Small molecule	Binds to and blocks ferroportin	Oral frequency of administration unknown
Sapablursen (ISIS 702843, <i>TMPRSS6</i> -L _{Rx})	Ionis Pharmaceuticals Inc	GalNAc-ASO	Binds to <i>TMPRSS6</i> mRNA causing its degradation leading to up-regulation of <i>HAMP</i>	SC monthly
SLN124	Silence Therapeutics	GalNAc-siRNA	Binds to <i>TMPRSS6</i> mRNA causing its degradation leading to up-regulation of <i>HAMP</i>	SC frequency of administration unknown

LJPC-401 failed to induce iron restriction because of inadequate dosing. Mild to moderate injection site reactions were reported with the drug [46].

TMPRSS6 as a Therapeutic Target

Inhibition of TMPRSS6, the negative regulator of hepcidin transcription, is an attractive option because its effects in humans are exemplified by studies of IRIDA, a genetic disease in which the function of TMPRSS6 is impaired as a result of mutations, most often involving both copies of the gene. From the studies of these patients and related mouse models, it appears that iron restriction is the sole clinical effect of the loss of TMPRSS6 function. As protease activity is not required for hepcidin suppression by TMPRSS6 [16], therapeutic targeting of TMPRSS6 will likely have to focus on decreasing the overall levels of TMPRSS6 or interfering with its allosteric function rather than solely on inhibiting its protease activity. SLN124 and sapabursen target TMPRSS6 by suppressing its synthesis through hepatocyte-targeted siRNA and anti-sense mechanisms, respectively.

Hemochromatosis

As described in previous sections, hemochromatosis is a group of genetic diseases most commonly caused by absolute or relative hepcidin deficiency culminating in whole-body iron overload. Removal of iron through phlebotomy remains the mainstay of treatment for this disease and consists of an intensive deironing phase with weekly or biweekly removal of 1 unit of blood (approximately 200–250 mg of iron each), typically for a year or more, followed by a maintenance phlebotomy regimen once accumulated iron excess is removed [47].

Mouse Models of Hemochromatosis

In the most common forms of hemochromatosis, pharmacologic restoration of normal hepcidin concentrations is expected to stop the progression of the disease, as has been demonstrated in various mouse models of hemochromatosis. Thus, early replacement of hepcidin in hepcidin-deficient patients, before substantial iron accumulation occurs, would effectively cure this disease. However, because of the low natural rate of iron loss from the body, therapeutic hepcidin replacement does not significantly reverse the lifetime accumulation of iron in various organs. In mouse models of the most common form of hemochromatosis caused by the C282Y mutation in *HFE*, genetic ablation of *TMPRSS6* or its decrease by administration of ASOs or siRNAs prevented development of iron overload [48–51]. These studies provide the proof of concept that decreasing TMPRSS6 levels is a rational therapeutic approach to increase hepcidin and prevent iron loading in patients with hemochromatosis.

Thalassemias

Forms of anemia such as β - or α -thalassemia are characterized by active proliferation of erythroid progenitors and low hepcidin expression [52, 53]. In particular, in β -thalassemia, mutations in the β -globin gene lead to a relative excess of α -globin, which, together with heme, forms hemichromes, molecules with toxic potential to trigger generation of reactive oxygen species (ROS), apoptosis of erythroid progenitors, and short-lived erythrocytes [54]. This results in anemia, chronically high levels of erythropoietin, and ineffective erythropoiesis—overproduction of erythroid progenitors that fail to generate mature erythrocytes [55]. β -Thalassemia is currently categorized as either transfusion-dependent (TDT) or non-transfusion dependent (NTDT). However, even when transfusions are not required, the

manifestations of the disease can be severe, including chronic anemia and splenomegaly as well as pulmonary hypertension and an increased risk of thrombosis [56]. Anemia and ineffective erythropoiesis also cause increased intestinal iron absorption mediated by duodenal hypoxia and erythroid-mediated suppression of the iron regulator hepcidin [27, 55, 57]. The resulting iron overload adds to morbidity by promoting cirrhosis, cardiomyopathy, diabetes, and hepatocarcinoma and exacerbating erythroid cell damage, apoptosis, and ineffective erythropoiesis [54]. Even NTDT patients with β -thalassemia may require iron chelation to avoid the morbidity and mortality associated with severe iron overload [54, 56]. Similar features are also observed in some patients affected by α -thalassemia.

Mouse Models of Non-Transfusion-Dependent β -Thalassemia

The *Hbb*^{th3/+} mouse model of β -thalassemia mimics the NTDT condition, showing increased proliferation and decreased differentiation of the erythroid progenitors, apoptosis of erythroblasts due to the presence of toxic hemichromes, reticulocytosis, shorter life span of RBCs in circulation, splenomegaly, extramedullary hematopoiesis, anemia, and iron overload [48, 52]. Treatment of *Hbb*^{th3/+} mice with TMPRSS6-ASO (an antisense oligonucleotide directed to TMPRSS6) decreased the formation of insoluble membrane-bound globins, ROS, and apoptosis and improved anemia. These animals also exhibited a significant amelioration of liver iron overload, ineffective erythropoiesis, and splenomegaly and an increase in total hemoglobin levels [48]. Similar results were observed with hepcidin mimetics and siRNA against TMPRSS6 [45, 49, 58, 59]. In addition, TMPRSS6-ASO in combination with iron chelators and erythropoietin-stimulating agents ameliorated ineffective erythropoiesis and iron overload in this model [60, 61]. These data suggest that ASOs targeting TMPRSS6 could be beneficial in individuals with β -thalassemia and related disorders, such as α -thalassemia.

Sickle Cell Disease

In sickle cell disease (SCD), a single base mutation in the globin gene (*HBB*) generates sickle hemoglobin (HbS), which upon deoxygenation can polymerize within the cell producing the characteristic sickle-shaped erythrocyte that defines the disease. Hemoglobin polymerization, leading to erythrocyte rigidity and vaso-occlusion, is central to the pathophysiology of this disease, but chronic anemia, hemolysis, and vasculopathy also contribute to chronic morbidity and premature mortality. In SCD, there is a delay between deoxygenation and the initiation of sickling that is dependent on the intracellular concentration of HbS [62]. Case reports and small studies have suggested associations among iron deficiency, decreased mean corpuscular Hb concentration (MCHC), and improvement in several clinical parameters, including decreased sickling and hemolysis and decreased hospitalization for pain crises [63, 64].

Mouse Models of Sickle Cell Disease

Additional support for iron restriction as a therapeutic approach has been obtained using mouse models of SCD, where the α - and β -globin genes are replaced by fragments of the human α - and sickle β^S -globin loci [65]. In the first study, iron deficiency (consequent to an inactivating mutation of the intestinal *Hif2 α* gene) led to better RBC survival and less severe anemia when compared with iron-sufficient wild-type mice [66]. In the second study, SCD mice were exposed to an iron-restricted diet. Compared to mice fed a standard diet, animals with an iron-restricted diet exhibited decreased serum iron concentrations, decreased MCHC, increased number of circulating RBCs, improved hematocrit (HCT), and reduced sickling under condition of hypoxia [67]. Administration of a small molecule inhibitor of ferroportin, vamifeport, in SCD mice also caused iron restriction and reduced sickling without worsening anemia [68]. Altogether, these observations and studies suggest that

iron restriction may decrease the clinical complications arising from both sickling and hemolysis in SCD.

Polycythemia Vera

Polycythemia vera (PV) is a myeloproliferative neoplasm (MPN) characterized by erythrocytosis, thrombocytosis, leukocytosis, and splenomegaly [69]. Almost all patients have a single driver mutation (~ 95% *JAK2 V617F*) in the Janus kinase 2 (*JAK2*) gene. The *JAK2 V617F* mutation results in constitutive activation of hematopoietic signal transduction and exuberant hematopoiesis, leading to the PV-associated symptoms. It is a disorder of the elderly with a median age of onset of ~ 60 years old. PV is a chronic and progressive disorder, and patients often experience worsening of symptoms with time. The cardinal manifestation of PV is an increase in blood hemoglobin and corresponding increase in hematocrit.

PV management is focused on minimizing the thrombotic risk using aspirin and phlebotomy, along with cytoreduction therapies for high(er)-risk patients. Phlebotomy reduces iron availability to the erythroid cells and, as a result, reduces the rate of production of erythrocytes. Intensive treatment with phlebotomy to target hematocrit < 45% has been shown to reduce the composite endpoint of time to death from cardiovascular causes or major thrombotic events in patients with PV [70]. Almost all PV patients are iron deficient at presentation and/or during the course of their disease and may remain iron deficient because of high erythroid demand for iron and frequent phlebotomies [71]. Phlebotomy reduces hepcidin levels and in this sense counteracts the iron restriction produced by phlebotomy by facilitating iron absorption and re-cycling necessary for enhanced erythropoiesis. Some patients with PV experience a substantial symptom burden which may negatively affect

patient health-related quality of life in areas such as inactivity, dizziness, headache, and fatigue [72] and which might be a function of iron deficiency. A recent exploratory analysis on the relationship between correction of iron deficiency and improvements in concentration problems, cognitive function, dizziness, fatigue, headaches, and inactivity during treatment with ruxolitinib showed a greater benefit in PV patients that were iron deficient at baseline [73].

Mouse Models of Polycythemia Vera

Controlling HCT is of critical importance in PV management. Pharmacologically induced iron-restricted erythropoiesis may be superior to intermittent phlebotomy, where the removal of RBCs is rapidly compensated. Such agents may treat the erythrocytosis of PV by limiting iron availability to erythroid precursors, thereby inhibiting *JAK2*-stimulated erythropoiesis and reducing RBC production. A mouse model of PV (carrying the *JAK2V617F* conditional knock-in allele [74]) has been utilized to test whether iron restriction would be beneficial in this disease. Administration of a hepcidin agonist or a *TMPRSS6*-ASO reverted erythrocytosis and normalized the HCT level in PV mice [59, 75]. These results suggest that *TMPRSS6*-ASO therapy could be used as a “medical phlebotomy” to provide continuous control of accelerated erythropoiesis to treat PV, while possibly avoiding the adverse consequences of total body iron depletion characteristic of chronic phlebotomy. Because of the association between high HCT levels and poor outcomes, continuous control of HCT levels using *TMPRSS6*-ASO could be highly clinically meaningful if clinical trials show that it can be done safely. Preliminary data from a clinical trial using a hepcidin mimetic developed by Protagonist Therapeutics [43] are encouraging in this regard.

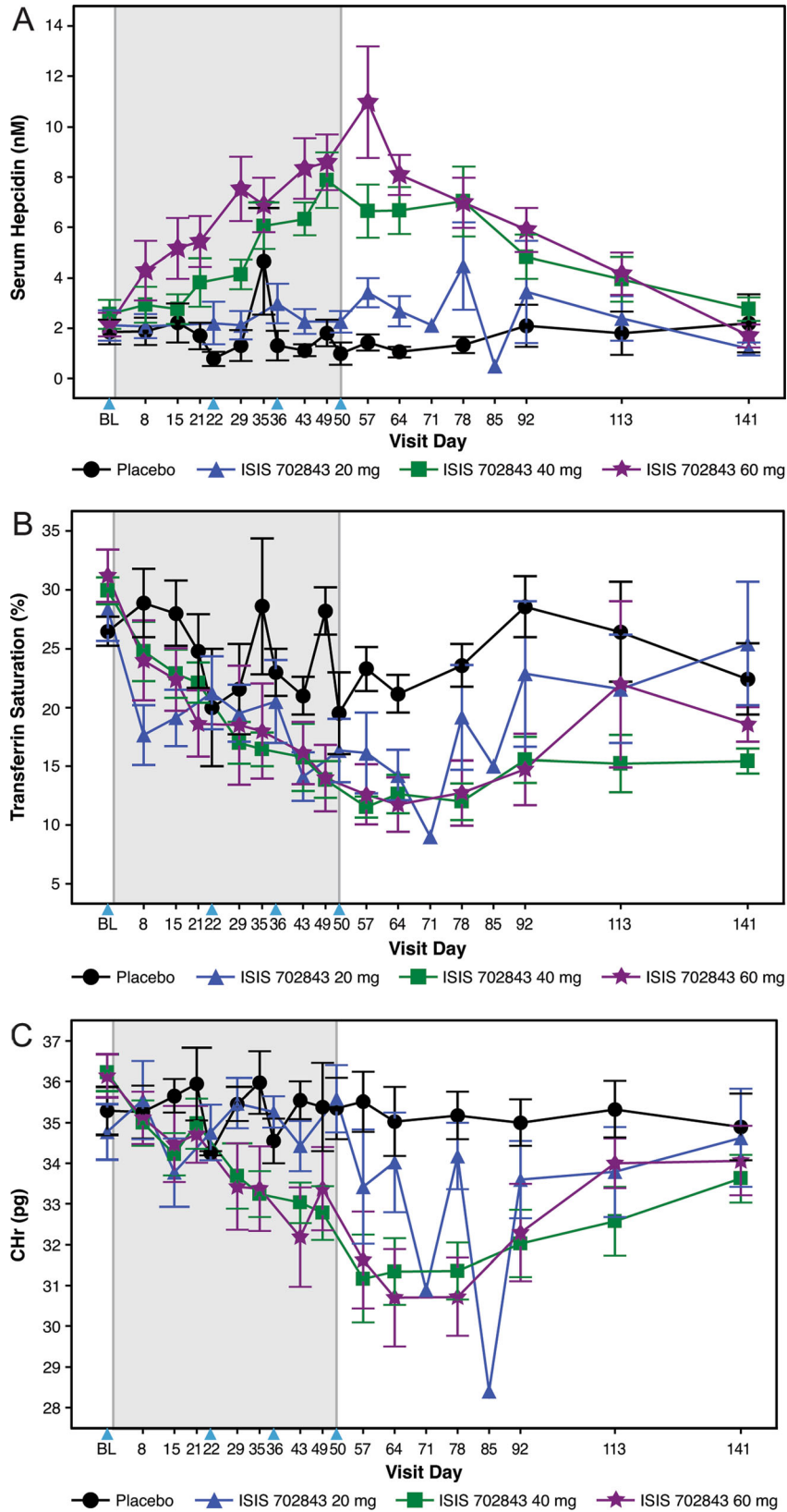
Effects of TMPRSS6 Antisense Oligonucleotide Therapy in Healthy Human Volunteers

Sapablursen (ISIS 702843) is an *N*-acetylgalactosamine-modified antisense oligonucleotide (GalNAc-ASO) that targets *TMPRSS6* mRNA. Sapablursen matches both human and monkey *TMPRSS6* sequences perfectly. In non-human primates, sapablursen treatment was well tolerated after chronic dosing. Dose-dependent reduction of *TMPRSS6* was accompanied by decreases in serum iron, transferrin saturation, and subsequent hemoglobin reduction, as expected for *TMPRSS6* deficiency in non-human primates and mice [76].

The importance of *TMPRSS6* inhibition in regulating hepcidin production in humans was assessed in healthy volunteers (NCT03165864). The study was approved by Institutional Review Board Services (Victoria, Australia) and conducted in compliance with the World Medical Association Declaration of Helsinki (October 2002), Good Clinical Practice (GCP) guidelines, and all national, state, and local laws of the appropriate regulatory authorities. All participants provided written informed consent prior to participation in the study. The treatment cohorts, $n = 9$ each, were well balanced in demographic and baseline characteristics. Overall, the mean age was 35 years (range 18–64); most subjects were male (91.7%), white (72.2%), and not Hispanic or Latino (91.7%) with a BMI 24.6 kg/m² (range 17.2–31.8). Participants received a single dose of 20, 40, or 60 mg sapablursen (or placebo 0.9% saline) on day 1, followed by three additional doses of the same strength on days 22, 36, and 50. Each dose was administered by subcutaneous injection. The single dose of 60 mg sapablursen increased serum hepcidin compared to placebo (Fig. 1A). Subsequent administration of three additional doses of 40 or 60 mg on days 22, 36, and 50 caused dose- and time-dependent increases in serum hepcidin relative to placebo (Fig. 1A). In the repeated dose phase, serum iron and

transferrin saturation were reduced in a time-dependent fashion by all three dose levels (Fig. 1B). The cellular concentration of hemoglobin in reticulocytes was reduced at the higher dose levels (40 and 60 mg) at later time points, indicating that the availability of iron to the erythroid was reduced (Fig. 1C). A trend towards a reduction in blood hemoglobin and hematocrit was observed but neither was significantly reduced in this short, healthy volunteer study (data not shown). While a reduction in blood hemoglobin is expected in healthy volunteers and PV patients, correcting ineffective erythropoiesis in patients with β -thalassemia might be expected to increase blood hemoglobin. No clinically significant changes were observed on other hematologic parameters, and platelet count was not changed. Three volunteers discontinued sapablursen because of a sustained reduction in transferrin saturation, and dosing was temporarily withheld in two additional volunteers for the same reason. Withholding the dose or discontinuing treatment with of sapablursen was mandated by the protocol if transferrin saturation was reduced to $< 10\%$ (absolute). Sapablursen was safe and well tolerated in healthy volunteers during both the single and repeated dose phases. The terminal pharmacokinetic half-life of sapablursen was 2–4 weeks, supporting once monthly dosing in further clinical studies.

Sapablursen is currently being assessed in two on-going clinical studies in patients with non-transfusion-dependent β -thalassemia (NCT04059406) and polycythemia vera (NCT05143957). The β -thalassemia study aims to assess the impact of sapablursen on blood hemoglobin and liver iron content over a treatment period of up to 2 years in 36 patients. The study is fully enrolled. The PV study is actively recruiting 40 patients and will assess the impact of two dose levels of sapablursen on the frequency of phlebotomy and the Myeloproliferative Neoplasm Symptom Assessment Form-Total Symptom Score (MPN-SAF-TSS) after 37 weeks of treatment. Additional indications are also being explored.



◀**Fig. 1 A–C** Subcutaneous administration of sapablursen (ISIS 702843) increases serum hepcidin concentration (A), reduces transferrin saturation (B), and reduces cellular hemoglobin content in reticulocytes (C) in healthy volunteers. A single dose of 20, 40, or 60 mg was administered on day 1, followed by three additional doses of the same strength on days 22, 36, and 50. The planned administration schedule for sapablursen is indicated by a triangle on the x-axis. Deviations from the planned administration schedule occurred because of scheduling issues with individual volunteers and transient or sustained reduction of TSAT < 10% in five subjects receiving sapablursen. All cohorts included nine healthy volunteers; however, for various reasons (e.g., early/late sample collection, missed sample collection) the number of samples at different timepoints varies. Values are \pm standard error of the mean [SEM] with n typically > 6 for all groups; however, individual values range from $n = 1$ to 9. The study was conducted in a dose-escalating fashion, with lower doses tested prior to testing higher doses. Some volunteers were administered placebo in each cohort. Data for these volunteers were pooled to form a single placebo group

CONCLUSIONS

Iron restriction represents a unique precision medicine approach for treatment of hematologic disorders affecting the erythroid lineage. Erythrocyte precursors are exquisitely sensitive to iron restriction, conferring a high level of selectivity to the approach. Heparin is the key regulator of iron absorption and recycling, making modulation of hepcidin activity or expression a highly effective way to restrict iron availability to erythroid precursors. Targeting TMPRSS6 as a negative regulator of hepcidin expression has been shown to be effective in animal models of hematologic disease and shows promise in early clinical trials. The ongoing clinical trials will be critical to determine whether this approach can deliver new treatments with better risk-benefit profiles for patients with erythroid disorders.

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Data Availability. The datasets generated during and/or analyzed during the current study are not publicly available as they contain data proprietary to Ionis Pharmaceutical Inc and mechanism and standards for sharing these data are not available institutionally.

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