Combination of *Tmprss6*-ASO and the iron chelator deferiprone improves erythropoiesis and reduces iron overload in a mouse model of beta-thalassemia intermedia

Beta-thalassemia is one of the most frequently inherited disorders caused by mutations in the beta globin gene or its promoter, leading to reduced or absent beta globin synthesis. Ineffective erythropoiesis (IE) and consequent extramedullary hematopoiesis, splenomegaly and systemic iron overload are major features of this disease. The disease course can be associated with severe anemia and need for lifelong transfusion therapy (thalassemia major, TM) or relatively less severe anemia (non-transfusion-dependent thalassemia, NTDT, or thalassemia intermedia, TI). Patients affected by beta-thalassemia intermedia do not require chronic blood transfusions for survival. However, transfusion-independence is still associated with a variety of serious clinical morbidities.[1-3] In NTDT the master regulator of iron homeostasis, hepcidin (*Hamp*), is chronically repressed.[4-7] Therefore, patients absorb abnormally high levels of iron, requiring iron chelation to prevent the clinical sequelae associated with iron overload. Iron homeostasis needs to be carefully regulated in order to avoid toxicity due to its excess. If untreated, iron overload leads to organ failure and death. For this reason, in beta-thalassemia and other iron-related disorders, the management of iron overload has become the main focus. Chelation therapy, however, does not target the mechanism responsible for abnormal iron absorption, which is low levels of *Hamp* expression and synthesis. It has been shown that in mice affected by NTDT ($Hbb^{th3/+}$ or *th3*/+), second generation antisense oligonucleotides (*Tmprss6*-ASO) or lipid nanoparticle (LNP)-formulated siRNAs can reduce the expression of transmembrane serine protease *Tmprss6*, one of the major suppressors of hepcidin expression.[8,9] Suppression of *Tmprss6* led to an increase in hepcidin synthesis and hemoglobin levels. These observations were also associated with a net reduction in splenomegaly, iron overload, transferrin saturation (TfSat), formation of insoluble membrane-bound globins (hemichromes) and reactive oxygen species (ROS).[9] Thus, we hypothesized that the simultaneous use of the iron chelator deferiprone (DFP) with *Tmprss6*-ASO (*Tmprss6*-ASO+DFP) could combine the positive effects of *Tmprss6*-ASO on erythropoiesis and iron absorption with the chelation benefit on organ iron content. In this study, 3- to 4-month-old $Hbb^{th3/+}$ females were treated with 50 mg/kg of *Tmprss6* antisense oligonucleotide (*Tmprss6*-ASO, twice a week for 6 weeks) or *Tmprss6*-ASO in combination with the oral iron chelator DFP dissolved in the drinking water at 1.25 mg/ml, using either a commercial diet (normally used in the facility where animals were housed) containing 200 ppm of iron, or a physiological diet containing 35 ppm of iron. The majority of the animals available were treated using the commercial diet and just a few animals per group received the physiological one. With both diets we obtained the same trend in behavior, but considering that the numbers were not comparable, we decided to show only the data obtained from the 200 ppm diet.

As expected, *Tmprss6*-ASO treatment, alone or in combination with DFP, suppressed *Tmprss6* expression in the liver reaching an 82% decrease (P<1.8E-08) (Figure 1A). This was associated with a significant increase in *Hamp* expression (Figure 1B). DFP alone did not induce any changes in Tmprss6 or Hamp levels (Figure 1A,B).

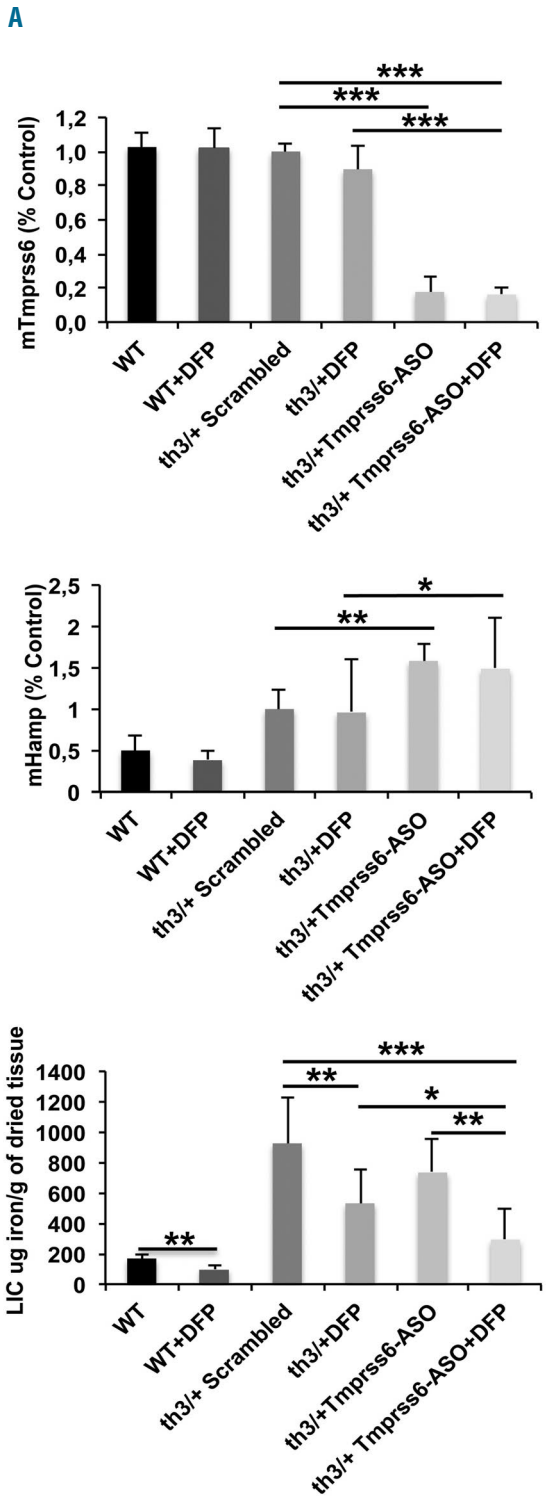

Figure 1. Reduced *Tmprss6* expression and increased hepcidin levels are associated with significant reduction in liver iron concentration following administration of *Tmprss6*-ASO alone, or in combination with DFP: $Hbb^{th3/+}$ (indicated in figures as *th3*/+) females were treated twice a week for 6 weeks with: Scrambled-ASO (n=5-9), *Tmprss6*-ASO (n=6), DFP (n=9-11), *Tmprss6*-ASO+DFP (n=7). *Tmprss6*-ASO with or without DFP significantly reduced mTmprss6 expression (A). Increased *Hamp* expression was achieved only when animals received *Tmprss6*-ASO with or without DFP (B), and greater reduction in liver iron concentration was observed in animals treated with both *Tmprss6*-ASO and DFP (C). *Tmprss6*-ASO sequence, 5′-GCTTAGAG-TACAGCCCACTT-3′. Results represent mean ± SD. Statistical significance was determined using Student's *t*-test (*P<0.05, **P<0.01, ***P<0.001).

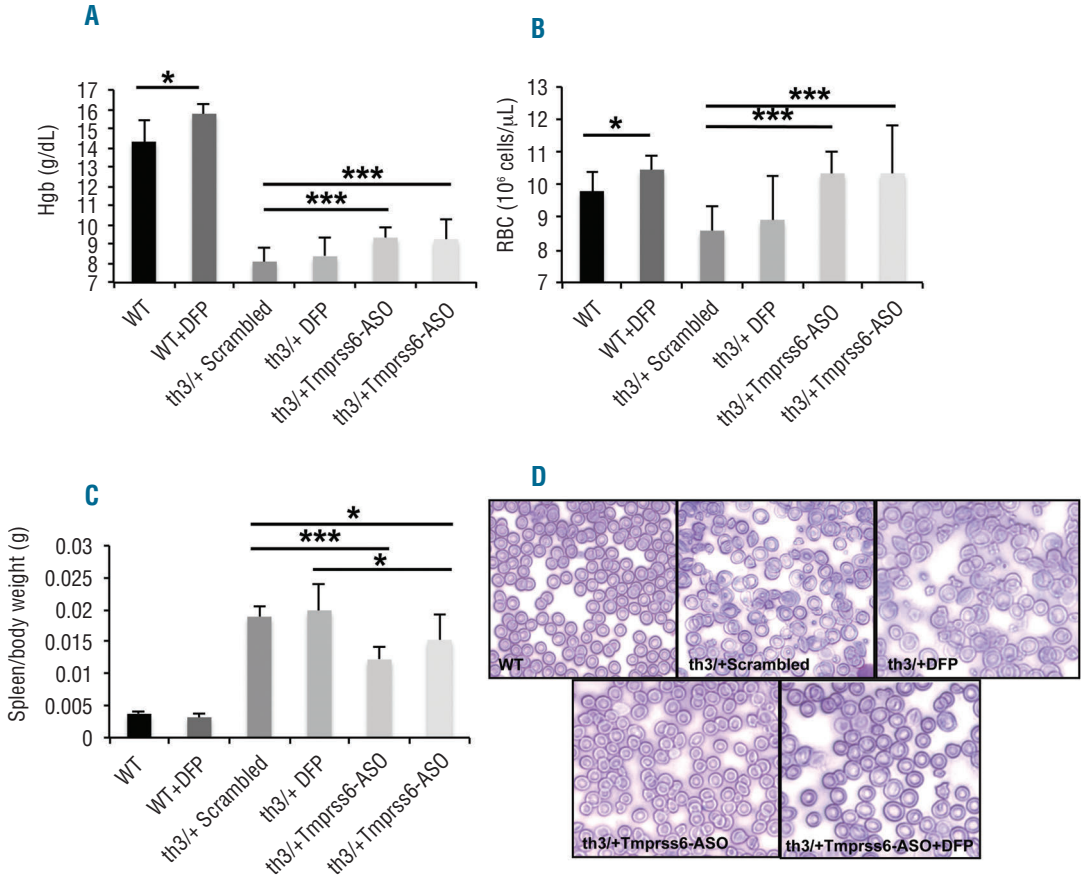

Figure 2. DFP alone is not sufficient to improve IE. *Tmprss6*-ASO alone or in combination with DFP increased Hgb levels (≥1.5 g/dL) (A) and RBC count. (B). Splenomegaly was reduced in *Tmprss6*-ASO and *Tmprss6*-ASO+DFP-treated mice compared to $Hbb^{th3/+}$ controls and DFP-treated animals (C). RBC morphology was improved following treatment as can be seen in a representative example of Giemsa staining of peripheral blood smears (D). Results represent mean ± SD. Statistical significance was determined using Student's *t*-test (*P<0.05, **P<0.01, ***P<0.001).

However, as expected, DFP was efficient in reducing liver iron content both in wild-type (WT) and $Hbb^{th3/+}$ animals (Figure 1C). *Tmprss6*-ASO alone showed a trend towards reducing liver iron concentration when compared with scrambled-ASO treated animals (Figure 1C). The level of reduction achieved by *Tmprss6*-ASO alone is lower than that which we previously published. This could be due to a number of reasons. Animals used in this study were younger and showed a lower level of iron accumulation to start with. In addition, we observed a bigger variability in the results, mostly in scrambled-ASO treated animals, which apparently reduces the effect of the *Tmprss6*-ASO. Regardless, in combination with DFP the antisense oligonucleotide achieved a significantly greater reduction in iron content when compared with each agent separately or with scrambled-ASO controls. Of note, similar results were also observed by Schmidt PJ, *et al.* in their recent publication using siRNAs against *Tmprss6* in combination with DFP in the same mouse model of thalassemia intermedia.[10] The ratio between *Hamp* expression and liver iron concentration (LIC) (Table 1), highlights the concept that to achieve iron restriction in this mouse model of beta-thalassemia increased *Hamp* expression is necessary. Even though administration of DFP alone was successful in decreasing liver iron content, with a reduction of 38% (Figure 1C), it failed to improve parameters of erythropoiesis such as hemoglobin (Hgb) levels (Figure 2A), red blood cell (RBC) production (Figure 2B) spleen weight (Figure 2C) and RBC morphology (Figure 2D). All these parameters were significantly improved in animals treated with *Tmprss6*-ASO alone or combined with DFP, reflecting improved erythropoietic efficiency. When compared with $Hbb^{th3/+}$ controls, chelation therapy alone was associated with increases in serum iron and transferrin saturation levels (Figure 3A,B). Mice treated with *Tmprss6*-ASO alone or in combination with DFP, however, exhibited reduction in serum iron and transferrin saturation *versus* $Hbb^{th3/+}$ animals treated with or without DFP (Figure 3A,B). DFP is able to spontaneously transfer iron to extracellular transferrin, which

Table 1. Ratio between Hamp expression and liver iron concentration (LIC).

	mHamp (%control)	LIC (ug/g)	mHamp/LIC*1000
WT	0.499	168.800	2.956
WT+DFP	0.381	98.000	3.888
$Hbb^{th3/+}$ Scrambled	1.000	926.125	1.080
$Hbb^{th3/+}$ DFP	0.958	532.091	1.800
$Hbb^{th3/+}$ Tmprss6- ASO	1.582	734.500	2.154
$Hbb^{th3/+}$ Tmprss6- ASO+DFP	1.488	293.286	5.074

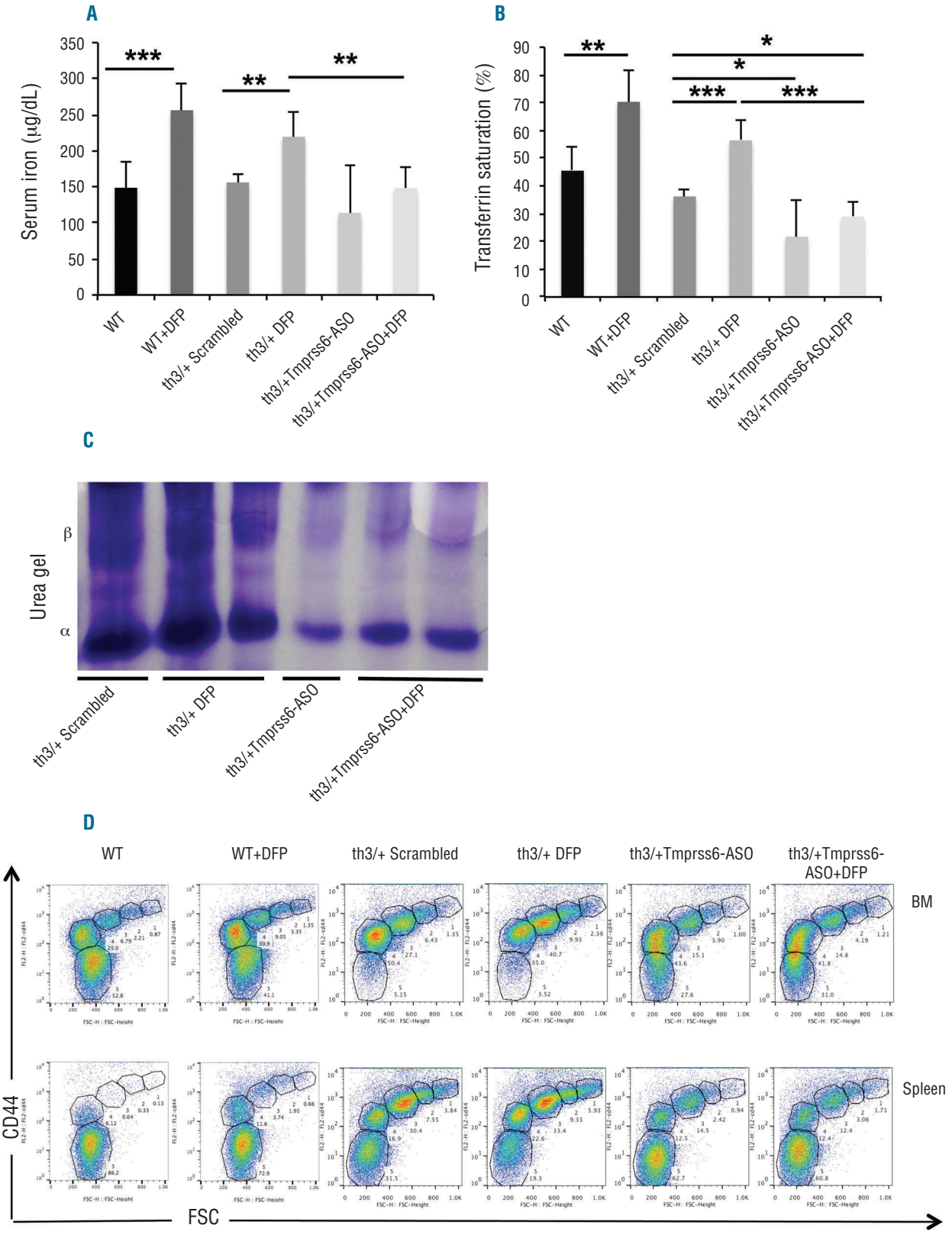

Figure 3. Amelioration of erythropoiesis requires decreased erythroid iron intake and hemichrome formation: Serum iron (A) and transferrin saturation (B) were decreased in animals that received *Tmprss6*-ASO alone or in combination with DFP, while increases were observed in DFP-treated animals. As a consequence, hemichrome formation (C, urea gel electrophoresis) was decreased only in animals treated with *Tmprss6*-ASO or *Tmprss6*-ASO+DFP, resulting in markedly improved IE (D). More than 5 mice per group were analyzed. Each plot shown is from a representative mouse for each group. Results represent mean ± SD. Statistical significance was determined using Student's *t*-test. (**P*<0.05, ***P*<0.01, ****P*<0.001).

causes an increase in serum iron and transferrin saturation. The resulting holotransferrin is biologically active, since it is recognized by the transferrin receptor. This holotransferrin-bound iron can be transferred and become available for hemoglobin synthesis.[11] Amelioration of erythropoiesis in this model of NTDT requires decreased erythroid iron intake and hemichrome formation.[9] Since DFP did not decrease TfSat, we postulated that hemichrome formation was not decreased in this setting. In fact, hemichrome levels were unchanged in DFP-treated animals compared to *Hbb*$^{th3/+}$ controls, while they were reduced in animals that received *Tmprss6*-ASO alone or *Tmprss6*-ASO+DFP (Figure 3C). It is known that IE in thalassemia is characterized by abnormal erythroid marrow expansion (extramedullary hematopoiesis). Although the erythron is expanded, only a limited number of erythroid progenitors give rise to mature RBCs. This is a result of limited differentiation and increased apoptosis of erythroid precursors due to chain imbalances, observed as increased hemichrome formation.[12] In this study, only upon hemichrome reduction there was a correlation with improvement of IE, observed as reduced proportions of immature erythroid cells. Using Ter119 and CD44 antibodies on bone marrow (BM) and spleen cells we were able to perform FACS analysis which allowed us to discriminate different stages of erythroid differentiation (Figure 3D). With this assay we could separate erythroid cells into distinct populations corresponding to proerythroblasts (fraction 1), basophilic (2), polychromatic (3), orthochromatic cells and reticulocytes (4), and mature RBC (5) (Figure 3D). *Hbb*$^{th3/+}$ animals are characterized by higher percentages of erythroid progenitors (fractions 1 to 4) and a lower percentage of mature RBC (fraction 5) when compared with WT animals. DFP alone was not able to revert this phenotype. In contrast, in *Tmprss6*-ASO and *Tmprss6*-ASO+DFP treated animals the percentage of more immature erythroid cells was markedly reduced while the RBC number was increased, indicating reduced IE and increased differentiation. This is consistent with the observed increase in peripheral RBC count (Figure 2B), decrease in red cell distribution width (RDW) (*data not shown*) and improvement in red cell morphology (Figure 2D). This effect was stronger in the spleen in comparison to the bone marrow. Taken together, our study shows that an antisense oligonucleotide targeting *Tmprss6* combined with the oral iron chelator DFP, is more powerful in reducing hepatic iron stores than either therapy alone, independently from dietary iron content. Furthermore, our study shows that improved erythropoiesis is achieved only with the administration of *Tmprss6*-ASO, in the presence or absence of DFP. In conclusion, the powerful effect of this combined therapy strongly suggests that *Tmprss6*-ASO could be extremely helpful in improving the management of iron chelation as well as anemia in NTDT.

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