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

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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, 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\textsuperscript{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 (hemichrome) 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\textsuperscript{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 35ppm 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 the data obtained from the 200ppm diet only.

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) (Fig 1A). This was associated with a significant increase in Hamp expression (Fig. 1B). DFP alone did not induce any changes in Tmprss6 or Hamp levels (Fig. 1A and 1B). However, as expected, DFP was efficient in reducing liver iron content both in WT and Hbbth3/+ animals (Fig. 1C). Tmprss6-ASO alone showed a trend towards reducing liver iron concentration when compared with scrambled-ASO treated animals (Fig. 1C). The level of reduction achieved by Tmprss6-ASO alone is lower than what 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 and colleagues 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 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 (Fig. 1C) (reduction of 38%) it failed to improve parameters of erythropoiesis such as hemoglobin (Hgb) levels (Fig. 2A), RBC production (Fig. 2B) spleen weight (Fig. 2C) and RBC morphology (Fig. 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 Hbbth3/+ controls, chelation therapy alone was associated with increases in serum iron and transferrin saturation levels (Fig. 3A and 3B). Mice treated with Tmprss6-ASO alone or in combination with DFP, however, exhibited reduction in serum iron and transferrin saturation versus Hbbth3/+ animals treated with or without DFP (Fig 3A and 3B). DFP is able to spontaneously transfer iron to extracellular transferrin, which 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. 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 Hbbth3/+ controls, while they were reduced in animals that received Tmprss6-ASO alone or Tmprss6-ASO+DFP (Fig. 3C). It is known that IE in thalassemia is characterized by abnormal erythroid marrow expansion (extramedullary erythropoiesis). Although the erythron is expanded, only a limited number of erythroid progenitors give rise to mature RBC. This is a result of limited differentiation and increased apoptosis of erythroid precursors due to chain imbalances, observed as increased hemichrome formation.12
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 BM and spleen cells we were able to perform FACS analysis which allowed us to discriminate different stages of erythroid differentiation (Fig. 3D). With this assay we can separate erythroid cells into distinct populations corresponding to proerythroblasts (fraction 1), basophilic (2), polychromatophilic (3), orthochromatophilic cells and reticulocytes (4), and mature RBC (5) (Fig. 3D). *Hbbth3* 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 (Fig. 2B), decrease in red cell distribution width (RDW, not shown) and improvement in red cell morphology (Fig. 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 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.

**Authorship**

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**Contribution:** C.C., M.A. and P.R.O. performed research and analyzed the results. S.R., C.C., S.G. and B.M. designed research, analyzed results and wrote the paper.

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References


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Table 1. Ratio between \textit{Hamp} expression and liver iron concentration (LIC).
Figure legend

Figure 1: Reduced Tmprss6 expression and increased hepcidin levels are associated with significant reduction in liver iron concentration after administration of Tmprss6-ASO alone or in combination with DFP: Hbb<sup>th3/+</sup> 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 (1A). Increased Hamp expression was achieved only when animals received Tmprss6-ASO with or without DFP (1B) and greater reduction in liver iron concentration was observed in animals treated with both Tmprss6-ASO and DFP (1C). Tmprss6-ASO sequence, 5'-GCTTAGAGTACAGCCCACTT-3'. Results represent mean ± SD. Statistical significance was determined using Student 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) (2A) and RBC counts (2B). Splenomegaly was reduced in Tmprss6-ASO and Tmprss6-ASO+DFP-treated mice compared to Hbb<sup>th3/+</sup> controls and DFP-treated animals (2C). RBC morphology was improved after treatment as can be seen in a representative example of Giemsa staining of peripheral blood smears (2D). Results represent mean ± SD. Statistical significance was determined using Student t-test (*P< 0.05, **P< 0.01, ***P< 0.001).

Figure 3: Amelioration of erythropoiesis requires decreased erythroid iron intake and hemichrome formation: Serum iron (3A) and transferrin saturation (3B) 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 (3C, urea gel electrophoresis) was decreased only in animals treated with Tmprss6-ASO or Tmprss6-ASO+DFP resulting in markedly improved IE (3D). 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 t-test. (*P< 0.05, **P< 0.01, ***P< 0.001).