# Iron-deficiency anemia from matriptase-2 inactivation is dependent on the presence of functional Bmp6.

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**Anne Lenoir [1] , Jean-Christophe Deschemin [1] , Léon Kautz [2] , Andrew J. Ramsay [3] , Marie-Paule Roth [2] , Carlos Lopez-Otin [3] , Sophie Vaulont [1] , Gaël Nicolas [1 *]**

[1] *Institut Cochin INSERM : U1016 , CNRS : UMR8104 , Université Paris Descartes , Paris,FR*

[2] *Centre de Physiopathologie Toulouse Purpan INSERM : U563 , IFR30 , Université Paul Sabatier - Toulouse III , IFR150 , Hopital de Purpan Place du Docteur Baylac 31024 TOULOUSE CEDEX 3,FR*

[3] *Departamento de Bioquímica y Biología Molecular Instituto Universitario de Oncología , Universidad de Oviedo , Facultad de Medicina, calle Fernando. Bongera s/n, 33006-Oviedo,ES*

* Correspondence should be adressed to: Gaël Nicolas <gael.nicolas@inserm.fr >

## Abstract

**Hepcidin is the master regulator of iron homeostasis. In the liver, iron-dependent hepcidin activation is regulated through Bmp6 and its membrane receptor hemojuvelin (Hjv) whereas, in response to iron deficiency, hepcidin repression seems to be controlled by a pathway involving the serine protease matriptase-2 (encoded by Tmprss6 ). To determine the relationship between Bmp6 and matriptase-2 pathways, Tmprss6 $^{-/-}$ mice (characterized by increased hepcidin levels and anemia) and Bmp6 $^{-/-}$ mice (exhibiting severe iron overload due to hepcidin deficiency) were intercrossed. We showed that loss of Bmp6 decreased hepcidin levels, increased hepatic iron and, importantly, corrected hematological abnormalities in Tmprss6 $^{-/-}$ mice. This suggests that elevated hepcidin levels in patients with familial iron-refractory iron deficiency anemia are due to excess signaling through the Bmp6/Hjv pathway.**

**MESH Keywords** Anemia, Iron-Deficiency ; metabolism ; Animals ; Antimicrobial Cationic Peptides ; metabolism ; Bone Morphogenetic Protein 6 ; metabolism ; Female ; Iron ; metabolism ; Iron, Dietary ; metabolism ; Liver ; metabolism ; Membrane Proteins ; metabolism ; Mice ; Mice, Knockout ; Serine Endopeptidases ; metabolism ; Signal Transduction ; physiology

**Author Keywords** hepcidin ; hemojuvelin ; bmp6 ; matriptase2 ; tmprss6

## Introduction

Body iron supply is provided both by iron recycling from senescent erythrocytes within the reticuloendothelial system and dietary iron absorption by duodenal enterocytes[1 ]. The liver-iron regulatory hormone, hepcidin, controls these two iron-delivery pathways via its targeted degradation of the cell surface iron exporter, ferroportin. As a consequence, iron availability in the circulation is decreased, leading to hypoferraemia[2 ,3 ]. Neither the lack of hepcidin nor its excess can be compensated for by the body, the results of which ultimately manifest in either iron overload or iron deficiency anemia, respectively.

Hepcidin gene expression is tightly regulated by body iron status and is dependent upon bone morphogenetic protein 6 (Bmp6) and hemojuvelin (Hjv). Binding of the iron-regulated Bmp6 ligand[4 ] to its receptors activates a signaling cascade leading to hepcidin transcription via phosphorylation of son of mother against decapentaplegic (Smad) 1/5/8 effectors[5 ]. Hjv, a GPI-linked membrane protein synthesized by the hepatocytes, is a Bmp6 coreceptor[5 ]. The critical role of the Bmp6/Hjv/Smad pathway in iron homeostasis is supported by the loss of hepcidin expression and massive parenchymal iron overload observed in Bmp6 $^{-/-}$ and Hjv $^{-/-}$ mice as well as in mice with targeted liver deletion of Smad4[6 –9 ].

Recently, the serine protease matriptase-2 (also known as TMPRSS6) has been connected to this iron-pathway[10 –12 ] due to its proteolysis of Hjv[13 ]. Matriptase-2 is a type II serine protease that is predominately expressed in the liver (for review[14 ]). Matriptase-2 deficient mice[10 ,12 ] have very high levels of hepcidin, which lead to the inhibition of dietary iron absorption and cause a severe iron deficiency anemia phenotype. Matriptase-2 was thus characterized as a negative regulator of hepcidin gene expression. Accordingly, Du et al. demonstrated that overexpression of normal matriptase-2 protein in hepatoma cells suppresses the activation of hepcidin expression[10 ] . The anemic phenotype of matriptase-2 deficient mice is mirrored in patients with matriptase-2 mutations who present with iron-refractory iron deficiency anemia (IRIDA)[11 ]. Indeed, IRIDA patients show inappropriately high hepcidin levels[11 ,14 ,15 ], which explain lack of dietary iron absorption and partial response to parenteral iron treatment[16 ].

The goal of this study was to characterize the in vivo relationship between matriptase-2 and the iron-regulated ligand of Hjv, Bmp6, by analyzing the role of Bmp6 in the setting of anemia in mice deficient for matriptase-2. Towards this purpose, we intercrossed matriptase-2 and Bmp6-deficient mice and compared the iron status of the double mutant mice with that of wild-type controls or single mutant mice.

## Material and methods

$Tmprss6^{tm1Otin}$ mice on a mixed 129/Ola x C57BL/6 background[12 ] were mated to $Bmp6^{tm1Rob}$ mice on an outbred CD1 background[6 ]. F1 mice, heterozygous for both the $Tmprss6^{tm1Otin}$ (hereafter referred to as $Tmprss6^{+/-}$) and the $Bmp6^{tm1Rob}$ alleles (referred to as $Bmp6^{+/-}$) were then intercrossed and the F2 progeny genotyped as previously described[6 ,12 ]. As expected, the nine possible genotypic combinations were observed among the F2 mice.

Mice were cared for in accordance with the «European convention for the protection of laboratory animals». Animals were given free access to tap water and standard laboratory mouse chow diet (AO3, iron content 280 mg/kg, UAR, France). Mice used in this study were 8 to 13-week-old females and had a mixed 129/Ola × C57BL/6 × CD1 background. Hematological parameters as well as plasma and liver iron were obtained as previously described[17 ].

RNA extraction and real-time quantification of the hepcidin and β actin transcripts were performed as reported in[18 ]. Standardized genetic nomenclature for mouse hepcidin is Hamp and Atcb for β actin.

Student t tests were used to compare quantitative traits between mouse groups. P values less than .05 were considered as statistically significant.

## Results and discussion

To investigate the role of Bmp6 in the pathogenesis of iron-refractory iron deficiency anemia, we intercrossed matriptase-2 and Bmp6-deficient mice and analyzed iron metabolism in their F2 progeny.

As recently published[6 ,7 ], liver hepcidin expression was repressed in $Bmp6^{-/-}$ mice compared with $Bmp6^{+/+}$ controls (Fig. 1A ), leading to increased liver and plasma iron levels (Fig. 1B & C ). Id1 , a marker of activation of the Bmp/Smad signaling pathway, had an expression pattern similar to that of hepcidin in these $Bmp6^{-/-}$ mice (Fig. 1D ). However, their expression of Tmprss6 was not significantly different from the wild-type controls (data not shown). Furthermore, as shown in Table 1 , hematological parameters of $Bmp6^{-/-}$ mice were similar to those of $Bmp6^{+/+}$ controls.

Conversely, hepcidin and Id1 gene expressions were significantly up-regulated in $Tmprss6^{-/-}$ mice (Fig. 1A & D ), and as expected[12 ], these mice had reduced hepatic and plasma iron indices, compared with $Tmprss6^{+/+}$ controls (Fig. 1B & C ). In addition, $Tmprss6^{-/-}$ mice were anemic and presented with significantly decreased Hb levels, as well as RBC, Hct and MCV (Table 1 ). In addition, as previously reported[19 ], they exhibited lower Bmp6 gene expression than wild-type controls (data not shown).

Interestingly, in double mutant $Bmp6^{-/-}$ $Tmprss6^{-/-}$ mice, hepcidin expression was repressed to the same extent as in $Bmp6^{-/-}$ mice (Fig. 1A ). A similar pattern of expression was observed for Id1 , although the comparison did not reach statistical significance, due to a slightly higher variability of gene expression levels between mice for Id1 than for hepcidin (Fig. 1D ). In addition, phosphorylation of Smad1/5/8 appeared similarly decreased in $Bmp6^{-/-}$ $Tmprss6^{+/+}$ mice and in $Bmp6^{-/-}$ $Tmprss6^{-/-}$ mice, compared with wild-type controls (data not shown), which is concordant with the similar reduction we observed in their levels of hepcidin expression. However, although liver and plasmatic iron levels were higher in $Bmp6^{-/-}$ $Tmprss6^{-/-}$ mice than in $Bmp6^{+/+}$ mice, these levels remained significantly lower than in $Bmp6^{-/-}$ mice (Fig. 1B & C ). Lastly, and most importantly, iron deficiency anemia observed in the $Tmprss6^{-/-}$ mice was completely rescued by Bmp6 deficiency. As shown in Table 1 , Hb, Hct and MCV values observed in $Bmp6^{-/-}$ $Tmprss6^{-/-}$ mice were comparable to control values. Furthermore, heterozygous loss of Bmp6 in $Tmprss6^{-/-}$ mice was able to partially correct systemic iron homeostasis by decreasing hepcidin gene expression and increasing plasma and liver iron (Fig. 1 ).

Although hematological parameters were found to be normal in mice deficient for both Hjv and matriptase-2[19 ,20 ], supporting the role of matriptase-2 as a regulator of Hjv expression at the hepatocyte membrane, the role of Bmp6 in this process was not clearly defined. The data obtained in this study indicate that hepcidin overexpression which results from matriptase-2 inactivation requires the presence of Bmp6. Indeed, neither the activation of the Hjv/Smad signaling pathway nor the establishment of the anemic phenotype were observed in double knockout mice.

However, in contrast to $Hjv^{-/-}$ $Tmprss6^{-/-}$ mice[19 ,20 ] which had a phenotype very similar to $Hjv^{-/-}$ mice, we found that loss of matriptase-2 in $Bmp6^{-/-}$ $Tmprss6^{-/-}$ mice attenuates the effects of Bmp6 deficiency on hepatic and plasma iron accumulation. It could be speculated that, due to the lack of matriptase-2, Hjv is stabilized at the hepatocyte plasma membrane and can serve as a coreceptor for ligands other than Bmp6. However, neither hepcidin nor Id1 gene expression were found up-regulated in the double mutant female mice compared with $Bmp6^{-/-}$ mice. Alternatively, matriptase-2 could, in absence of Bmp6, regulate other iron-related proteins or initiate a signaling pathway involved in maintaining hepatic iron balance and/or systemic iron regulation, independently of hepcidin.

In conclusion, the present data further support that Bmp6 is the physiological ligand of Hjv and demonstrate that the regulation of Hjv membrane expression by matriptase-2 serves to tightly control the signaling pathway induced by Bmp6.

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## Footnotes:

**Author contribution:** AL: designed and performed research, analyzed data JCD: designed and performed research, analyzed data LK: provided the Bmp6 deficient mice and performed research AJR: generated the Tmprss6 deficient mice MPR: provided the Bmp6 deficient mice and contributed to the writing of the paper CLO: provided the Tmprss6 deficient mice and contributed to the writing of the paper SV: designed research, analyzed data and wrote the paper GN: designed research, analyzed data and wrote the paper

The authors declare no conflict of interest.

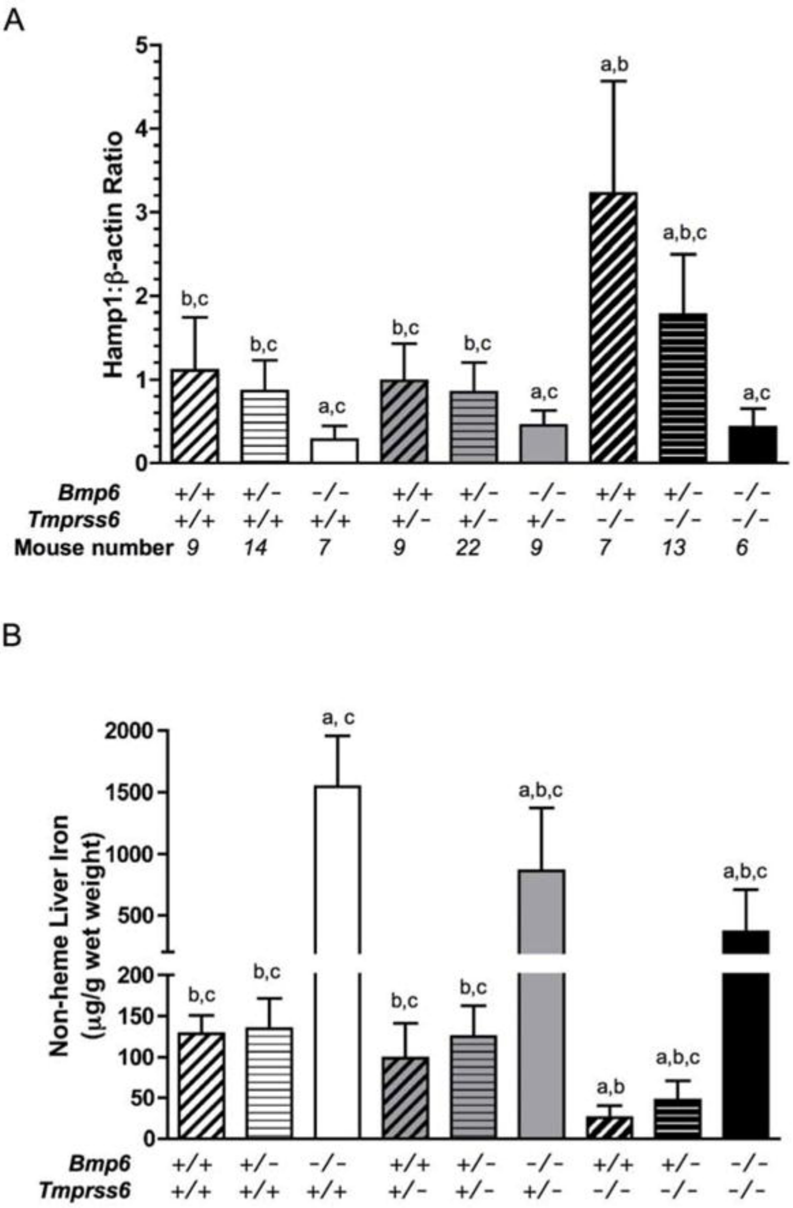
## References:


- 1 . Andrews NC , Schmidt PJ . Iron homeostasis . Annu Rev Physiol . 2007 ; 69 : 69 - 85
- 2 . De Domenico I , McVey Ward D , Kaplan J . Regulation of iron acquisition and storage: consequences for iron-linked disorders . Nat Rev Mol Cell Biol . 2008 ; 9 : 72 - 81
- 3 . Viatte L , Vaulont S . Hepcidin, the iron watcher . Biochimie . 2009 ; 91 : 1223 - 1228
- 4 . Kautz L , Meynard D , Monnier A . Iron regulates phosphorylation of Smad1/5/8 and gene expression of Bmp6, Smad7, Id1, and Atoh8 in the mouse liver . Blood . 2008 ; 112 : 1503 - 1509
- 5 . Babitt JL , Huang FW , Wrighting DM . Bone morphogenetic protein signaling by hemojuvelin regulates hepcidin expression . Nat Genet . 2006 ; 38 : 531 - 539
- 6 . Meynard D , Kautz L , Darnaud V , Canonne-Hergaux F , Coppin H , Roth MP . Lack of the bone morphogenetic protein BMP6 induces massive iron overload . Nat Genet . 2009 ;
- 7 . Andriopoulos B Jr , Corradini E , Xia Y . BMP6 is a key endogenous regulator of hepcidin expression and iron metabolism . Nat Genet . 2009 ;
- 8 . Huang FW , Pinkus JL , Pinkus GS , Fleming MD , Andrews NC . A mouse model of juvenile hemochoromatosis . J Clin Invest . 2005 ; 115 : 2187 - 2191
- 9 . Wang RH , Li C , Xu X . A role of SMAD4 in iron metabolism through the positive regulation of hepcidin expression . Cell Metab . 2005 ; 2 : 399 - 409
- 10 . Du X , She E , Gelbart T . The serine protease TMPRSS6 is required to sense iron deficiency . Science . 2008 ; 320 : 1088 - 1092
- 11 . Finberg KE , Heeney MM , Campagna DR . Mutations in TMPRSS6 cause iron-refractory iron deficiency anemia (IRIDA) . Nat Genet . 2008 ; 40 : 569 - 571
- 12 . Folgueras AR , de Lara FM , Pendas AM . Membrane-bound serine protease matriptase-2 (Tmprss6) is an essential regulator of iron homeostasis . Blood . 2008 ; 112 : 2539 - 2545
- 13 . Silvestri L , Pagani A , Nai A , De Domenico I , Kaplan J , Camaschella C . The serine protease matriptase-2 (TMPRSS6) inhibits hepcidin activation by cleaving membrane hemojuvelin . Cell Metab . 2008 ; 8 : 502 - 511
- 14 . Ramsay AJ , Hooper JD , Folgueras AR , Velasco G , Lopez-Otin C . Matriptase-2 (TMPRSS6): a proteolytic regulator of iron homeostasis . Haematologica . 2009 ; 94 : 840 - 849
- 15 . Camaschella C , Silvestri L . New and old players in the hepcidin pathway . Haematologica . 2008 ; 93 : 1441 - 1444
- 16 . Finberg KE . Iron-refractory iron deficiency anemia . Semin Hematol . 2009 ; 46 : 378 - 386
- 17 . Viatte L , Nicolas G , Lou DQ . Chronic hepcidin induction causes hyposideremia and alters the pattern of cellular iron accumulation in hemochromatotic mice . Blood . 2006 ; 107 : 2952 - 2958
- 18 . Chung B , Verdier F , Matak P , Deschemin JC , Mayeux P , Vaulont S . Oncostatin M is a potent inducer of hepcidin, the iron regulatory hormone . Faseb J . 2010 ; 24 : 2093 - 2103
- 19 . Finberg KE , Whittlesey RL , Fleming MD , Andrews NC . Down-regulation of Bmp/Smad signaling by Tmprss6 is required for maintenance of systemic iron homeostasis . Blood . 2010 ; 115 : 3817 - 3826
- 20 . Truksa J , Gelbart T , Peng H , Beutler E , Beutler B , Lee P . Suppression of the hepcidin-encoding gene Hamp permits iron overload in mice lacking both hemojuvelin and matriptase-2/TMPRSS6 . Br J Haematol . 2009 ; 147 : 571 - 581

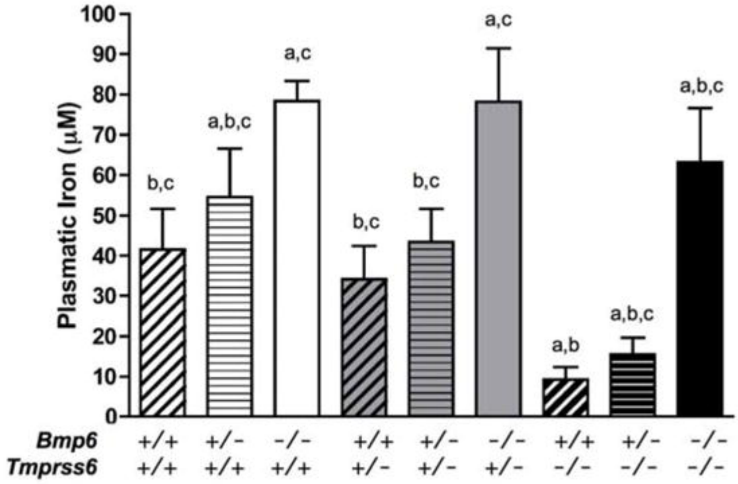

**Figure 1**

Phenotypic analysis of female mice according to their Bmp6/Tmprss6 genotypes

The phenotypes analyzed include hepcidin mRNA expression relative to β-actin (A), non-heme iron concentration of liver (B), plasmatic iron (C) and Id1 mRNA expression relative to β-actin (D). Data are presented as mean ± SD. a, $p<0.05$ when compared with Bmp6$^{+/+}$ Tmprss6$^{+/+}$ controls. b, $p<0.05$ when compared with Bmp6$^{-/-}$ Tmprss6$^{+/+}$ mice. c, $p<0.05$ when compared with Bmp6$^{+/+}$ Tmprss6$^{-/-}$ mice.


A
Hamp1:β-actin Ratio
5
4
3
2
1
0
b,c
b,c
a,c
b,c
b,c
a,c
a,b
a,b,c
a,c
Bmp6 +/+ +/− −/− +/+ +/− −/− +/+ +/− −/−
Tmprss6 +/+ +/+ +/+ +/− +/− +/− −/− −/− −/−
Mouse number 9 14 7 9 22 9 7 13 6
B
Non-heme Liver Iron
(µg/g wet weight)
2000
1500
1000
500
200
150
100
50
0
b,c
b,c
a, c
b,c
b,c
a,b,c
a,b
a,b,c
a,b,c
Bmp6 +/+ +/− −/− +/+ +/− −/− +/+ +/− −/−
Tmprss6 +/+ +/+ +/+ +/− +/− +/− −/− −/− −/−

C


Plasmatic Iron (µM)
100
90
80
70
60
50
40
30
20
10
0
b,c
a,b,c
a,c
b,c
b,c
a,c
a,b
a,b,c
a,b,c
Bmp6
+/+ +/- -/- +/+ +/- -/- +/+ +/- -/-
Tmprss6
+/+ +/+ +/+ +/- +/- +/- -/- -/- -/-

D

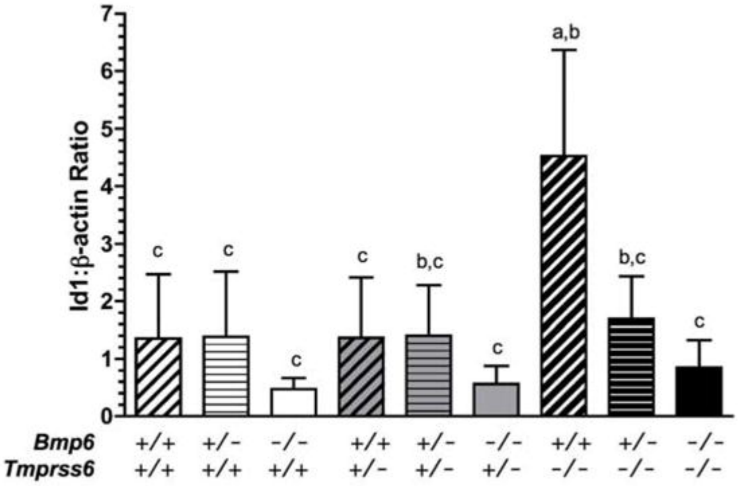

Id1:β-actin Ratio
7
6
5
4
3
2
1
0
c
c
c
c
b,c
c
a,b
b,c
c
Bmp6
+/+ +/- -/- +/+ +/- -/- +/+ +/- -/-
Tmprss6
+/+ +/+ +/+ +/- +/- +/- -/- -/- -/-

**Table 1**

Hematological parameters of female mice according to their Bmp6/Tmprss6 genotypes.

| Genotype | RBC ($10^{12}$/L) | Hb (g/dL) | Hct (%) | MCV (fL) |
|---|---|---|---|---|
| Bmp6 $^{+/+}$ Tmprss6 $^{+/+}$ | 9,77 ± 0,35[c] | 16,22 ± 0,63[c] | 48,12 ± 2,36[c] | 49,28 ± 1,86[c] |
| Bmp6 $^{+/-}$ Tmprss6 $^{+/+}$ | 9,59 ± 0,49[c] | 16,01 ± 0,62[c] | 47,96 ± 2,46[c] | 50,04 ± 1,62[c] |
| Bmp6 $^{-/-}$ Tmprss6 $^{+/+}$ | 9,52 ± 0,48[c] | 16,40 ± 0,66[c] | 48,61 ± 2,01[c] | 51,13 ± 2,12[c] |
| Bmp6 $^{+/+}$ Tmprss6 $^{+/-}$ | 9,54 ± 0,37[c] | 15,60 ± 0,45[a b c] | 46,27 ± 1,44[b c] | 48,52 ± 1,86[b c] |
| Bmp6 $^{+/-}$ Tmprss6 $^{+/-}$ | 9,53 ± 0,35[c] | 16,08 ± 0,59[c] | 47,67 ± 2,69[c] | 50,10 ± 2,26[c] |
| Bmp6 $^{-/-}$ Tmprss6 $^{+/-}$ | 9,45 ± 0,45[c] | 16,51 ± 0,72[c] | 48,98 ± 2,57[c] | 51,82 ± 1,62[a c] |
| Bmp6 $^{+/+}$ Tmprss6 $^{-/-}$ | 6,99 ± 1,1[a b] | 11,97 ± 1,44[a b] | 32,06 ± 4,86[a b] | 45,94 ± 1,02[a b] |
| Bmp6 $^{+/-}$ Tmprss6 $^{-/-}$ | 8,45 ± 0,63[a b c] | 13,48 ± 0,85[a b c] | 38,40 ± 2,54[a b c] | 45,47 ± 0,63[a b] |
| Bmp6 $^{-/-}$ Tmprss6 $^{-/-}$ | 9,28 ± 0,35[a c] | 15,92 ± 1,13[c] | 46,88 ± 4,08[c] | 50,42 ± 2,61[c] |

RBC indicates red cell count; Hb, hemoglobin; Hct, hematocrit; MCV, mean corpuscular volume. Data are presented as mean ± SD.

**a** ,$p<0.05$ when compared with Bmp6$^{+/+}$ Tmprss6$^{+/+}$ controls.

**b** $p<0.05$ when compared with Bmp6 $^{-/-}$ Tmprss6$^{+/+}$ mice.

**c** $p<0.05$ when compared with Bmp6$^{+/+}$ Tmprss6 $^{-/-}$ mice.