Romiplostim Administration Shows Reduced Megakaryocyte Response-Capacity And Increased Myelofibrosis In a Mouse Model Of MYH9-RD

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Short title: Effects of romiplostim in Myh9-/– mice

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Abstract (200 words)

Macrothrombocytopenia in MYH9-related disease (MYH9-RD) results from defects in nonmuscular myosin-IIA function. Thrombopoietin receptor agonists (eltrombopag; romiplostim) seem to improve hemostasis, but little is known about their biological effects in MYH9-RD. We administered romiplostim to Myh9-/- mice (100µg/kg, every 3 days, during 1 month). Megakaryocytes increased to similar numbers in Myh9-/- and wild type (WT) mice (with an increase in immature megakaryocytes), but Myh9-/- platelet count response was much less (2.5-fold vs. 8-fold increase). A strong increase in megakaryocyte nuclei emboli in the lung, in WT and Myh9-/- mice, indicates increased transmigration of megakaryocytes from the bone-marrow. Prolonged (but not acute) treatment with romiplostim decreased expression of GPIb-IX-V complex and GPVI, but not of GPIIbIIIa and bleeding time increased in WT mice. Microcirculation was not altered by the increased number of large platelets in any of the assessed organs, but in Myh9-/- mice a much stronger increase in bone marrow reticulin fibres was present after 4 weeks of romiplostim treatment vs WT mice. These data further encourage short term use of thrombopoietic agents in MYH9-RD patients, however, myelofibrosis has to be considered as a potential severe adverse effect during longer treatment. Reduction of GPIbIX/GPVI expression by romiplostim requires further studies.

Key words: MYH9-related disorder; hereditary thrombocytopenia; myelofibrosis, thrombopoietin receptor agonists, romiplostim, platelets
Introduction

MYH9-related disorders (MYH9-RD) are a group of rare diseases characterized by congenital macrothrombocytopenia that results from mutations in the MYH9 gene encoding the non-muscle myosin IIA, the only isoform of myosin present in platelets. Thrombocytopenia ranges from mild to severe and remains relatively stable in an individual throughout life. Patients may suffer from easy bruising, epistaxis, and menorrhagia, in some rare occasions requiring blood transfusion. Other manifestations may occur later in life such as cataract, hearing loss and nephropathy, the mechanism leading to these additional symptoms being presently unknown. In these patients, thrombocytopenia results from defective platelet production, probably resulting from impairment in marrow megakaryocyte maturation due to decreased or abnormal myosin IIA, leading to a strong decrease in their capacity to extend proplatelets.

Second-generation thrombopoietic agents, which stimulate megakaryocytopoiesis by binding to the thrombopoietin (TPO) receptor, may be an option for increasing the platelet count in MYH9-RD. Two of these TPO receptor agonists have completed phase III trials in primary immune thrombocytopenia, eltrombopag, a nonpeptide TPO receptor agonist, and romiplostim, a peptide TPO receptor agonist and had been recently approved in several countries for treatment of certain patients with immune thrombocytopenia.

Eltrombopag administration has recently been tested in MYH9-RD patients in a phase II, multicenter trial, showing moderate increase in platelet counts and reduction in bleeding tendency, suggesting that these new thrombopoietic agents could also represent an interesting therapeutic option for MYH9-RD patients. However, platelets in MYH9-RD differ from platelets in ITP and it is unknown whether stimulation of megakaryocytopoiesis by thrombopoietin receptor agonists may cause additional changes in these MYH9-RD platelets. Furthermore, many of the giant platelets are larger than the diameter of capillaries and little is
known about their rheology. Increasing the number of circulating giant platelets may increase the risk for microthrombotic events. This is especially relevant in MYH9 related disorders, as these patients are also at risk to develop renal insufficiency. Clustering of giant platelets in the capillaries of the glomerula might increase the risk for damage of the renal tissue, potentially aggravating the risk of renal failure.

To evaluate the possibility that an increase in giant platelets alters the microcirculation, we took advantage of a mouse model of MYH9-related macrothrombocytopenia. Romiplostim was injected into Myh9/- mice during a one-month period and the consequences in terms of platelet production and microcirculation have been evaluated. We found a much less pronounced response of platelet count increase and increased myelofibrosis compared to wild type (WT) mice, but no evidence of impairment of the microcirculation induced by the increased number of large platelets. Interestingly, romiplostim induced a decrease in platelet GPIbIX and GPVI expression in both WT and Myh9/- mice.
Material and Methods

**Animals.** Myh9-/- mice have already been described and are on a C57BL/6 background (backcrossed for 11 generations). C57BL/6 mice were used as control.

**Protocol design.** Ten-week old WT and Myh9-/- mice (5 males and 5 females in each group) received s.c. injections of either vehicle (saline) or romiplostim (100µg/kg body weight; AMGEN, Thousand Oaks, CA, USA) every 3 days, during 1 month. Doses and administration schedule were selected based on previous studies in mice. Blood samples were analyzed for platelet count and platelet volume before the onset of the experiment and at day 8, 18, and 29. At days 33, mice were sacrificed. Blood was drawn from the abdominal aorta for platelet isolation and electron microscopy observations. Organs (brain, lungs, pancreas, kidneys, psoas muscles, gut and bone marrow) were removed and immersed into 4% paraformaldehyde (PFA) for histological analysis.

**Bleeding time.** Bleeding time was performed on other groups of one-month, 10-days or 6h treated mice, by sectioning 3 mm of the tail tip and immersing the tail in saline at 37°C, as previously described.

**Platelet count, volume determination, P-selectin, glycoprotein level, annexin V and mitochondrial potential measurement.** Blood was taken from the tail tip of isoflurane anesthetized mice and anticoagulated with EDTA (6 mM). Platelet count was determined using an automated platelet counter (scil Vet abc, scil animal care company Holtzeim, France) and platelet volume modifications were evaluated by flow cytometry (Gallios, Beckman Coulter France, Roissy, France) following GPIbβ (RAM1 antibody) labeling. Platelet P-selectin exposure, GPIIbIIIa activation and glycoprotein levels were evaluated by flow cytometry in whole blood by labeling with an anti-P-selectin antibody (Becton-Dickinson), or
with Jon/A-PE antibody (Emfret, Wurtzburg, Germany) or with monoclonal antibodies directed against GPIbα (RAM6), GPIbβ (RAM1), GPV (Gonc2, Emfret, Würzburg, Germany), GPIIbIIIa (RAM2), or GPVI (JAQ1, Emfret, Würzburg, Germany) in blood samples taken after 28 days of romiplostim treatment. The data are presented as mean fluorescence intensity (MFI), in arbitrary units. Exposition of negatively charged phospholipids was checked by flow cytometry using FITC-annexin V labeling and putative modifications of the mitochondrial potential was evaluated using the fluorescent probe TMRM followed by flow cytometry analysis.

**Platelet isolation and electron microscopy.** ACD anticoagulated whole blood was centrifuged and platelet rich plasma washed in Tyrodes’s buffer as described. Platelets were fixed with 2.5% glutaraldehyde in 0.1M cacodylate buffer, pH 7.2, containing 2% sucrose and processed as described previously and ultrathin sections were examined under a Philips CM120 Biotwin electron microscope (FEI, Eindhoven, The Netherlands) at 120 kV. For ultrastructural observation of bone marrow, femurs were flushed, fixed and processed as mentioned above.

**Western Blot.** Platelet lysates were prepared by resuspending washed platelets (200 × 10⁹/L) in SDS buffer (1% SDS final concentration). Proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) under reducing or non-reducing conditions, transferred to PVDF membranes, and incubated with the primary antibody directed against GPIIIa (LucA5, Emfret, Würzburg, Germany), GPIbα (RAM6), GPIbβ (RAM1), GPVI (JAQ1, Emfret, Würzburg, Germany), or actin for normalization.

**Histology.** The mouse tissue samples were dehydrated and embedded in paraffin. Slides of 2 - 3 µm thickness were cut and stained by H&E and afterwards digitized with the NanoZoomer 2.0 (Hamamatsu, Hersching, Germany). The amount of megakaryocytes in the spleen was
counted in an area of 2.62 mm² using the ImageScope software (http://www.aperio.com). For this purpose we counted every megakaryocyte which showed a nucleus within the analysed area.

To quantify the amount of megakaryocyte emboli in the lung we used relative units to compare the surface of the lung with their varying ventilation ¹³. We quantified the surface area as described ¹⁴. In brief, four parallel lines with a distance of 7 cm were superimposed on the computer screen (789-fold magnification). First we counted the intersections of the alveolar walls with the lines and thereafter we separately counted the megakaryocyte emboli within this section. Finally we calculated the ratio of megakaryocyte emboli per 500 intersections of alveolar walls with the lines.

Myelofibrosis was detected on bone marrow paraffin sections using the Gomori stain for reticular silver staining. Scores were attributed according to the absence, moderate or abundant presence of fibers. The observations were made on 4-5 mice per experiment.

Statistics. Statistical analyses were performed using one way ANOVA and bonferroni as post-test. *, p<0.05; **, p<0.01; ***, p<0.0001.

Ethics. All animal studies were performed according to the regulations of the Université de Strasbourg.
Results

Romiplostim increases the platelet count in Myh9-/- mice less than in WT mice. Romiplostim (100 µg/kg) injection to WT mice every 3 days led to a 4-fold increase in platelet count 8 days following the beginning of the protocol for both males and females (Fig. 1A). Platelet count further increased after 1 month of treatment, reaching an 8-fold increase for females and 6-fold increase for males (Fig. 1A). Romiplostim also increased platelet counts in Myh9-/- mice, though to a lesser extent, with a 2.5-fold increase for females and 1.7-fold increase for males (Fig. 1A). As expected platelet volume was higher for Myh9-/- mice at baseline, and a transient increase in the mean platelet volume was observed in both genotypes after start of romiplostim treatment (Fig. 1B).

Effect of romiplostim on platelet morphology. Romiplostim treatment led to the formation of circulating platelet debris as observed by flow cytometry (Fig. 2A). These debris were more numerous in WT-treated mice, probably reflecting the stronger increase in platelet count. By electron microscopy after one month treatment, the increased platelet size was still evident in romiplostin-treated WT mice (Fig. 2B). In addition platelet fragments were present, together with poorly contrasted platelets (Fig. 2B, arrows). Myh9-/- platelet morphology was altered as previously shown, with larger and ovoid platelets, presenting heterogeneity in organelle content and distribution. Romiplostim treatment did not improve or modify their morphology, but the presence of debris was less evident compared to wild type mice.

Romiplostim increases megakaryocyte numbers in the spleen. Quantification of megakaryocytes in the spleen using histology sections (Fig. 3A) indicates that, as previously reported for bone marrow 15, the number of megakaryocytes was higher in control Myh9-/-
mice compared to control WT mice (11.7 megakaryocytes/field vs. 30.2 megakaryocytes/field for control WT and Myh9-/- spleen, respectively) (Fig. 3A, panels a and c, and Fig. 3B).

Romiplostim treatment strongly increased the megakaryocyte number in both, WT and Myh9-/- mice, compared to their respective controls. The absolute number of megakaryocytes was even higher in Myh9-/- mice compared to WT mice, with a mean value of 400 megakaryocytes/field for treated Myh9-/- mice and 319.3 megakaryocytes/field in the treated WT mice (Fig. 3A panels b and d and Fig. 3B). However, the relative increase in megakaryocyte numbers during administration of romiplostim was much lower in Myh9-/- mice (13 fold) than in wild type mice (27 fold). This may explain in part the lower increase in Myh9-/- platelet count. As a likely consequence of increased extra-medullary megakaryocytopoiesis, the spleen was enlarged following romiplostim injections in both Myh9-/- mice and WT mice (Fig. 3C). Romiplostim-treated mice also showed an increased number of cluster-forming megakaryocytes in the spleen, with some megakaryocytes being in mitosis (Fig. 3A panel b).

Romiplostim increases the proportion of immature megakaryocytes. Romiplostim also increased total megakaryocyte numbers of the bone marrow to a similar level in WT and Myh9-/- mice (Fig. 4A). Ultrastructural observations revealed that the proportion of immature vs. mature megakaryocytes was modified by the treatment, with a higher proportion of immature megakaryocytes (Fig. 4B).

However, the number of visible naked nuclei remained very low and did not increase in the romiplostim–treated marrow. Thus when calculating their percentage in the bone marrow, it was decreased in romiplostim treated marrow since the number of megakaryocytes increased (Fig. 4C). This observation was surprising in the view of the large increase in megakaryocyte numbers and the 8-fold increase in platelet count in WT mice treated with...
romiplostim and suggests that MK nuclei elimination is either increased to maintain a low level of naked nuclei by some regulation or that an increased number of intact megakaryocytes transmigrate into the sinusoid circulation.

**Romiplostim-induced megakaryocyte nuclei emboli in the pulmonary microvasculature.** Since the presence of megakaryocytes in lung capillaries from thrombopoietin-treated mice was previously observed \(^{16}\), we investigated whether megakaryocytes were also present in the lungs from romiplostim-treated mice. Histological lung sections showed a large amount of emboli composed mostly of megakaryocyte nuclei present in the capillaries of both WT and Myh9-/- mice treated with romiplostim (Fig. 5A, B). This observation favors an increased transmigration of intact megakaryocytes to the sinusoid circulation in romiplostim-treated mice and explains the above finding of a low number of naked megakaryocyte nuclei despite a major increase in total megakaryocyte numbers. Of note, non-treated control Myh9-/- mice had slightly more emboli than non-treated WT control mice, though the difference was not significant (Fig. 5B), likely resulting from the increased baseline levels of megakaryocytes in Myh9-/- mice.

**Romiplostim treatment did not lead to obstruction of the microvasculature in other organs.** In order to investigate whether an increase in the number of circulating large platelets could lead to obstruction of the microcirculation, organs, in particular brain, gut, pancreas, lungs, spleen, kidney and psoas muscle, were removed for histological analyses. No occlusions were observed in all examined tissues either from WT mice or from Myh9-/- mice following one month of romiplostim treatment.
Romiplostim induces a relative deficiency of the GPIb-IX-V complex and of GPVI but no increase in P-selectin exposure or GPIIbIIIa activation. Expression of GPIb-IX-V complex and GPVI decreased during treatment with romiplostim for 28 days, as assessed by flow cytometry. In contrast, expression of GPIIbIIIa did not change significantly (Fig. 6A a, b, c, d,e). No difference of GPIb complex or GPVI surface expression was observed after 6h romiplostim treatment (not shown). This indicates that reduction of GPIbα and GPVI expression is not caused by direct platelet activation by romiplostim, but by a more complex mechanism. Western blot experiments to visualize total platelet GPIbα, GPIbβ, GPIIIa, and GPVI protein also showed a slight decrease of GPVI following romiplostim treatment but no significant decrease in GPIbα or GPIbβ, suggesting that the decrease in surface expression of the GP is more likely caused by cleavage or internalization rather than by romiplostim-induced decrease of protein synthesis (Fig. 6B). However, no increase in P-selectin exposure could be detected following romiplostim treatment, nor signs of GPIIbIIIa activation as measured by Jon/A-PE labeling, indicating no major platelet activation (Fig. 6C).

Effect of romiplostim on bleeding time. Romiplostim treatment did not reduce the bleeding time in Myh9-/- mice after one month of treatment. Surprisingly, the bleeding time was increased in WT mice. This prolongation of bleeding time persisted throughout romiplostim treatment and was already present after only 10 days treatment (not shown) (Fig. 6D). On the contrary, romiplostim administration for only 6 hours did not modify the bleeding time in WT mice (not shown).

Romiplostim enhances reticulin fibres in the bone marrow of Myh9-/- mice to a larger extent than in WT mice. We also observed a difference in the amount of reticulin fibres in the bone marrow of Myh9-/- mice compared to WT mice after romiplostim treatment. None of the non-
treated WT mice showed reticulin fibres in the bone marrow, and only one of the control
Myh9-/- mice showed mild fibrosis. However, in sections from four of five independent
Myh9-/- bone marrows a much stronger increase in reticulin fibres was present after 4 weeks
of romiplostim treatment, compared to sections from bone marrow of four WT mice (Fig. 7).
This finding is very important for safety considerations in regard to long term treatment of
MYH9-RD patients with thrombopoietin receptor agonists.
Discussion

In this study we made three important observations. First we show that the increase in the number of giant platelets in Myh9-/− mice by the thrombopoietin receptor agonist romiplostim does not affect the microcirculation to an extent causing changes in histology. Second, our study provides first in-vivo evidence that the risk for increased myelofibrosis induced by romiplostim treatment might be enhanced in MYH9-RD patients compared to patients with immune-thrombocytopenia; and third, romiplostim induces a reduction of the GPIbIX complex and of GPVI in Myh9-/− and WT mice.

Especially the first two findings are important in view of a recent clinical evaluation of another thrombopoietin receptor agonist, namely eltrombopag in MYH9-RD patients. This pivotal study suggested that second-generation thrombopoietic agents could represent new therapeutic strategies in MYH9-RD patients presenting with symptomatic bleeding tendency. While the increase in giant platelet numbers reduced the bleeding tendency clinically, it remained unclear whether the increase in circulating giant platelets could have deleterious consequences for the microcirculation, especially of the renal glomerula.

We evaluated the effects of administration of romiplostim over one-month to Myh9-/− mice. These mice differ from patients with MYH9-RD, as they lack the non muscular myosin IIA protein, whereas the protein is present albeit structurally changed in patients. However, the mice reproduce the macrothrombocytopenia of MYH9-RD patients. Although romiplostim was effective in these mice in terms of an increase in platelet counts, the effect was only modest (2 fold) compared to WT mice (7 fold). This was surprising as the absolute maximum number of megakaryocytes in the spleen of Myh9-/− mice after romiplostim treatment was even higher than in WT mice. In this regard, the mouse model reflects the observation of the clinical study with eltrombopag in MYH9-RD patients. These patients also showed a moderate increase in platelet counts only, and some of the patients did not respond
to eltrombopag at all\textsuperscript{9}. Our study provides evidence that the reason for the moderate response to thrombopoietin receptor agonists could be in part the limited response dynamic of the Myh9-/- bone marrow due to the increased baseline number of megakaryocytes that results from a higher amount of circulating TPO\textsuperscript{15}. Therefore the mean fold increase in megakaryocyte numbers both in the spleen and in the bone marrow was significantly lower for Myh9-/- mice compared to WT mice. Another reason is probably also the increased mortality and the intrinsic decreased capacity of Myh9-/- megakaryocytes to effectively produce platelets as previously reported\textsuperscript{4}. As observed by electron microscopy, the ultrastructural defects already observed in Myh9-/- bone marrow\textsuperscript{15} were still present following romiplostim treatment, namely the invasive/leaky aspect, absence of peripheral zone and abnormal development/organization of the DMS (data not shown), indicating that stimulation of megakaryocytopoiesis does not improve Myh9-/- megakaryocyte maturation or their ability to release platelets.

A major concern was whether the increase in numbers of circulating giant platelets in Myh9-/- mice may cause tissue infarcts due to occlusion of microvessels. Indeed MYH9-RD does not necessarily protect against cardiovascular diseases and thromboses despite reduced platelet number as observed in a few patients\textsuperscript{17-19}. Thus, agents that increase platelet count may indeed be considered with caution. To evaluate this possibility, histology on various tissues where performed to check for the presence of microthrombi. Histology of the microcirculation in brain, gut, pancreas, lungs, spleen, kidney, heart, and psoas muscle did not reveal any signs of microthrombi. These data suggest that increasing platelet counts in macrothrombocytopenic patients may not worsen microcirculation and argue in favor of the use of thrombopoietic agents in MYH9-RD patients when needed.

However, our study also raises the very relevant issue that the romiplostim-induced increase in bone marrow reticulin fibres seems to be much more enhanced in Myh9-/- mice
compared to WT mice. Given the relatively good prognosis related to morbidity due to major bleeding in MYH9-RD patients, this animal experiment finding indicates that a very careful risk-benefit assessment is necessary before MYH9-RD patients are treated for longer periods of time with thrombopoietin receptor agonists. Furthermore, this animal experiment finding advises to control the bone marrow in such patients to recognize patients with an increased risk for myelofibrosis in time. The observation of increased myelofibrosis is plausible and consistent with our finding of an increased death rate of Myh9-/- megakaryocytes in the bone marrow. These dying cells likely release substances which promote myelofibrosis. That impaired storage of platelet alpha granule contents causes myelofibrosis is well known from the gray platelet syndrome 20-22.

Our study also provides further information on more settle effects of romiplostim treatment. The romiplostim-induced increase in bone marrow and spleen megakaryocyte numbers was accompanied by an increase in megakaryocyte transmigration as revealed by the higher number of megakaryocyte nuclei emboli counted in pulmonary vasculature (Fig.5). The presence of megakaryocytes or megakaryocyte nuclei in the lungs has long been reported 16, 23, 24 and is particularly increased in cases of reactive thrombopoiesis or following TPO treatment 16 in agreement with the present observations. In addition, infusion of mature megakaryocytes into mice mostly localized to the pulmonary vasculature where they release platelets 25. This indicates that the pulmonary vascular bed represents indeed a major trap for circulating megakaryocytes. Our observations that the number of naked nuclei was not increased in the bone marrow following romiplostim administration may suggest that most of the megakaryocytes produced in response to romiplostim exit the bone marrow. A similar number of megakaryocyte emboli were found in WT and Myh9-/- mouse lungs, indicating that Myh9-/- megakaryocytes are able to transmigrate to a similar extent as WT ones. In non-
treated animals, only Myh9-/- mice exhibited a few megakaryocyte-nuclei emboli that may reflect the basal higher megakaryocyte number present in their bone marrow.

Interestingly, for both genotypes we observed an increase in platelet size after start of romiplostim treatment, which was present until day 28 in wild type mice, while it was reversible in Myh9-/- mice (Fig. 1B). This suggests that the stimulation of megakaryocytopoiesis results in a change of platelet production with formation of larger platelets and potentially also in changes in the platelet membrane. The reversible pattern of platelet size increase in the Myh9-/- mice could be due to an increase in platelet debris, which overall may result into a decrease in mean platelet volume. When assessed by electron microscopy, also the Myh9-/- platelets appeared larger at day 33 (data not shown).

This prompted us to measure the expression of platelet membrane glycoproteins which showed a reduced expression of the GPIbIX complex and of GPVI after start of romiplostim in both WT and Myh9-/- mice. This effect has not been described for thrombopoietin receptor agonists so far. As we also observed in parallel a small increase in platelet size, the absolute decrease of receptor expression might even be underestimated. Reduction of membrane GPs can result from shedding by metalloproteases, from internalization, or from apoptosis. Both, GPIbα and GPVI are cleaved by metalloproteinases after platelet activation. However, we found no evidence for platelet activation as indicated by absence of P-selectin exposure or GPIIbIIIa activation. Although GPVI was reduced in the Western blots, likely due to shedding, no decrease in GPIbα and GPIbβ was observed, which rules out putative romiplostim-induced decrease in protein synthesis. Platelet apoptosis is also unlikely to explain the decreased glycoproteins surface expression since both annexin V labeling and mitochondrial potential were unaffected by romiplostim treatment (not shown).

GPIb shedding might also be the reason for the observed decrease in the GPIb/GPIIbIIIa ratio in patients with MYH9-RD, who have increased thrombopoietin
levels, which might already trigger a reduction of GPIb expression. As recent studies imply a potentially important role of the GPIb-IX complex for thrombin generation\textsuperscript{28, 29}, the observed decrease in GPIb-V-IX complex on platelet function and thrombin generation potential should be further assessed in patients receiving thrombopoietin receptor agonists.

Romiplostim treatment did not decrease the bleeding time in the Myh9-/- mice, indicating that in our model, platelet dysfunction is not overcome by doubling the platelet count. Surprisingly, the bleeding time even increased in the treated WT mice. Though we have no evidence why this phenomenon occurs, it may be due to a combination of effects. These may include of course the decrease in the GPIb complex, and in GPVI, but also an increase in VWF consumption resulting from the high amount of circulating platelets, as already reported for thrombocytemia\textsuperscript{30}. A direct effect of romiplostim on platelet function is unlikely as romiplostim administration for 6h did not increase the bleeding time.

A potential limitation of our study is that we used Myh9-/- mice and not mice showing a heterozygous mutation of the Myh9 gene, which would reflect the exact situation in patients with MYH9-RD. Furthermore, in our mouse model Myh9 is knocked out in the megakaryocytic lineage only, while in affected humans also other organs are affected. Thus our study is primarily informative about platelet and megakaryocyte related effects. However, as the platelet phenotype observed in MYH9-RD is autosomal dominant, and since Myh9-/- mice present with platelet characteristics of the human disease, including enlarged platelets, our model should sufficiently rule out the main issue of impairment of the microcirculation by increasing the number of giant platelets. Having an even more severe platelet phenotype in terms of total absence of contractile functions and higher mean platelet volume, compared to a heterozygous mutation as reported recently by Zhang et al.\textsuperscript{31}, allows drawing stronger conclusions regarding the potential risks of such a treatment. A second note of caution relates to the duration of observation. As our study was performed over a period of one month, the
data do not exclude more pronounced adverse effects on myelofibrosis and microcirculation during long term treatment.

In conclusion, we showed that romiplostim is able to increase, though modestly, the number of circulating platelets in a murine MYH9-RD model. Despite the presence of increased circulating large platelets, no occlusion of the microcirculation was observed whatever the organs examined. These data, together with a previous study using eltrombopag in patients are encouraging and further suggest that a short term use of thrombopoietic agents could have a role in reducing the bleeding tendency in MYH9-RD patients without the risk for major adverse effects. However, during longer treatment of MYH9-RD patients with thrombopoietic agents, myelofibrosis has to be considered as a potential severe adverse effect and patients should be carefully monitored. A more generally relevant finding is the reduction of GPIbIX and GPVI expression on platelets during treatment with thrombopoietin receptor agonists. This needs to be further assessed in patients receiving thrombopoietin receptor agonists.
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Conflict of interest statement:

AMGEN provided romiplostim. The company had no role in planning or performing the study, evaluating the data or writing the manuscript.

The authors have no conflict of interest to declare

Author contribution

C.L., F.P., P.L. and A.E. performed the mouse experiments and the electron microscopy studies

K.E. and F.D. performed the histology and megakaryocyte quantifications

C.L., C.G. and A.G. designed the study, supervised the experiments, interpreted results

C.L., C.G., and A.G wrote the manuscript

All authors contributed to the final version of the manuscript.
References


Legends to Figures

Figure 1. Romiplostim-induced increase in platelet number and size. (A) Platelet counts of WT and Myh9-/− mice, before (day 0) and after 8, 18, and 28 days of romiplostim administration. Each bar represents the mean±sem of 5 animals. (B) Relative platelet size variation during romiplostim treatment as visualize by flow cytometry (forward scatter parameter) in the GPIbβ-positive population. Mean±sem of 10 animals. Of note, the platelet size increase after start of romiplostim was transient in the MYH9-/− mice, indicating that it is not only the effect of thrombopoietin receptor stimulation.

Figure 2. Romiplostim-induced increase in circulating platelet debris. (A) Presence of GPIbβ-positive debris as observed by flow cytometry in the romiplostim-treated WT mice. (B) Transmission electron microscopy showing platelet ultrastructure of romiplostim-treated animals compared to controls. Arrows show poorly contrasted platelets in the romiplostim-treated WT mice.

Figure 3. Romiplostim-induced increase in splenic megakaryocytes.
(A) H & E stained paraffin sections of the spleen of romiplostim-treated and control mice. Animals treated with romiplostim (b, d) show a clearly visible increase of megakaryocytes in the spleen compared with the untreated animals (a, c). Of note, the number of megakaryocytes was already slightly increased in MYH9-/− mice at baseline (c). The arrow in panel b shows a mitosis of a megakaryocyte (original magnification x 789).

(B) Quantification of megakaryocyte numbers in the spleen (mean±sem of 9-10 animals per column). (C) Spleen weight; mean±sem of 9-10 animals per column.

Figure 4. Romiplostim-induced an increase in immature megakaryocytes. (A) Quantification of bone marrow megakaryocytes as observed by electron microscopy, per surface unit (s.u.) (12,945 µm²). Each bar represents the mean±sem of 3 bone marrows, for a total number of 99-205 counted megakaryocytes. (B) Classification of the megakaryocytes according to their maturation stages: stage I (presence of granules), stage II (developing DMS not yet organized), stage III (DMS organized in platelet territories). Data are represented as the percentage of total megakaryocytes. (C) Quantification of naked megakaryocyte nuclei.
inside the bone marrow. Each bar represents the percentage of naked nuclei relative to the total number of megakaryocytes counted.

**Figure 5. Romiplostim-induced an increase in megakaryocyte nuclei lung emboli.** (A) H&E-stained lung tissue of a wild-type animal treated with romiplostim showing a representative lung embolus (arrow).

(B) Quantification of the emboli per capillary in lung tissue. Each bar represents the mean±sem of 10 animals.

**Figure 6: Romiplostim induces a reduction of GPVI and GPIb-IX-V complex expression.** (A) Flow cytometry experiment showing in both WT mice and Myh9-/- mice a reduced surface expression of GPVI and GPIb-IX-V complex during romiplostim treatment (a, b, c, d), while expression of GPIIbIIIa (e) remained normal. Blood samples were taken after 28 days of romiplostim treatment. Results are expressed as the mean fluorescent intensity ± sem (n=8 for GPVI and n=4 for other proteins). (B) Western Blot showing total GPVI, GPIbβ and GPIIIa expression (lysate from a pool of 8-10 mice, after 1 month treatment, representative of 2 separate experiments). (C) Flow cytometry experiment showing absence of P-selectin exposure or Jon/A-PE labeling following romiplostim treatment (n=4-5). (D) Bleeding time, measured by the tail tip sectioning, was not reduced in one-month romiplostim treated Myh9-/- mice, while it was increased in treated WT mice.

**Figure 7. Myosin-deficiency increases myelofibrosis induced by romiplostim.** Histological sections stained for reticulin fibers (appearing black, arrows) showing absence of fibrosis in control WT or Myh9-/- bone marrow, compared to the presence of fibrosis following 1-month romiplostim treatment. Quantification was performed according to the absence of fibers (-), presence of a few fibers (+) or presence of numerous fibers (+). Fibrosis was more extensive in Myh9-/- mice. Very similar data were obtained with a second series of animals treated with the same romiplostim protocol (not shown).
Figure 1: Romiplostim-induced increase in platelet number and size

A

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Platelets/µL ($x 10^3$)

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FSC (Y-Med)

(days)
Figure 2: Romiplostim-induced increase in circulating platelet debris

A

WT

Myh9

B

WT control

Myh9-/- control

WT + Romiplostim

Myh9-/- + Romiplostim
Figure 3: Romiplostim-induced increase in splenic megakaryocytes

A

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<tr>
<td>c</td>
<td>d</td>
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For personal use only.
Figure 3 B and C

B

Megakaryocyte number / 2.62 mm²

WT NaCl  WT Romi  Myh9-/- NaCl  Myh9-/- romiplostim

C

Spleen weight (mg)

WT NaCl  WT romiplostim  Myh9-/- NaCl  Myh9-/- romiplostim
Figure 4: Romiplostim-induced increase in immature bone marrow megakaryocytes

A

![Graph showing the increase in megakaryocytes per s.u. in different genotypes after romiplostim treatment.]

B

![Graph showing the percentage of megakaryocytes in different stages after romiplostim treatment.]

C

![Graph showing the percentage of naked nuclei after romiplostim treatment.]

**Note:** For personal use only.
Figure 5: Romiplostim-induced increase in megakaryocyte nuclei lung emboli
Figure 6: Romiplostim induces a reduction of GPVI and GPIb-IX-V complex expression.
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Figure 7. Myosin IIa deficiency increases myelofibrosis induced by romiplostim

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Romiplostim administration shows reduced megakaryocyte response-capacity and increased myelofibrosis in a mouse model of MYH9-RD

Catherine Léon, Katja Evert, Frank Dombrowski, Fabien Pertuy, Anita Eckly, Patricia Laeuffer, Christian Gachet and Andreas Greinacher