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Preclinical studies on the use of a P-selectin-blocking monoclonal antibody to halt progression of myelofibrosis in the Gata1low mouse model

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1 **Preclinical studies on the use of a P-selectin blocking monoclonal antibody to halt progression**  
2 **of myelofibrosis in the *Gata1<sup>low</sup>* mouse model**

3  
4 **Paola Verachi<sup>1</sup>, Francesca Gobbo<sup>1,2</sup>, Fabrizio Martelli<sup>3</sup>, Mario Falchi<sup>4</sup>, Antonio di Virgilio<sup>5</sup>,**  
5 **Giuseppe Sarli<sup>2</sup>, Celine Wilke<sup>6</sup>, Andreas Bruederle<sup>6</sup>, Anirudh Prahallad<sup>6</sup>, Francesca Arciprete<sup>7</sup>,**  
6 **Maria Zingariello<sup>7</sup> and Anna Rita Migliaccio<sup>7,8</sup>**

7  
8 <sup>1</sup> Department of Biomedical and Neuromotor Sciences, University of Bologna, Italy;

9 <sup>2</sup> Department of Veterinary Medical Sciences, University of Bologna, Italy;

10 <sup>3</sup>National Center for Preclinical and Clinical Research and Evaluation of Pharmaceutical Drugs,  
11 Istituto Superiore di Sanità, Rome, Italy;

12 <sup>4</sup>National Center for HIV/AIDS Research, Istituto Superiore di Sanità, Rome, Italy; <sup>5</sup>Center for  
13 animal experimentation and well-being, Istituto Superiore di Santà, Rome, Italy;

14 <sup>6</sup>Novartis (United States), East Hanover, USA

15 <sup>7</sup>Unit of Microscopic and Ultrastructural Anatomy, University Campus Bio-Medico, Rome, Italy;

16 <sup>8</sup>Altius Institute for Biomedical Sciences, Seattle, WA, USA.

17

18 **Corresponding Author:** Anna Rita Migliaccio, PhD; Altius Institute for Biomedical Sciences,  
19 Seattle, WA, USA. Email: amigliaccio@altius.org

20 **Running Title:** P-selectin as target for myelofibrosis

21 **Keywords:** Myeloproliferative Neoplasms, P-selectin, Fibrosis, megakaryocytes, TGF- $\beta$

22

23 **Abstract**

24 The bone marrow (BM) and spleen from myelofibrosis patients, as well as those from the *Gata1<sup>low</sup>*  
25 mouse model of the disease, contain increased numbers of abnormal megakaryocytes. These cells  
26 express on their surface high levels of the adhesion receptor P-selectin that, by triggering a  
27 pathological neutrophil emperipolesis, lead to increased bioavailability of TGF- $\beta$  in the

28 microenvironment and disease progression. *Gatal*<sup>low</sup> mice develop with age a phenotype similar to  
29 that of patients with myelofibrosis, the most severe of the Philadelphia-negative myeloproliferative  
30 neoplasms. We previously demonstrated that *Gatal*<sup>low</sup> mice lacking the *P-selectin* gene do not  
31 develop myelofibrosis. In the current study, we test the hypothesis that pharmacological inhibition of  
32 P-selectin may normalize the phenotype of *Gatal*<sup>low</sup> mice which have already developed  
33 myelofibrosis. To test this hypothesis, we have investigated the phenotype expressed by aged  
34 *Gatal*<sup>low</sup> mice treated with the anti-mouse monoclonal antibody RB40.34, alone or in combination  
35 with Ruxolitinib. The results indicate that RB40.34 in combination with Ruxolitinib normalize the  
36 phenotype of *Gatal*<sup>low</sup> mice with limited toxicity by reducing fibrosis, and TGF- $\beta$  and CXCL1 (two  
37 drivers of fibrosis in this model) content in the BM and spleen and by restoring hematopoiesis in the  
38 bone marrow and the architecture of the spleen. In conclusion, we provide pre-clinical evidence that  
39 treatment with an antibody against P-selectin in combination with Ruxolitinib may be more effective  
40 than Ruxolitinib alone to treat myelofibrosis in patients.

41

## 42 **1 Introduction**

43 Myelofibrosis (MF) is the most severe of Philadelphia chromosome negative myeloproliferative  
44 neoplasms (MPN). The complex phenotype of the MF patients includes fibrosis and hematopoietic  
45 failure in bone marrow (BM), stem/progenitor cell mobilization, development of extramedullary  
46 hematopoiesis with splenomegaly and their clinical course is associated with increased risk of  
47 thrombosis, bleeding and evolution to acute leukemia (1–4). MF may be driven by gain of function  
48 mutations in several genes of the thrombopoietin axes such as *MPL*, the thrombopoietin receptor,  
49 *JAK2*, the first element of the MPL signaling, and *calreticulin*, a chaperon protein that when mutated  
50 binds MPL on the cell surface, inducing conformational changes which lead to ligand independent  
51 constitutive activation of the receptor (5). Regardless of the driver mutation, it has been recognized  
52 that MF has a distinctive cellular signature. In fact, both the BM and spleen from these patients  
53 contain numerous clusters of morphologically immature megakaryocytes (MK)(6) endowed with  
54 great proliferation potential (7,8). In MF, MK are retained immature by a mutation-driven RSP14  
55 ribosomopathy that impairs the translation of the mRNA for GATA1 (9,10), the transcription factor  
56 which plays a pivotal role in the progression of MK maturation (11). The causative role of the  
57 resulting abnormal MK in the pathogenesis of this disease is strongly supported by experiments in  
58 mice indicating that those carrying a hypomorphic mutation which selectively reduces GATA1 in

59 MK (*Gata1<sup>low</sup>* mice) develop myelofibrosis with age (12) while transgenic mice expressing  
60 *JAK2V617F*, one of the driver mutations of the disease(1–4), only in MK develop myelofibrosis even  
61 if their hematopoietic stem cells are normal (13,14). As first hypothesized by Schmitt et al (7),  
62 mechanistically, malignant MK are thought to drive MF by engaging in a pathological process of  
63 emperipolesis with the neutrophils which increases the bioavailability of transforming growth factor-  
64  $\beta$  (TGF- $\beta$ ), and possibly of other pro-inflammatory cytokines, in the BM of MF patients and animal  
65 models (8,15–17) The pathobiological role of TGF- $\beta$  in the development of myelofibrosis has been  
66 further confirmed by the observation that in animal models development of myelofibrosis is  
67 prevented by genetic ablation of the *TGF- $\beta$*  gene (17,18) and reverted by treating myelofibrosis mice  
68 with a small TGF- $\beta$  receptor-1 kinase inhibitors (19) or with the TGF- $\beta$  trap AVID200 (20). The  
69 TGF- $\beta$  trap AVID200 is currently in clinical phase-1/2 clinical trials for MF who are resistant to  
70 therapy with the JAK1/2 inhibitor Ruxolitinib (Rux)(21).

71 Later studies have indicated that the adhesion receptor P-selectin (P-SEL) may represent an element  
72 upstream to TGF- $\beta$  in the pathobiological pathway leading to MF. In fact, the MK abnormalities  
73 observed in this disease include abnormal cytoplasmic trafficking of P-SEL which instead to be  
74 partitioned in the granules is displayed on the cell surface(22). The high levels of P-SEL on the cell  
75 surface, by interacting with its ligand (P-selectin glycoprotein ligand-1, PSGL-1) expressed by the  
76 neutrophils (23,24) has been hypothesis to drive a process of pathological emperipolesis between the  
77 neutrophils and the MK which leads to death of the MK by para-apoptosis and release of TGF- $\beta$  in  
78 the microenvironment (25,26). This hypothesis has been mechanistically demonstrated by the  
79 observation that the TGF- $\beta$  bio-availability in the BM of *Gata1<sup>low</sup>* mice lacking the *P-sel* gene is  
80 normal and that these mice do not develop myelofibrosis with age and live, on average, 2 months  
81 longer than their *Gata1<sup>low</sup>* littermates (27). These findings support the hypothesis that in MF, the  
82 disease is established and progresses thanks to a pathological P-SEL/TGF- $\beta$  circuit established by the  
83 malignant MK(28). Whether inhibition of P-SEL would also be effective in reverting to normal  
84 myelofibrosis once the disease is established has not been demonstrated as yet.

85 Recently, the P-SEL inhibitor Crizanlizumab (SEG101) has been demonstrated to reduce the  
86 frequency of vaso-occlusive crises in patients with Sickle Cell Disease with limited toxicity (29).  
87 Based on these observations, in November 2019, the Federal Drug Administration approved the use  
88 of Crizanlizumab for the treatment of pain crisis in Sickle Cell Disease. The rationale for the clinical  
89 study with Crizanlizumab had been provided by a pre-clinical study that evaluated the effects of the  
90 commercially available monoclonal antibody RB40.34 targeting the murine P-SEL as antithrombotic

91 agent in a mouse model of Sickle Cell Disease (30). Using the fact that Crizanlizumab had been  
92 already approved for clinical use and that conditions for effective treatment of mice with RB40.34  
93 had been already described, we test here the hypothesis that pharmacological inhibition of P-SEL  
94 with RB40.34, alone or in combination with Rux, is effective in reverting the myelofibrotic  
95 phenotype expressed by *Gata1*<sup>low</sup> mice.

96

## 97 **2 Materials and Methods**

98 **2.1 Mice.** *Gata1*<sup>low</sup> mice are bred in the animal facility of Istituto Superiore di Sanità as described  
99 (31). Littermates are genotyped at birth by PCR and those found not to carry the mutation are used as  
100 wild-type (WT) controls. All the experiments, including the size of the experimental groups, are  
101 performed according to the protocols D9997.121 approved by the Italian Ministry of Health on  
102 September 2<sup>nd</sup> 2021, and according to the European Directive 86/609/EEC.

103 **2.2 Treatments.** A total of 47 *Gata1*<sup>low</sup> mice were implanted with 14mm micro-chips (one  
104 chip/mouse) (AVID, Norco, CA, USA) and divided into two separate experiments (**Figure S1**). In  
105 the first experiment, 24 11-months-old mice were randomly divided in four groups (3 males and 3  
106 females each) that were treated as follows: Group 1: Vehicle (2% v/v DMSO by gavage, negative  
107 control for group 3 and 4); Group 2: Biotin-conjugated rat anti-mouse CD62P (RB40.34, Cat.  
108 n. 553743, BD Pharmigen, San Diego, CA, USA; 30 µg/mouse per day x three days per week by iv,  
109 as described (30), until day 45, and then ip); Group 3: Rux (Novartis Pharma AG, Basel, Switzerland;  
110 45mg/Kg twice per day x 5 days a week by gavage as described (32)); Group 4: biotin-labeled  
111 RB40.34 and Rux in combination. On Day 5, all the mice were weighed and bled for blood cell  
112 counts determinations and detection of RB34.40 on platelets. Mice were sacrificed at day 5 (males)  
113 and day 12 (females) and BM and spleen collected for cell signaling and histopathological  
114 determinations. In the second experiment, 23 8-months-old *Gata1*<sup>low</sup> mice were divided in the same  
115 groups described above and treated for 7 weeks. In this experiment, we used the purified RB40.34  
116 (Cat. n. 553742, BD Pharmigen). The treatment was interrupted for two weeks during the holiday  
117 break (from day 24 to day 43). On day 54, all the mice were weighed, bled for blood counts  
118 determination, and sacrificed for histopathology observations of their BM and spleen.

119 **2.3 Blood counts determination.** Mice were topically anesthetized with Novesina (Cat. n. s01ha02,  
120 Novartis, Basel, CH, one drop/eye) and blood collected from the retro-orbital plexus into heparinized

121 microcapillary tubes. Blood counts were evaluated on deidentified samples by an accredited  
 122 commercial laboratory which provide diagnostic services for laboratory animals (Plaisant Laboratory,  
 123 Rome, Italy).

124 **2.4 Flow cytometry.** Binding of RB40.34 to platelets. Platelet-enriched plasma was prepared by  
 125 centrifugation of 200 $\mu$ L of heparinized blood (10,000rpm for 20min with the Eppendorf™  
 126 Centrifuge 5425/5425 R, Eppendorf, Milan, Italy) and the binding of biotinylated RB40.34 to  
 127 platelets measured by flow cytometry following incubation with PE-Cy7 Streptavidin (ca.  
 128 no. 557598, BD Pharmingen). Platelets were identified based on size (FS: forward scatter) and  
 129 internal cell complexity (SS: side scatter), as described(22). MK identification and binding to  
 130 RB40.34. BM and spleen cells were resuspended in Ca<sup>++</sup> Mg<sup>++</sup>-free PBS containing 0.5% (v/v) fetal  
 131 bovine serum (FBS, Cat. n. F7524, Sigma-Aldrich) and incubated with PE-CD41, FITC-CD61 and  
 132 PE-Cy7-streptavidin. Cells were then divided by flow cytometry into four populations corresponding  
 133 to non-MK (CD41<sup>neg</sup>/CD61<sup>neg</sup>); immature MK (CD41<sup>neg</sup>/CD61<sup>high</sup>); mature MK (CD41<sup>high</sup>/CD61<sup>high</sup>)  
 134 and very mature MK (CD41<sup>high</sup>/CD61<sup>neg</sup>), as described(19). The levels of PE-Cy7-streptavidin  
 135 staining was assessed as a measure of biotinylated-RB40.34 binding to the MK. Hematopoietic  
 136 stem/progenitor cell determinations. Mononuclear BM and spleen suspensions were incubated with  
 137 a cocktail of antibodies including APC-CD117, APC-Cy7-Sca1, PE-Cy7-CD150, biotin-labeled anti-  
 138 mouse CD48 and biotin-labeled anti-lineage antibodies. After 30min of incubation on ice, cells were  
 139 washed and incubated with streptavidin-PE-Cy5 (all from BD Pharmingen). Hematopoietic  
 140 progenitor cells were defined as lineage negative cells (Lin-). Hematopoietic stem cells were defined  
 141 as LSK (Lin-/CD48<sup>neg</sup>/c-Kit<sup>pos</sup>/Sca-1<sup>pos</sup>) while long-term repopulating hematopoietic stem cells were  
 142 defined as SLAM (Lin-/CD48<sup>neg</sup>/c-Kit<sup>pos</sup>/Sca-1<sup>pos</sup>/CD150<sup>pos</sup>) as described(27,33). Nonspecific signals  
 143 and dead cells were excluded, respectively, by appropriate fluorochrome-conjugated isotype and  
 144 propidium iodide staining. All the flow cytometry analyses were performed with the Gallios analyzer  
 145 (Beckman Coulter) and the results analyzed with the Kaluza analysis program, version 2.1 (Beckman  
 146 Coulter).

147 **2.5 Western blot analysis.** BM and spleen from *Gatal*<sup>low</sup> mice treated for 5 days were dissolved in  
 148 lysis buffer containing protease and phosphatase inhibitors and stored at -80°C. Protein extracts were  
 149 separated by electrophoresis under denaturing conditions using 7.5-10% mini-Protean TGX pre-  
 150 casted gels (Bio-Rad, CA, USA) and transferred to nitrocellulose filters with the Transblot-Turbo  
 151 system (Bio-Rad, Hercules). Filters were probed with antibodies against proteins of the canonical  
 152 (SMAD2/3, cat no. 8685, Cell Signaling, Boston, MA, USA), p-SMAD2/3 (cat no. 8828, Cell

153 Signaling), TGF- $\beta$ RII (cat no. ab186838, Abcam, Cambridge, UK) and non-canonical (p38, cat no.  
 154 9212; p-p38, cat no. 4511; ERK1/2, cat no. 9102; p-ERK1/2, cat no. 9101; all from Cell Signaling)  
 155 TGF- $\beta$  signaling and of the JAK/STAT signaling (JAK2 (cat. No 3230, Cell Signaling), STAT5 (cat  
 156 no. sc-74442, Santa Cruz, Dallas, Texas, USA), pJAK2 (Phospho-Tyr1007/1008 JAK2, cat no. 3771,  
 157 Cell Signaling) and p-STAT5 (cat no, 9351, Cell Signaling). GAPDH (cat no. G9545, Sigma  
 158 Aldrich) was used as a loading control. The bands were quantified with the ImageJ 1.52q software  
 159 (National Institutes of Health, Bethesda, MD, USA) and normalized against GAPDH. Stoichiometry  
 160 determinations of phospho-proteins levels were obtained by normalizing the content of the  
 161 phosphoprotein with that of the corresponding total protein.

162 **2.6 Histological analyses.** Femurs were fixed in formaldehyde (10% v/v with neutral buffer), treated  
 163 for 1h with a decalcifying kit (Osteodec; Bio-Optica, Milan, Italy) and included in paraffin. Spleens  
 164 were fixed in formaldehyde and then included in paraffin (19). Paraffin-embedded tissues were cut  
 165 into consecutive 3 $\mu$ m sections and stained either with Hematoxylin-Eosin (H&E; cat no.  
 166 01HEMH2500 and 01EOY101000, respectively; Histo-Line Laboratories, Pantigliate, MI, Italy),  
 167 Gomori silver or Reticulin staining and Mallory Trichrome staining (cat no. 04-040801, 04-040802, 04-  
 168 020802 respectively; Bio-Optica). For immune-microscopy, BM sections were incubated with anti-CXCL1  
 169 (cat no. ab86436, Abcam), anti-TGF- $\beta$ 1 (cat no. sc-130348, Santa Cruz Biotechnology) antibodies and  
 170 reactions detected by avidin-biotin immune-peroxidase staining and 3,3'-diaminobenzidine (0.05% w/v)  
 171 (Vectastain Elite ABC Kit, Vector Laboratories, Burlingame, CA, USA). Slides were counterstained  
 172 with Papanicolaou's hematoxylin (Histo-Line Laboratories). Images were acquired with the optical  
 173 microscope Eclipse E600 (Nikon, Shinjuku, Japan) equipped with the Imaging Source "33" Series  
 174 USB 3.0 Camera (cat no. DFK 33UX264; Bremen, DE) and the signal quantified acquiring at least 5  
 175 different areas/femur/mouse from at least 4 mice per group using the ImageJ program (version 1.52t)  
 176 (National Institutes of Health), as described(34,35). For Immuno-fluorescence microscopy -  
 177 determinations, three micron-thick BM sections were dewaxed in xylene and antigens were retrieved  
 178 by treatment with EDTA buffer (pH=8) for 20' in a pressure cooker (110-120°C, high pressure) and  
 179 incubated with antibodies against CD42b (cat no. ab183345, Abcam), GATA1 (cat no. sc-265, Santa  
 180 Cruz), CD3 (cat no. ab16669, Abcam) and CD45R/B220 (cat no. 553085, BD-Pharmingen) over  
 181 night at 4°C. Primary antibodies were visualized with the secondary antibody goat anti rat Alexa  
 182 Fluor 488 (cat no. ab150165, Abcam) or goat anti rabbit Alexa Fluor 555 (cat no. ab150078, Abcam).  
 183 All sections were counterstained with DAPI (cat no. D9542-5MG, Sigma-Aldrich, Darmstadt, DE),



184 mounted with Fluor-shield histology mounting medium (cat. F6182-10MG, Sigma-Aldrich), and  
185 examined using a Nikon Eclipse Ni microscope equipped with filters appropriate for the  
186 fluorochrome to be analyzed. Images were recorded with a Nikon DS-Qi1Nc digital camera and NIS  
187 190 Elements software BR 4.20.01 and quantified with the ImageJ program by counting the number  
188 of cells that exceeded the intensity set as threshold, as described(35). Reconstruction of the image of  
189 the all femur was obtained by the combining the entire set of stack images (15 images at 20x or 34  
190 images at 63x) with the Zen Blue software (Zeiss, Oberkochen, DE). Microvessel density was  
191 determined by incubating bone marrow and spleen sections with anti-CD34 (cat no. MAB7100,  
192 AbNova, Taiwan, primary) and Alexa Fluor 568-conjugated donkey anti-rat (Invitrogen, Carlsbad,  
193 CA, USA, secondary) and with Hoechst 33342 (Thermo Fisher Scientific, Waltham, MA, USA).

194 **2.7 Data analysis.** Data were analyzed and plotted using GraphPad Prism 8.0.2 software (GraphPad  
195 Software, San Diego, CA, USA) and presented as Mean ( $\pm$ SD) or as box charts, as more appropriate.  
196 All the data had a normal distribution, as assessed by Shapiro Wilk T test. Values between two  
197 groups were compared by T test while those among multiple groups were compared by Tukey's  
198 multiple comparisons test or Anova, as indicated. Kaplan–Meier survival curves were compared by  
199 log-rank (Mantel-Cox) test. Differences were considered statistically significant with a  $p < 0.05$ .

200

### 201 **3 Results**

202 **3.1 The RB40.34 antibody readily binds to platelets in the blood and reaches the fibrotic BM of**  
203 ***Gata1<sup>low</sup>* mice.** Since the underlying fibrosis in the BM of *Gata1<sup>low</sup>* mice may restrain the RB40.34  
204 antibody to reach the BM, we conducted a feasibility study to determine whether the biotinylated-  
205 RB40.34 was detectable in BM sections from mice treated for 5 days. In addition, since we, and  
206 others, have demonstrated that platelets from *Gata1<sup>low</sup>* mice express greater levels of P-SEL on their  
207 surface(22,36), we determined whether biotinylated-RB40.34 was detectable on platelets present in  
208 the blood 5hr after its administration as control of the persistence of the antibody in the circulation  
209 after its injection (**Figure 1**). Biotin is produced in the liver and is present, albeit at low levels, in  
210 several cell types(37). Therefore, it is not surprising that the APC-Cy7 Streptavidin signal is detected  
211 also on platelets and BM sections from mice in the vehicle and Rux groups which did not receive  
212 biotinylated-RB40.34. However, the signals on platelets and BM sections from mice which had  
213 received biotinylated-RB40.34 is clearly greater than background levels (**Figure 1**).

214 **3.2 Five days treatment with RB40.34 in combination with Rux reduces TGF- $\beta$  signaling in**  
215 **bone marrow and JAK2/STAT5 signaling in the spleen from *Gata1<sup>low</sup>* mice.** To investigate the  
216 effects of the treatments on the signaling state of the BM and spleen from *Gata1<sup>low</sup>* mice, western-  
217 blot analyses of these organs from untreated *Gata1<sup>low</sup>* mice and from mice treated for 5 days were  
218 performed. These studies used a panel of antibodies which target SMAD2/3, and TGF- $\beta$ RII  
219 (canonical TGF- $\beta$  signaling); p38, p-p38, ERK1/2 and p-ERK1/2 (non-canonical TGF- $\beta$  signaling)  
220 and JAK2 and STAT5 (JAK/STAT signaling). Untreated WT mice were analyzed in parallel as  
221 control (**Figures 2,3 and S3**). pJAK2 and pSTAT5 were not investigated because the phosphorylation  
222 of these two proteins in extracts from primary tissues is very sensitive to degradation upon storage  
223 (**Figure S4**).

224 The BM from untreated *Gata1<sup>low</sup>* mice expresses levels of TGF- $\beta$ RII significantly greater than WT  
225 mice, which are likely a reflection of the increased number of MK present in this organ. Treatment  
226 with RB40.34 or Rux alone had no effect to the levels of SMAD2/3 and TGF- $\beta$ RII proteins which  
227 remain similar to that of untreated *Gata1<sup>low</sup>* mice. By contrast, treatment with RB40.34 in  
228 combination with Rux reduce the content of SMAD2/3 and of TGF- $\beta$ RII in the BM down to levels  
229 expressed by BM from WT mice, suggesting that the treatment reduced the canonical TGF- $\beta$   
230 signaling in this organ (**Figure 2A,C**). The levels of total ERK and p38, that are two elements of the  
231 MAPK-dependent non canonical TGF- $\beta$  signaling (38), and of their phosphorylated forms in the BM  
232 of untreated *Gata1<sup>low</sup>* mice are not significantly greater than normal. Although none of the treatments  
233 affected the p38 content/activation state, treatment with RB40.34 in combination with Rux reduces to  
234 barely detectable levels the activation of p-ERK, an indication that this treatment is reducing the non-  
235 canonical TGF- $\beta$  signaling possibly responsible for fibrosis in the BM (**Figure 2B,C**). By contrast,  
236 the content and activation state of canonical and non-canonical TGF- $\beta$  signaling in the spleen from  
237 untreated *Gata1<sup>low</sup>* mice are similar to that of spleen from WT mice and are not significantly affected  
238 by any of the treatments (**Figure S3**).

239 JAK2 is not detected in BM from untreated *Gata1<sup>low</sup>* and WT littermates and, with the exception of  
240 two out of three mice in the vehicle and Rux alone groups, remains undetectable in BM from the  
241 treated groups as well. The content of STAT5 is instead robust and not statistically different across  
242 all groups in BM (**Figure 3A,C**). By contrast, the content of JAK2 and STAT5 in the spleen from  
243 untreated *Gata1<sup>low</sup>* mice is significantly greater than that from WT littermates (**Figure 3B,D**),  
244 possibly reflecting the great levels of extramedullary hematopoiesis occurring in the spleen of the

245 mutant animals(39). The levels of STAT5 in the spleen from *Gatal<sup>low</sup>* mice remain robust after  
246 treatment with either Rux or RB40.34 alone or in combination. By contrast, the levels of JAK2 in the  
247 spleen of the mutant mice are drastically reduced upon the combined treatment with Rux and  
248 RB40.34, but not with either of the drugs alone. These data suggest that the combination of  
249 Rux+RB40.34 is targeting the extramedullary hematopoiesis in spleen.

250 These data indicate that treatment for only 5 days with Rux+RB03.34 in combination induces  
251 detectable biochemical changes in the BM and spleen from *Gatal<sup>low</sup>* mice. Additional experiments,  
252 associated with expression profiling of individual cell populations, are necessary to assess whether  
253 these biochemical changes are due to alterations in cell composition and/or in the signaling cascade  
254 of individual cell populations in these organs.

### 255 **3.3 All the treatments are well tolerated with no significant effects on survival and body weight.**

256 Encouraged by the results described above, we performed longer treatments (day 12 and day 54) to  
257 assess whether these drugs may affect the myelofibrosis phenotype expressed by *Gatal<sup>low</sup>* mice. To  
258 determine safety, all the treated mice were daily monitored by a veterinarian who recorded no  
259 significant modifications in physical activity and behavior (no lethargy, no excessive grooming, no  
260 change in coat luster) during all the period of observation. None of the treatments affected the  
261 weight of the animals which remains similar to that observed before treatment in all the experimental  
262 groups (**Figure S5A**). Although few deaths were recorded during treatment (**Table S1**), overall log-  
263 rank test of the Kaplan-Meier survival curves of the treated *Gatal<sup>low</sup>* mice showed no significance  
264 difference in death rate among groups (**Figure S5B**).

### 265 **3.4 RB40.34, alone or in combination with Rux, reduces anisocytosis and lymphocyte counts.**

266 Hct levels remained within normal ranges in all experimental groups for all the duration of the  
267 treatments (**Table 1**). A significant raise in red blood cell (RBC) distribution width (RDW) at levels  
268 that meet the criteria for anysocytosis is observed in some of the mice treated with vehicle and in all  
269 of those treated with Rux for 54 days while the RDW in the groups treated with RB40.34 alone or in  
270 combination with Rux is within normal ranges (**Figure 4A**).

271 None of the treatments rescue the platelet deficiency of *Gatal<sup>low</sup>* mice which remains significantly  
272 lower than normal in all the groups (**Table 1**).

273 The difference in WBC counts between untreated *Gatal<sup>low</sup>* and WT littermates is not statistically  
274 significant (**Table 1**) and none of the drugs investigated induces significant changes in the WBC  
275 counts since even the two-fold reductions observed at day 54 in the Rux and RB40.54 plus Rux

276 groups are not statistically significant by Tukey multiple comparison test with those of untreated  
 277 mice. However, a comparison of the frequencies of the different WBC subpopulations reveals that  
 278 RB40.34 in combination with Rux significantly decreases the lymphocytes counts by day 54 (**Figure**  
 279 **4B**).

280 In conclusion, none of the treatments induced anemia nor rescued thrombocytopenia of *Gata1*<sup>low</sup>  
 281 mice. However, treatment for 54 days with RB40.34 in combination with Rux reduced anisocytosis  
 282 and lymphocyte counts.

283 **3.5 Treatment with RB40.34 in combination with Rux reduces fibrosis and restores**  
 284 **hematopoiesis in the bone marrow from *Gata1*<sup>low</sup> mice.** By 8-11 months of age, the femur of  
 285 *Gata1*<sup>low</sup> mice is hypocellular and contain great levels of fibrosis (**Figure S6**). None of the treatments  
 286 alters the BM cellularity (which remains lower than normal) and the level of fibrosis observed by day  
 287 5 (data not shown). By day 12, however, although the BM from all the groups remains hypocellular  
 288 (data not shown), the level of fibrosis in the diaphysis of the femur from mice treated with RB40.34  
 289 and Rux in combination is reduced (**Figure S7**), suggesting that this combination is starting to be  
 290 effective. In agreement with this hypothesis, by day 54, the femur from mice of the RB40.34 plus  
 291 Rux group appears reddish, a sign of improved erythropoiesis (**Figure 5A**), and contains significantly  
 292 greater number of cells than that from the vehicle group (**Figure 5B**). This increased cellularity is  
 293 also evident by hematoxylin/eosin staining of the BM sections (**Figure 5C**). In addition, reticulin  
 294 staining indicates strong reductions of fibrosis in the BM of the entire femur of mice treated with  
 295 RB40.34 plus Rux for 54 days (**Figure 5C,D**). By contrast, single treatment with RB40.34  
 296 significantly reduces fibrosis, but does not increase BM cellularity while, as previously reported(32),  
 297 treatment with Rux alone does not increase BM cellularity and does not reduce fibrosis in *Gata1*<sup>low</sup>  
 298 mice. The reason while, by contrast with our data, Rux is effective in reducing fibrosis in the  
 299 *JAK2*<sup>V617F</sup>-driven mouse model (40) is unclear and deserves to be further investigated.

300 The abnormal *Gata1*<sup>low</sup> MK release several bone morphogenic proteins that are responsible for  
 301 increased bone formation starting at 1-months of age(12,41,42). In spite of the increased collagen  
 302 deposition, the bone from the *Gata1*<sup>low</sup> mice remains immature with poor Ca<sup>++</sup> deposition and,  
 303 similarly to what observed in MF patients, the mice develop osteopetrosis(43,44). Differences in the  
 304 integrity of the femur from mice treated with the different drug combinations (**Figure 5C**) suggest  
 305 possible differences in the levels of osteopetrosis expressed in the four experimental groups. To test  
 306 this hypothesis, we analyzed by Mallory trichrome staining femur from untreated WT and *Gata1*<sup>low</sup>

307 littermates and from *Gata1<sup>low</sup>* mice treated with the different drug combination (**Figure S8**). As  
308 expected, the cortical bone of WT mice is characterized by red-mature lamellar bone with limited  
309 blue areas of osteoids rich in collagen fibers but poor in Ca<sup>++</sup>. By contrast, the cortical bone of both  
310 the epiphysis and the diaphysis from *Gata1<sup>low</sup>* mice contains large blue areas with unmineralized  
311 osteoids and limited areas of red-mature lamellar bone. The diaphysis of the mutant mice also  
312 contains large areas of trabecular unmineralized bone protruding in the medulla. These results are  
313 similar to those published in (40, 41). After 54 days of treatment, the histopathology of the femur  
314 from the vehicle and RB40.34 group is similar to that of untreated *Gata1<sup>low</sup>* mice of comparable age.  
315 However, the medulla of the femur from the mice treated with Rux, alone and in combination with  
316 RB40.34, contains significant less areas of neo-bone formation while the maturation of the cortical  
317 bone from the femurs of mice treated with Rux is normal (**Figure S8**).

318 Another of the features associated with myelofibrosis which is conserved in animal models is  
319 increased neo-angiogenesis (12). To assess whether the treatments had reduced the neo-angiogenesis in  
320 the bone marrow and spleen from *Gata1<sup>low</sup>* mice, confocal microscopy studies with CD34, which in  
321 mice recognize endothelial cells, and Hoechst, to identify the nucleated cells, were performed  
322 (**Figure 6**). Indeed, by day 54, the vessel density of all the treatment groups was significantly lower  
323 than in the vehicle.

324 The BM hematopoietic failure associated with the myelofibrotic phenotype of *Gata1<sup>low</sup>* mice includes  
325 barely detectable levels of hematopoietic stem/progenitor cells in this organ(27). To confirm that  
326 RB40.34 in combination with Rux improves hematopoiesis in BM, the frequency and total numbers  
327 of progenitor (Lin-) and short term (LSK) and long (SLAM) term repopulating stem cells in the BM  
328 from mice treated for 54 days with the various drug combination was evaluated (**Figures 5E and S9**).  
329 Indeed, the BM from mice treated with RB40.34 in combination with Rux contains significantly  
330 greater frequency of Lin- and LSK and greater total numbers of all three populations than that from  
331 the vehicle-treated group.

332 **3.6 Treatment with RB40.34 in combination with Rux reduces fibrosis, extramedullary**  
333 **hematopoiesis and restores the architecture of the spleen from *Gata1<sup>low</sup>* mice.** Given the great  
334 relevance of JAK2 signaling in hematopoiesis(45), the observation that treatment with RB40.34 and  
335 Rux in combination greatly reduces the JAK2 content in the spleen suggest that this treatment  
336 decreases hematopoiesis in this organ. In agreement with this hypothesis, we observe marked  
337 reductions of fibrosis in spleen from *Gata1<sup>low</sup>* mice treated with RB40.34 plus Rux for only 12 days  
338 (**Figure S7**). By day 54, a trend toward reduction in spleen size (both as weight, as ratio between

339 spleen weight and body weight, and as cell numbers) is observed in the group treated with RB40.34  
340 plus Rux (**Figure 7A-C**). Furthermore, a significant reduction in the total number of hematopoietic  
341 progenitor cells (Lin- cells) was observed at day 54 in the spleen from this group (**Figures 7D** and  
342 **S9**), supporting the hypothesis that RB40.34 and Rux in combination reduces extramedullary  
343 hematopoiesis in this organ.

344 The architecture of the spleen from *Gata1<sup>low</sup>* mice is greatly altered by the fibrosis and by the  
345 underlying extramedullary hematopoiesis (**Figure 8A,B,D**). Significant reductions in fibrosis are  
346 observed in mice treated with RB40.34 alone, Rux alone and RB40.34 and Rux in combination,  
347 although the greater reductions are observed in mice treated with the combination (**Figure 8C,E**). As  
348 expected(46), CD45R/CD3 staining indicates that the architecture of the spleen from WT mice is  
349 characterized by the presence of large aggregates of lymphoid cells and a well-developed white pulp.  
350 Red blood cells are embedded in the reticular connective tissue which contains few megakaryocytes  
351 and supporting trabeculae. The T (CD3pos, in red) and B (CD45Rpos, in green) lymphocytes are  
352 numerous and localized around the central arterioles: T lymphocytes form a sleeve around the central  
353 arteriole, the periarteriolar lymphoid sheath, while B cells are mainly localized in the outer region of  
354 the white pulp, defined the marginal zone. By contrast, the spleen from *Gata1<sup>low</sup>* mice contains a  
355 hypoplastic white pulp and its periarteriolar lymphoid sheath contains a markedly reduced number of  
356 T cells. In addition, the red pulp appears disorganized by the presence of numerous MK and fibrosis  
357 (**Figure 8A-C**). Treatment of *Gata1<sup>low</sup>* mice with RB40.34 and Rux in combination, and to a lesser  
358 extent by the two drugs as single agents, restores the normal architecture of the spleen with a great  
359 expansion of white pulp and a nearly normal organization of the periarteriolar lymphoid sheath and  
360 of the marginal zone (**Figure 8C**).

361 **3.7 RB40.34 in combination with Rux for 54 days improves MK maturation but does not**  
362 **decrease the MK content in the bone marrow and spleen from *Gata1<sup>low</sup>* mice.** The process of  
363 terminal megakaryocyte maturation involves a series of precursors that progressively acquire features  
364 of mature cells that release platelets(16,47). As these precursors progress along the maturation  
365 pathway, they express increased levels of CD41 and CD61 on their surface(47,48). Therefore, flow  
366 cytometry analyses for CD41 and CD61 expression divides MK precursors into three classes:  
367 immature (CD41negCD61pos), mature (CD41posCD61pos) and very mature (CD41posCD61low)  
368 while non-MK are negative for both markers. On the basis of this flow cytometry criteria, we  
369 determined whether the treatments rescued the defective MK maturation of *Gata1<sup>low</sup>* mice (**Figure 9**).

370 Since the BM of *Gatal<sup>low</sup>* mice contains great number of MK (12), it is not surprising that CD61pos  
371 cells represent almost 30% of the total cells of BM and spleen from mutant mice (**Figure 9**). The total  
372 frequency of the CD61pos cells in the BM and spleen from all the experimental groups remains high  
373 for all the duration of the treatments. By day 5 and 12, very few of the MK in the BM and spleen  
374 from *Gatal<sup>low</sup>* mice in all the experimental groups had the very mature CD41bposCD61low  
375 phenotype. The frequency of very mature CD41bposCD61low MK is low and that of immature  
376 CD41bnegCD61pos MK high also in BM and spleen from mice treated with vehicle for 54 days. By  
377 contrast, by day 54, the frequency of MK with the very mature and immature phenotypes in the  
378 groups treated with RB40.34, Rux or RB40.34 and Rux in combination is, respectively, significantly  
379 greater and lower than in the vehicle group. These results suggest that the drugs, although ineffective  
380 in reducing the proliferation of the MK, are improving their maturation.

381 The abnormal maturation of MK from mouse models and MF patients includes localization of P-SEL  
382 on the DMS instead than in the  $\alpha$ -granules (22,25,26). Since the DMS increases with maturation, the  
383 amount of P-SEL exposed to the extracellular space also increases during this process. Therefore, the  
384 biotinylated RB40.34 which reach the BM should bind great numbers of *Gatal<sup>low</sup>* MK and its binding  
385 should be greater as these cells mature. To assess whether the improved MK maturation induced by  
386 the treatments for 54 days included rescue of the altered cell surface expression of P-SEL, the  
387 binding of PE-Cy7-streptavidin to MK from the BM and spleen of mice treated for 5, 12 or 54 days  
388 was determined (**Figures 9A-C**). As expected, PE-Cy7-streptavidin binding is barely detected on BM  
389 and spleen cells from mice treated with either vehicle or Rux alone which had not received the  
390 antibody in any of the time points. The low levels PE-Cy7-streptavidin binding observed in these  
391 group probably represent background signals due to endogenously produced biotin and are not  
392 informative on the levels of P-SEL expressed by MK. By day 5 and 12, PE-Cy7-streptavidin binding  
393 is detected in cells both in the non-MK and in the MK gate. The binding of PE-Cy7-streptavidin to  
394 the non-MK cells is possibly related to the presence in this population of endothelial cells, also  
395 known to express P-SEL (17). By day 5 and 12, great numbers of MK from the BM and spleen of  
396 mice treated with RB40.34 alone or in combination with Rux bind PE-Cy7-streptavidin. As expected,  
397 the MFI of the binding increases in cells with a more mature phenotype, a reflection of the greater  
398 levels of P-SEL on the cell surface of *Gatal<sup>low</sup>* MK as they mature. In addition, increases in MFI are  
399 also observed among MK of comparable maturation stage analyzed at day 5 and day 12, a possible  
400 reflection of increased bioavailability of the antibody in the microenvironment due to the reduction of  
401 fibrosis induced by the treatments. By contrast, PE-Cy7-streptavidin is found barely bound to MK

402 from BM and spleen of mice treated with RB40.34 alone or in combination with Rux by day 54.  
403 These last results indicate that the improved MK maturation induced by these two drug combinations  
404 may include reduced localization of P-SEL on the DMS. By day 54, PE-Cy7-streptavidin binding is  
405 also barely detectable in the non-MK populations from mice treated with RB40.34 alone or in  
406 combination with Rux. Since the cells responsible for binding PE-Cy7-streptavidin in the non-MK  
407 population are probably endothelial cells and that P-SEL expression in endothelial cells is up-  
408 regulated by inflammation(49), these data provide further support for the hypothesis that the  
409 treatments are reducing the inflammatory milieu of the BM and spleen from *Gata1<sup>low</sup>* mice.

410 **3.8 Treatment with Rux increases the frequency of MK expressing detectable levels of GATA1**  
411 **in the BM from *Gata1<sup>low</sup>* mice.** The abnormal maturation of the MK which is thought to drive  
412 myelofibrosis in patients and mouse models is driven by defective content of GATA1(6,9,10), the  
413 transcription factor which plays a pivotal role in supporting MK maturation(6,11). As expected,  
414 confocal microscopy analyses with antibodies against GATA1 and CD42b (as a marker of MK)  
415 indicated that the BM from *Gata1<sup>low</sup>* mice contains great numbers of MK the nuclei of which are not  
416 stained by the GATA1 antibody (**Figure S10**). To generate insights on the possible mechanism(s)  
417 that rescues MK maturation in mice treated with RB40.34 and Rux, alone or in combination, we  
418 performed confocal microscopy analyses with the same antibodies of BM section from mice treated  
419 for 54 days with either vehicle, RB40.34 alone, Rux alone or the two drugs in combination (**Figure**  
420 **10**). These analyses confirm the indications provided by flow cytometry (**Figure 9**) that none of the  
421 treatments affected the number of MK (as CD42bpos cells) present in the BM, which remains high.  
422 As expected, very few of the MK from mice treated with vehicle contain GATA1. By contrast, a  
423 significant number of MK in the BM from mice treated with either RB40.34 or Rux alone contain  
424 detectable levels of GATA1 in their nuclei (**Figures 10** and **S11**). It is surprising instead that GATA1  
425 is not detected in MK in the BM from mice treated with RB40.34 and Rux in combination.

426 **3.9 Treatment with RB40.34 in combination with Rux reduces the TGF- $\beta$  and CXCL1 content**  
427 **of the BM of *Gata1<sup>low</sup>* mice.** Increased bioavailability of the pro-inflammatory cytokines TGF- $\beta$  and  
428 CXCL1, the murine equivalent of human IL-8 has been suggested to represent the driver for fibrosis  
429 and hematopoietic failure in BM of MF patients and mouse models(4,50,51). In previous studies we  
430 demonstrated that the cells responsible for increasing the bioavailability of these two cytokines in the  
431 BM (and spleen) from *Gata1<sup>low</sup>* mice are the abnormal MK(35). To test whether treatment with  
432 RB40.34 in combination with Rux decreases the proinflammatory milieu of the BM



433 microenvironment of *Gata1<sup>low</sup>* mice, we performed histochemical evaluations with antibodies against  
434 TGF- $\beta$  and CXCL1 of BM sections from *Gata1<sup>low</sup>* mice treated for 54 days with either vehicle,  
435 RB4034 alone, Rux alone or the two drugs in combinations (**Figure 11**). As expected, the BM from  
436 *Gata1<sup>low</sup>* mice treated with vehicle contains great levels of TGF- $\beta$  and CXCL1. The levels of TGF- $\beta$   
437 are significantly decreased by treatment with both Rux alone or in combination with RB40.34  
438 (**Figure 11A, B**). Morphological analyses of the cells which expressed TGF- $\beta$  indicates that the  
439 reductions are mainly due to reduced numbers of MK expressing this factor (**Figure 11C**). CXCL1  
440 instead is reduced only by RB40.34 in combination with Rux and the numbers of MK which express  
441 this factor remain high in all the groups (**Figure 11A-C**). Since in addition to MK, CXCL1 is  
442 expressed by many other cell types, we suggest that the two-drugs in combination reduce the pro-  
443 inflammatory milieu of *Gata1<sup>low</sup>* mice by targeting not only the MK but also additional cells in the  
444 microenvironment.

445

#### 446 4 Discussion

447 The hypomorphic *Gata1<sup>low</sup>* mutation deletes only one of the three major hypersensitive sites which  
448 regulate the expression of the gene (52). After birth, the hematopoietic cells of these mice activate the  
449 expression of the gene from the two regulatory sites not affected by the mutation so that the levels of  
450 *Gata1* mRNA in the hematopoietic cells are overall normal (39). However, the thrombocytopenia  
451 induced by the mutation activates the TPO/Mpl axis(32) which results in a RSP14 ribosomopathy,  
452 similar to that observed in MF patients(10), which reduces the efficiency of the translation of *Gata1*  
453 mRNA reducing the content of the protein. The mechanism(s) linking the TPO/Mpl axis to the  
454 RSP14 ribosomopathy in MF, and in animal models including *Gata1<sup>low</sup>* mice, is still unknown. It has  
455 been suggested that it is represented by aurora kinases since their inhibition increase GATA1 in the  
456 MK while reducing fibrosis in animal models and in MF patients(53).

457 *Gata1<sup>low</sup>* mice, although they do not carry any of the MF driver mutations, are considered a bona fide  
458 animal model of MF because their HSC express an activated TPO/MPL axis, which may be drugged  
459 by JAK inhibitors, and a RSP14 ribosomopathy, which is responsible for low GATA1 content and  
460 altered MK maturation and P-SEL expression. In turn, altered P-SEL expression on the MK is  
461 responsible for the pathological cell interaction that increase the bioavailability of pro-inflammatory  
462 cytokines and drives fibrosis. Over the years we, and others(54–56), have extensively used *Gata1<sup>low</sup>*  
463 mice as a tool to identify lesions which may be targeted to normalize their myelofibrosis phenotype.

464 Based on previous observations indicating that deletion of *P-sel* prevents *Gata1<sup>low</sup>* mice for  
465 developing myelofibrosis (27), we establish here whether inhibition of P-SEL, alone or in  
466 combination with Rux, may also normalize the phenotype of *Gata1<sup>low</sup>* mice that have already  
467 established myelofibrosis. A summary of the results is presented in **Table S2**.

468 We first demonstrated that after short term-treatment, RB40.34 is bound to the platelets and the MK  
469 from the BM of *Gata1<sup>low</sup>* mice, suggesting that the drug is retained in the circulation for at least 5h  
470 and that, in spite of fibrosis, reaches the BM of the animals. We also found that after 5 days, RB40.34  
471 in combination with Rux normalizes non only the abnormal non-canonical TGF- $\beta$  signals, which is a  
472 signature of a pro-fibrotic microenvironment, but also the abnormal canonical TGF- $\beta$  signature,  
473 which indicates reduced hematopoiesis in the BM. These data suggests that after only 5 days the  
474 combination of RB40.34 and Rux is more effective than any of the two drugs alone in suppressing  
475 the cells responsible for fibrosis while reactivating hematopoiesis in the BM. The drug combination  
476 was also more effective than the two drugs as single agent in reducing JAK2 in the spleen from the  
477 mutant mice, suggesting that it is reducing extramedullary hematopoiesis in this organ.

478 Encouraged by these results, we analyzed the effects of long-term treatments with RB40.34 and Rux,  
479 alone or in combination, on the myelofibrotic phenotype expressed by *Gata1<sup>low</sup>* mice using a vast  
480 range of clinically relevant end points. The results indicate that none of the treatments induce anemia  
481 nor rescue the thrombocytopenia of *Gata1<sup>low</sup>* mice. However, treatment for 54 days with RB40.34 in  
482 combination with Rux, and to a less extent the two drugs alone, reduced anisocytosis, expression of  
483 P-SEL on MK, and probably on endothelial cells, and lymphocyte counts. Since the iron metabolism  
484 of old *Gata1<sup>low</sup>* mice is normal (Stefano Rivella, unpublished observation) and the mean corpuscular  
485 volume and Hb content of the RBC remain within normal values in all the treated mice (**Table S3**), it  
486 is unlikely that the high RDW detected in *Gata1<sup>low</sup>* mice is a sign of impaired iron metabolism.  
487 Anisocytosis without raises in mean corpuscular volume is induced by pro-inflammatory cytokines in  
488 several benign and malignant disorders, including MF where it has been proposed as a marker that  
489 predict inferior survival (57). We hypothesize that the reduction in anisocytosis observed at day 54 in  
490 the RB40.34 alone or in combination with Rux groups reflects reductions in the proinflammatory  
491 cytokines TGF- $\beta$  and/or CXCL1 that drives myelofibrosis in this model. Since TGF- $\beta$  and CXCL1  
492 are well known to affect directly (TGF- $\beta$ ) or indirectly (through neutrophil activation, CXCL1)  
493 lymphocyte counts (58–61), this hypothesis is also consistent with the reduced lymphocyte counts  
494 observed by day 54 in the mice treated with the two drugs in combination. Reduction in

495 microenvironment bioavailability of pro-inflammatory cytokines was directly tested by showing that  
496 RB40.34 and Rux in combination significantly reduces the TGF- $\beta$  and CXCL1 content of the BM.

497 Finally, treatment for 54 days with RB40.34 in combination with Rux also reduced fibrosis in BM  
498 and spleen while improving effective hematopoiesis in BM and reducing extramedullary  
499 hematopoiesis restoring the architecture of the spleen.

500 Treatments for 54 days with RB40.34 and Rux, alone or in combination, were ineffective in reducing  
501 the proliferation of the MK which may be driven in our model, as well as in the patients, by the  
502 activated TPO/Mpl axis. They were, however, all effective in improving the maturation profile of the  
503 MK, including reducing the abnormally high level of cell surface expression of P-SEL and TGF- $\beta$   
504 content. RB40.34 and Rux alone were also effective in increasing the GATA1 content in a proportion  
505 of CD42bpos MK. The mechanistic interpretation of these data is complicated by the recent single  
506 cell profiling indicating that murine (and human) BM contains four distinctive MK subpopulations,  
507 each one exerting a different function (47,62–64). The BM of adult mice and men contains at least  
508 three subpopulations: the platelet producing MK, the niche supportive MK, and the immune MK.  
509 Only platelet producing MK have the morphology of mature MK. Niche supportive MK and immune  
510 MK have instead the morphology of immature MK. In addition, by characterizing the MK  
511 subpopulations present in the embryos, Wang et al(63) identified a fourth subpopulation, which they  
512 defined niche-poised-MK that has an immature morphology and is characterized by high expression  
513 of extracellular matrix genes such as *COL1A1*, *COL3A1*, and *COL6A2* and enrichment of the  
514 “response to TGF-  $\beta$  signature”, which indicate that this subpopulation is sustained by TGF- $\beta$ . Of  
515 interest for this paper, reduced GATA1 content blocks the maturation of platelet producing MK but  
516 not favors the maturation of the other three subpopulations(47,62–64). This new knowledge indicates  
517 that the nature of the immature GATA1<sup>low</sup> MK found in great numbers in MF is presently not known.  
518 Preliminary observations indicating that great numbers of the morphologically immature MKs in the  
519 BM from *Gata1*<sup>low</sup> mice, as well as that of MF patients, express collagen(65,66) suggests that at least  
520 some of these MK are represented by niche poised MK the maturation of which is reactivated by  
521 TGF- $\beta$ (19). It is, therefore, possible, that low levels of GATA1 drive the disease not only by  
522 retaining platelet forming MK immature (leading to the thrombocytopenia), but also by increasing  
523 the frequency of other megakaryocyte subtypes. According to this hypothesis, although RB40.34 and  
524 Rux in combination did not induce detectable increase in GATA1 content in MK (and did not  
525 increased platelet counts), it reduced the frequency of MK containing TGF- $\beta$ , which may correspond  
526 to niche supporting MK. Reduced TGF- $\beta$  bioavailability may have then limit the number of niche

527 MK, which are sustained by this growth factors and are responsible for. On the other hand, Rux  
528 alone, which induced the greater increase of GATA1 in MK, also reduced the TGF- $\beta$  containing MK  
529 but did not altered fibrosis, suggesting that the number of MK expressing collagen was not  
530 significantly affected. These data support the need of further studies, clearly outside the purpose of  
531 the current manuscript, to clarify the MK subpopulations which are altered in MF, which of them is  
532 responsible for the different traits of the myelofibrosis phenotype and how they are affected by  
533 RB40.34 and Rux alone or in combination.

534 In conclusion, these data provide pre-clinical evidence that treatment with the RB40.34 antibody in  
535 combination with Rux is more effective than the use of Rux alone for reverting the myelofibrotic trait  
536 in the *Gatal<sup>low</sup>* mouse model and encourage clinical studies to validate the effects of Crizanlizumab,  
537 in combination with Rux, for the treatment of human PMF.

## 538 **5 Conflict of Interest**

539 PV, FG, FM, MF, AV, GS and MZ declare no conflict. CW, AB and AP are employee of Novartis  
540 Pharmaceutical Corporation. ARM received research funds from Novartis Pharmaceutical  
541 Corporation.

## 542 **6 Author Contributions**

543 PV, FG, FM, MF, FA and AV performed experiments and analyzed the data. PV and FG performed  
544 statistical analyses. GS reviewed all the histopathological determinations. ARM and MZ designed the  
545 study, interpreted the data and wrote the manuscript. CW, AB and AP revised the data and wrote the  
546 manuscript. All the authors read the manuscript and concur with its content.

## 547 **7 Data Availability Statement**

548 The individual data for each mouse are available on request.

## 549 **8 Disclosure**

550 The content of the manuscript was presented as a poster at the 62rd ASH Annual Meeting &  
551 Exposition (Paola Verachi, Fabrizio Martelli, Maria Zingariello, Shalini Chaturvedi, Celine Wilke,  
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565

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749

750 **LEGEND to FIGURES**

751 **Figure 1. At day 5, RB40.34 is readily detected on the platelets present in the blood and on**  
752 **sections of bone marrow from *Gata1*<sup>low</sup> mice.** (A) Flow cytometry analyses with APC-Cy7-  
753 streptavidin of platelets present in the blood from *Gata1*<sup>low</sup> mice after 5 days of treatment. The mice  
754 had been treated 5hr earlier with vehicle, biotinylated-RB40.34, Rux or the two drugs in  
755 combination, as indicated. Platelets are recognized based on size (FS: forward scatter) and internal  
756 cell complexity (SS: side scatter). Representative FS/SS gating and histograms of the APC-Cy7-  
757 streptavidin staining are presented on the left. Mean Fluorescence Intensity (MFI) ( $\pm$ SD) of APC-  
758 Cy7-streptavidin staining and values in individual mice (each symbol a mouse) are presented on the  
759 right. p values are calculated with Tukey multiple comparison test and significant differences are  
760 indicated in the panels. (B) Representative sections of BM from *Gata1*<sup>low</sup> mice treated with either  
761 vehicle (first panel) or biotinylated-RB40.34, Rux and Rux+ biotinylated-RB40.34 incubated with  
762 APC-Cy7-streptavidin (top panels). The panel on the bottom shows the computer-generated signal  
763 specific for mAb RB40.34 obtained by subtracting the background from vehicle with the ImageJ  
764 program. Areas exciding the threshold are artificially labeled in red. Detail of the ImageJ processing  
765 of the images are provided in **Figure S2**. Magnification 40x. The intensity of APC-Cy7 staining as  
766 percent of areas above the threshold in sections from the BM of multiple mice are presented on the  
767 right.

768 **Figure 2. Treatment with RB40.34 in combination with Rux restores the abnormal canonical**  
 769 **and non-canonical TGF- $\beta$  signaling observed in the bone marrow from *Gata1*<sup>low</sup> mice. (A-C)**  
 770 Western blot analyses for the content of elements downstream to the canonical and non-canonical  
 771 TGF- $\beta$  signaling of the BM of untreated wild-type (WT) and *Gata1*<sup>low</sup> mice and from *Gata1*<sup>low</sup>  
 772 treated with vehicle, RB40.34, Rux or the two drugs in combination, as indicated. Blots are presented  
 773 on the right and quantifications on the left. Quantitative values are presented as Means ( $\pm$  SD) and as  
 774 individual values for each mouse. Total protein levels are normalized toward the corresponding  
 775 GPDH levels while, to take into account differences in total protein, the levels of the phosphoproteins  
 776 are expressed stoichiometrically as a ratio with the total level of the corresponding protein. p values  
 777 were calculated with Tukey multiple comparison test and statistically significant differences are  
 778 indicated in the panels.

779 **Figure 3. Treatment with RB40.34 in combination with Rux restores the abnormal**  
 780 **JAK2/STAT5 signaling observed in the spleen from *Gata1*<sup>low</sup> mice.** Western blot analyses for  
 781 JAK2, STAT5 and GAPDH (as loading control) of BM (A,C) and spleen (B,D) from untreated wild-  
 782 type (WT) and *Gata1*<sup>low</sup> mice and from *Gata1*<sup>low</sup> mice treated with vehicle, RB40.34 and Rux, alone  
 783 or in combination. Blots are presented on the right and quantifications on the left. In C and D,  
 784 quantitative values are presented as Means ( $\pm$  SD) and as individual values for each mouse. p values  
 785 are calculated with Tukey multiple comparison test and statistically significant differences are  
 786 indicated in the panels.

787 **Figure 4. Treatment for 54 days with RB40.34 in combination with Rux reduces the frequency**  
 788 **of red blood cell anisocytosis and lymphocyte counts in the blood from *Gata1*<sup>low</sup> mice. (A)** Red  
 789 blood cell distribution width (RDW, in %) detected in the blood from *Gata1*<sup>low</sup> mice treated with  
 790 vehicle, RB40.34, Rux or the two drugs in combination for 54 days, as indicated. Data are presented  
 791 as Mean ( $\pm$ SD) and as individual values from each mouse. The asterisks indicate the groups  
 792 containing the deidentified samples flagged for anisocytosis by the accredited laboratory. (B)  
 793 Lymphocyte counts observed in the blood from *Gata1*<sup>low</sup> mice treated with vehicle, RB40.34, Rux or  
 794 the two drugs in combination for 54 days, as indicated. Data are presented as Mean ( $\pm$  SD) and as  
 795 individual values from each mouse. p values are calculated with Tukey multiple comparison test and  
 796 statistically significant differences ( $p < 0.05$ ) are indicated in the panels.

797• **Figure 5. Treatment for 54 days with RB40.34 in combination with Rux increases the**  
 798 **cellularity, reduces fibrosis and restores hematopoiesis in the bone marrow from *Gata1*<sup>low</sup> mice.**  
 799 A) Photographs of the femur and tibia from representative mice treated for 54 days with either

800 Vehicle or RB40.34 in combination with Rux, as indicated. B) Number of cells per femur observed at  
 801 day 54 in *Gata1<sup>low</sup>* mice treated with either vehicle, RB40.34, Rux or the two drugs in combination.  
 802 C) Hematoxylin/Eosin (H&E) and Reticulin staining of femurs from representative *Gata1<sup>low</sup>* mice  
 803 treated for 54 days with either vehicle, RB40.34, Rux or the two drugs in combination, as indicated.  
 804 The femurs are presented as stack images (at 4x) and as representative sections at 4 and 20x  
 805 magnification, as indicated. D) Levels of fibrosis quantified by image analyses of the reticulin  
 806 staining of BM sections from *Gata1<sup>low</sup>* mice treated for 54 days as indicated above. E) Frequency and  
 807 total number of Lin-, LSK and SLAM cells in the femur of *Gata1<sup>low</sup>* mice treated with the various  
 808 drug combinations. In B, D and E, results are presented as Mean ( $\pm$ SD) and as values per individual  
 809 mice (each symbol a mouse) and were analyzed by Tukey's multiple comparisons test. Statistically  
 810 significant groups are indicated within the panels.

811 • **Figure 6. Treatment for 54 days with RB40.34 and Rux, alone or in combination, reduces the**  
 812 **vessel density in the bone and spleen from *Gata1<sup>low</sup>* mice.** Confocal microscopy with CD34 and  
 813 Hoechst (to counterstain the nuclei) of bone marrow (A) and spleen (B) sections from *Gata1<sup>low</sup>* mice  
 814 treated for 54 days with either vehicle or with RB40.34 and Rux alone and in combinations. The  
 815 panels in the first and third lanes are at 20x magnification, and the area depicted in the rectangles are  
 816 shown at 60x in the corresponding panels in the second and third lanes. At 60x magnification,  
 817 microvessels (dashed lines) are identified as structures surrounded by CD34pos cells (indicated by  
 818 arrows) and containing red cells (autofluorescent cells not counterstained by Hoechst, asterisks).  
 819 Quantitative results are shown on the right as Mean ( $\pm$ SD) and as values per individual mice (each  
 820 symbol a mouse). Statistical analysis was performed by Tukey's multiple comparisons test and  
 821 significant p-values are indicated within the panels.

822 **Figure 7. Treatment for 54 days with RB40.34 in combination with Rux decreases**  
 823 **hematopoiesis of the spleen from *Gata1<sup>low</sup>* mice.** A) Photographs of representative spleens treated  
 824 for 54 days with the various drug combinations, as indicated. B,C) Spleen size, as weight and ratio  
 825 between spleen weight and body weight (B), and total cell numbers (C), of mice treated for 54 days  
 826 with the various drug combinations. D) Frequency and total number of Lin- and LSK cells in the  
 827 spleen of *Gata1<sup>low</sup>* mice treated with the various drug combinations. SLAM cells are not presented  
 828 because they are almost 100% of the LSK cells detected in the spleen (see **Figure S9**). In B, C and D,  
 829 results are presented as Mean ( $\pm$ SD) and as values per individual mice (each symbol a mouse) and

830 are analyzed by Tukey's multiple comparisons test. Statistically significant groups are indicated  
831 within the panels.

832 **Figure 8. Treatment for 54 days with RB40.34 in combination with Rux decreases fibrosis and**  
833 **restores the architecture of the spleen from *Gata1<sup>low</sup>* mice.** A) Hematoxylin/Eosin (H&E) and  
834 reticulin staining of spleen from representative 8-11-months old WT and *Gata1<sup>low</sup>* mice. WT spleens  
835 are characterized by the presence of large aggregates of lymphoid cells, well developed white pulp,  
836 with the presence of red blood cells embedded in reticular connective tissue containing few  
837 megakaryocytes and supporting trabeculae. By contrast, *Gata1<sup>low</sup>* spleen is characterized by  
838 hypoplastic white pulp and red pulp rich of megakaryocytes. Reticulin staining of the consecutive  
839 section indicates that fibrosis is localized mostly in the red pulp. Results are representative of those  
840 observed in at least three WT and three *Gata1<sup>low</sup>* littermates, all 11-months old. B) Triple  
841 immunofluorescent analyses for CD3 (as a marker of T cells, red), CD45R (B220, as a marker for B  
842 cells, green) and DAPI (nuclei) of spleen sections from representative WT and *Gata1<sup>low</sup>* mice, as  
843 indicated. As expected (50), the white pulp of WT spleen contains numerous T and B lymphocytes,  
844 organized around central arterioles. T lymphocytes form a sleeve around the central arteriole, the  
845 periarteriolar lymphoid sheath while B cells are mainly localized in the outer white pulp region, the  
846 marginal zone. In *Gata1<sup>low</sup>* spleens the white pulp is smaller than that in the WT organ and the  
847 periarteriolar lymphoid sheath contain a markedly reduced number of T cells. Magnification 4x, 10x  
848 and 20x, as indicated. C) Hematoxylin/Eosin (H&E), reticulin staining and triple staining with  
849 CD45R (green), CD3 (red) and DAPI (blue) of sections from the spleen of representative *Gata1<sup>low</sup>*  
850 mice treated for 54 days with the various drug combinations, as indicated. Images are presented at 4x  
851 and 20x magnification. D) Levels of fibrosis quantified by image analyses of the reticulin staining of  
852 spleen sections from untreated 8-11-months old WT and *Gata1<sup>low</sup>* littermates, as indicated. E) Levels  
853 of fibrosis, quantified by image analyses of the reticulin staining, in spleen sections, from *Gata1<sup>low</sup>*  
854 mice treated for 54 days, as indicated. In D and E) results are presented as Mean ( $\pm$ SD) and as values  
855 per individual mice (each symbol a mouse) and were analyzed by t test. Statistically significant  
856 groups are indicated within the panels.

857 **Figure 9. Treatment for 54 days with RB40.34 and Rux alone in combination improves the**  
858 **maturation profile of the MK from the bone marrow and spleen of *Gata1<sup>low</sup>* mice.** A)  
859 Representative dot-plots and histograms of MK from the bone marrow (left quadrant) and spleen  
860 (right quadrant) of one representative mouse from each experimental group treated for 5 days. MKs  
861 were labeled with CD41, CD61 and PE-Cy7-streptavidin. The a, b, c and d gates identify non-MK,

862 immature MK, mature MK and very mature MK, respectively. The levels of APC-Cy7-streptavidin  
 863 bound to the MKs at their different stage of maturation is presented by histograms. Since P-sel is  
 864 abnormally expressed at high levels on the surface of *Gata1<sup>low</sup>* MK, the APC-Cy7-streptavidin  
 865 signal identifies the MK expressing P-sel which have bound the biotinylated RB40.34 injected 5 hr  
 866 earlier in the mice. B,C) Frequency of cells in the non-MK (a) and immature (b), mature (c) and very  
 867 mature (d) MK gate (% in percent of total cell number) and percentage and MFI of the events  
 868 positive for PE-Cy7-streptavidin staining in each gate in the BM (A) and spleen (B) from *Gata1<sup>low</sup>*  
 869 mice treated for 5, 12 and 54 days with either vehicle or the different drug combinations, as  
 870 indicated. Values were reported as Means ( $\pm$ SD) of those detected in at least three mice per  
 871 experimental group. Data are analyzed by Tukey's multiple comparisons and statistically significant  
 872 differences among groups are indicated within the panels.

873 **Figure 10. Treatment for 54 days with RB40.34 or Rux alone, but not in combination, increases**  
 874 **the GATA1 content in the MK from the BM of *Gata1<sup>low</sup>* mice.** A) Merged GATA1 (FITCH-green)  
 875 and CD42b (TRITCH-red, as a marker of MKs) images of the confocal microscopy analyses with the  
 876 corresponding antibodies in BM sections from representative *Gata1<sup>low</sup>* mice treated for 54 days with  
 877 either vehicle, RB40.34 alone, Rux alone, or the two drugs in combination, as indicated. The  
 878 corresponding images acquired in the single channels, in the channel for DAPI (as indication of the  
 879 nuclear localization of GATA1) and in the bright field (to exclude autofluorescence) are presented in  
 880 **Figure S11.** Magnification 40x. B) Frequency of MK (CD42b positive cells) and percentage of MK  
 881 positive for GATA1 in BM sections from *Gata1<sup>low</sup>* mice treated for 54 days as indicated. Data are  
 882 presented as Mean ( $\pm$ SD) and as values in individual mice (each symbol a mouse). Results were  
 883 analyzed by Tukey's multiple comparisons test and significant differences among groups indicated  
 884 within the panels.

885 **Figure 11. Treatment for 54 days with RB40.34 and Rux in combination decreases the TGF- $\beta$ ,**  
 886 **mainly in the MK, and CXCL1 content of bone marrow from *Gata1<sup>low</sup>* mice.** A) BM sections  
 887 from representative mice treated for 54 days with either vehicle, RB40.34, Rux or the two drugs in  
 888 combination immune-stained for TGF- $\beta$  or CXCL1, as indicated. Representative MK are indicated  
 889 by arrows. Magnification 40x. B) Quantification by computer assisted imaging of the TGF- $\beta$ 1 and  
 890 CXCL1 content in the BM from *Gata1<sup>low</sup>* mice treated for 54 days as indicated. C) Frequency of MK  
 891 and percentage of MK expressing high levels of TGF- $\beta$ 1 and CXCL1 in BM sections from *Gata1<sup>low</sup>*  
 892 mice treated as indicated. MK were identified on the basis of size (10 times greater than that of any

893 other cell type in the section) and the polylobate morphology of their nuclei. In B and C, data are  
 894 presented as Mean ( $\pm$ SD) and as values per individual mice (each symbol a different mouse) and are  
 895 analyzed by Tukey's multiple comparisons test. Values statistically different are indicated within the  
 896 panels.

### 897 10.1 Tables

898 **Table 1.** Hematocrit (Hct, %), platelets (plt) and white blood cells (WBC) counts determinations  
 899 at day 5, 12 and 54 in *Gata1*<sup>low</sup> mice treated with vehicle, RB40.34, Rux or with the combination of drugs.  
 900 Historical values from *Gata1*<sup>low</sup> untreated mice and WT littermates are reported. <sup>(n)</sup>: number of mice.  
 901 Values among the treatment groups are not statistically different by Tukey multiple comparison test.  
 902

Hct (%)	Controls	Day 5	Day 12	Day 54
WT	39.65 $\pm$ 0.49 <sup>(2)</sup>			
<i>Gata1</i> <sup>low</sup> untreated	21.10 $\pm$ 6.64 <sup>(16)</sup>			
Vehicle		33.03 $\pm$ 4.23 <sup>(6)</sup>	44.30 $\pm$ 0.10 <sup>(3)</sup>	33.52 $\pm$ 3.08 <sup>(5)</sup>
RB40.34		32.15 $\pm$ 5.51 <sup>(6)</sup>	43.25 $\pm$ 0.21 <sup>(2)</sup>	33.47 $\pm$ 0.23 <sup>(3)</sup>
Rux		38.12 $\pm$ 1.46 <sup>(6)</sup>	44.43 $\pm$ 0.81 <sup>(3)</sup>	29.46 $\pm$ 5.00 <sup>(5)</sup>
Rux + RB40.34		38.00 $\pm$ 1.58 <sup>(6)</sup>	44.07 $\pm$ 0.58 <sup>(2)</sup>	29.19 $\pm$ 1.23 <sup>(3)</sup>

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Plt (10 <sup>3</sup> / $\mu$ L)	Controls	Day 5	Day 12	Day 54
WT	775 $\pm$ 466.69 <sup>(2)</sup>			
<i>Gata1</i> <sup>low</sup> untreated	115.06 $\pm$ 42.10 <sup>(16)</sup>			
Vehicle		138.67 $\pm$ 40.23 <sup>(6)</sup>	100.67 $\pm$ 17.67 <sup>(3)</sup>	92.40 $\pm$ 26.34 <sup>(5)</sup>
RB40.34		111 $\pm$ 46.23 <sup>(6)</sup>	79 $\pm$ 19.80 <sup>(2)</sup>	65.00 $\pm$ 28.48 <sup>(3)</sup>
Rux		137.83 $\pm$ 33.81 <sup>(6)</sup>	105.33 $\pm$ 26.41 <sup>(3)</sup>	94.60 $\pm$ 59.32 <sup>(5)</sup>
Rux + RB40.34		142.60 $\pm$ 26.45 <sup>(6)</sup>	123 $\pm$ 31.43 <sup>(2)</sup>	126.33 $\pm$ 34.59 <sup>(3)</sup>

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Plt (10 <sup>3</sup> / $\mu$ L)	Controls	Day 5	Day 12	Day 54
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<b>WT</b>	$775 \pm 466.69^{(2)}$		
<b>Gata1<sup>low</sup> untreated</b>	$115.06 \pm 42.10^{(16)}$		
<b>Vehicle</b>	$138.67 \pm 40.23^{(6)}$	$100.67 \pm 17.67^{(3)}$	$92.40 \pm 26.34^{(5)}$
<b>RB40.34</b>	$111 \pm 46.23^{(6)}$	$79 \pm 19.80^{(2)}$	$65.00 \pm 28.48^{(3)}$
<b>Rux</b>	$137.83 \pm 33.81^{(6)}$	$105.33 \pm 26.41^{(3)}$	$94.60 \pm 59.32^{(5)}$
<b>Rux + RB40.34</b>	$142.60 \pm 26.45^{(6)}$	$123 \pm 31.43^{(2)}$	$126.33 \pm 34.59^{(3)}$

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