Splenectomy ameliorates portal pressure and anemia in animal models of cirrhotic and non-cirrhotic portal hypertension

Philipp Schwabl[1,2,3], Berit Anna Seeland[1,2], Florian Riedl[1,2], Tim Lukas Schubert[1,2], Philipp Königshofer[1,2,3], Ksenia Brusilovskaya[1,2,3], Oleksandr Petrenko[1,2,3,4,5], Benedikt Hofer[1,2,3], Ana-Iris Schiefer[6], Michael Trauner[1], Markus Peck-Radosavljevic[1,2,7], Thomas Reiberger[1,2,3,4,5]

[1] Division of Gastroenterology & Hepatology, Department of Internal Medicine III, Medical University of Vienna, Vienna, 1090, Austria

[2] Vienna Hepatic Hemodynamic Experimental (HEPEX) Laboratory, Medical University of Vienna, Vienna, 1090, Austria

[3] Christian-Doppler Laboratory for Portal Hypertension and Liver Fibrosis, Medical University of Vienna, Vienna, 1090, Austria

[4] Ludwig Boltzmann Institute for Rare and Undiagnosed Diseases, Ludwig Boltzmann Institute, Vienna 1090, Austria

[5] CeMM Research Center for Molecular Medicine, Austrian Academy of Sciences, Vienna 1090, Austria

[6] Division of Pathology, Department of Clinical Pathology, Medical University of Vienna, Vienna, 1090, Austria

[7] Innere Medizin und Gastroenterologie (IMuG), Klinikum Klagenfurt am Wörthersee, Klagenfurt, 9020, Austria

Corresponding author:

Prof. Dr. Thomas Reiberger,

Division of Gastroenterology & Hepatology

Department of Internal Medicine III

Medical University of Vienna

Währinger Gürtel 18-20

1090 Vienna

Austria

eMail: thomas.reiberger@meduniwien.ac.at

Fax: +43 1 40400-47350

Telephone: +43 1 40400-65890

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Author contributions

Study design: Philipp Schwabl, Berit A Seeland, Markus Peck-Radosavljevic and Thomas Reiberger. Data collection: Philipp Schwabl, Berit A Seeland, Florian Riedl, Tim L Schubert, Philipp Königshofer, Ksenia Brusilovskaya, Oleksandr Petrenko, Benedikt Hofer, Ana I Schiefer and Thomas Reiberger. Statistical analysis and interpretation of data: Philipp Schwabl and Thomas Reiberger. Manuscript preparation: Philipp Schwabl and Thomas Reiberger. Literature search: Philipp Schwabl and Thomas Reiberger. Funds collection: Philipp Schwabl and Thomas Reiberger. Study supervision: Michael Trauner, Markus Peck-Radosavljevic and Thomas Reiberger. Critical revision for important intellectual content of the manuscript and final approval of the article: all authors.

ABSTRACT

Purpose: Portal hypertension (PH)-associated splenomegaly is caused by portal venous congestion and splanchnic hyperemia. This can trigger hypersplenism, which favors the development of cytopenia. We investigated the time-dependent impact of splenectomy on portal pressure and blood cell counts in animal models of non-cirrhotic and cirrhotic PH.

Materials and methods: Ninety-six rats underwent either partial portal vein ligation (PPVL), bile duct ligation (BDL), or sham operation (SO), with subgroups undergoing additional splenectomy. Portal pressure, mean arterial pressure, heart rate, blood cell counts and hemoglobin concentrations were evaluated throughout 5 weeks following surgery.

Results: Following PPVL or BDL surgery, the animals presented a progressive rise in portal pressure, paralleled by decreased mean arterial pressure and accelerated heart rate. Splenectomy curbed the development of PH in both models (PPVL: 16.25 vs. 17.93 mmHg, p=0.083; BDL: 13.55 vs. 15.23 mmHg, p=0.028), increased mean arterial pressure (PPVL: +7%; BDL: +9%), and reduced heart rate (PPVL: -10%; BDL: -13%). Accordingly, splenectomized rats had lower von Willebrand factor plasma levels (PPVL: -22%; BDL: -25%). Splenectomy resulted in higher hemoglobin levels in PPVL (14.15 vs. 13.08 g/dL, p<0.001) and BDL (13.20 vs. 12.39 g/dL, p=0.097) animals, and significantly increased mean corpuscular hemoglobin concentrations (PPVL: +9%; BDL: +15%). Thrombocytopenia only developed in the PPVL model and was alleviated in the splenectomized subgroup. Conversely, BDL rats presented with thrombocytosis, which was not affected by splenectomy.

Conclusions: Splenectomy improves both cirrhotic and non-cirrhotic PH, and ameliorates the hyperdynamic circulation. Hypersplenism related anemia and thrombocytopenia were only significantly improved in the non-cirrhotic PH model.

Keywords: Splenectomy; Portal hypertension; Bile duct ligation; Partial portal vein ligation; Cytopenia

1 INTRODUCTION

Portal hypertension (PH) develops as a result of increased vascular resistance and mostly affects patients with liver cirrhosis [1]. Yet, PH may also occur in non-cirrhotic patients, as observed in case of obstructive portal vein thrombosis or in portosinusoidal vascular disease [2]. Typical clinical sequelae of PH are development of portosystemic collaterals [3] (e.g. esophageal varices) and hypersplenism [4, 5]. Consequentially, PH increases the risk of ascites, variceal bleeding, hepatic encephalopathy and cytopenia, which are all factors affecting clinical outcomes [1, 6]. In PH, splenic blood inflow may account for up to 30% of the portal blood volume [7, 8], thus significantly contributing to portal pressure. Moreover, splenomegaly contributes to the PH syndrome by modulating the mesenteric vascular tone and affecting renal function [9]. Furthermore, congestive splenomegaly leads to increased splenic blood cell sequestration (i.e. hypersplenism), resulting in cytopenia [4, 5]. Importantly, the spleen may even facilitate the progression of liver fibrosis, as it is a source for transforming growth factor β, it influences the hepatic immune cell composition and can hamper liver regeneration [5]. Hence, (partial) splenic artery embolization [8] or splenectomy [10, 11], have been used in patients with pronounced splenomegaly to counteract its aggravating impact on PH. Indeed, both measures are able to reduce, at least temporarily, the portal blood flow, portal pressure and ameliorate cytopenia. However, the pathophysiologic mechanisms leading to splenomegaly and the associated hemodynamic and hematologic changes remain poorly characterized.

While there exist several animal models of PH [12], the impact of splenectomy on the time course of portal pressure, systemic hemodynamics and blood cell counts in such models has not been studied yet. We therefore compared the time course of these respective readouts in models of cirrhotic and non-cirrhotic (prehepatic) PH.

2 MATERIAL AND METHODS

2.1 Study design

Wee randomly assigned 96 rats to undergo either bile duct ligation (BDL), partial portal vein ligation (PPVL) or sham operation (SO). In each group, half of the animals also underwent splenectomy (SPL) during the same surgical procedure. The observation time lasted 1, 3, 5, 7, 14, 21, 28 and 35 days in PPVL and SO rats, and focused on day 14, 21, 28 and 35 in BDL rats, yielding a total of 40 subgroups comprising 2 or 3 animals each. The exact numbers of rats in the respective groups are outlined in Supplementary Table 1. The different observation times relate to the time course of PH development, which is instantaneous in the PPVL model and gradual in the BDL model. After the observation period, hepatic and systemic hemodynamics,

as well as a hematology panel were assessed in order to portray the natural time course of the respective disease models of (cirrhotic and non-cirrhotic) PH and to illustrate the impact of splenectomy (Figure 1). Additionally, splenic and bone marrow megakaryopoiesis, and von Willebrand factor (vWF), a biomarker for endothelial stress and PH, were assessed.

2.2 Animals

The study was conducted in 6-8 weeks old, male Sprague-Dawley rats (substrain "Him:OFA"), after 2 weeks of local acclimatization. The animal protocol was designed to minimize pain or discomfort. The animals were housed under controlled and standardized conditions in Makrolon type IV cages (3 animals per cage) with aspen wood bedding material. Rats were housed in a 12h/12h day/night cycle and had free access to standard laboratory rodent chow and water. The animal health status was regularly monitored by certified animal keepers, study personnel and veterinarians. Interventions were performed under anesthesia, and at the study endpoint all animals were euthanized by pentobarbital overdose for blood and tissue collection.

2.3 Ethical issues

The study was reviewed and approved by the local animal ethics committee of the Medical University of Vienna, Austria (Zl. 494/115-97/98 aus 2011/12) and the Austrian Federal Ministry of Science and Research (EK Nr. GZ 66.009/0331-II/3b/2011).

The experiments were performed according to the EU Directive 2010/63/EU for animal experiments and are reported according to ARRIVE guidelines.

2.4 Surgery

PPVL, BDL and SO, were performed under sterile conditions in an operating theatre. Initially, all animals received weight-adapted anesthesia intramuscularly, containing ketamine (100 mg/kg) and xylazine (12 mg/kg). After shaving and disinfection, a median laparotomy was performed and the upper abdominal area was surgically prepared. PPVL was performed as previously described [12]. Briefly, a single ligature of silk (3-0) was placed around the portal vein and a 21G blunt-tipped needle. Once the ligature was firm, the needle was removed, leaving a calibrated constriction of the portal vein and causing pre-hepatic PH. For BDL, the common bile duct was first isolated along its course and then ligated by one ligature at the proximal and one at the distal end, as previously described [12]. The section between the two ligatures was resected to ensure interruption of biliary drainage into the duodenum. The resulting mechanical cholestasis in BDL rats ultimately leads to development of cirrhotic PH.

In SO animals, the portal vein and bile duct were similarly isolated but the silk was only pulled through and no ligations were made. Splenectomy was performed in the same surgical session, right after the PPVL, BDL or SO procedure. As previously described [13], first, the main splenic vessels were ligated twice. Subsequently, ligaments were separated using electrocauterization to mobilize the spleen and excise it. After the surgery, animals recovered under a heating lamp and received piritramide analgesia (3 mg/kg), followed by 3 days of piritramide (0.1 mg/mL) in drinking water *ad libitum*.

2.5 Hemodynamic studies

For the follow-up assessment, animals were administered a ketamine (100mg/kg, i.p.) anesthesia. Adequate depth of anesthesia was controlled by toe or tail pinching. Body temperature of 37.0 ± 0.5 °C was monitored by a rectal temperature probe and maintained by using a heating pad. Mean arterial pressure, heart rate and portal pressure, were measured as previously described [12]. Briefly, the femoral artery was prepared and cannulated with a PE-50 catheter to assess blood pressure and heart rate. Portal pressure was measured by cannulating an ileocolic vein and advancing the catheter tip into the proximal portal vein. Hemodynamic parameters were registered on a multichannel recorder (ML870 PowerLab 8/30, ADInstruments Inc., Colorado Springs, CO, USA).

2.6 Blood analysis

Blood was sampled after completion of the invasive hemodynamic studies via cardiac puncture. A complete blood panel assessment was performed using a Cobas c311 analyzer (Roche Diagnostics International AG, Rotkreuz, Switzerland): white blood cell (WBC) count, hemoglobin, platelets, neutrophils, lymphocytes, monocytes, eosinophils, basophils, red blood cell count, hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC). In 5 rats of each group, vWF antigen levels were quantified in blood plasma using an enzyme-linked immunosorbent assay (ELISA Asserachrom VWF; Diagnostica Stago, Asnières, France) according to the manufacturers' instructions.

2.7 Hematopathological assessment of megakaryopoiesis

Megakaryocyte density was evaluated in periodic acid–Schiff (PAS) staining of femoral bone marrow by a blinded hematopathologist. The number of megakaryocytes was counted at 200X magnification in 3 different examination areas of 0.64 mm^2. Mean values per low power field

were calculated. Additionally, in non-SPL animals, extramedullary thrombocytopoiesis was assessed in spleen sections and reported as low (+ scattered), moderate (++ frequent) or high (+++ plenty).

2.8 Statistics

The primary outcome parameters were changes in portal pressure and platelet counts comparing splenectomized and non-splenectomized animals across the 3 models of PPVL, BDL and SO. All other parameters were secondary and exploratory. The averaged time point values are presented as mean +/- standard deviation. Group comparisons were performed using the Mann-Whitney U test of the averaged time point values and are reported using median and interquartile range. The normal range was defined as the respective standard deviation in the SO group. A two-sided p-value <0.05 denoted statistical significance. The data that support the findings of this study are available from the corresponding author upon reasonable request.

3 RESULTS

3.1 Portal hypertension development in the PPVL and BDL models

In contrast to SO rats, animals undergoing PPVL or BDL developed distinct changes in hepatic and systemic hemodynamics and also presented hematological alterations. In PPVL rats, portal pressure increased rapidly and peaked on day 3 (21.3±0.6 mmHg), and from then on it showed a steady decline until day 35. In BDL rats, portal pressure increased steadily and reached 17.9±1.2 mmHg on day 35 (Figure 2A). The mean arterial pressure curve developed inversely to the portal pressure readings. In PPVL animals, it reached its lowest point on day 7 (71.5±5.0 mmHg), and in the BDL cohort, the mean arterial pressure was steadily decreasing until reaching 71.8±6.6 mmHg on day 35 (Figure 2B). In contrast, heart rate followed blood pressure in an opposite manner to compensate for arterial hypotension. Accordingly, PPVL animals had their peak heart rate on day 5 (362±48 bpm), and in BDL rats, the heart rate increased to 358±16 bpm on day 35 (Figure 2C).

Levels of vWF, a biomarker for endothelial stress and PH, were statistically significantly higher in PPVL (920 [890-1173] ng/mL; p=0.008) or BDL (972 [847-1011] ng/mL; p=0.008) rats, compared to healthy controls (654 [626-810] ng/mL) (Figure 2D). In SO rats, hemoglobin, WBC and platelet counts remained stable throughout the observation period. In contrast, PPVL and BDL animals had statistically significantly lower hemoglobin, while the red blood cell count did not change (Figure 3A and B). PPVL animals presented low hemoglobin values from day 1 onwards and showed a slight increase over time, whereas in BDL animals, hemoglobin

declined continuously and reached 11.3±0.35 g/dL on day 35 (Figure 3A). In both models, the amount of hemoglobin per red blood cell was reduced, as seen by lower MCH and MCHC values, whereas MCV remained unchanged (Supplementary Figure 1). While in PPVL rats WBC values stayed within normal limits, BDL animals developed a marked and progressive leukocytosis (Figure 3C). However, in both groups the WBC dynamics were symmetric among all leukocyte subtypes (Supplementary Table 2). The platelet counts were statistically significantly increased in the BDL rats. Yet, in the PPVL model the number of thrombocytes was slightly reduced (Figure 3D), which was paralleled by a diminished number of megakaryocytes in the bone marrow and in the spleen (Supplementary Figure 2).

3.2 Impact of splenectomy on hemodynamics in PPVL and BDL rats

In healthy controls, splenectomy changed neither hemodynamic parameters nor hemoglobin or platelet levels (Supplementary Figure 3). In contrast, splenectomy reduced portal pressure in both, PPVL-SPL and BDL-SPL animals by 9.36% (p=0.083) and 11.03% (p=0.028), respectively (Figure 4A). In line, splenectomized animals had a trend towards higher blood pressure throughout the timeline. In PPVL-SPL animals, the mean arterial pressure increase was 6.2% (p=0.053) and in BDL-SPL rats it was 21.0% (p=0.057) (Figure 4B). Accordingly, splenectomy also attenuated the compensatory tachycardia. In PPVL-SPL rats, the heart rate was 9.6% slower (p=0.054) and in BDL animals a heart rate reduction of 12.3% was observed (p=0.028) (Figure 4C). In PPVL animals, the beneficial effects of splenectomy on hemodynamics were not immediately visible on day 1, but started to manifest in the subsequent days. After day 7, in both models, the hemodynamic curve development showed a constant shift between splenectomized and non-splenectomized rats. The hemodynamic improvements in splenectomized rats were also mirrored by a decrease in vWF levels. Both, PPVL-SPL (711 [651-938] ng/mL vs. 920 [890-1173] ng/mL in PPVL; p=0.087) and BDL-SPL (728 [309-903] ng/mL vs. 972 [847-1011] ng/mL in BDL; p=0.008) rats presented lower vWF serum levels compared to non-splenectomized animals (Figure 4D).

3.3 Impact of splenectomy on blood cell counts in PPVL and BDL rats

In PPVL rats, splenectomy statistically significantly increased hemoglobin levels. The median hemoglobin in PPVL-SPL was 14.15 (13.83-15.35) g/dL, as compared to 13.08 (12.73-13.57) g/dL in non-splenectomized PPVL animals (p<0.001) (Figure 5A). While red blood cell count (Figure 5B) and MCV did not change, a statistically significant rise in MCH and MCHC was noted, indicating higher hemoglobin levels per erythrocyte (Supplementary Figure 4). Neither

total WBC (Figure 5C), nor leukocyte subtype composition (Supplementary Table 2) changed in the PPVL-SPL rats. However, splenectomized PPVL animals presented with 22% higher platelet counts, compared to PPVL controls (p=0.065) (Figure 5D). Yet, no changes in the bone marrow megakaryopoiesis were noted (Supplementary Figure 5).

In BDL animals, splenectomy also improved hemoglobin levels (13.20 [12.66-13.30] vs. 12.39 [11.55-12.65] g/dL; p=0.097), but to a lesser extent as in the PPVL group (Figure 5A) and without affecting red blood cell counts (Figure 5B). In regard to red cell indices, particularly MCHC was elevated in the BDL-SPL group (Supplementary Figure 4). The progressive leukocytosis observed in the BDL animals, was further fueled by splenectomy, especially towards the end of the observation period (i.e. on day 35) (Figure 5C). This reaction led to an even rise of differential WBC counts (Supplementary Table 2). In the BDL-SPL rats, no major change of platelet counts was noted, as compared to the BDL group (Figure 5D). Ultimately, there were also no differences in the bone marrow megakaryopoiesis between the BDL and BDL-SPL animals (Supplementary Figure 5).

3.4 Safety and animal wellbeing

The standardized surgeries were well tolerated. During the postsurgical convalescence period most animals presented a dip in the blood pressure. In line, a transient body weight loss was noted. However, body weight increased steadily in all groups and no group-related differences were apparent (Supplementary Figure 6).

In two subgroups (SO-d7 and SO-SPL-d1), we had a total loss of all animals, which occurred due to a heating plate defect on that particular surgery day. Moreover, in the PPVL-SPL group we lost 1 animal in the d3, d5 and d7 subgroups, each due to postsurgical complications. The total number of included animals in the final analysis is shown in the Supplementary Table 1.

4 DISCUSSION

4.1 Discussion of hemodynamic readouts

In this animal study, we examined the impact of splenectomy on the PH syndrome and blood cell counts in models of cirrhotic and non-cirrhotic PH. Splenectomy effectively decreased portal pressure in both models, which is in line with previous investigations in animals [14] or humans [10, 11]. The underlying principle is a reduction of arterial and consequently venous blood flow into the splanchnic area [15], due to ligation of the splenic vessels [7, 10, 11]. Accordingly, the decrease in portal pressure is independent of the type of PH (pre-, intra-, or

post-hepatic), as seen in our study, where in both models a portal pressure reduction of about 10% was achieved. However, splenic hemodynamics has been reported to change during progression of PH, from a hyperdynamic to a congestive state [16]. Hence timing of splenectomy appears to be important for hemodynamic outcomes. While most clinical reports investigate splenectomy in a therapeutic setting, where PH is fully developed, our study focused on a preventive design, with splenectomy being performed at the same time as PH induction. Accordingly, in the PPVL model, differences in portal pressure were not immediately evident, but emerged 3 to 5 days after splenectomy. Yet, in both models, the splenectomy-related hemodynamic improvements remained robust throughout the observational period. Thus, the contrast to experimental [13] and clinical studies [17, 18] is not surprising, in which the beneficial impact of splenectomy on portal pressure is promptly visible, yet the sustainability of this effect is uncertain as conflicting reports exist [19].

Notably, in both models, splenectomy additionally improved systemic hemodynamics, as demonstrated by a lower heart rate and higher mean arterial pressure. Based on the current understanding of PH pathophysiology, we assume that the reduction of splenic blood flow into the portal system and consequent preservation of the splenic blood flow equivalent into the systemic circulation, improved the PH-associated hyperdynamic circulation. Yet, in clinical studies on splenectomy or splenic artery embolization, data on blood pressure or heart rate are either not reported [10, 11, 17, 18] or indicate no significant changes [7]. We assume that again the different timing of the intervention accounts for these differences. Our findings indicate that during PH development the spleen contributes not only to the portal pressure increase, but also to the progression of a hyperdynamic circulation, which both appear to become more autonomous in advanced disease stages.

In our study, the hemodynamic measurements were complemented by assessment of vWF plasma levels, which mirrored the improvements caused by splenectomy in both models. We chose to investigate vWF as it is a promising biomarker for the diagnosis of clinically significant PH [20] and because the spleen appears to play no significant role in vWF dynamics [21, 22]. Our findings suggest that vWF-levels might still be a useful biomarker in cirrhotic patients, even after splenectomy, which however requires further clinical investigations. Yet, it was interesting to observe increases in vWF, not only in the cirrhotic BDL model, but also in the non-cirrhotic PPVL rats, which may have been caused by splanchnic/portal venous shear stress [23] and vascular strain [24]. Importantly, higher vWF levels were also found in patients with non-cirrhotic idiopathic portal hypertension (NCIPH), however, this has rather been attributed

to ADAMTS13 deficiency [25, 26]. Hence, it remains to be elucidated, whether vWF may also be used as a biomarker for disease severity in patients with non-cirrhotic portal hypertension.

4.2 Discussion of hematological readouts

In addition to influencing the splanchnic blood distribution, the spleen is an important hemato-immunological organ that responds distinctively to the development of PH. Typically, the spleen enlarges progressively, becomes stiffer and hyperactive [27, 28]. A clinical consequence of hypersplenism is cytopenia, caused by splenic blood cell pooling and sequestration [4, 5]. However, splenic size and cytopenia do not strictly correlate with PH. In our study, both animal models presented no changes in the red blood cell counts, but showed significantly reduced hemoglobin levels and decreased MCHC. Indeed, anemia is common in cirrhotic [6] and non-cirrhotic PH [29], yet the development is multifactorial. Contributors to anemia in PH are lack of iron and/or vitamin B12, (chronic) gastrointestinal bleedings, hemodilution, and splenic retention and degradation of erythrocytes [6, 30, 31]. Therefore, prophylactic splenectomy was expected to primarily improve the latter factors. However, the red blood cell count did not increase in splenectomized animals, indicating, that neither splenic erythrocyte degradation, nor splenic hemodilution appear to play a major role. Instead, we noted a significant increase in hemoglobin levels and MCHC. This is interestingly in line with clinical data, where splenectomy also had no significant effect on red blood cell counts, but increased hemoglobin concentrations [32-34]. We thus speculate, that in the studied setting, the spleen [35] and/or portal pressure changes [6, 31] might have an impact on iron and thus hemoglobin metabolism, but unfortunately our study lacks data on transferrin, ferritin, reticulocyte counts or erythropoietin levels to further dissect this topic.

In PH, thrombopenia is a characteristic finding that strongly correlates with portal pressure [1]. Hypersplenism and bone marrow suppression are regarded as contributing factors [4]. Yet, in our study, the BDL model rather developed thrombocytosis and presented no significant bone marrow alterations. Here we might have missed to observe a transient decrease in platelet counts, which has been reported in mice to last about 3 days following BDL [36]. Irrespective of that, splenectomy did not affect platelet counts or megakaryopoiesis in the BDL model. In contrast, the PPVL model presented with altered megakaryopoiesis, and 2 weeks after surgery, with decreasing platelet counts, which was less pronounced in splenectomized rats. However, the impact of splenectomy on platelet counts was modest in our animal study, compared to many clinical reports, where splenectomy led to a strong and also sustainable increase of platelets [11, 32, 33, 37, 38].

The studies mentioned above also coherently show a beneficial impact on WBC counts [11, 32, 33, 37, 38]. However, in our experiments, the PPVL rats did not show any changes in WBC at all, and in the BDL rats, we instead observed progressive leukocytosis, which was further exacerbated by splenectomy. Leukocytosis in BDL rats has also been reported in other BDL studies [39] and is likely attributed to extensive cholestatic hepatitis.

4.3 Limitations of the study

Indeed, we have to acknowledge inherent limitations of our animal study. Both models are aggressive and show a rapid PH development, which is rarely observed in patients. Especially the long-term BDL setting might hamper studying the subtle effects of splenectomy on hematological changes, as the pronounced hepatic injury seems to trigger a pro-inflammatory systemic reaction that also has an impact on blood cell counts. Accordingly, the BDL model is not recommended to be studied beyond 6 weeks [12]. Moreover, we realized that short-term effects of splenectomy on hematological parameters are difficult to study, due to surgery-related alterations (e.g. blood loss or inflammation in the surgical area). Importantly, the clinical translatability of our findings in terms of a pre-emptive treatment is limited, because we would not propose splenectomy prior to developing PH. What is more, splenectomy bares the risk of (post)-surgical complications, including portal vein thrombosis, bleeding or infections [40, 41]. Yet, splenectomy may still have its role not only to treat severe hypersplenism-induced cytopenia but also to improve PH, which has been shown to provide clinical benefit in selected patients undergoing simultaneous liver transplantation and splenectomy [42, 43]. While the idea of performing splenectomy to ameliorate congestive hypersplenism exists for over 100 years [44], less invasive and more efficient treatment options, such as partial splenic arterial embolization, have been developed [1]. Partial splenic arterial embolization combines the beneficial impact on hemodynamics and cytopenia, while mitigating the risks of surgery and asplenia [7, 45, 46]. Yet, the impact on blood cell counts appears to be more transient, as compared to splenectomy [45].

4.4 Future research

Although the role of the spleen in the PH syndrome is undisputed, it does not seem to be in the focus of current research. Indeed, future trials are needed to further refine the conditions, under which splenic interventions are most effective and beneficial for the patient. The invasiveness of splenectomy limits its use in PH therapy, but the spleen, or the splenic vasculature, respectively, can serve as a starting point for more refined therapeutic strategies. Accordingly,

pharmacological advances have shown promising results as spleen-targeted treatment options in the setting of PH [5, 47, 48]. Future experimental studies may use telemetric assessment of hemodynamics and include subsequent blood withdrawals to reduce the number of animals and inter-individual variations, thus following the 3R (Replacement, Reduction and Refinement) principles. Models, where cirrhosis induction is slower and which do not entail hematologic toxicity (such as carbon tetrachloride [49]) might help to elucidate more subtle effects of splenic interventions.

5 CONCLUSIONS

In conclusion, we demonstrate that splenectomy decreases portal pressure and improves the hyperdynamic circulation in two different models of cirrhotic and non-cirrhotic PH. These splenectomy-related improvements in hemodynamics were mirrored by reduced vWF levels. Moreover, splenectomy also led to increases in hemoglobin levels and mean corpuscular hemoglobin concentrations in both models, whereas thrombocytopenia only improved in the non-cirrhotic setting. Considering clinical evidence and our experimental data, the impact of surgical or functional splenectomy on PH-related complications and hypersplenism-associated cytopenias appears to be a promising target and should be further investigated.

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The Author Contribution

Study Design: Philipp Schwabl, Berit A Seeland, Michael Trauner, Markus Peck-Radosavljevic, Thomas Reiberger

Data Collection: Philipp Schwabl, Berit A Seeland, Florian Riedl, Tim L Schubert, Philipp Königshofer, Ksenia Brusilovskaya, Oleksandr Petrenko, Benedikt Hofer, Ana I Schiefer, Thomas Reiberger

Statistical Analysis: Philipp Schwabl, Thomas Reiberger

Data Interpretation: Philipp Schwabl, Thomas Reiberger

Manuscript Preparation: Philipp Schwabl and Thomas Reiberger

Literature Search: Philipp Schwabl, Thomas Reiberger

Funds collection: Philipp Schwabl, Thomas Reiberger

Declaration of Competing Interest

Philipp Schwabl received consulting fees from PharmaIN.

Michael Trauner served as consultant and/or advisory board member for Albireo, Boehringer Ingelheim, Bristol-Myers Squibb, Falk, Genfit, Gilead, Intercept, MSD, Novartis, Phenex, Regulus and Shire, and received travel support from AbbVie, Falk, Gilead, and Intercept, as well as grants from Albireo, Cymabay, Falk, Gilead, Intercept, MSD, and Takeda.

Michael Trauner is co-inventor of patents on the medical use of 24-norursodeoxycholic acid.

Markus Peck-Radosavljevic served as advisor and/or speaker for Shionogi and Sobi.

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FIGURE LEGENDS

Figure 1. Study design. Rats underwent either bile duct ligation (BDL), partial portal vein ligation (PPVL) or sham operation to induce cirrhotic portal hypertension, non-cirrhotic portal hypertension or to serve as healthy control, respectively. Additionally, in each group a subgroup of animals was splenectomized. The postsurgical observation lasted between 1 and 35 days, which was concluded by invasive hemodynamic measurements, hematology panel analysis, and assessment of megakaryopoiesis and von Willebrand factor serum concentrations.
To design Figure 1 we used royalty-free resources from Flaticon.com.

Figure 2. Course of the portal hypertensive syndrome in PPVL and BDL rats. A, **B**: Compared to SO animals, PPVL and BDL rats had elevated portal pressure and reduced mean arterial pressure. **C**: The heart rate was increased in PPVL and BDL animals. **D**: Accordingly, von Willebrand factor serum levels were higher in PPVL and BDL rats, compared to SO controls.
[a] $p<0.001$, [b] $p<0.01$, [c] $p<0.05$ *vs* healthy control (SO), data is shown as mean ± standard deviation.
Abbreviations: BDL: bile duct ligation; PPVL: partial portal vein ligation; SO: sham operation.

Figure 3. Course of blood cell counts in PPVL and BDL rats. A: Hemoglobin levels were depleted in PPVL and BDL animals, compared to SO controls. **B**: However, no changes were seen in red blood cell counts. **C**, **D**: BDL rats presented with a strong elevation in white blood cell and platelet counts.
[a] $p<0.001$, [b] $p<0.01$ *vs* healthy control (SO), data is shown as mean ± standard deviation.
Abbreviations: BDL: bile duct ligation; PPVL: partial portal vein ligation; SO: sham operation.

Figure 4. Impact of splenectomy on the portal hypertensive syndrome. A: In splenectomized rats the portal pressure decreased in both models of portal hypertension. **B**, **C**: Accordingly, there was an increase in mean arterial pressure and a reduction of heart rate. **D**: In line, von Willebrand factor serum levels decreased.
The normal range indicates the standard deviation of the respective values in sham operated animals.
[b] $p<0.01$, [c] $p<0.05$, [d] $p<0.10$ *vs* respective splenectomized groups, data is shown as mean ± standard deviation.

Abbreviations: BDL: bile duct ligation; BDL-SPL: bile duct ligation with splenectomy; PPVL: partial portal vein ligation; PPVL-SPL: partial portal vein ligation with splenectomy.

Figure 5. Impact of splenectomy on blood cell counts in experimental portal hypertension.
A: In both models the hemoglobin levels were higher in the splenectomized groups. **B**: However, no changes in red blood cell counts were obvious. **C**: White blood cell counts did not change significantly, yet on day 28 and 35 BDL-SPL rats presented with much higher values compared to the BDL group. **D**: In the PPVL model, splenectomized animals presented a trend towards lower platelet counts.
The normal range indicates the standard deviation of the respective values in sham operated animals.
[a] $p<0.001$, [d] $p<0.10$ *vs* respective splenectomized groups, data is shown as mean ± standard deviation.
Abbreviations: BDL: bile duct ligation; BDL-SPL: bile duct ligation with splenectomy; PPVL: partial portal vein ligation; PPVL-SPL: partial portal vein ligation with splenectomy.

Figure 1. Study design

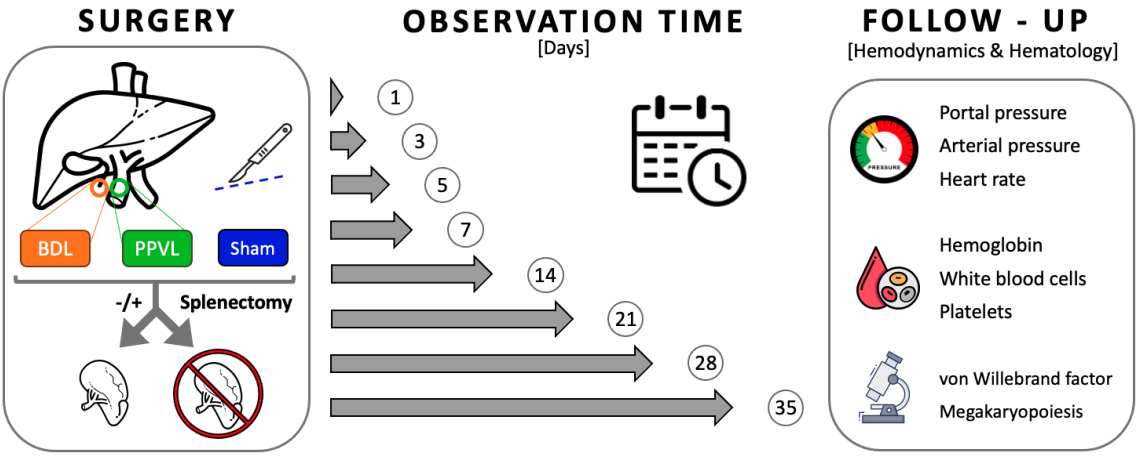

Figure 1. Study design.

Rats underwent either BDL, PPVL or sham operation to induce cirrhotic portal hypertension, non-cirrhotic portal hypertension or serve as healthy control, respectively. Additionally, in each group a subgroup of animals was splenectomized. The following observation time lasted between 1-35 days and concluded with invasive hemodynamic measurements, a hematology panel analysis, and assessment of megakaryopoiesis and von Willebrand factor serum concentrations.

Abbreviations: BDL, bile duct ligation; PPVL, partial portal vein ligation.

Figure 2. Course of the portal hypertensive syndrome in PPVL and BDL rats

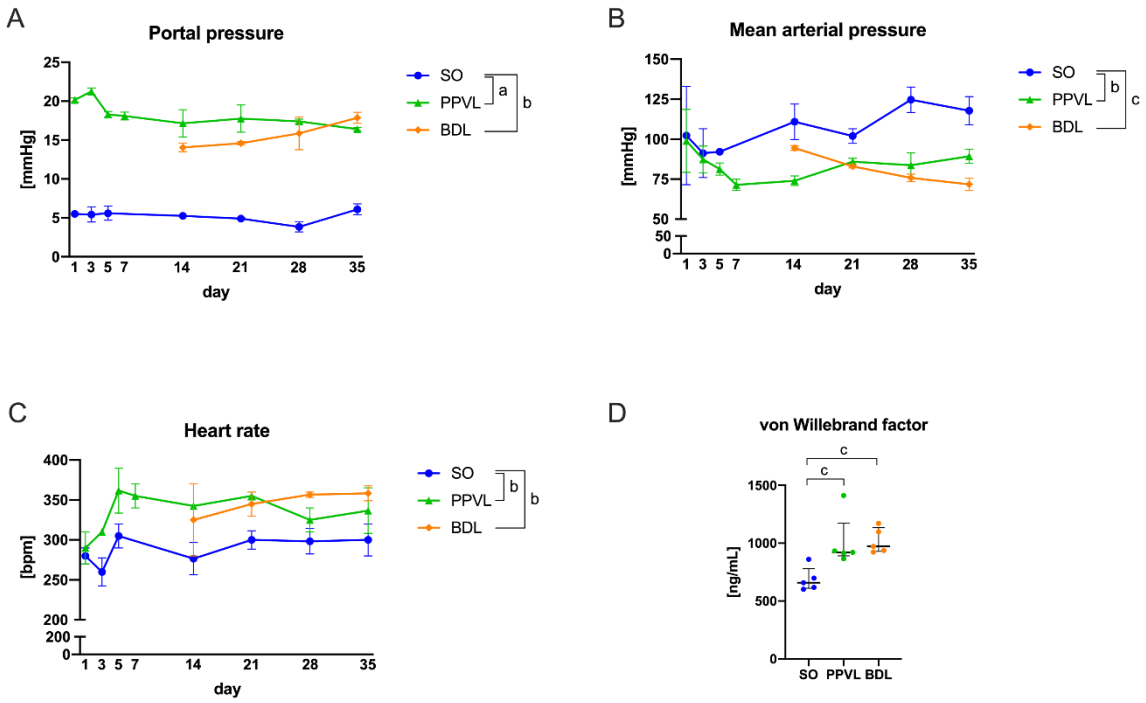

Figure 2. Course of the portal hypertensive syndrome in PPVL and BDL rats. Compared to SO animals, PPVL and BDL rats had **(A)** elevated portal pressure and **(B)** reduced mean arterial pressure. **(C)** The heart rate was increased in PPVL and BDL animals. **(D)** Accordingly, von Willebrand factor serum levels were higher in PPVL and BDL rats, compared to SO controls.

Abbreviations: BDL, bile duct ligation; PPVL, partial portal vein ligation; SO, sham operation; [a] $p<0.001$; [b] $p<0.01$; [c] $p<0.05$

Figure 3. Course of blood cell counts in PPVL and BDL rats

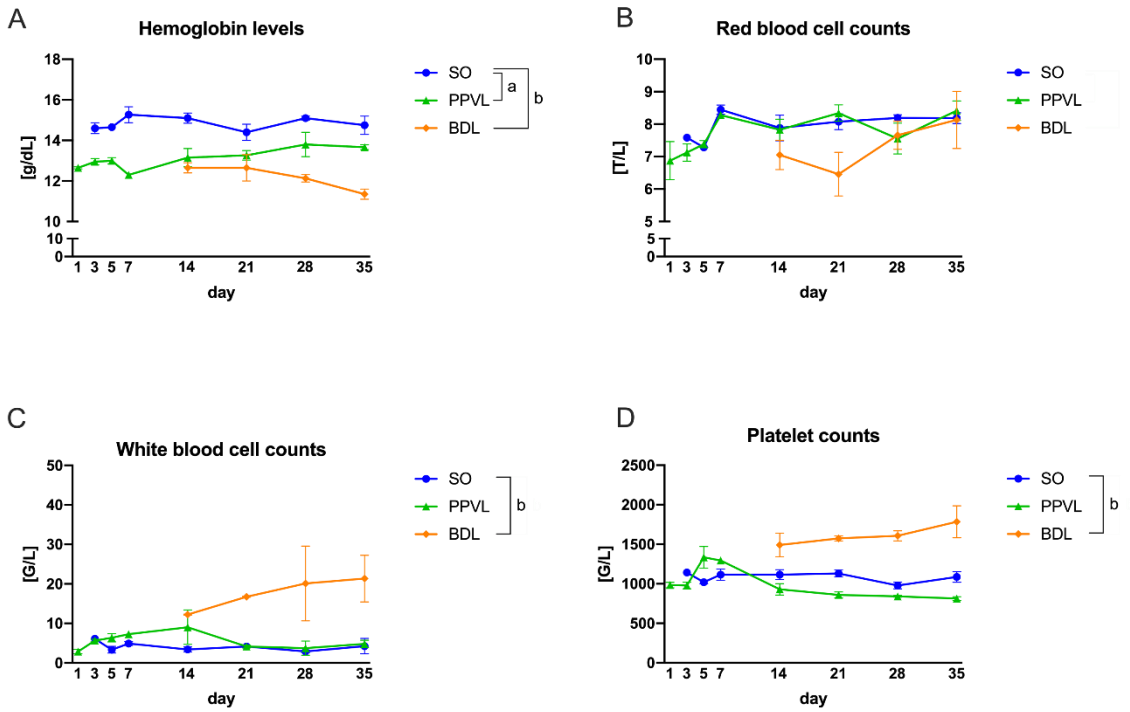

Figure 3. Course of blood cell counts in PPVL and BDL rats.

(A) Hemoglobin levels were depleted in PPVL and BDL animals, compared to SO controls, whereas **(B)** no changes were seen in red blood cell counts. BDL rats presented with a strong elevation in **(C)** white blood cell and **(D)** platelet counts.

Abbreviations: BDL, bile duct ligation; PPVL, partial portal vein ligation; SO, sham operation; [a] $p<0.001$; [b] $p<0.01$

Figure 4. Impact of splenectomy on the portal hypertensive syndrome

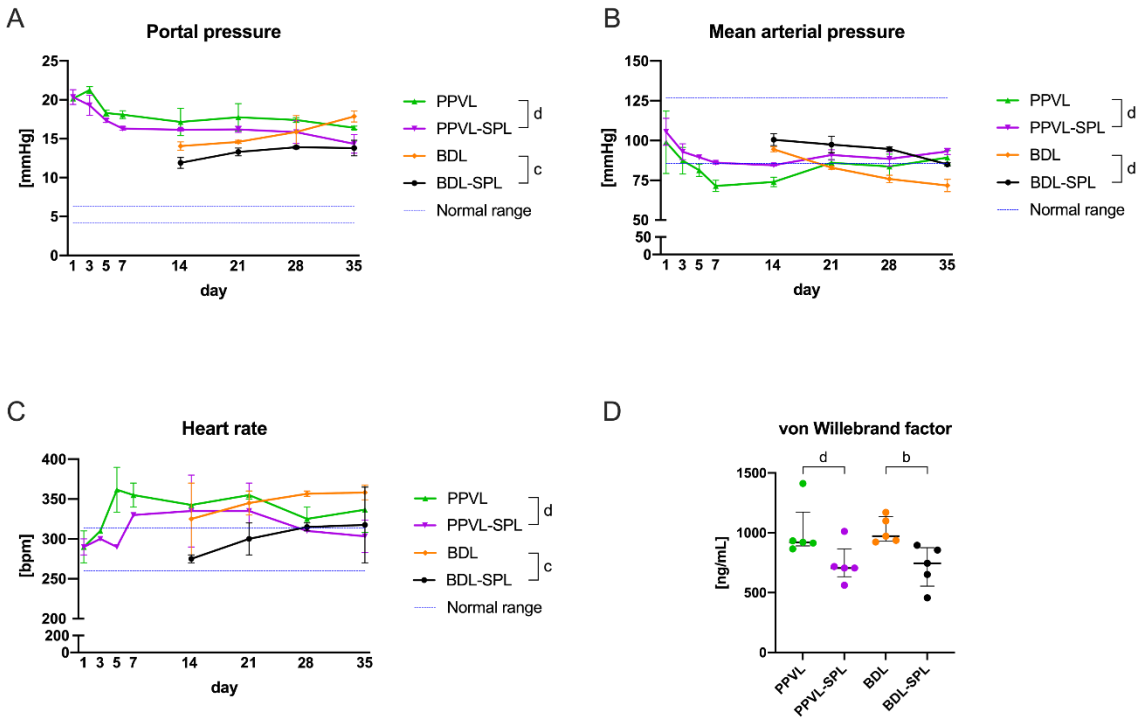

Figure 4. Impact of splenectomy on the portal hypertensive syndrome.

(A) In splenectomized rats the portal pressure decreased in both models of portal hypertension. Accordingly, there was an increase in **(B)** mean arterial pressure and **(C)** a reduction of heart rate. **(D)** In line, von Willebrand factor serum levels decreased. The normal range indicates the standard deviation of the respective values in sham operated animals.

Abbreviations: BDL, bile duct ligation; BDL-SPL, bile duct ligation with splenectomy; PPVL, partial portal vein ligation; PPVL-SPL, partial portal vein ligation with splenectomy; [b] $p<0.01$; [c] $p<0.05$; [d] $p<0.10$

Figure 5. Impact of splenectomy on blood cell counts in experimental portal hypertension

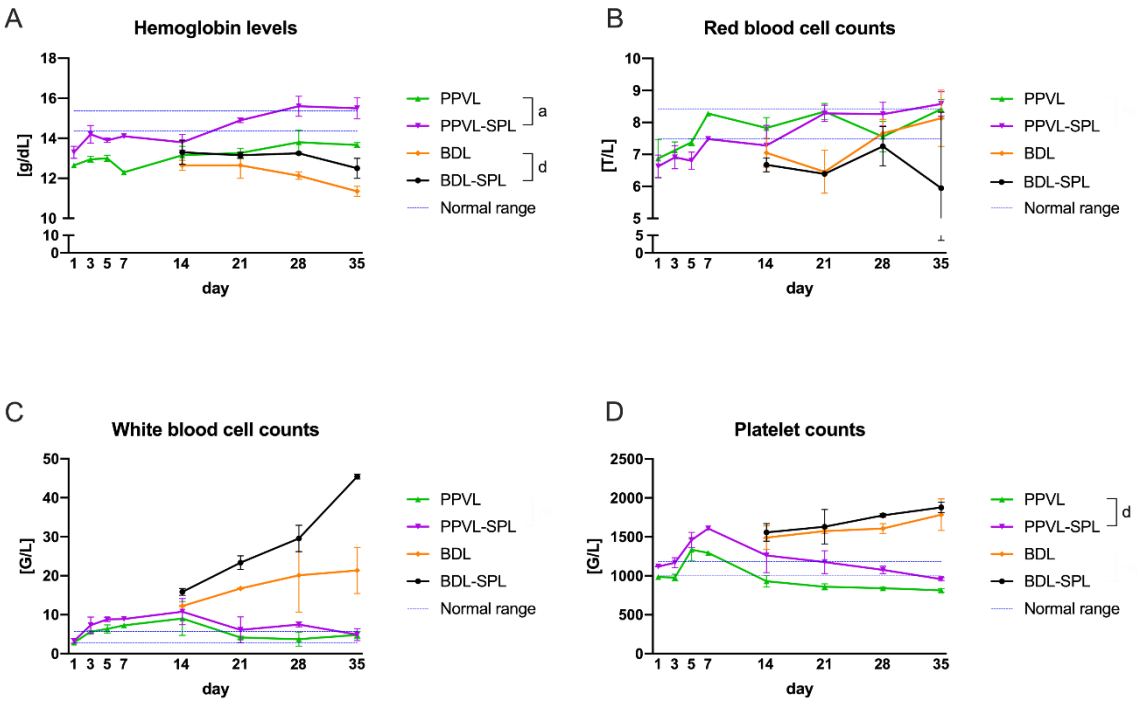

Figure 5. Impact of splenectomy on blood cell counts in experimental portal hypertension. **(A)** In both models the hemoglobin levels were higher in the splenectomized groups, **(B)** however no changes in red blood cell counts were obvious. **(C)** White blood cell counts did not change significantly, yet on day 28 and 35 BDL-SPL rats presented with much higher values compared to BDL group. **(D)** In the PPVL model, splenectomized animals presented a trend towards lower platelet counts. The normal range indicates the standard deviation of the respective values in sham operated animals.

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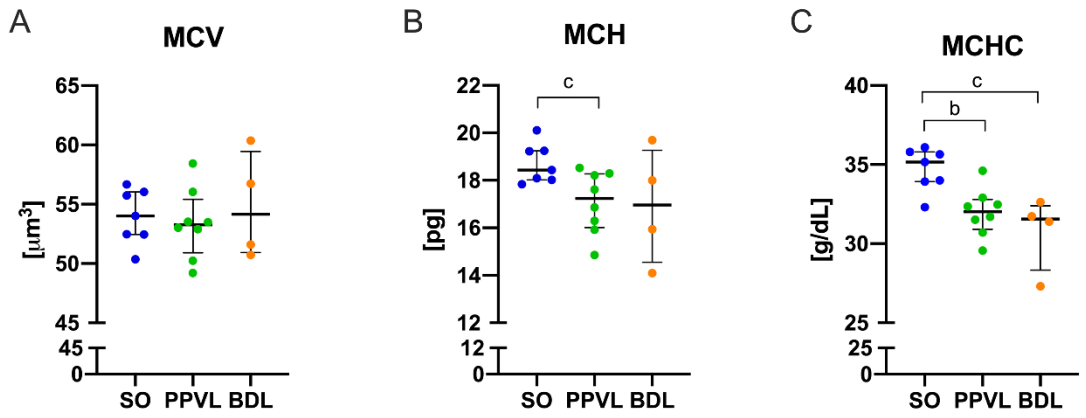
Supplementary Figure 1. Changes in red blood cell indices in experimental models of portal hypertension

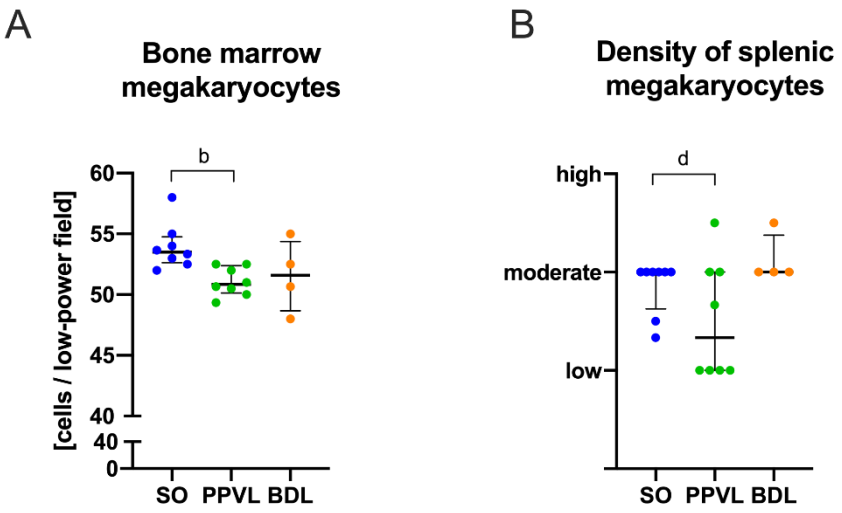


Supplementary Figure 1. Changes in red blood cell indices in experimental models of portal hypertension.

(A) MCV values remained similar among all three groups. **(B)** MCH was significantly decreased in the PPVL group. **(C)** Both, PPVL and BDL rats presented with lower MCHC compared to SO controls.

Abbreviations: BDL, bile duct ligation; PPVL, partial portal vein ligation; SO, sham operation; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; ^{b} $p<0.01$; ^{c} $p<0.05$

Supplementary Figure 2. Changes in bone marrow megakaryocytes in experimental models of portal hypertension

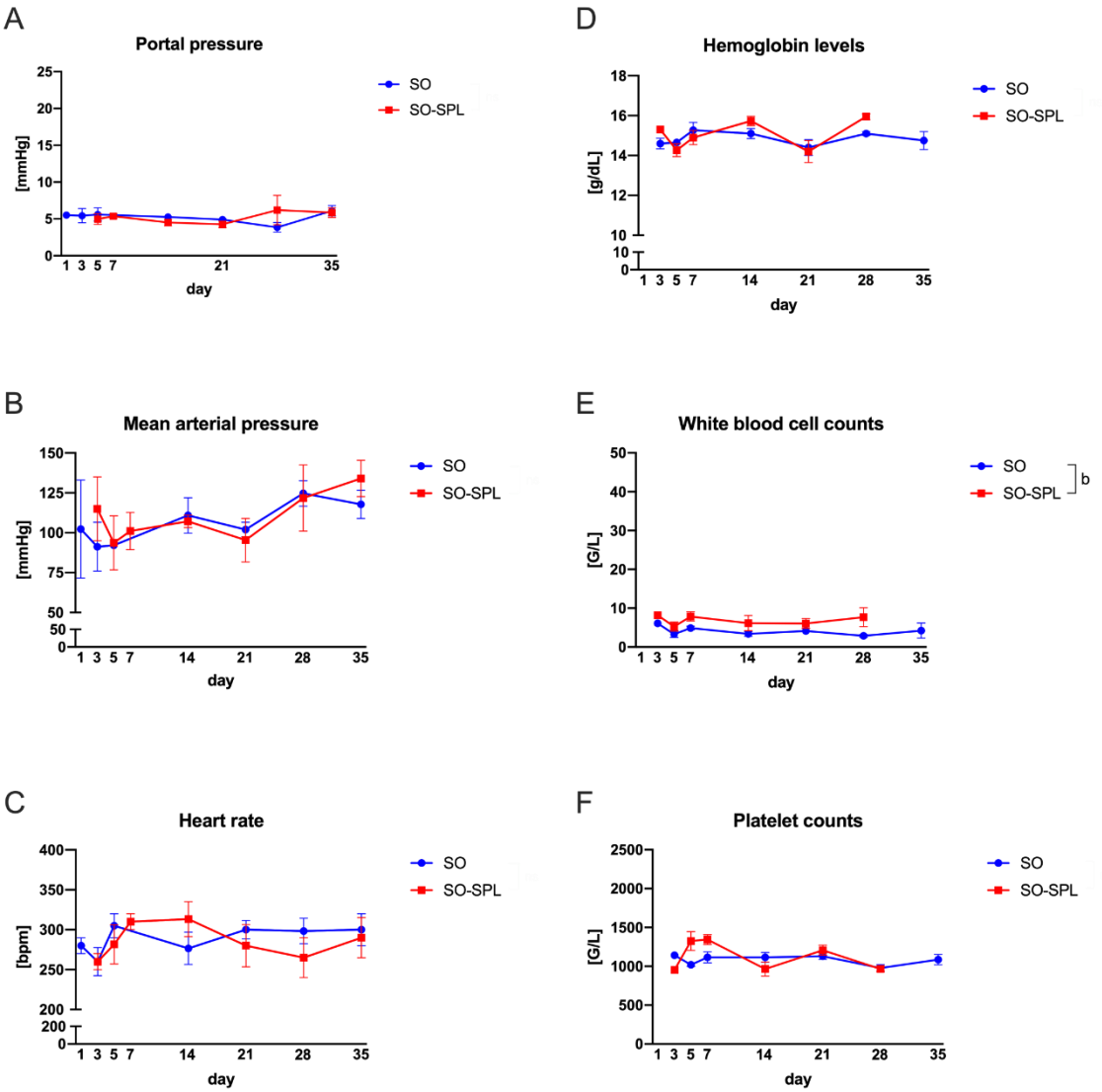


Supplementary Figure 2. Changes in bone marrow megakaryocytes in experimental models of portal hypertension.

PPVL rats presented with **(A)** a reduced number of bone marrow megakaryocytes and **(B)** a lower density of splenic megakaryocytes. In contrast, in BDL animals the megakaryocyte counts were not significantly altered, compared to SO controls.

Abbreviations: BDL, bile duct ligation; PPVL, partial portal vein ligation; SO, sham operation; [b] $p<0.01$; [d] $p<0.10$

Supplementary Figure 3. Course of hemodynamics and blood cell counts in sham operated control animals

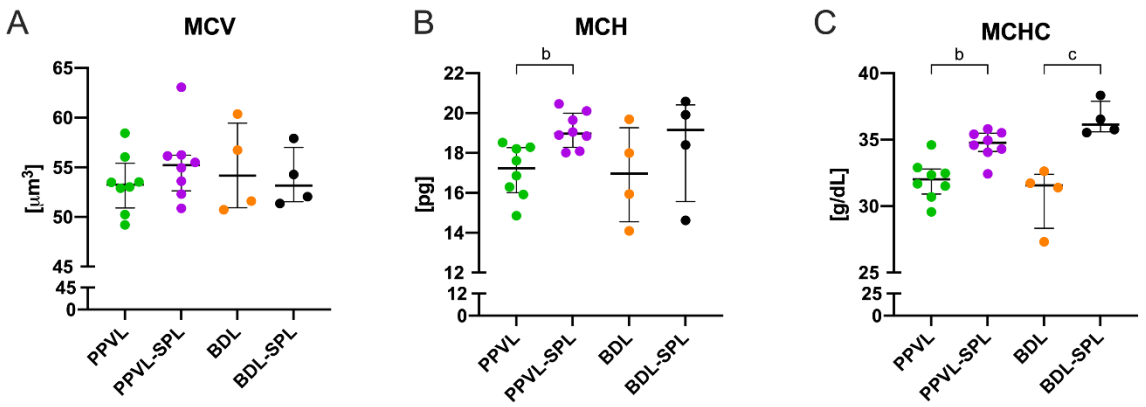


Supplementary Figure 3. Course of hemodynamics and blood cell counts in sham operated control animals.

Sham operated rats, who also underwent splenectomy presented similar **(A)** portal pressure, **(B)** mean arterial pressure and **(C)** as non-splenectomized SO rats. While **(D)** hemoglobin levels and **(F)** platelet counts remained unchanged, **(E)** SO-SPL animals had consistently higher white blood cell counts.

Abbreviations: SO, sham operation; SPL-splenectomy; [b] $p<0.01$

Supplementary Figure 4. Impact of splenectomy on red blood cell indices

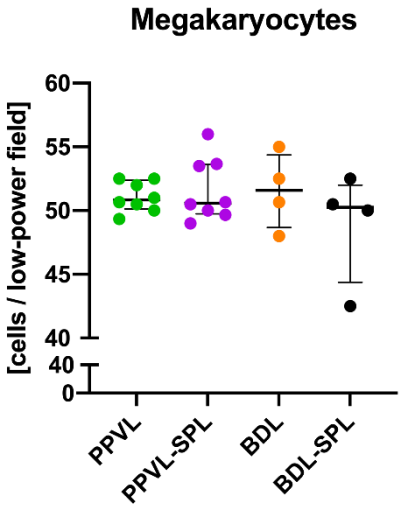


Supplementary Figure 4. Impact of splenectomy on red blood cell indices.

(A) In splenectomized rats, the MCV values did not change significantly. In contrast, PPVL-SPL animals had a higher **(B)** MCH and **(C)** MCHC. The latter one was also elevated in splenectomized BDL rats.

Abbreviations: BDL, bile duct ligation; BDL-SPL, bile duct ligation with splenectomy; PPVL, partial portal vein ligation; PPVL-SPL, partial portal vein ligation with splenectomy; [b] $p<0.01$; [c] $p<0.05$

Supplementary Figure 5. Impact of splenectomy on bone-marrow megakaryocyte numbers

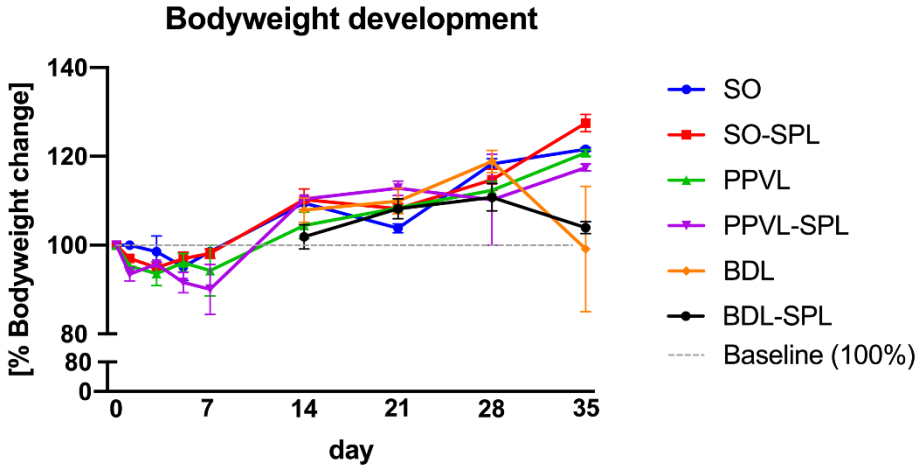


Supplementary Figure 5. Impact of splenectomy on bone-marrow megakaryocyte numbers.

Splenectomy did not affect the number of bone marrow megakaryocytes, neither in the PPVL nor in the BDL group.

Abbreviations: BDL, bile duct ligation; BDL-SPL, bile duct ligation with splenectomy; PPVL, partial portal vein ligation; PPVL-SPL, partial portal vein ligation with splenectomy.

Supplementary Figure 6. Relative body weight development in all subgroups across the timeline



Supplementary Figure 6. Relative body weight development in all subgroups across the timeline.

After a short, postsurgical decrease, all groups presented a positive body weight development. No significant differences among the different groups were notable. The gray dotted line illustrates the group baseline on the surgery day.

Abbreviations: SO sham operation; BDL, bile duct ligation; PPVL, partial portal vein ligation; SPL, splenectomized.

Supplementary Table 1. Study groups and number of animals

Timepoint	SO	SO-SPL	PPVL	PPVL-SPL	BDL	BDL-SPL
day 1	2	0*	2	2		
day 3	3	2	2	1[#]		
day 5	2	3	3	1[#]		
day 7	0*	3	2	1[#]		
day 14	3	3	2	2	2	2
day 21	3	3	2	3	2	2
day 28	3	2	2	2	3	2
day 35	2	3	3	3	3	2

Supplementary Table 1. Study groups and number of animals.

In this study in total 40 subgroups (n= 2-3) were defined. * In two groups, SO_{d7} and $SO\text{-}SPL_{d1}$, a heating plate defect led to postsurgical death of all animals. [#] In $PPVL\text{-}SPL_{d3}$, $PPVL\text{-}SPL_{d5}$ and $PPVL\text{-}SPL_{d7}$ post-surgical animal drop outs occurred leading to a diminished group size.

Abbreviations: SO, sham operation; BDL, bile duct ligation; PPVL, partial portal vein ligation; SPL, splenectomy.

Supplementary Table 2. Median white blood cell differential counts

Parameter	SO	PPVL	BDL	PPVL-SPL	BDL-SPL
WBC	4.1 [3.3-4.9]	5.2 [3.8-7.0]	18.4 [b] [13.4-21.0]	7.4 [5.1-8.8]	26.5 [17.7-41.4]
Neutrophils	0.89 [0.48-1.08]	0.77 [0.69-2.12]	5.85 [c] [3.75-7.81]	1.09 [0.93-1.42]	6.50 [4.95-16.28]
Lymphocytes	3.7 [2.2-4.1]	3.7 [3.0-5.2]	10.7 [b] [8.8-14.7]	5.6 [4.2-7.6]	18.4 [11.8-35.1]
Monocytes	0.065 [0.050-0.116]	0.103 [0.073-0.201]	0.875 [b] [0.521-1.011]	0.135 [0.101-0.286]	0.995 [0.565-2.644]
Eosinophils	0.070 [0.055-0.097]	0.050 [0.045-0.079]	0.138 [c] [0.119-0.151]	0.070 [0.052-0.098]	0.180 [0.129-0.401]
Basophils	0.003 [0.000-0.006]	0.010 [c] [0.010-0.018]	0.060 [a] [0.034-0.139]	0.020 [0.006-0.029]	0.135 [0.044-0.777]

Supplementary Table 2. Median white blood cell differential counts.

The median and interquartile range values of the respective white blood cell differential counts are listed in G/L. BDL rats presented with a global elevation of WBC, affecting all leukocyte subgroups. In contrast, the only deviations seen in PPVL rats was an increase in basophils not affecting the total WBC. The splenectomized PPVL and BDL groups presented no statistically significant changes, compared to the non-splenectomized PPVL and BDL groups, respectively.

Abbreviations: SO, sham operation; BDL, bile duct ligation; PPVL, partial portal vein ligation; SPL, splenectomy; WBC, white blood cell counts; [a] $p<0.001$; [b] $p<0.01$; [c] $p<0.05$