Diplomarbeit

Establishment of an animal model of venous thrombosis in inflammatory bowel diseases

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Zusammenfassung

EINLEITUNG Chronische entzündliche Darmerkrankungen (CED) repräsentieren eine Gruppe von chronischen Entzündungen, welche hauptsächlich den Darm befallen. Über die letzten Jahrzehnte stieg die Inzidenz des Morbus Crohn und der Colitis ulcerosa kontinuierlich an, welche daher vermehrt ein ernstzunehmendes gesundheitliches Problem in der westlichen Gesellschaft darstellen. Weiters sind Patienten, die an CED leiden, häufiger von venösen Thrombosen und deren potentiell letalen thromboembolischen Komplikation betroffen, welche de facto eine der häufigsten Todesursachen bei diesen Patienten ist. Die Ursache für die vermehrte Thrombogenese bei chronisch entzündlichen Darmerkrankungen ist nicht hinreichend geklärt. Um die ätiologischen Mechanismen weiter zu untersuchen, haben wir in diesem Projekt die Entstehung und Auflösung venöser Thromben bei Mäusen mit Darmentzündung beobachtet. Dabei haben wir ein etabliertes Kolitismodell mit einem venösen Ligaturmodell kombiniert mit der Hypothese, dass der Schweregrad der Darmentzündung die Entstehung bzw. Auflösung venöser Thromben beeinflusst.

METHODEN Die Pilotstudie beinhaltet die Untersuchung von zwei verschiedenen Mäusegruppen, nämlich fünf Interleukin-10 Knockout (IL-10 KO) und fünf Wildtyp (WT) Tieren. Zunächst wurde eine Darmentzündung durch die Gabe von Dextran-Natriumsulfat (DSS) im Trinkwasser getriggert. Anschließend folgte die Ligatur der unteren Hohlvene (IVC) unterhalb der Nierenvenen. Der verlangsamte Blutfluss führte zur Entstehung eines Thrombus vor dem stenotischen Bereich, das heißt herzfern der Ligatur. Der Thrombus wurde mittels Duplex Ultraschall zu wiederholten Zeitpunkten untersucht. Drei Wochen nach der Operation wurden die Mäuse getötet und die entnommenen Organe sowie Thromben histologisch untersucht.

ERGEBNISSE Überraschenderweise waren die WT Mäuse durch die Behandlung mit DSS und der operativen Ligatur stärker betroffen, was zum vorzeitigen Tod von drei Mäusen führte. Das Gewicht der WT Mäuse fiel kurze Zeit nach der Induktionsphase drastisch ab, wohingegen die IL-10 KO Mäuse weniger Symptome zeigten. Es kann nicht ausgeschlossen werden, dass hier ein Käfigeffekt vorliegt, der sich durch unterschiedliche Größen der Trinkbehältnisse erklärt. Trotz der milderen klinischen Ausprägung der akuten Kolitissymptome bei den IL-10 KO Tieren, fanden sich nach drei Wochen eine ausgeprägtere chronische Entzündung im Darm [Disease Activity Index: 1.8 (IL-10 KO); 1.25 (WT)] sowie größere organisierte Thromben in der IVC [Volumen: 0.8 mm^3 (IL-10

KO); 0.3 mm^3 (WT)]. Auch die sonographischen Untersuchungen zeigten im zeitlichen Verlauf größere Thromben bei den IL-10 KO Mäusen. Die Maus Genotypisierung bestätigte die korrekte Zuordnung der Mäuse zu den beiden Gruppen.

DISKUSSION Die Daten dieser Pilotstudie zeigen, dass die Durchführung einer IVC Ligation bei DSS behandelten Mäusen mit einer hohen Belastung der Tiere einhergeht, aber grundsätzlich möglich ist. Aufgrund der stark reduzierten Gesundheit bei manchen Mäusen und der relativ hohen Anzahl an verstorbenen Tieren sollten in zukünftigen Experimenten Versuchsanpassungen, wie die Verlängerung der Erholungszeit nach der DSS Induktion, in Betracht gezogen werden. Des Weiteren sollten auf idente Untersuchungsbedingungen bei beiden Tiergruppen geachtet werden (Käfige, Trinkflaschen, etc.) um potentielle Fehlerquellen zu vermeiden. Weitere Untersuchungen mit einer größeren Anzahl an Mäusen sind notwendig, um diese vorläufigen Ergebnisse zu bestätigen. Das neue Tiermodell kann in Zukunft für die Testung verschiedener pharmakologischer Substanzen genutzt werden.

Abstract

INTRODUCTION Inflammatory bowel diseases (IBD) represent a group of recurring inflammatory diseases mainly affecting the intestines. Over the past decades, the incidence of Crohn's disease and ulcerative colitis have continued to increase, which therefore poses a serious health problem in Western society. Furthermore, patients suffering from IBD are more frequently affected by venous thrombosis and its potentially lethal thromboembolic complication, which is one of the most common causes of death in these patients. The cause for the increased thrombogenesis in chronic inflammatory diseases remains mostly unknown. In order to further investigate the etiological mechanisms, we investigated the formation and resolution of venous thrombi in mice suffering from intestinal inflammation. Therefore, we combined an established model of colitis with a venous ligation model hypothesizing that the severity of colitis influences the formation or resolution of venous thrombi.

METHODS The conducted pilot study involved the selection of 10 murine animals, namely five interleukin-10 knockout (IL-10 KO) and five wild type (WT) mice. Initially, intestinal inflammation was triggered by the administration of dextran sodium sulfate (DSS) mixed into drinking water. Subsequently, the inferior vena cava (IVC) of the animals was ligated below the renal veins. The reduced blood flow led to the formation of a thrombus before (caudal of) the stenotic region. The thrombus was examined using sonographic imaging at specific time points. Three weeks after the ligation, the mice were sacrificed and the removed organs as well as thrombi were histologically examined.

RESULTS To our surprise, the WT group was affected to a greater extent by the combination of DSS treatment and ligation of the IVC resulting in the premature death of three mice. The weight of the WT animals plunged shortly after the induction period, whereas the IL-10 KO mice showed little symptoms. It cannot be ruled out that a cage phenomenon occurred, which is explained by different sizes of the drinking containers. Nevertheless, despite milder clinical manifestations of colitis in the IL-10 KO, a more severe chronic inflammation of the intestines [disease activity index: 1.8 (IL-10 KO); 1.25 (WT)] as well as larger organized thrombi in the IVC [volume: 0.8 mm^3 (IL-10 KO); 0.3 mm^3 (WT)] were observed three weeks following the ligation. The sonographic examinations also depicted greater clots over the course of time. Mouse genotyping confirmed the correct allocation of the mice to the two groups.

DISCUSSION The data from this pilot study show that the implementation of IVC ligation in DSS-treated mice is accompanied by a high degree of stress on the animals, but is generally feasible. Due to the severely reduced health in some mice and the relatively high number of deceased animals, experimental adjustments, such as the extension of the recovery time after DSS induction, should be considered in future experiments. Additionally, identical experimental conditions (cages, drinking bottles, etc.) should be achieved in order to avoid potential sources of error. Further studies with a larger number of mice are necessary to confirm these preliminary results. The murine model can be further used to test various pharmacological substances.

Glossar und Abkürzungen

CD	Crohn's disease
DOAC	Direct oral anticoagulant
DSS	Dextran sodium sulfate
DVT	Deep vein thrombosis
IBD	Inflammatory bowel diseases
IL	Interleukin
IL-10 KO	Interleukin-10 knockout
IVC	Inferior vena cava
LMWH	Low molecular weight heparin
PE	Pulmonary embolism
TNF	Tumor necrosis factor
UC	Ulcerative colitis
UH	Unfractionated heparin
VKA	Vitamin K antagonist
VTE	Venous thromboembolism
vWF	von Willebrand factor
WT	Wild type

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1 Introduction

1.1 Inflammatory bowel diseases

Inflammatory bowel diseases (IBD) are a group of complex inflammatory conditions with unknown etiology and not fully understood pathogenesis, characterized by chronic inflammation and ulceration of the gut mucosa (1). Crohn's disease (CD) and ulcerative colitis (UC) represent the two main forms of IBD. IBD mostly affect the colon as well as the small intestine. Though it should be noted that they are associated with extensive extra intestinal manifestations such as skin, joint or ocular involvement (2).

Both major types have distinct characteristics (3). On the one hand, CD can cause transmural inflammation affecting any part of the gastrointestinal tract. Due to the transmural colitis, the intestine in patients suffering from CD is more prone to develop abscesses, fistulas and strictures. Unlike UC, the inflammation in CD doesn't disseminate continuously. On the other hand, UC is characterized by an uninterrupted mucosal inflammation limited to the colon. Although the etiology and pathogenesis of the IBD remain mostly unknown, research over the past decades points out that the external environment, the genetic composition and intestinal microbial flora play a major role in developing these relapsing intestinal inflammations (4).

In general, these inflammatory diseases are very complex to treat and challenge specialists around the world (5). The treatment mainly focuses on conservative measures, modulation of the immune system as well as surgical interventions.

1.1.1 Epidemiology

CD and UC are chronic, relapsing and progressive diseases with an estimated 2.2 million people being affected in Europe (6). These inflammatory diseases are a global health-care issue with a constantly increasing incidence. IBD is linked to a high geographical variation with the highest European incidence in the northern parts and the lowest in countries bounded to the Mediterranean Sea. Furthermore, Burisch et al (7) managed to provide data suggesting an East-West gradient in Europe with Western countries depicting annual incidence rates twice as high as rates collected from Eastern centers. As a matter of fact,

Caucasians and Ashkenazic Jews are more likely to be affected by IBD than people from other ethnic and racial backgrounds (8).

Over the past 20 years, the incidence of UC as well as CD has risen (9). In Europe, the incidence of UC varies from 0.9 to 24.3 per 100,000 person-years in comparison to CD which ranges from 0.5 to 10.6 cases per 100,000 person-years (10). Generally, both diseases are more likely to occur during the young adult life. UC first develops in 20 to 35-year old patients, whereas CD manifests itself in 15 to 35-year-olds (11). Interestingly, the incidence curve of IBD has a second peak in patients above 40 years of age.

Due to the fact that genetic factors play an important role in the pathogenesis of these chronic relapsing immune-mediated diseases, family members of patients are at higher risk of suffering from IBD (11). Siblings of affected patients are 15 (for UC) to 30 (for CD) times more likely to develop CD or UC than the general population.

1.1.2 Etiology

As already mentioned above, the inflammatory diseases present a challenge for most clinicians as the etiology as well as the pathomechanisms have not been fully discovered yet (4, 5). Nonetheless, there is a high correlation between genetic, environmental, microbial and immunological factors (Figure 1). Years of research have concluded that IBD is a disease of the intestinal barrier function which results in an abnormal and continuous immune response to the microbial flora of the intestine, amplified by the genetic predisposition of the individual (12). Therefore, it seems appropriate to highlight each component involved.

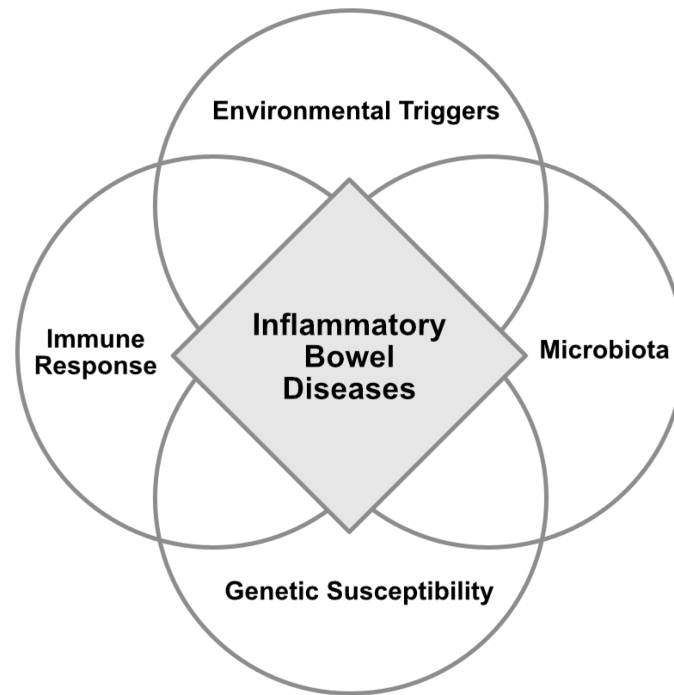

Figure 1: Etiological factors of Crohn's disease and ulcerative colitis

The figure depicts the main interacting factors leading to IBD, namely an abnormal immune response to external triggers, amplified by disrupted microbiota and combined with a genetic predisposition.

1.1.2.1 Genetics

Due to the technological improvements in DNA analysis and sequencing over the past decades, there has been huge progress in the understanding of genetic susceptibility to IBD (12). Recent studies have shown that over 110 gene loci are associated with both CD and UC, NOD2 being the most familiar one. Different alleles of nucleotide-binding oligomerization domain 2 (NOD2) exist and are associated with a geographical variation of frequency (13).

Patients, who carry a homozygous NOD2 gene mutation, are 17 times more likely to suffer from CD (11). To the present-day, there are two major hypotheses of NOD2 variations leading to an increased risk of CD (14). The first aspect proposes that NOD2 plays an essential role in the production of α-defensin. Human enteric α-defensins are small antimicrobial proteins produced by Paneth cells in the intestine and participate in the innate defense system of the intestines (15). These peptides pose an important part of the intestinal

immunity and thus sufficient protection is not achievable in NOD2 deficiency. Secondly, many studies have shown the crucial role of toll-like receptor signaling and its regulation. It is likely that the deficiency of NOD2 results in a colitis due to an improper stimulation of toll-like receptors (14).

Both hypotheses manage to show the risk of NOD2 variants as the alteration of the gene affects the expression of α-defensin as well as the regulation of toll-like receptors. Hence, NOD2 variants lead to increased inflammation through impaired mucosal immunity (14).
In conclusion, the growing number of genes susceptible to IBD illustrate the critical genetic influence on the pathogenesis of the disease (12).

1.1.2.2 Environment

Taking into account the multifactorial origin of IBD, environmental factors play without doubt a major role in the pathogenesis of CD as well as UC. Multiple components have an influence on the development of IBD, the most important being smoking, medications, nutritional regimen, appendectomy, psychological state, stress and geographical location (16). Smoking has been one of the most studied risk factors and was first described in 1982 where it showed the inverse effect on the two main IBD forms (17). Lakatos et al (18) found that smoking increased the risk of CD, although having a protective influence on UC.

The effects of antibiotics on the gastrointestinal tract and, in particular, the microbiome have been well identified (12). Especially, pediatric patients are more susceptible to IBD when receiving antibiotics during the first year of life (19).

Stress and its components, including anxiety and depression, are also known as major modulators in the pathogenesis of IBD and may mediate inflammatory flares. Goodhand et al (20) came to the conclusion in their retrospective study that antidepressants have a beneficial impact on the course of IBD as the medication reduces the number of symptomatic relapses, use of steroids as well as endoscopies in the year after their introduction.

Due to the increase in IBD over the last decades, it has been hypothesized that processed foods containing emulsifiers promote inflammatory diseases (21). In a murine model the emulsifiers carboxymethylcellulose and polysorbate-80, commonly used in various foods,

were administered for 12 weeks. The application of these dietary detergents resulted in erosions of the mucus as well as alterations of the microbiota composition confirming the pro-inflammatory potential of emulsifiers (21).

1.1.2.3 Microbial factors

The endogenous intestinal microflora is an essential part of the health of an individual, making it a crucial "organ" consisting of roughly 1150 different bacterial species (22). Important functions of the microbial flora include regulating and protecting epithelial cells, stimulating the intestinal angiogenesis and developing the immune system (23).

Even though much research has been conducted, the specific function of the gut flora in IBD has not been identified yet. However, many studies show a correlation between classic infectious enteritis and IBD, proposing that a variety of bacteria habiting the complex microflora take part as etiological factors in the physiopathology (24). Over the past decades researchers have tried to analyze the intestinal flora by cultivating fecal samples. Yet, classical bacterial culture only managed to identify 20 - 30 % of bacteria of the intestinal microbiota, suggesting to develop new methods in order to target more microbial agents (12). The analysis of variability in the 16S rDNA has been established as a culture independent method for classifying bacteria (24). Ott et al (24) concluded that patients suffering from IBD had a different composition of bacterial species in comparison to the healthy control group with a significant reduction of anaerobic bacteria. In patients with CD, the study of Martinez-Medina (25) showed a higher prevalence of *Escherichia coli* and a reduction of *Faecalibacterium prausnitzii*. A specific *E. coli* which is highly associated with CD is the *adherent-invasive E. coli* (AIEC) (26). AIEC manage to invade into epithelial cells and survive in the phagosomes of macrophages due to the low pH where they are able to replicate on a large scale. In addition, this species induces the aggregation of lymphocytes forming granulomatous structures, distinctive histopathological features of CD. Thus, AIEC may induce an inflammatory process in humans (26).

1.1.2.4 Immunological factors

Our immune system can be divided into an innate and adaptive immune response (27). The innate immune system is known as the non-specific defense against pathological stimuli. It

consists of various cells such as neutrophils, dendritic cells, monocytes, macrophages and natural killer cells who quickly react to pathogens. In contrast, the adaptive immune system is composed of mainly lymphocytes, such as B and T cells, which are highly specific to stimuli and take days to develop a response.

The mucosal immune system including the response of T cells has been studied extensively in order to explore the pathogenesis of IBD. Studies show that the intestinal inflammation is caused by a deregulation of innate and adaptive immune responses, concluding different pathways for the two main forms of IBD (12). CD is associated with an increased expression of T-helper 1 (Th1) cytokines produced by lymphocytes localized in the lamina propria. In CD, Th1 produce high amounts of proinflammatory IL-2 and IFN-γ (28). As opposed to CD, UC is driven by an atypical T-helper 2 response and great production of IL-13 by natural killer T cells (29). Geremia et al (30) showed in their murine study that IL-23, produced by Th17 cells, is also a key cytokine in inducing chronic intestinal inflammation and damaging the tissue, suggesting a role in the pathogenesis of IBD. Additionally, the intestinal mucosal barrier represents an essential part of preventing pathogens to enter and harm the tissue (31). CD and UC are both associated with a disrupted barrier resulting in an increased permeability. This leakiness aggravates the response of the immune system leading consequently to an inflamed tissue. The primary cytokine responsible for the barrier defects is tumor necrosis factor (TNF) alpha in CD, while IL-23 and IL-13 are linked to the evolvement of UC (31). Thus, modern therapies are directed against TNF alpha.

1.1.3 Clinical presentation

In general, there are a major range of symptoms shown in IBD, depending on the extent and severity as well as the location of the disease (32). Moreover, it should be noted that the symptoms are influenced by ongoing therapies as well as possible enteric pathogens causing superinfections. IBD also causes a number of extra intestinal symptoms, the most common being enteropathic arthritis. The classification of IBD involves anatomical location, disease behavior and age of onset as described by the “Vienna and Montreal classification for Crohn's disease” (33, 34).

Both CD and UC may present with systemic manifestations such as tachycardia, fever and weight loss (32). However, they are more common in CD patients during relapse periods, whereas patients suffering from UC only acquire these symptoms at a severe stage.

The cardinal sign of UC is diarrhea mixed with visible blood and occurs in over 90 % of active colitis (35). Patients suffering from proctitis usually present with classical complaints such as rectal bleeding, tenesmus and sometimes even constipation, while the main symptoms of left-sided or extensive UC are bloody diarrhea with crampy abdominal pain and nocturnal defecation. The pain (tenesmus) is mostly relieved by defecation.

Patients with CD show similar symptoms as UC (32). On one side, blood stools don't occur as frequently and can only be seen in patients with colonic CD. On the other side, patients mostly present with recurrent diarrhea and abdominal pain. The clinical manifestations of CD show similarities with the symptoms of appendicitis, namely colicky pain in the lower right quadrant with a possible pressure sensitive mass (11). Anal lesions and persistent perianal involvement such as recurrent or complex fistulae should always raise the suspect of CD (35).

1.1.3.1 Extra intestinal manifestations

As already mentioned, patients suffering from IBD may show numerous extra intestinal manifestations (EIM) involving multiple systems including skin, eyes, joints and liver. The EIM are more likely to occur in patients with CD and are influenced by the duration and progress of the IBD (32). In addition, the extra intestinal symptoms are dependent on the type of IBD, and therefore present supportive assistance in diagnosing the colitis.

The most frequent EIM is arthritis, having a higher prevalence in CD (20 %) than in UC (11 %) (32). The arthropathies typically manifest during relapse episodes and are occasionally associated with skin symptoms, such as erythema nodosum. Primary sclerosing cholangitis (PSC) is an inflammatory disease of the bile ducts and is more commonly linked to UC than CD (32). Boonstra et al (36) noted that 77 % of PSC patients have an association with UC, the majority (64 %) being male. Other disorders include uveitis, osteopathy, psoriasis, anemia and venous thromboembolism (VTE)(32).

1.1.4 Therapy

As IBD are a set of complex diseases with unknown pathogenesis, the therapy poses a challenge for many gastroenterologists. However, due to the fact that these relapsing inflammatory conditions increase in prevalence the therapy takes on an essential role for clinicians (32). The treatment regime includes conservative, pharmaceutical and surgical measures. The main therapeutic goal is reached by improving the quality of life through reducing relapses and maintaining remission phases (37). Complications and nutritional deficits need to be treated in order to prevent an aggressive course of the diseases. Additionally, patients should be given the possibility of psychological assistance.

Conservative treatment includes dietary and supportive measures (11). Patients with CD, whose small intestines have been affected, may suffer from malabsorption syndrome. This disorder should be handled by substituting proteins, electrolytes, vitamins (especially liposolubles) and calcium. Patients benefit from vitamin D and calcium as it proves to reduce the risk for osteoporosis. Furthermore, low fiber diet seems to be beneficial in acute exacerbations of CD. Smoking has an inverse effect on the two main IBD forms, being favorable for UC (17, 18). However, smoking should be studiously avoided as it doubles the risk of relapses in CD (11).

Pharmaceutical medication can be divided into the following five groups: anti-inflammatory drugs, immunomodulatory agents, biological agents, antibiotics and supportive medication (37). These drugs thrive to reduce the inflammatory process by repressing crucial targets.
Mesalazine, the active form of sulfasalazine, is the most established anti-inflammatory drug used in IBD and is particularly advantageous for the treatment of UC (37). Meta-analysis has been conducted in order to evaluate the efficiency of aminosalicylates in comparison with placebo, corticosteroids and other aminosalicylates in CD (38). The findings concluded 5-aminosalicylates show little benefit for the treatment of active CD. Corticosteroids represent a highly effective group of drugs as they suppress inflammation on a wide spectrum of molecular pathways (37). Therefore, they make up the basis of treating patients suffering from IBD, especially by inducing remission in both active UC and CD. It should be emphasized that corticosteroids have numerous side-effects and are not useful for maintaining remission phases in IBD.

Immunosuppressive drugs have an effect on the immune system by preventing the activation and proliferation of lymphocytes (37). The main representatives of this group used for IBD are azathioprine, 6-mercaptopurine (6-MP), methotrexate, cyclosporine and tacrolimus. Azathioprine and 6-MP are considered beneficial for inducing and maintaining remission in patients with active CD. Cyclosporine and tacrolimus are immunosuppressant used in patients with UC during severe flares, when refractory to conventional steroid therapy.

Another powerful pharmaceutical group for IBD are the biological agents with focus on TNF alpha blockers (37). TNF-α plays a vital role as a cytokine in regulating immune cells and furthermore inducing inflammation in patients with IBD. Thus, the inhibition of this protein proves to be a powerful tool in treating IBD. Infliximab is one of the anti-TNF-α monoclonal antibodies and well established in treating both CD and UC. Hanauer et al (39) found in their randomized controlled ACCENT I study that infliximab proved to be efficacious as a maintenance treatment among patients who responded to a single infliximab infusion. In addition, infliximab proves to be useful for treating perianal fistulas in patients with CD (40). The studies ACT 1 and ACT 2 managed to evaluate the superiority of IFX in comparison with placebo as patients with UC were more likely to achieve remission and mucosal healing (41). In conclusion, in 2017 biological agents play a key role in improving the patient's life and can considerably reduce complications and the risk of undergoing surgery. Further anti-TNF strategies involve adalimumab and golimumab. In addition, vedolizumab (directed against the a4b7 integrin) and ustekinomab (directed against the IL-12 receptor) have been recently introduced in the Austrian market.

Surgical measures include the resection of intestinal segments in patients with CD as well as the proctocolectomy with ileo pouch-anal anastomosis in UC, which is often linked to a good outcome (11).

1.2 Venous thrombosis

Thrombosis is described as the formation of a blood clot inside a blood vessel, consequently obstructing the blood flow through the circulatory system and eventually leading to infarction of the tissue supplied by that vessel (42). Deep vein thrombosis (DVT) and pulmonary embolism (PE) together define the term venous thromboembolism (VTE). Arterial thromboses occur in arteries and are most commonly known for their consequences,

namely myocardial infarction and stroke. Venous thrombosis has a high incidence rate of 1 per 1000 per year and is especially lethal in elderly (43). In addition, 5 % of patients suffer from recurring thrombus formation (44).

Over the past years, numerous risk factors have been identified for developing venous thrombosis (42). They all have the alteration of blood flow, activation of the vessel endothelium as well as the increase of blood coagulability in common. Most frequently thrombus formations are located in the valve pocket sinus. Nevertheless, this clotting disease remains still incompletely understood with the precise mechanisms not being fully clarified (44). However, new treatments could be developed through understanding the pathogenesis of venous thrombosis which is of high interest to the pharmaceutical industry.

Recurrent venous thrombosis is usually linked with a lifelong anticoagulation and thereby increases health care costs as well as provides potential for serious side effects such as internal and external bleeding (45). Reducing the risk of recurrent thrombosis would lead to a minimized risk of suffering from adverse effects due to the anticoagulant therapy. The purpose of the following chapters is to outline the importance as well as the pathogenesis of venous thrombosis and its therapeutic methods.

1.2.1 Epidemiology

Venous thrombosis is a severe disease with over half a million associated deaths per annum in European countries (44). VTE is caused by the formation of a clot in the deep veins of the legs or elsewhere (46). The thrombus can be dragged into the pulmonary circulation where, depending on its size, it may be lethal. Surprisingly, despite modern treatment regimens and improved diagnostic possibilities the mortality rate hasn’t changed in the past 20 years (46). Wendelboe et al (47) conducted a global survey in order to analyze the public awareness of VTE. The data gathered from 800 respondents showed that the basic knowledge of symptoms and risks of thrombosis in general was low. Fewer people were aware of DVT and PE than of other vascular diseases such as heart attack and stroke suggesting an increase in public campaigns to prevent this thrombotic disorder.

The incidence of venous thrombosis is approximately 1 per 1000 person per year (43). A Norwegian study analyzed the residents of Nord-Trøndelag county for 6.5 years and came

to the conclusion that the incidence rates increased exponentially with the age of the patients (43). Additionally, they showed that during the childbearing years the incidence was higher in women than in men. However, at the age of 60 the incidence rate turned around and became slightly higher in men than in women. Furthermore, multiple studies have proven a substantially lower incidence in Asian countries than in Europe, the reason for the different rates still remaining unknown (44).

Cancer is known as a major risk factor for developing venous thrombosis with an increased risk by 7 fold in patients suffering from malignancies than in people without cancer (48). Especially, the first months after diagnosing the malignancy are linked to a high risk.

1.2.2 Risk factors

The awareness of risk factors leading to venous thrombosis is a necessity to prevent this disease in individuals at higher risk (49). Intrinsic patient characteristics (genetics, age, obesity, cancer) can be distinguished from trigger factors such as surgery, immobility and pregnancy. Many risks are avoidable, unlike genetic predisposition and increasing age, and are depicted in Table 1.

Table 1: Risk factors associated with the occurrence of venous thrombosis (49)

Older Age	Immobility	Hospitalization	Oral Contraceptives
Obesity / Overweight	Varicose Veins	Cancer	Long Travel
Personal History of Previous Thrombosis	Pregnancy	Myeloproliferative Disease	Genetic Factors Affecting Coagulation Balance
Family Member with Venous Thrombosis	Postmenopausal Hormone Therapy	Trauma / Injury / Surgery	Antiphospholipid Syndrome

The genetic factors can be divided into three groups, that is strong, moderate and weak (50). The first group encompasses rare diseases leading to a hypercoagulable state including deficiencies of antithrombin, protein C, and protein S. These strong genetic risk factors are only seen in 1 % of the population (50). The moderate group of genetic risks is represented by factor V Leiden, prothrombin 20210A, fibrinogen 10034T and non-type O blood. Factor

V Leiden is a common mutation affecting around 5 % of Caucasians and is found in 20 % of patients suffering from venous thrombosis (50). Individuals with O blood group have lower levels of von Willebrand factor (vWF) and factor VIII reducing the risk of venous thrombosis. Weak genetic risk factors include numerous variants that have a small effect on thrombus formation.

Acquired factors such as obesity are known to double the risk of suffering from thrombosis (51). Furthermore, venous thrombosis is most likely to occur when a combination of risk factors arises at the same time (49). A population based study investigated the interaction of factor V Leiden and the use of oral contraceptives in premenopausal women (52). They concluded that female individuals heterozygous for factor V Leiden who used oral contraceptives were extremely susceptible to venous thrombosis. The risk for these women was as high as the multiplication of each relative risk resulting in a 30-fold increase for developing venous thrombosis.

1.2.3 Pathophysiology

In general, the underlying pathophysiology of thrombus formation can be well understood by Virchow's postulated triad (Figure 2): stasis, hypercoagulability and changes in the endothelial cells of the vessels (53). Due to the ease of obtaining blood, the composition of blood and its role in developing venous thrombosis has been well studied (53). In contrast, the mechanisms of stasis and changes in the vessel wall leading to a thrombus still remain in most parts unclear. The following chapter gives an overview over the impact of the alteration of blood flow, blood composition and blood vessel wall on the clot formation.

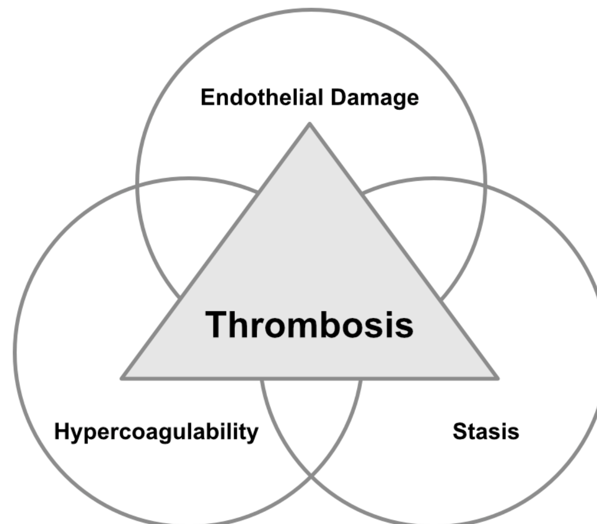

Figure 2: Virchow's triad for demonstrating the trigger factors of thrombosis

The etiological factors leading to the formation of thrombi include injury of the endothelium, reduced blood flow as well as a hypercoagulant blood composition.

1.2.3.1 Changes in the blood vessel wall

The endothelial cells of the vessel wall play a crucial role in preventing thrombus formation as they prohibit cells and proteins from attaching to the surface (42). The relationship between injury of the vessel wall and the formation of a clot is much clearer in arteries as the rupture of an atherosclerotic plaque exposes procoagulant ligands for thrombocytes initiating coagulation (53). Additionally, the core of an arterial thrombus and its connection to the vessel wall is mainly made up of platelets, whereas fibrin is the mediator attaching venous thrombi to the wall. Arterial thrombi contain many platelets, thus the name "white clot", and are therefore treated with drugs inhibiting the platelet aggregation (42). On the other hand, as venous thrombi include a lot of fibrin, the therapy consists of anticoagulant medication.

However, in an autopsy study from 1974, Sevitt et al (54) analyzed the structure of 50 venous thrombi concluding that, with one exception, there were no vessel wall injuries identified. This conclusion supports a tissue factor initiation over a platelet mediated adhesion resulting in fibrin rich thrombi (46).

Moreover, studies suggest that local inflammation is characterized by activating endothelial cells and thus initiating the coagulation cascade (53). The activation of the endothelium results in a downregulation of the anticoagulant thrombomodulin, while upregulating the expression of various adhesive molecules, such as tissue factor, P-selectin, E-selectin, and vWF (42). Subsequently these molecules bind and recruit platelets, leukocytes and microvesicles leading to the risk of developing a clot.

1.2.3.2 Stasis

Reduced blood flow in the vessels leads to an accumulation of prothrombotic substances such as thrombin (53). The aggregation of procoagulant proteases may tip the homeostasis of coagulation and anticoagulation towards thrombosis. The association of stasis and coagulation has been well established and multiple studies show an increased rate of DVT in bedridden patients (53). It has been established that paralyzed patients as well as people who travel long distances are at higher risk of suffering from VTE (42). Pregnant women are also associated with a higher rate of VTE as the fetus compresses the left common iliac vein resulting in a left sided reduced blood flow.
Numerous studies, including autopsies as well phlebographies, demonstrate that clots originate in the venous valve sinus and thus are thought to be the starting point of thrombus formation (46). The combination of hypoxia and vortical flow allow thrombi to form in the deep recess of valvular sinus. Interestingly, the rate of DVT correlates directly with the amount of valves (46).

Additionally, reduced blood flow leads to the desaturation of hemoglobin in the erythrocytes resulting in hypoxia which stimulates leukocytes, platelets and the endothelium (53). A study conducted in 1981 by Hamer et al (55) analyzed the PO_2 in valve pockets of two patients and eight dogs. They found that the blood became hypoxic quickly under static flow conditions. During the state of decreased blood flow, the endothelial cells facing the valvular pocket became hypoxic and after just two hours the first thrombi formed. Hypoxia is known to initiate thrombus formation as many pathways have been identified (53). On the one hand, reduced oxygen activates the endothelial cells as well as the release of Weibel-Palade bodies containing procoagulant vWF and P-selectin. The study of Closse et al (56) showed that the surface expression of P-selectin, a known adhesion molecule, was increased when exposed to hypoxia reaching a maximum after 90 minutes. On the other hand, hypoxia induces the

synthesis of tissue factor (TF) on macrophages as well as concomitantly releasing small membrane vesicles containing high amounts of TF (57). Tissue factor is known as an essential protein in initiating the coagulation cascade.

1.2.3.3 Hypercoagulability

Hypercoagulability makes up the last component of Virchow's triad. Thrombophilia is simply defined as a blood abnormality resulting in an increase of risk to develop thrombosis (42). There are numerous causes leading to a prothrombotic state, such as an increase in procoagulant proteins, decrease in anticoagulant proteins or decreased break down of blood clots (42). Genetic factors are well associated with hypercoagulable states such as factor V (FV) Leiden which is a disorder present in 5 % of Caucasians (50). FV Leiden is a variant of the procoagulant factor V and thereby resistant to the inactivation by the anticoagulant activated protein C. Pregnant women are also known to be more susceptible to thrombosis as they are in a transient hypercoagulable state produced by hormones (58). This has most likely evolved in order to protect women from bleeding at childbirth or in a miscarriage. Normal pregnancies are associated with a significant increase in numerous coagulation factors such as plasma fibrinogen, factors VII, VIII, IX, X, XII and vWF (58). The anticoagulant protein S is reduced in pregnancy though its contribution to an increased incidence of thromboembolism remains questionable. Middeldorp et al (59) showed that oral contraceptives significantly increase the levels of factor VII, X, fibrinogen and prothrombin. Furthermore, studies have shown that surgery is associated with an elevated expression of tissue factor by monocytes one day after tumor surgery as well as after total knee arthroplasty (42). The Austrian Cancer and Thrombosis Study (CATS) identified thrombocytosis as an individual risk parameter in cancer patients for VTE (60). High platelet counts are thought to increase the risk by 3-fold.

At last, obesity is also known as a risk for VTE as the body mass index (BMI) correlates positively with factor VII, factor VIII, vWF and fibrinogen (61). Moreover, plasminogen activator inhibitor-1 is elevated in individuals with increased BMI and thus it can be stated that obesity is linked to a hypofibrinolytic state.

1.2.4 Clinical presentation

It is important to diagnose DVT as patients are at risk of suffering from PE if they remain untreated. However, the clinical examination as well as the patient history are not reliable to diagnose DVT (62).

Common symptoms of DVT include warmth, redness, pain and swelling of the body part, most often the lower limb, evolving over a couple of days (63). Nevertheless, these signs can develop in a few hours as well as over weeks. Patients suffering from DVT may also be asymptomatic. A well-known sign was named after the physician who first described it, Homans (63). His method produces discomfort in the upper part of the calf by sudden dorsiflexion of the ankle.

However, multiple studies have shown that symptoms and signs are not useful when it comes to distinguishing between patients with and without DVT (63). The combination of symptoms and other information about the patient, such as the presence of risk factors for venous thrombosis, may be considerably more accurate for the diagnosis of DVT (63). Hence, further diagnostic methods are needed to reliably evaluate the existence of deep venous thrombosis (64).

The diagnosis of PE based on the clinical examination is inaccurate as symptoms and signs, which include dyspnea, cough, chest pain, syncope and/or hemoptysis, are non-specific (65, 66). Miniati et al (67) conducted a thorough study analyzing the clinical manifestations and their importance in diagnosing PE. The results showed that no symptom per se was reliable for the diagnosis. However, over 95 % of patients with confirmed PE showed manifestations such as tachypnea, chest pain and/or dyspnea whereas only a minor part of patients suffering from PE had no major symptoms. Thus, the signs may be unspecific but the identification is useful to do further testing for the diagnostic workup in affected patients.

1.2.5 Therapy

The therapy of venous thrombosis and its complications can be divided into short term as well as long term goals (68). Initially, it is important to prevent the detachment of the thrombus and the consequent embolization of the lung arteries. In the long run, complications such as recurring thromboses, post thrombotic syndrome and pulmonary

hypertension should be prevented and studiously avoided (69). For many years, the first line medical treatment of acute venous thrombosis and PE has been the application of low molecular weight heparin (LMWH), unfractionated heparin (UFH) and fondaparinux (69, 70). Concomitantly and following the heparin treatment, patients have taken vitamin K antagonists (VKA) for several months. The direct oral anticoagulants (DOAC) represent a new group of anticoagulants and will be presented in an own chapter.

1.2.5.1 Heparin and vitamin K antagonists

LMW heparin is known as the most frequent first line anticoagulation medication in the treatment of deep venous thrombosis (71). It can be administered subcutaneously by the patient itself, doesn't need monitoring and is therefore excellent for outpatients. Nevertheless, heparin comes in a second form: unfractionated heparin. In contrast to LMWH, UFH is most commonly administered intravenously and its blood clotting factors need to be monitored continuously (72). Van Dongen et al (72) conducted a meta-analysis in order to examine the effectiveness of both heparin forms for treating VTE. They came to the result that subcutaneous LMWH at a fixed dose is more advantageous as it is linked to a reduction of mortality and major hemorrhage in comparison to UFH. Furthermore, LMW heparin shows more benefits as it has a lower risk of developing heparin-induced thrombocytopenia which by itself increases the risk of clot formation (71). However, due to the fact that LMWH is predominantly excreted by the kidneys, UFH is preferably used in patients suffering from severe renal failure (68). In general, LMWH outweighs the advantages of UFH and therefore its use for treating DVT is well established (71).

VKA such as warfarin are used for the long term treatment of recurrent thrombotic events after initial anticoagulation with heparin has been performed (69). Though they are associated with a high effectiveness compared to placebo, the international normalized ratio (INR) needs to be monitored closely as these anticoagulants depict a narrow therapeutic range (70). The duration of warfarin treatment is dependent on the risk of a recurring deep venous thrombosis and normally varies between 3 to 6 months (68). It should be noted that the administration of VKA such as warfarin are contraindicated in pregnancy due to their teratogenic risks and thus replaced by LMWH (68).

In conclusion, the standard therapeutic regime includes 5-7 days of heparin with simultaneous administration of VKA (68, 69). The overlap of both anticoagulants is needed in order for VKA to affect the vitamin K dependent clotting factors. Heparin is discontinued after at least 5 days when the international normalized ratio reaches the therapeutic range of 2.0 to 3.0.

1.2.5.2 Direct oral anticoagulants

As already mentioned, LMWH and VKA show many advantages, however it should be mentioned that the subcutaneous administration of LMW heparin may be unpleasant for patients (69, 70). In addition, the downside of VKA is reinforced by the strict coagulation monitoring needed due to their association with major hemorrhagic complications.

Thus, the modern DOAC have been introduced. DOAC strive to be more practical with the same efficiency, can be orally administered and don't need to be monitored constantly (73). These anticoagulants, such as rivaroxaban, apixaban and dabigatran, function by inhibiting specific clotting factors of the coagulation cascade directly. Rivaroxaban and apixaban target activated factor X whereas dabigatran suppresses the role of thrombin. Multiple studies have analyzed the effectiveness and practicability of DOAC in treating VTE (70). The two large EINSTEIN studies, published in the New England Journal of Medicine, were conducted in order to compare the efficiency of oral rivaroxaban with subcutaneous enoxaparin followed by VKA for the treatment of DVT and PE (74, 75). Both studies concluded that rivaroxaban was non-inferior to the standard treatment for VTE with similar risks for major bleeding. Therefore, rivaroxaban appears to be a safe and easily administered drug for the acute and long term treatment of venous thrombosis. The second factor X inhibitor is apixaban which in contrast to the other DOAC has the lowest renal excretion (73). Agnelli et al (76) set up a large double blinded AMPLIFY study with 5395 patients comparing oral apixaban with the standard treatment regime, including LMWH and VKA. The results demonstrated, similar to the EINSTEIN study, non-inferiority of apixaban to the conventional treatment. A significant reduction of the risk for major bleeding was evaluated in the AMPLIFY study making this drug an effective alternative for treating VTE. Additionally, a meta-analysis has shown that apixaban is superior to enoxaparin for prophylaxis in orthopedic patients as it is associated with a reduced risk of bleeding and developing venous thrombosis (73). Dabigatran is characterized by its direct thrombin inhibition and is, contrary to apixaban,

mainly eliminated via the kidneys (73). Similar to the two prior DOAC, large studies named RE-COVER I and II have been carried out to identify the effectiveness compared to warfarin (77, 78). The results are again very promising proposing dabigatran as an alternative medication for treating VTE.

To conclude, these new DOAC outperform the conventional agents as they reach their full anticoagulant potential quickly, have a shorter half-life and don't need to be monitored constantly (73). Their practicality makes them an additional treatment tool for VTE. However, it should be noted that their main downside is the lack of antidotes.

1.3 Inflammatory bowel disease mouse model

In order to study the mechanisms and treatment of IBD, animal models of chronic gut inflammation are needed. Since 1957, 66 animal models have been established with the aim to study IBD and develop therapeutic interventions (79). The major breakthrough on IBD model research was the discovery of three different kinds of knockout mice. The interleukin (IL)-2, IL-10 and T cell receptor (TCR) ∝ knockout mice were able to spontaneously develop colitis (79). Knockout mice are genetically modified mice in which the expression of a gene has been delete ("knocked-out"). The process consists of inserting a modified DNA sequence into a specific genetic locus resulting in an inactive gene (80). The method of targeting genes was made possible due to the discovery of homologous recombination as well as the isolation of embryonic stem cells. Homologous recombination is a process in which nucleotide sequences are exchanged between similar DNA strands and occurs exemplary during meiosis. Thus, this genetic recombination can be used to insert an engineered mutation into a designated genetic locus of murine embryonic stem cells by using electroporation (80). After injecting these totipotent cells into a blastocyst, they are able to differentiate into various cell types.

The development of intestinal inflammation in knockout animals may lead to the suggestion that IBD is mediated by genetic mechanisms. The interleukin-10 knockout (IL-10 KO) mouse lack the IL-10 gene and therefore are able to develop a spontaneous enterocolitis after 3 months of age (79). IL-10 represents an inhibitory cytokine which plays a central role in controlling the inflammation (81). In humans, germline mutations in both IL-10 receptor genes were associated with early onset enterocolitis as well (82). In order to enhance the

speed and severity of inflammation in murine IL-10 KO models, colitis is triggered by the application of nonsteroidal anti-inflammatory drug (83). The treatment with piroxicam, for an example, produces a rapid development of severe chronic enterocolitis in IL-10 deficient animals compared to wild type (WT) mice. The IL-2 KO mice, in contrary, are characterized by the development of distal colitis, hepatitis, pneumonia and nephritis leading to the death of half the animals in the weeks after birth (79, 84). The remaining mice develop severe manifestations exhibiting chronic diarrhea, intestinal bleeding as well as rectal prolapse. The IL-2 deficiency leads to severe alterations in the murine immune system initiating an inflammatory process resembling the human UC closely (84). The intestinal inflammation is characterized by a massive lymphocytic infiltration of T and B cells in the colonic mucosa. The cytokine enhances T cell proliferation as well as suppresses excessive T cell reactions in order to obtain homeostasis (79, 85). As the IL-2 gene is located within a genetic locus susceptible for intestinal inflammations, the suggestion of its influence on IBD is strengthened. Both IL-10 and IL-2-deficient animals remain healthy when kept in a completely germ-free conditions compared to a specific pathogen-free environment (86). The characteristic suggests intestinal microbiota to be important for development of the disease phenotype. Another knockout model prone to the development of colitis involves the deficiency of T cell receptors (TCR) which is made up of alpha and beta chains (79). TCR-∝ KO animals result in the development of specific T cells producing IL-4 and consequently developing colitis. Interestingly, the animal group is associated with UC by involving various etiological characteristics. When exposing carbon monoxide to the animals, imitating smoking in humans, the intestinal inflammation appears to be inhibited (87). Additionally, by performing appendectomy which protects from UC in humans, the knockout animals show suppression of IBD development (88). The most frequently used murine model of IBD is the colitis induction with dextran sodium sulfate (DSS). DSS is composed of negatively charged glucose polymers with sulfate groups (89). For administration, DSS is mixed with drinking water until a concentration between 1 - 5 % is reached (79). When looking at the effective location, DSS mainly affects the distal part of the colon and presents with clinical manifestations including diarrhea, rectal bleeding and weight loss. Though the specific mechanisms of DSS remain unknown, the substance damages the intestinal barrier and consequently causes local inflammation (89). It acts as detergent that reduced the mucus layer and breaks tight junctions.

Multiple variants of applications exist in order to analyze intestinal injuries (79). First, it is possible to investigate acute colitis by continuously administering the mixture. The second regimen involves mice being treated for a few days with DSS and afterwards examining the recovery process as the mucosa begins to heal. The last application method comprises the repetitive treatment with DSS inducing chronic colitis. The DSS colitis model proves popular as it is easy applicable and cost-saving. A distinct feature of this model is that the gastrointestinal microbiota appear to suppress acute inflammation, whereas animals in a germ-free facility suffer from lethal intestinal bleeding after DSS treatment (90). The principal disadvantage of the DSS murine model is presented by the great variation of inflammatory severity from animal to animal (79). In fact, the extent and localization of intestinal inflammation can vary substantially among the same breed.

Various IBD mouse models exist and a selected group have been described shortly. Each model shows specific benefits as well as negative aspects. However, no model can imitate human IBD completely and thus, the right model or combined model systems should be considered (79). This diploma thesis involves the combination of an IL-10 KO mouse treated with DSS in order to trigger intestinal inflammation faster. Due to the reason that the study analyses the progress of thrombus resolution, a colitis induction with a nonsteroidal anti-inflammatory drug may result in distorted values as it influences the blood clotting. Hence, the colitis induction in our trials is achieved by the treatment with DSS.

1.4 Mouse models of venous thrombosis

Murine models analyzing the development as well as the resolution of venous thrombosis are useful to understand mechanisms of disease, and identify targets of treatment. Hence, various models have been established and have since increased our knowledge of thrombosis and thrombus resolution (91). Several approaches are focused on manipulation of the mouse inferior vena cava (IVC) (92). The IVC represents the largest vessel in the mouse. The use of the large vein is easy for ligation and yields sufficient material for analysis (91). To visualize the IVC, animals are anaesthetized and subsequently the abdominal wall is cut open in the midline (92). After moving the intestinal structures to the side, the IVC is visualized. At this point, various models exist based on the method of thrombus induction, for example plain ligation versus physical or pharmacologic endothelial injury.

Thrombosis is induced by chemicals such as ferric chloride or the photoreactive Rose Bengal (93). Administration of these substances exerts oxidative damage of the endothelium resulting in immediate occlusive thrombosis. The animal model using ferric chloride has been described over 25 years ago and was used for induction of arterial thrombosis in the carotid arteries of rats (94). When applying this method to a murine model, a small piece of filter paper is soaked with 3.5 % ferric chloride and placed on the exposed IVC (91). After a few minutes the filter paper is removed, followed by the development of a thrombus due to transmural vessel injury. The extent of thrombus size is dependent on the concentration as well as application time of the ferric chloride. Rose Bengal represents a photoreactive substance that is injected into the vein followed by the formation of oxygen radicals upon exposure to mercury light (93). The resulting injury of the endothelial layer leads to thrombus formation. The main disadvantage of both substances is the damage of the vessel wall which is not generally physiologic (91). Another model causes thrombus formation by applying an electric current. First, all lateral branches caudal the renal veins of the IVC are ligated using Prolene (95). Then, a 25-gauge needle connected to a copper wire is inserted into the IVC and a 250µA direct current is applied to the vessel wall over 15 minutes. This technique leads to the generation of free radicals which activate endothelial cells. Thrombi do not completely occlude the IVC. Electrostimulation is associated with a high survival rate and good reproducibility. Furthermore, it represents the only tool to develop a thrombus by using an internal stimulus to activate the epithelium (95). The downside is expenses for equipment and longer operation time. All IVC models closely mimic DVT in humans, both by time course and histological features.

The method described in this diploma thesis represents a subtotal ligation of the IVC (91). The classic IVC stasis model creates a total occlusion through the ligation of the IVC and its infrarenal branches. This model mimics thrombi that are completely occlusive in a clinical environment. In C57BL/6 mice, the ligation results in large reproducible clots that remain for up to 21 days (91). Thus, the method is favorable when analyzing thrombus resolution in a chronic process. The alternative ligation technique creates a stenosis (91). After performing a midline laparotomy and visualizing the IVC, a needle or suture is placed lengthwise the vessel and tied by a Prolene suture until complete stasis is reached. Subsequently, the longitudinal thread is removed allowing blood to flow through the stenosis. Varying from author to author, lateral branches are ligated to further enhance stasis and thrombus formation. A significant advantage of the stenosis model is the formation of thrombi under

existing circulation as well as intact endothelium (92). It is most widely used to analyze acute and chronic venous thrombosis as well as the effectiveness of pharmacological substances (91). A major disadvantage of this model is a variation of thrombus size.

1.5 Link between IBD and VTE

The two disorders IBD and VTE, including DVT and PE, have been illustrated thoroughly in the previous chapters. However, the connection between the diseases, which is the main focus of this diploma thesis, has not been elucidated yet. The risk of developing venous thrombosis in IBD patients is 3-fold higher than in the general population making VTE an extra intestinal complication of IBD (96). While there is still lack of awareness of the association between IBD and VTE among physicians, VTE is linked to significant morbidity and mortality in these patients (97). Furthermore, the guidelines for thromboprophylaxis in patients suffering from IBD are poorly executed due to the concerns about safeness of the pharmacological prevention.

1.5.1 Epidemiology

The risk of VTE is 1.5 to 3.5 times greater in IBD patients than non IBD patients (98). A large population-based study conducted by Bernstein et al (99) aimed to evaluate the incidence of VTE in IBD patients. The results showed that UC and CD had similar incidences for DVT of approximately 30 – 31 per 10000 person-years, whereas the incidence for PE was 14.9 per 10000 person-years (10.3 for CD and 19.8 for UC patients) (99). The overall risk for IBD patients to be hospitalized due to VTE was 3.5 times higher than in the general population, with no gender differences. Additionally, it seems that patients younger than 40 years of age were at particular risk of suffering from VTE compared with controls. Another large, more recent, cohort study has been carried out by Grainge et al (100) to quantify the risk of VTE in IBD patients with relation to disease activity. The findings showed that the patients had a distinctive increase in risk (hazard ratio of 8.4) during periods of disease flares with a higher relative risk during ambulatory periods than during periods of hospitalization. These studies seem to confirm that VTE is a specific feature of IBD (97). Furthermore, Miehsler et al (101) compared the risk of VTE in patients suffering from IBD with other chronic inflammatory diseases. They showed that VTE was specific for IBD

because neither rheumatoid arthritis nor coeliac disease were accompanied with an increased risk for VTE. Another Austrian study analyzing 2811 IBD patients demonstrated that venous thrombosis occurred more commonly as DVT than as cerebral, portal, splenic or mesenteric vein thrombosis (102). Moreover, the study confirmed previous findings that most VTEs occur in ambulatory patients. Taking a look at the mortality, Nguyen et al (103) concluded in their analysis that VTE in IBD carried a 2.1 fold higher mortality than in the absence of IBD.

To summarize, multiple studies support the hypothesis that patients suffering from UC or CD are at an increased risk for thrombosis. As discussed above, DVT and PE are more likely to occur in younger patients as well as in patients during periods of active disease (99, 100). Unfortunately, venous thrombosis in patients with UC or CD are linked to a significant mortality (103).

1.5.2 Mechanisms

Thrombosis results from a concerted action of proteases within the coagulation cascade, resulting in the formation of fibrin (42). The underlying mechanisms resulting in thrombosis can be summarized by the Virchow's triad, namely vascular stasis, hypercoagulability and endothelial lesions (53)(Figure 2, page 13). The risk factors for IBD have been analyzed thoroughly and include the combination of genetic, environmental, microbial and immunological factors (12)(Figure 1, page 3). The etiology for the increased formation of clots in IBD patients is not fully understood, however multiple risk factors have been identified (104). IBD patients are exposed to prolonged immobilization, inflammation, surgery, dehydration and steroid medication. Vitamin deficiencies are also common in patients suffering from IBD, leading to an elevated level of homocysteine in the blood which is an additional risk factor for thrombosis. Interestingly, in the majority of IBD patients at least one risk factor for DVT/PE is present (105).

Some genetic mutations of thrombosis may be present in IBD patients (106). However, available literature has demonstrated that the common genetic variants including factor V Leiden, G20210A prothrombin gene mutation and others are not more common in patients with IBD than in the general population. Nevertheless, IBD patients with a prothrombotic

genetic risk factor are at even higher risk for VTE than non IBD patients with similar mutations (106).

IBD are characterized by intestinal and systemic inflammation associated with a hypercoagulable and prothrombotic state (107). The underlying pathways leading to thrombosis are complex and not yet understood, however the hypothesis of a connection between coagulation and inflammation is substantiated (107). Numerous studies have shown alterations in quantity and quality of enzymes playing a role in the coagulation cascade (108). It has been described that the prothrombotic condition in IBD is initiated and maintained by increased levels of factor V, VII, VIII, fibrinogen as well as thrombin antithrombin complex (104). Additional abnormalities of the coagulation cascade in IBD patients are characterized by the decrease of anticoagulant factors including antithrombin III, protein C and protein S. The hypercoagulable state in CD and UC may also be explained by disturbances in the fibrinolytic system. The elevated numbers of enzymes inhibiting fibrinolysis, such as plasminogen activator inhibitor (PAI) and thrombin-activatable fibrinolysis inhibitor (TAFI), and the decrease of tissue-type plasminogen activator (tPA), the main activator of the fibrinolysis system, lead to hypofibrinolysis (104). Another mechanism leading to a hypercoagulable state is the increase in the number of circulating platelets, so called secondary thrombocytosis (107-109). Platelet hyperactivation is thought to be mediated by elevated surface expression of CD40 ligands (110). Plasma of IBD patients shows significant increases of soluble CD40L levels compared to healthy individuals which is associated with increased risk for vascular events. Thus, it has been established that the activation of the CD40 pathway results in an increased risk of developing VTE (108, 110). Recent studies have linked secondary thrombocytosis in IBD to iron deficiency, which is found in up to 60 % of patients with IBD (111, 112). This is most surprising because iron deficiency leads to G1-arrest of cells. Other mechanism may involve increase in megakaryopoiesis, megakaryocyte ploidy, HIF2alpha stabilization and VEGF (113). Secondary thrombocytosis has been identified also as independent risk factor for VTE in patients with cancer (60). It is intriguing to speculate that secondary thrombocytosis as a response to iron deficiency should counteract chronic intestinal blood loss to prevent potentially lethal intestinal blood loss.

Last, activation of the endothelium has been proposed as a trigger of clot formation in patients suffering from IBD (108, 114). Under physiological conditions the vascular

endothelium has a thrombo-resistant surface and prevents clot formation. However, when damage occurs the endothelium transforms into an procoagulant surface. Proinflammatory cytokines, including IL-1, TNF-α and others induce the expression of cell adhesion molecules on the endothelial cells, such as VCAM-1, ICAM-1 and PECAM-1 which promote the acquisition of leukocytes (114). VCAM-1, ICAM-1 as well as vWF and thrombomodulin are markers of endothelial injury and are increased in IBD patients (104, 114). Furthermore, TNF-α prevents the breakdown of asymmetric dimethylarginine (ADMA), an endogenous NO synthase inhibitor, resulting in decreased nitric oxide generation (115). ADMA is elevated in IBD patients and is known to induce endothelial dysfunction by impairing vasodilatation and enhancing aggregation of platelets (116).
Factors leading to damage of the vascular endothelium are hypothesized to increase the risk of developing venous thrombosis in IBD patients (108). Despite numerous studies reporting on abnormalities and alterations in the coagulation system and hemostasis, the major cause for the increased risk of VTE remains unknown (104). The etiology is said to be multifactorial as no single cause has been found to date.

1.5.3 Management

Management of VTE in IBD patients includes primarily prophylaxis and secondly the treatment of thromboembolic events as thrombosis complicates the course of IBD (104, 114). As mentioned in the previous chapter, VTE in IBD is often associated with general acquired risk factors such as prolonged immobilization, surgery, dehydration and nutritional deficiencies. Thus, non-pharmacological prophylaxis can be achieved by reducing these prothrombotic risks regardless of whether the patient is treated with pharmacological prophylaxis (97). The supplementation of vitamin B6 and vitamin B12 results in a normalization of homocysteine plasma levels, which when elevated has been shown to induce thrombosis (104). Furthermore, adequate hydration as well as early mobilization after surgical procedures should be sought in hospitalized patients suffering from IBD (97). In addition, although no direct evidence exists, controlling the disease activity of IBD is thought to reduce the risk of VTE due to the decrease of procoagulant factors associated with the inflammation (117). Advantageously, numerous anti-inflammatory drugs, including mesalazine and azathioprine, used in the treatment of IBD have in vitro some anticoagulant characteristics (97). Furthermore, a retrospective study conducted by Higgins et al (118) analyzed 15,100 IBD patients in order to investigate the risk for VTE in two different

treatment regimes, namely biological agents versus corticosteroids. The results showed that biologicals were linked to a reduced risk of developing VTE events in comparison to patients receiving a corticosteroid therapy. The antithrombotic effect of biological agents, however, remains unclear.

To date, the most potent pharmacological prophylaxis of VTE includes the anticoagulation of IBD patients (117). This is demonstrated perfectly as all guidelines by scientific societies for the prevention of thrombosis in these patients (European Crohn's and Colitis Organization, American College of Gastroenterology, and Canadian Association of Gastroenterology) suggest primarily anticoagulants, such as UFH, LMWH or fondaparinux (119-122). However, the use of prophylactic anticoagulation is contraindicated in patients with severe gastrointestinal bleeding leading to hemodynamic instabilities. In this case the use of mechanical thromboprophylaxis, such as intermittent pneumatic compression, is preferred (119). Interestingly, there have been no randomized controlled trials carried out in order to investigate the efficacy of anticoagulant treatments to reduce the risk of developing thrombosis in IBD patients (117). Scarpa et al (123) conducted a study to analyze the effectiveness of standardized LMWH in IBD patients who underwent intestinal surgical procedures. In contrast to the guidelines, they showed that the anticoagulation regimen was not effective in patients suffering from CD or UC. However, the study lacked a control group and further testing with weight adapted LMWH dose would be advisable. Although evidence on the effectiveness of anticoagulation in reducing DVT is scarce, pharmacological thrombosis prevention remains the advocated therapy regime (97). The implementation of antithrombotic prophylaxis in the clinical setting is not well established despite the similar guideline recommendations among multiple societies (117). Ra et al (124) managed to show in their retrospective study analyzing 974 IBD admissions that patients were more likely to receive pharmacological VTE prophylaxis on the surgical service (96 % of patients), whereas on the gastroenterology service only half of the patients received prophylaxis. It is thought that the reasons for this diversification is due to the standardized protocols on the surgical service and the concerns of gastroenterologists about safety in IBD patients with rectal bleeding. Yet, the results showed that none of the patients who presented with rectal bleeding developed major bleeding while on thromboprophylaxis (124).

When encountering a thromboembolic event, the treatment regimens are similar between IBD and non-IBD patients (114). Initially, venous thrombosis is treated with a parental

anticoagulant drug, such as UH or LMWH, as bridge to warfarin, a VKA (117). When the VKA creates an international normalized ratio (INR) of 2 to 3, the initial drug (UH or LMWH) is discontinued. Anticoagulation with warfarin is extended for 3 to 6 months, depending on the risk factors for recurring VTE or major bleeding of the patient. The initial therapeutic steps with heparin and bridging are mostly done in a clinical setting (117). The duration of warfarin therapy, however, presents a highly discussed issue ranging from 3 months to lifelong as it is known that IBD patients have also an increased risk of recurrent VTE (114, 125). Nguyen et al (126) performed a study in IBD patients comparing 6 months with extended anticoagulation after a first episode of VTE. The results suggested that extended anticoagulation may be more beneficial than anticoagulant treatment over 6 months in patients with unprovoked VTE and absence of active disease. The risk of recurring thromboembolic events in patients without anticoagulation outweighed the risk of suffering from major bleeding while on anticoagulant drugs. Additionally, in non IBD patients, thrombolytic therapy and catheter-directed thrombolysis are possible treatment procedures especially in severe and life-threatening VTE (114). Tabibian et al (127) concluded from their study, analyzing 52 cases of VTE in IBD patients, that catheter-directed thrombolysis could become a potential therapeutic approach as the patients tolerated the intervention well. DOAC represent effective replacements of VKA, particularly in the outpatient setting, however, we still lack knowledge of these drugs on large scale patient populations (117).

One more important issue seems to be iron deficiency in this population, specifically when taking into account the association of iron deficiency and secondary thrombocytosis as well as the triggering of ongoing blood loss through long-term anticoagulant therapies. Parenteral iron therapy, however, is well established in IBD patients and can maintain iron homeostasis despite chronic blood loss (128). Its potential effect on the prevention of recurrent VTE has not been studied so far.

Multiple studies have depicted the higher risk of IBD patients developing VTE (96, 98, 99). Despite guideline recommendations, the knowledge of physicians, in particular gastroenterologists, on anticoagulant treatments in IBD patients is scarce (124). Thus, main focus should be laid on the successful implementation of thromboprophylaxis in these high risk patients in order to prevent thrombosis and its complications (117).

2 Materials and methods

The goal of this diploma thesis is to develop a mouse model for studying the formation and resolution of venous thrombi under conditions of intestinal inflammation. To better understand the mechanisms, we combined a well-established mouse model of chronic enterocolitis with a model of thrombus formation. Such model will help to better understand the mechanisms leading to venous thrombus formation or resolution and to test preventive measures as well as therapy regimen with relation to thrombogenesis.

2.1 IL-10 KO mice and WT controls

The laboratory animals used for this project were 8-week old female IL-10 KO mice (on a C57BL/6 background), which were bred under specific pathogen-free conditions at the Department of Laboratory Science and Genetics in Himberg, Austria, and weighed around 20 g at arrival. For breeding, homozygous animals were mated. The control group consisted of 8-week old female C57BL/6 WT mice, again weighing approximately 20 g. Over the course of the trials the animals were always maintained in the Biomedical Research Department under conventional housing conditions. The experiments were conducted in accordance with the criteria of the Good Laboratory Practice guidelines defined by the Medical University of Vienna. The protocol was approved by the animal ethics committee.

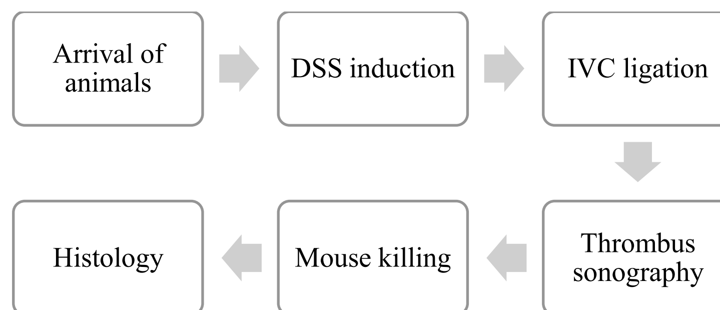

Figure 3: Procedural steps during the pilot study

The pilot study was composed of two groups, an IL-10 KO group and a WT control group (C57BL/6 strain), each consisting of 5 mice with a total of 10 animals. The two different breeds were placed in two individual cages with same build and characteristics. After the arrival of the animals from the breeding center in Himberg, their ears were pierced in order

to differentiate between each individual mouse. Next, they were left alone for a timeframe of 7 days to adapt to the new location at the Biomedical Research Department. Over the course of the study, mouse weights were monitored according to the severity of colitis (Table 2).

2.2 Dextran sodium sulfate induction

In order to trigger intestinal inflammation, DSS was administered to all animals. DSS is a water soluble polysaccharide, which ordinarily is administered with a molecular weight of approximately 40 - 50 kDa (129). DSS is thought to damage the epithelial barrier and increase intestinal permeability resulting in translocation of intestinal contents and consequently inflammation.

DSS was administered to both groups, the IL-10 KO and WT controls, over a period of 5 days (Monday – Friday). On the first day of administration, the DSS salt (MP Biomedicals) was weighed and mixed into 500 mL of autoclaved water at a concentration of 1.7 % and stirred until a clear solution was achieved. Subsequently, the drinking water was exchanged for DSS solution and placed in the cage with each group receiving it *ad libitum*. Each day around the same time the remaining amount in the bottle was measured. In the middle of the week (Wednesday) DSS powder was once more mixed with sterile water and replaced the old mixture. On day 5, the DSS water was removed from the cage and the bottles were filled again with plain drinking water.

Over the course of the pilot study, disease activity was monitored (Table 2). The disease activity index (DAI) is a commonly used score for measuring the clinical degree of colitis. It includes weight loss from starting weight, stool consistency and rectal bleeding (130). Furthermore, our modified score involved the presence of proctitis by evaluating the extent of rectal prolapse. The scoring system ranges from 0 to 4 as depicted in Table 2. Each parameter was measured and documented on a regular basis. Trend over time was analyzed.

Table 2: Disease Activity Index for scoring the extent of intestinal inflammation macroscopically

Score	Weight Loss	Stool Consistency	Bleeding	Proctitis
0	< 1 %	solid pellets	Negative	negative
1	1 - 5 %			
2	5 - 10 %	loose stool	positive	minimal prolapse
3	10 - 15 %			
4	> 15 %	diarrhea		overt prolapse

Next, mice were allowed to recover from DSS stress for 72 hours. Following the recovery phase, the ligation of the IVC was carried out. During the process of colitis induction, all animals had adequate access to standard animal chow. Furthermore, the animal care attendants cleaned the animal cages on a regular basis and looked after the well-being of the mice.

2.3 Ligation of inferior vena cava

To achieve the formation of thrombi, a standardized ligation of the IVC was performed. The vena cava ligation model depicts a distinctive method as the endothelial layer of the vessel remains intact (92). The thrombus forms due to the decreased blood flow.

In our murine model, the IVC was ligated 72 hours after the end of DSS treatment. Initially, a mixture of medetomidine, midazolam, fentanyl and ketasol was administered intraperitoneally in order to adequately anesthetize the animals. A midline laparotomy was performed and the infrarenal region of the IVC was sought. A 5-0 Prolene suture was placed lengthwise the vessel and tied by a 4-0 silk suture around the IVC including the Prolene resulting in a complete stenosis. Subsequently, the 5-0 Prolene was removed allowing a small blood flow to pass through the stenotic vessel. The final step included the suturing of the abdominal wall with 4-0 Vicryl. The complete surgery took place under sterile conditions and averaged 20 minutes per mouse depending on the difficulty of the preparation of the IVC. The thrombi were expected to form within hours after the ligation. Lastly, the animals received an analgesic mixture containing piritramide for 5 days to reduce the pain caused by the surgery and colitis.

2.4 Sonography of inferior vena cava

Sonography of the thrombi forming in the IVC represented a main outcome measurement of the study. Sonography was performed at the Preclinical Imaging Laboratory (PIL) according to Aghourian et al (131). We used the preclinical Vevo 2100 developed by VisualSonics, which is optimized for small animal research. On the first day after ligation of the IVC, each mouse was carefully taken from the cage, anesthetized and examined. Initially, the mouse was sedated with 5 % isoflurane gas in a closed chamber. As soon as the animal seemed motionless, the inhaling dose was reduced to approximately 2 %. Following, the animal was placed on the heating pad to prevent a drop of body temperature. As the eyes were susceptible to drying out during anesthesia, they were covered with ophthalmic ointment. While the mouse received isoflurane gas through a tube, it was fixated lying on its back with adhesive tape on electrodes positioned at each limb. The electrodes were able to take a real-time electrocardiogram and measure the breathing rate. Subsequently, the abdominal hair was shaved and preheated ultrasound transmission gel was applied as it is a necessity for transmission of ultrasound waves. For optimal imaging results, we used the high frequency linear array transducer MS550 with frequency ranges of 32-56 MHz. Sonography uses sound waves reflected from internal structures to measure differences in tissue densities and visualize them accordingly (131). Thrombus in the animals was mostly well identifiable as the clot was mainly composed of fibrin, platelets and compressed cellular components resulting in a different density than the surrounding blood flow.

The first step of sonography included a transversal view of the abdomen, followed by visualization of the IVC and aorta. The plate on which the animal was fixated allowed movements in x, y and z axis. After detecting the IVC, the plate was moved along the axis to analyze the range of the thrombus in the vessel and as a result finding the ligation site. At sight of the first thrombus part shortly distal the ligation site, an optimal freeze image was taken. The software of Vevo 2100 allowed to manually trace the border of the thrombus and to calculate the cross-sectional area of the clot from the data obtained. The working plate was then moved further distal of the stenosis along the axis and re-measurements of the clot were performed at each millimeter until the thrombus was not identifiable in the IVC. The Doppler mode registers the motion of blood cells using the Doppler effect and was especially used when a thrombus exposed as a difficulty to distinguish from surrounding flow. The software recognizes the direction of blood flow in relation to the transducer and assigns

colors accordingly. To better identify the extent of a clot, careful compression was applied with the ultrasound probe. Veins that didn't contain thrombi were, as a result of the high vessel elasticity, completely collapsible whereas existent coagulum stabilized the IVC. After the cross-sectional areas were calculated in the transversal view, Vevo software created an image series for 3D rendering of the thrombus. Lastly, the ultrasound probe was rotated 90 degrees in order to visualize a longitudinal view of the IVC. Again an optimal freeze image including the complete length of the clot was captured, followed by the calculation of the distance between ligation site and tail of thrombus.

The duration of the procedure including preparation, sedation and sonography averaged around 30 minutes per mouse. ECG parameters as well as the breathing rate were controlled consistently throughout the examination and if necessary adaptions, such as reducing isoflurane gas, were carried out instantly. After the sonographic imaging transmission gel was wiped off, and the animals placed back in the cage.

Consecutive ultrasound of the IVC was performed on each mouse. The process was repeated on days 1, 3, 7, 14, and 21 after ligation, and at each point the length as well as cross-sectional area for every millimeter of thrombus was noted. These parameters made it possible to calculate the volume by multiplying the length with the cross-sectional area. Furthermore, the change of thrombus volume was easily computed as the volume data were collected.

2.5 DNA extraction, real-time PCR and gel electrophoresis

In order to verify the loss of IL-10 in KO animals, tail samples of three mice per cage were collected. Further procedures involved the isolation of DNA, amplifying specific sequences and analyzing the DNA fragments by using gel electrophoresis. The isolation of the nucleic acids from the tail sample was facilitated by the Maxwell 16 Mouse Tail DNA Purification Kit by Promega. Polymerase chain reaction (PCR) was carried out on the ThermoScript PCR system by Thermo Fisher Scientific using a modified protocol. The amplification method is a revolutionizing product that allows to produce millions of copies of specific DNA sequences in a short frame of time (132). Primers (Table 3) used for the reaction allowed to amplify a specific portion of the DNA in order to differentiate between WT and IL-10 deficient mice. The primers were specific for IL-10 wild type or a neomycin resistance

cassette. Cycle conditions of the PCR are depicted in Table 4. Lastly, gel electrophoresis was performed to visualize the replicated DNA sequences.

Table 3: Primers with 5' - 3' sequence used for DNA genotyping

Primer	Sequence 5' – 3'
IL-10 T1.4 Sense	GCCTTCAGTATAAAAGGGGGACC
IL-10 T2.2 AS	GTGGGTGCAGTTATTGTCTTCCCG
IL-10 KO Neo5 AS	CCTGCGTGCAATCCATCTTG

Table 4: Cycle conditions used for PCR DNA genotyping

Cycle steps	Temperature (°C)	Time
Initiation	94	4 min
Denaturation	94	30 s
Annealing	64	30 s (12 cycles)
Elongation	72	35 s
Denaturation	94	20 s
Annealing	58	30 s (12 cycles)
Elongation	72	35 s
Amplification	72	5 min
Hold	4	∞

2.6 Blood analysis

On day 21 after ligation, following the last sonography, the murine cages were brought to the autopsy room. The anesthesia was carried out as a standard procedure by injecting 100 mg/kg ketamine and 5 mg/kg xylazine into the peritoneal cavity. After sedation, a lethal heart puncture was performed on each mouse and blood was drawn for further tests. The test tubes were prefilled with ethylene diamine tetra acetic acid (EDTA) preventing clot formation in the vacutainer. The animal was then terminally killed by cervical fracture using a surgical forceps. The subsequent extraction of internal organs was performed post mortem. Blood samples were immediately brought to the animal facility and a complete blood count was measured. The blood analysis included parameters for evaluating the severity of the

intestinal inflammatory process, i.e. thrombocyte count, hemoglobin levels as well as white blood cell count.

2.7 Histological examination of thrombus

After the animals had been sacrificed, organs, including the intestine, spleen and kidneys were removed for further investigation. Additionally, the thrombus which had formed (and resolved) in the IVC over the last three weeks was extracted. The initial step included a midline laparotomy and visualization of the exposed abdominal cavity. After taking out the organs of the peritoneum, the complete IVC was identified and dissected from the surrounding tissue. Carefully we tried to sense the thrombus through the vessel wall identifying the stretch of the clot inside the vein. Ideally the region of the IVC containing the thrombus was removed from the abdominal cavity. In the laboratory further processing was conducted to precisely strip off as much unneeded tissue as possible yielding a minor part of the vena cava and its clot. The thrombus was weighed and its length was measured. The material was placed in 10 % buffered formaldehyde for preservation and was further processed for histological analysis. First, the preparation was dehydrated in an ascending alcohol series. Next, the thrombus was soaked in paraffin wax and embedded in order to produce thin slices with a microtome.

After cutting the paraffin blocks, the thrombus underwent a staining process. In our project, we used a modified trichrome staining protocol for optimal quality. After incubating the sections, they were hydrated in a descending alcohol series and washed with specific stain mixtures as seen in Figure 4. Lastly, the histological preparations were dehydrated again, placed on an object slide and embed in Eukitt, a mounting medium for microscopy. For the microscopic evaluation, the specimens were colored using a modified trichrome staining process, combining Masson's trichrome and Verhoeff's stain (133). Thus, the protocol is able to differentiate fibrin, elastic fibers, and collagen. The combination of biebrich scarlet, ponceau 2r and acid fuchsin stains mature fibrin resulting in a red color. Early fibrin, on the other hand, and erythrocytes are visualized through the fast yellow stain. Collagen is represented by a green color.

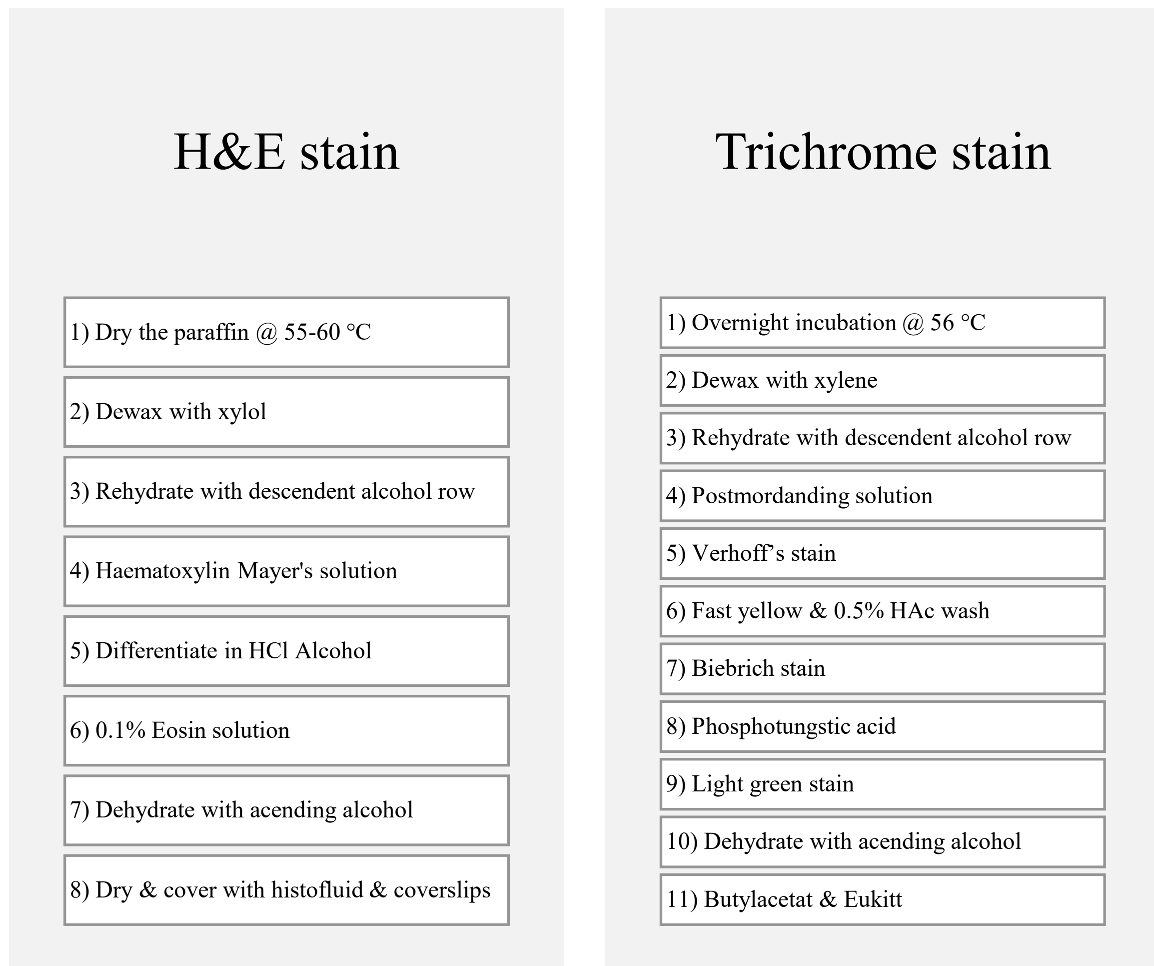

Figure 4: Staining processes for intestines (H&E stain) and thrombi (trichrome stain)

For histological thrombus analysis, the Olympus BX 50 microscope and the imaging software Axio by Carl Zeiss Vision was used. The software allowed measurement of thrombus area and consequently compute important arithmetic data. Initially, the IVC was optimally visualized on the screen and the border of the clot was traced with the cursor. The calculated parameters included cross-sectional area, perimeter and maximum diameter and were collected for each clot sample.

2.8 Histological examination of intestines

The severity of intestinal inflammation was analyzed clinically using the disease activity index (Table 2, page 31). Nevertheless, for precise information about the extent of inflammatory processes taking place in the intestines of the mice the histological analysis was inevitable. After the killing the pylorus of the stomach was identified and cut off. The intestinal structures were traced towards the rectum and carefully separated from the

surrounding tissue. When reaching the rectum, the colon was segregated delicately from the anus. Following the excision of the anus, the intestines were flushed with phosphate buffered saline (PBS) and coiled up to Swiss roles (Figure 5) allowing to study a large portion of the organ (134). The organs were fixed in 10 % buffered formalin solution and embedded in paraffin wax. After cutting 3-5 µm thick slides with the microtome, the specimens were stained using the hematoxylin and eosin stain (H&E stain), which remains one of the principal methods in histology that has been used over a century (135). Hematoxylin is depicted as a blue-purple color and stains nuclei of cells, whereas eosin, as a counterpart, colors proteins non-specifically in various shades of red. The specific schema used in our project is represented in Figure 4 (page 36). At first, the tissue was rehydrated with a descending alcohol series, followed by the staining with Mayer's hematoxylin and eosin solution. After dehydrating the slides in an ascending alcohol row, they were dried and similar to the trichrome staining covered with a mounting medium.

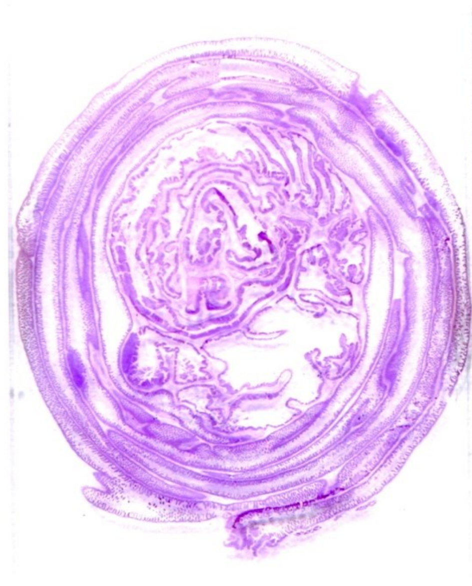

Figure 5: Specimen containing murine Swiss role after H&E staining

The figure shows a macroscopic specimen of large and small intestine aligned in a circle, so called "Swiss role". The method facilitates histological analysis.

The microscopic evaluation was conducted with an Olympus BX51 microscope equipped with the imaging software CellSens (Olympus). For scoring of the intestinal inflammation, the pilot project made use of two described methods (83, 136). Berg et al (83) investigated IL-10 deficient mice treated with nonsteroidal anti-inflammatory drugs and graded the

intestinal inflammation according to the number of lesions and their severity. A score of 0 represented a normal tissue, whereas grade 4 was marked by intense inflammation, ulcerations and crypt abscesses. Cooper et al (136) scored the inflammatory process from 0 to 4 and shows similar characteristics as the grading system described by Berg et al. However, the last-mentioned evaluation focuses more on the extent of tissue destruction and less on the various inflammatory cells involved.

2.9 Statistical analysis

Data was analyzed using SPSS (version 23.0) on Mac OS. The data collected from sonography and various other findings were inserted into the SPSS software and plotted as diagrams. No statistical tests were performed due to the nature of a pilot study with small number of animals.

3 Results

3.1 Animal characteristics

The pilot study initiated with two groups each consisting of 5 female mice weighing about 20 g. When arriving at the Medical University of Vienna, the animals had reached an age between 7 and 9 weeks. The mean weight on the day of arrival was 19.2 [18.3 - 19.9] grams for the IL-10 KO. The WT group had a comparable weight of 19.9 [19.1 - 21.1] grams. The weight trend over the course of the experiment is represented in Figure 6. The weight increased even during the 5-day period of colitis induction with DSS and dropped 16.4 [11.1 - 19.3] % for the WT group and 7.2 [4.4 - 9.2] % for the IL-10 KO group shortly after. Surprisingly, the weight of the WT group was affected more seriously. Both animal groups reached the lowest point on the day following the surgery with ligation of the IVC. Furthermore, the WT mice showed signs of rectal bleeding, proctitis and loose stool during induction of colitis. The IL-10 KO mice, in contrast, didn’t expose any manifestation of proctitis over their whole lifetime. It was unexpected that the control group responded stronger to DSS as both cages received the same concentration of DSS in the drinking water.

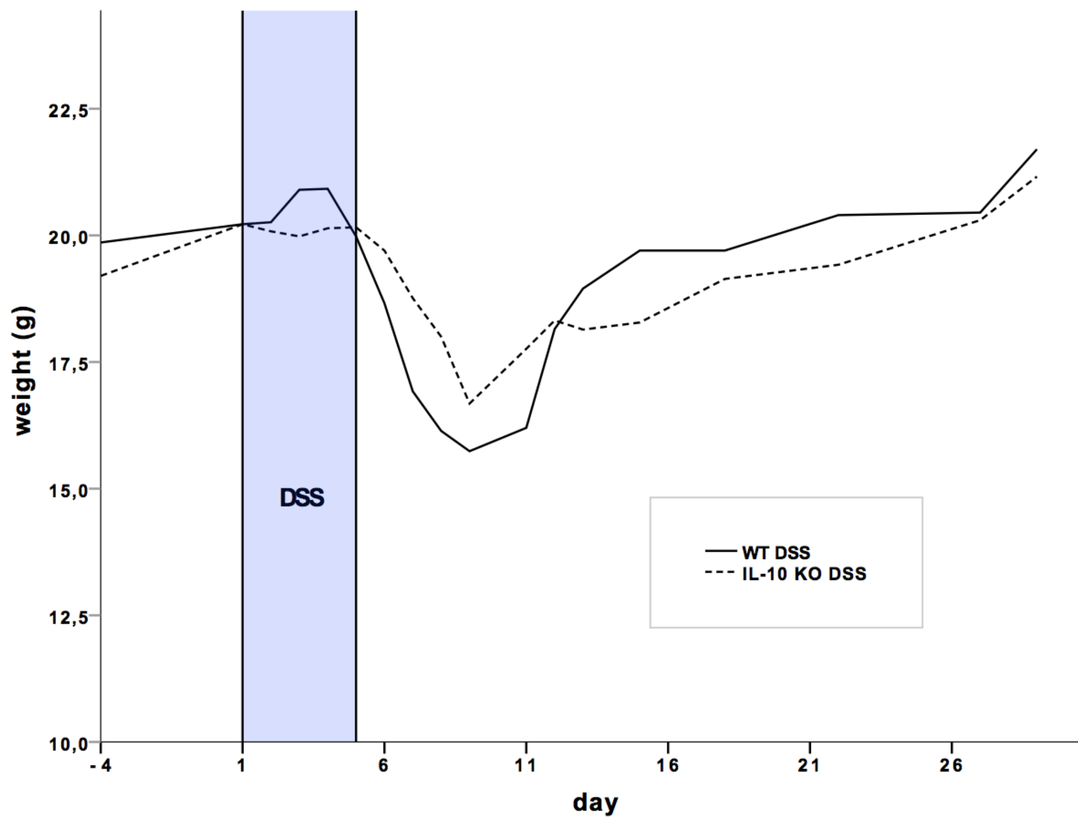

Figure 6: Mean animal weights throughout the study

The first measurement was taken five days before the induction of colitis with DSS. On day 8 the ligation of the IVC was achieved. The animals were weighed each day in the first week and three days after ligation.

When measuring the absolute amount of water consumption, the WT cage appeared to have drunk significantly more. At the end of the induction week, the WT animals had drunk 205 mL of DSS water, whereas the IL-10 KO had only consumed 70 mL of the mixture.

Three WT animals died within the first week following the surgery, i.e. day 2, 3 and 4 after ligating the IVC leaving solely two WT mice who were euthanized on the last day of examination. The factors leading to the premature death as well as a potential prolongation of the recovery phase will be discussed.

Interestingly, DSS and surgery did not cause any death in the IL-10 KO mice. This unexpected observation warrants a test for the according genetic designation of the animals (as described below). Nevertheless, the weight curve (Figure 6) clearly shows an excellent recovery of the two remaining WT mice. While the IL-10 KO mice didn't show such strong

symptoms as the WT mice and lost less weight, it seemed they had more difficulties in regaining health over the weeks following the IVC ligation. All mice were able to regain their weight. On the day of euthanasia, the animal weight averaged at 21.7 [20.4 - 23] grams for the WT and 21.2 [19.7 - 22.2] grams for the IL-10 KO mice.

As mentioned earlier, during the DSS treatment both groups were scored according to their symptoms in different degrees of severity (Table 2, page 31). On day 5 of DSS induction, the WT group averaged a DAI score of 1.4 [0 - 2] compared to 0.6 [0 - 1] for the IL-10 KO animals. Both groups showed phases of diarrhea and loose stool in particular on the days following the DSS treatment. On the day of ligation, all WT animals scored the maximum DAI of 4, while all IL-10 KO were assigned a score of 3. Furthermore, rectal bleeding was observable in the control animals at that time. As mentioned before, the animals were able to regain health and ultimately reached a score of 0 for the WT group and 0.2 [0 - 1] for the IL-10 KO on the day of euthanasia.

Overall, to outline the health trend of the animals, the WT group appeared to be affected worse than the IL-10 KO mice reaching DAI scores up to 4 compared to a maximum score of 3 in the IL-10 KO group.

3.2 Thrombus development and resolution

A major part of this pilot study consisted of the observation of thrombus formation with a sonographic imaging technique. The sonography was performed on all mice on the 1st, 3rd, 7th, 14th and 21st day following the ligation of the infrarenal region of the IVC. The animals were sedated with isoflurane gas and immediately after examined in order to keep the narcotization time as short as possible.

In both mice the clots developed on the first days (Figure 7, panel A + B) after surgery distal of the ligation site where the blood flow was reduced. Over the course of weeks, the thrombi decreased in size and in WT mice even dissolved at some point. The measured and analyzed parameters included thrombus area, volume, maximum area and change of volume over time.

The first day of sonographic examination was on the day after ligation and appeared to be quite demanding for the animals as they didn't recover from the surgery yet. Some mice were sedated easily by the isoflurane gas and as a result the concentration had to be reduced more often than later examination days. The breathing rate and heart rate were monitored closely. For an optimal anesthesia, a respiratory rate of 55 - 70 breaths per minute was targeted.

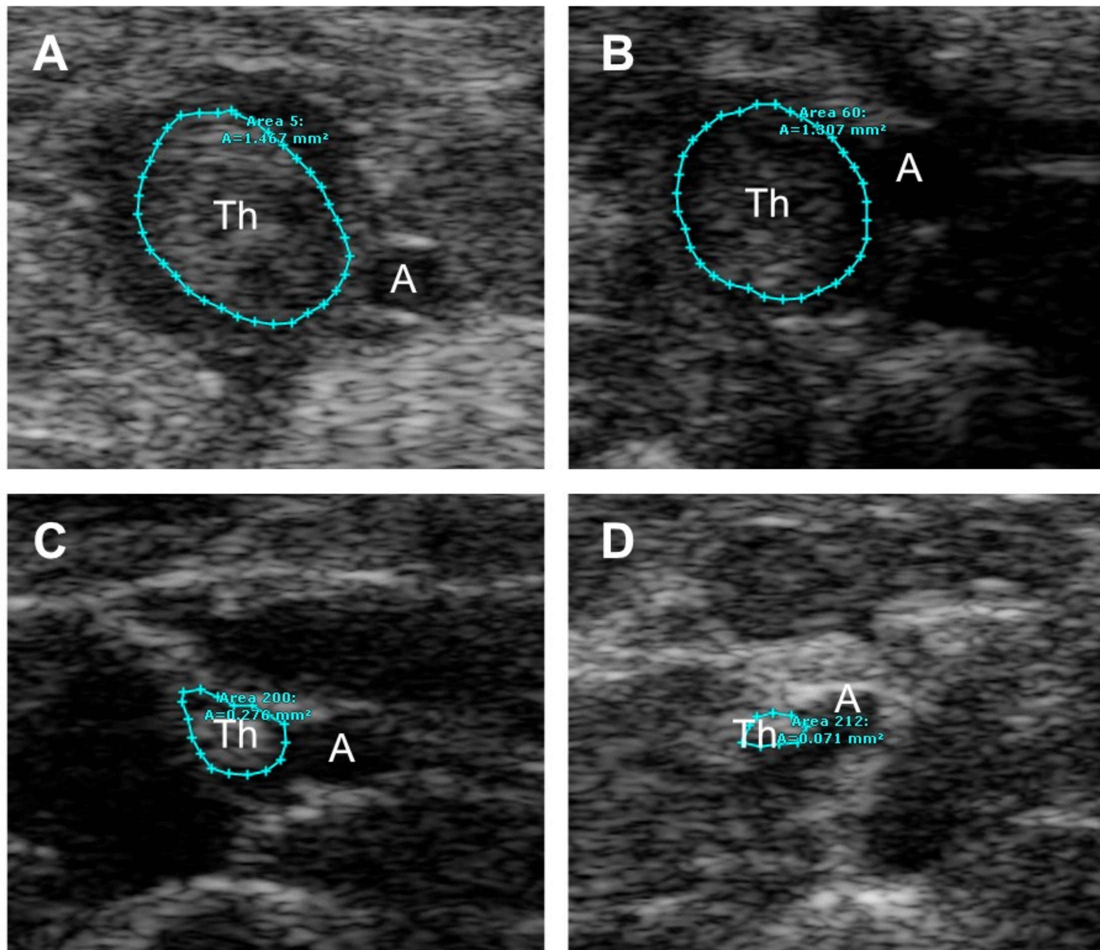

Figure 7: Sonographic examination of WT and IL-10 KO mice on the 1st and 21st day after ligation

A-D, transversal ultrasound view of IL-10 KO (panel A + C) and WT (panel B + D) animals. Each panel visualizes the aorta (A) as well as the thrombus (Th) inside the IVC. Panel A depicts an IL-10 KO thrombus with a size of 1.467 mm^2 on the first day of sonography after ligation. A WT thrombus of the same day is shown in panel B sizing 1.307 mm^2. Panel C (IL-10 KO) and D (WT) present remaining thrombi on the 21st day after surgery.

The sonography on the first day after ligation depicted evolved thrombi in all mice. When calculating the mean value, the IL-10 KO group showed clots with a slightly larger cross-

sectional area (WT: 1.03 [0.79 - 1.21] mm^2; IL-10 KO: 1.16 [1.03 - 1.37] mm^2), whereas the clots of the control turned out to be longer and hence plotting greater volumes (WT: 5.50 [2.11 - 8.24] mm^3; IL-10 KO: 5.25 [3.51 - 8.67] mm^3). The differences between the groups, however, are not significant and volumes ranged from 2.11 to 8.67 mm^3.

The next sonography examination was performed on the third day following the ligation at which two WT mice had already deceased. At this point the thrombus volume (WT: 4.63 [2.48 - 6.43] mm^3; IL-10 KO: 5.75 [3.57 - 11.32] mm^3) and area (WT: 0.94 [0.73 - 1.04] mm^2; IL-10 KO: 1.19 [0.75 - 1.70] mm^2) had plateaued with small variations between each group.

At the third sonographic examination a week after ligation, sonography was only able to be performed on two remaining WT mice of the control group and 5 IL-10 KO mice. At this point of time, the length (4.93 [3.45 - 6.41] mm), cross-sectional area (0.73 [0.70 - 0.75] mm^2) and volume (3.62 [2.43 - 4.81] mm^3) of the thrombus were already decreasing for the WT group. In contrast, the named variables (length: 4.79 [3.76 - 6.02] mm; area: 1.07 [0.81 - 1.23] mm^2; volume: 5.17 [3.45 - 7.21] mm^3) of the IL-10 KO mice seemed to continue plateauing.

At the next sonographic exam 14 days post ligation, the volumes dropped in both groups (WT: 1.44 [0.59 - 2.29] mm^3; IL-10 KO: 1.30 [0.43 - 2.47] mm^3; Figure 8). Interestingly, the thrombus area of one WT mouse was assessed bigger than the week before (0.70 -> 0.88 mm^2).

The last sonography took place three weeks after the surgery and showed the most astonishing results. While the clots of the two WT mice were hardly perceptible, the thrombi of the IL-10 KO mice were still well distinguishable. The clots of the IL-10 KO group averaged a volume of approximately 0.81 [0.28 - 1.37] mm^3 which represented a mean value more than twice as high as in the WT group (0.31 [0.20 - 0.42] mm^3). To summarize, within 24 hours after the ligation procedure thrombi of similar size had formed in both groups and thrombi resolution seems delayed in the IL-10 KO group.

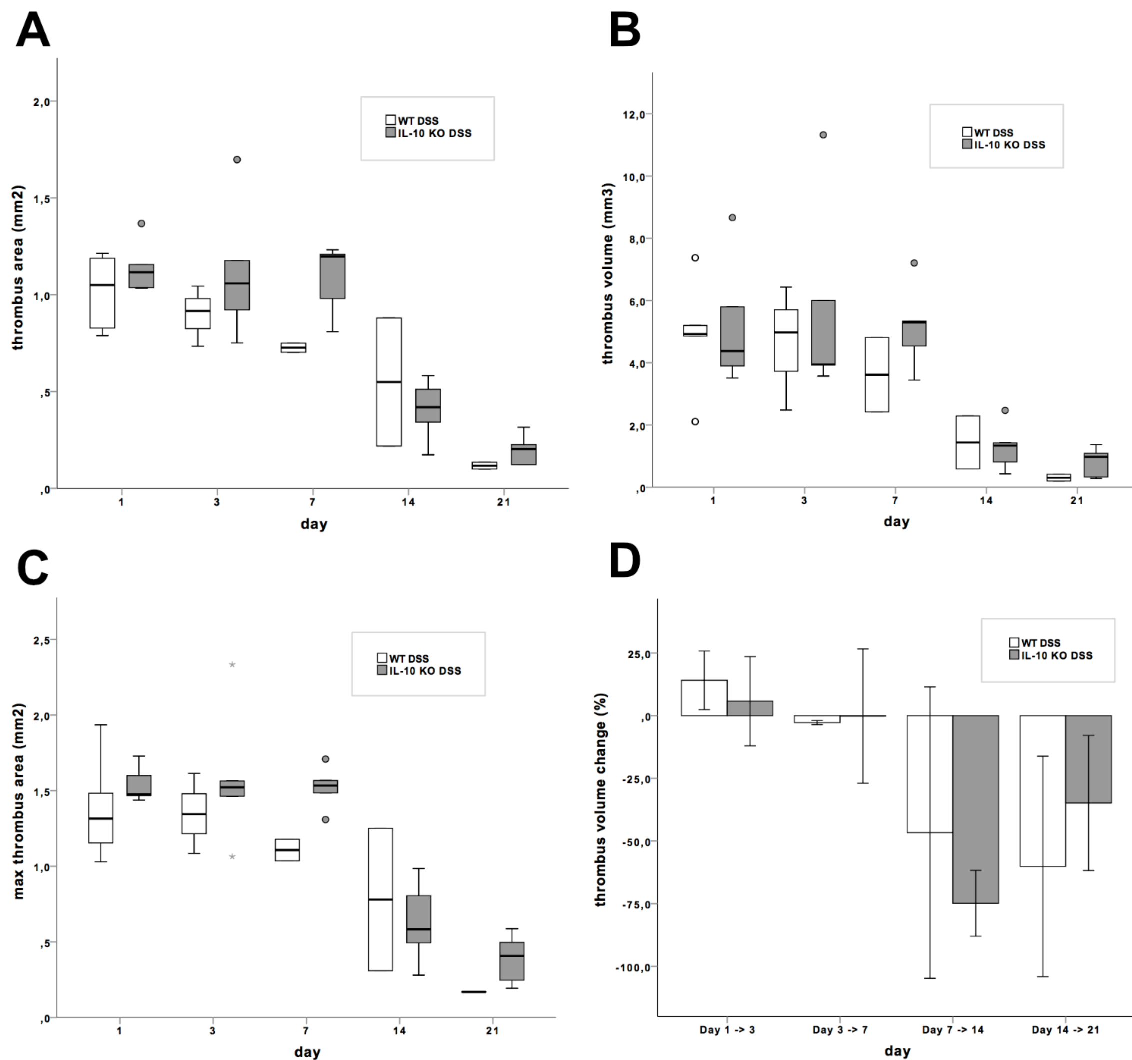

Figure 8: Summary of the calculated thrombus parameters using sonography

A-D, measurements taken at specific points of time (day 1/3/7/14/21 after ligation). A and B, graphs depicting the average thrombus area (mm^2) as well as volume (mm^3) for each mouse. Furthermore, the maximum thrombus area was calculated and represented in panel C. Chart D portrays the change in thrombus volume (%) between each imaging date.

3.3 Blood counts

Blood was taken from five mice on the day of euthanisation by cardiac puncture. In particular platelet count, hemoglobin and white blood cell count were investigated. The variables are influenced by the extent of intestinal inflammatory process and by iron deficiency. Due to the increased blood loss in mice with colitis, the level of hemoglobin was expected to be reduced as part of an anemic process. In consequence of the anemia a thrombocytosis may

have been observed as well. The reference values for hemoglobin levels in mice range from 10.9 to 18.1 g/dL whereas platelets depict normal values between 565 and 1849 K/µL. Normal values of white blood cells range from 3.9 to 13.9 K/µL. The blood cell counts are represented in the following table (Table 5).

Table 5: Blood counts from cardiac puncture

Breed (ear marking)	Hemoglobin (g/dL)	Platelets (x10^3 cells/µL)	WBC (x10^3 cells/µL)
WT (right)	8.9	33	2.13
WT (both)	9.2	98	4.27
IL-10 KO (right)	10.8	1237	3.30
IL-10 KO (left)	5.5	97	4.10
IL-10 KO (2x left)	10.9	935	2.31

All mice of both murine groups were found to be anemic. Interestingly the platelet count in the WT was reduced with values of 33 and 98 K/µL. The IL-10 KO mice posed higher levels of thrombocytes, however, most values were still in range of the reference parameters. Solely two animals depicted normal white blood cell levels, while the other mice showed reduced levels. Both groups averaged a WBC count of 3.2 K/µL which is slightly leukopenic. From two IL-10 KO mice the sample was too small for analysis.

3.4 Histological evaluation

3.4.1 Inferior vena cava thrombus evaluation

The part of the IVC containing the thrombus was removed after euthanizing the animals and stained using a modified trichrome protocol. Due to the fact that three WT mice deceased during the trials, it was only possible to analyze 7 clots. These specimens were initially separated from surrounding tissue leaving only the vein and its thrombus. Thereupon, weight and length were measured. The weight showed similar results across all samples ranging from 2.0 to 5.2 mg. At this point, it should be mentioned that the measurement included the mass of IVC, the thrombus and surrounding tissue. The lengths, in contrary, show slight differences between the two groups. The IL-10 KO mice exposed longer clots averaging 2.9 [2.75 - 3] mm in comparison to the control model with a mean value of 1.75 [1.5 - 2] mm. Simultaneously, weights of the spleens have been quantified. The two spleens of the WT

group weighed on average 0.15 [0.14 - 0.16] g, whereas the IL-10 knockouts depicted a slightly lower mean of 0.12 [0.10 - 0.13] g.

After staining the specimens, they were further assessed under the microscope. All IVC samples of the IL-10 KO group included clearly distinctive thrombi. The two WT samples, however, lacked clots as they were either lost during the histological preparation process or almost had completely resolved. A large organized thrombus had formed in an IL-10 KO mouse (Figure 9, panel B). The clot was easily definable and surrounded by a collapsed vessel wall. The other IL-10 KO mice showed highly developed thrombi as well though some had fused with the endothelial layer and a definite borderline could not be assessed. The mean thrombus area as measured under the microscope of the IL-10 KO clots averaged approximately 0.25 [0.05 - 0.78] mm^2. Unfortunately, comparable results from WT mice are not available as no clots were identified by histology. When comparing the mean value of the microscopically computed cross-sectional area with the average area measured on the last day of sonography, the results were closely related suggesting an accurate execution of sonography. The calculated average value with the ultrasound imaging technique amounted to 0.20 [0.12 - 0.32] mm^2.

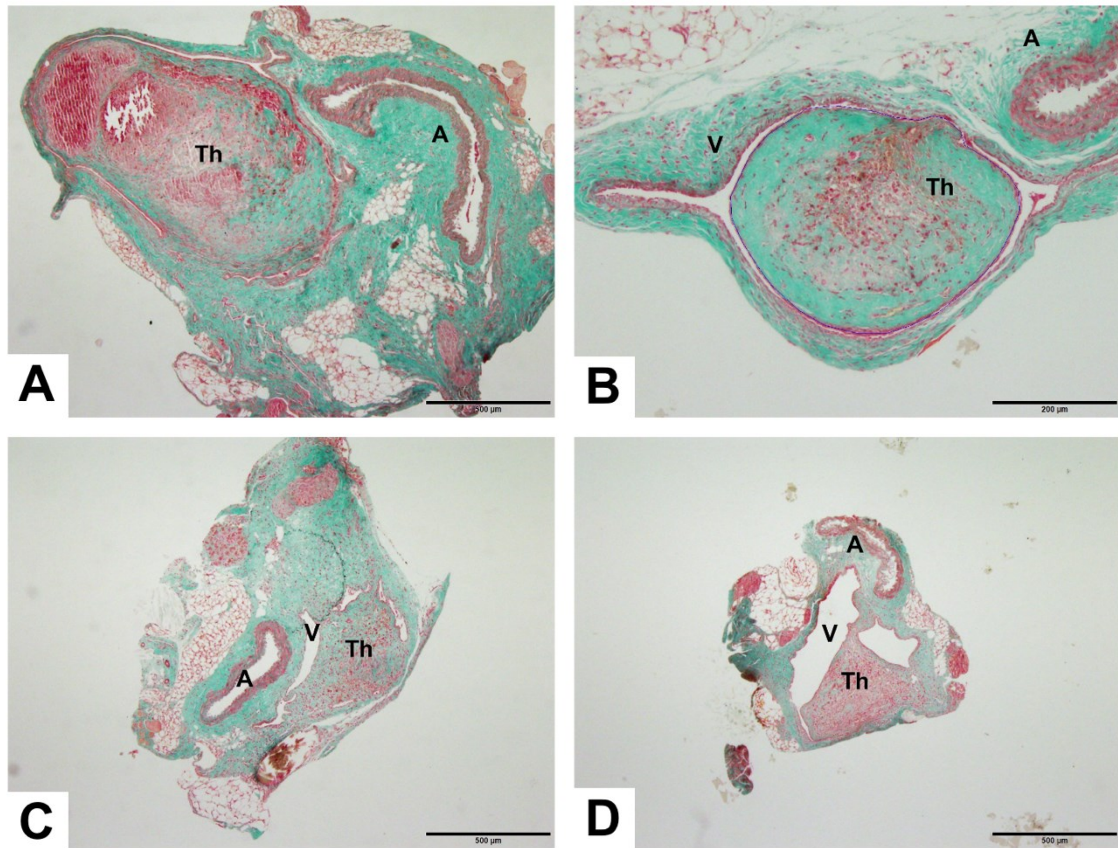

Figure 9: Different IL-10 KO thrombi after trichrome staining

A-D, histological images of distinctive thrombi of 4 different IL-10 KO mice taken with the Olympus BX 50 microscope after the killing on the 21st day. Each panel visualizes the aorta (A) as well as the thrombus (Th) inside the inferior vena cava (V) of different IL-10 KO mice. Panel B presents a zoomed specimen with a scale bar of 200µm, whereas the length of the reference bar in A, C, D is 500µm. The red color in the thrombus represents fibrin. Collagen is stained green.

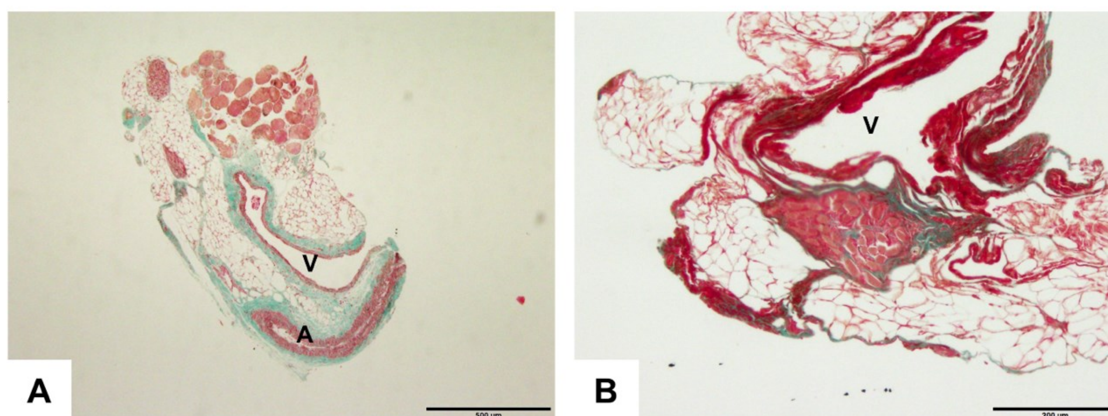

Figure 10: WT specimen after trichrome straining

A, B, histological specimens of the two remaining WT animals. Both inferior caval veins (V) do not contain identifiable thrombi. The scale bar measures 500µm in panel A and 200µm in panel B. Aorta (A).

3.4.2 Severity of intestinal inflammation

As stated thoroughly in the methods chapter, the degree of intestinal inflammation was assessed by combining two established scoring systems (83, 136). After staining the samples with the hematoxylin and eosin stain, intestinal structures were assessed under the microscope. The observations as well as scoring according to the extent of inflammation were conducted together with an experienced postgraduate researcher trained in gastrointestinal mouse pathology.

Table 6: Scoring of the intestinal inflammation with grades 0 - 4

WT	IL-10 KO
0	2.5
2.5	2
	1
	0.5
	3

The outcomes of the histological analysis are depicted in Table 6 and visualized in Figure 11. When looking at the average value of the evaluated combined scoring, the IL-10

knockout animals showed a mean score of 1.8 [1 - 3] whereas the WT mice were graded on average with 1.25 [0 - 2.5].

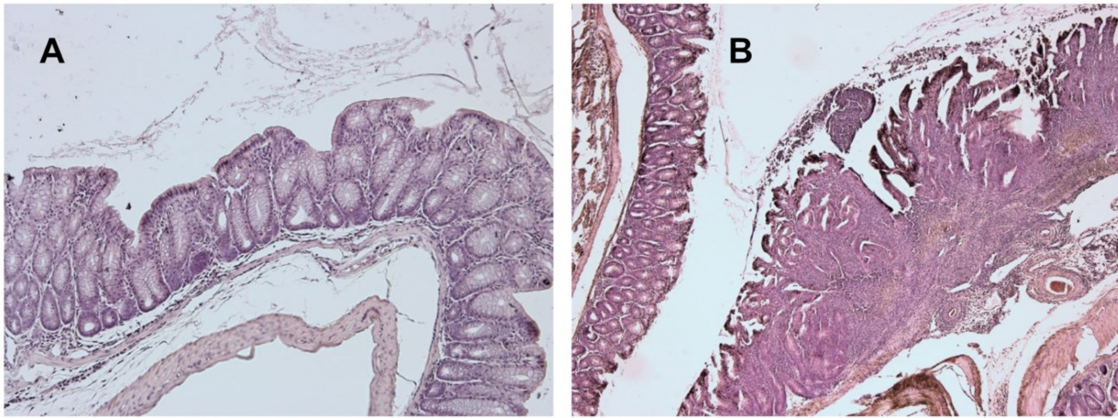

Figure 11: Histological analysis of WT and IL-10 KO intestines

The intestine of a WT animal is represented in panel A and depicts a normal intestinal tissue with grade 0 inflammation score. Crypts and epithelium of the mucosa are well identifiable. Panel B visualizes the intestine of an IL-10 KO mouse showing loss of crypts, inflammatory cell infiltration and edema with a 2.5 inflammation score.

Despite the lack of clinical signs in the IL-10 KO mice, the extent and severity of intestinal inflammation seemed to be well-marked among these with one mouse showing even a score of 3. Thus, it can be stated that in the IL-10 KO animals DSS-triggered colitis persisted throughout the experiment.

3.5 Mouse genotyping

To control for appropriate mouse allocation, tail samples were collected from three mice of each group. Tail DNA was extracted and genotyping was performed by two IL-10-specific PCR reactions. The amplicon was separated by gel electrophoresis (Figure 12). Four out of six samples showed successful DNA amplification and demonstrate the appropriate genetic alteration in IL-10 KO mice.

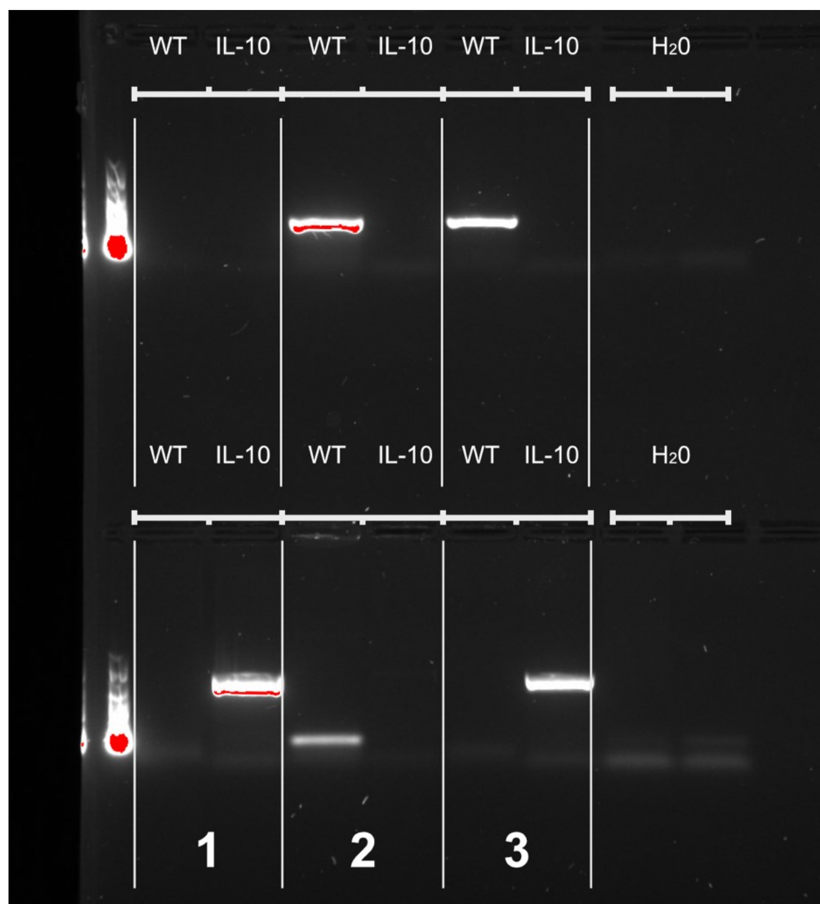

Figure 12: Gel electrophoresis of WT and IL-10 KO tail samples

The first row represents amplification of WT tail samples (WT), whereas the amplification of IL-10 KO samples is visualized in the second row. To the far left, a size marker is placed. The DNA segments of the first WT mouse (lane 1) as well as the second IL-10 KO (lane 2) animal were not amplified and thus show empty lanes. The amplification of the second IL-10 KO (lane 2) sample with WT primers has the wrong size and is most likely results from non-specific amplification or primer multimers.

4 Discussion

A magnitude of studies conducted on the topic of IBD and VTE have shown that patients suffering from chronic enterocolitis are at an increased risk of VTE (96, 98-100). The exact pathological mechanisms leading to this prothrombotic phenotype have not been fully understood yet (104). Furthermore, venous thromboembolic complications have a significant impact on mortality in these patients (103). A thorough understanding of thrombus formation and resolution in colitis is of particular importance (9). Thus, the study aimed to establish a mouse model for studying thrombus formation and resolution under conditions of chronic colitis. The mice used for the experiment were IL-10 KO as well as

WT females aging approximately 8 weeks. After induction of colitis with a five-day course of DSS, the animals underwent a surgical ligation of the IVC below the renal veins. The reduced blood flow lead to the formation of a clot which resolution trend was observed over three weeks using sonographic imaging. After euthanizing the animals, the intestines as well as the remaining clots were collected, stained and subsequently analyzed under the microscope. Histological and sonographic results showed distinctive clots in all IL-10 KO mice 3 weeks after ligation suggesting impaired thrombus resolution. WT animals were able to form thrombi in the days following the surgery, however, clots rapidly decreased in size. The pilot study was executed successfully, thus, a novel murine model has been established.

Thorough search of the PubMed database has shown that no in vivo murine study has been conducted for studying the formation and resolution of venous thrombi under conditions of intestinal inflammation. However, numerous clinical trials managed to depict a significant correlation between IBD and VTE (96, 99, 100). Thus, the idea originated to establish a mouse model to further investigate the mechanisms leading to increased venous thrombi formation in colitis. Additionally, such model may help testing preventive measures as well as therapeutic regimens.

The 5-day induction period with DSS lead to a drastic decrease of weight in the following days due to diarrhea. Interestingly, the WT animals experienced more severe weight loss than IL-10 KO mice. This unexpected result raised the question whether the cages had been switched during the experiment or the mouse genotype was flawed. Genotyping of tail DNA, however, proved appropriate mouse allocation. An alternative explanation for the more severe colitis induction in WT mice may be the larger amount of DSS water consumed by WT mice. In fact, a wider opening of the bottle lid was observed in the WT cage which may have led to an increased fluid consumption. The increase in fluid consumption may also be due to larger amounts of spontaneous fluid dripping. Thus, it remains unclear whether the higher fluid consumption in the cages of WT animals is the reason for the more pronounced DSS effect. Future experiments need to better control for this bias.

Surgery was carried out on all ten animals 72 hours after the end of colitis induction. The operation included anesthesia, midline laparotomy and the ligation of the IVC resulting in an almost complete stenosis. Following the ligature, the mice were given analgesic treatment and observed accurately. The first day of sonography was carried out on all animals,

however, the WT group showed severe symptoms of colitis including rectal bleeding. Furthermore, the animals had reached their weight nadir. As a consequence, three mice were not able to recover from the procedure and were lost. The selective death of WT mice is believed not to be due to the genotype but rather the more severe colitis. The interval between colitis induction, surgical procedures and ultrasound measurements is likely too short and should be lengthened in future experiments. The longer pause would also give the possibility to better analyze chronic colitis in IL-10 KO mice because WT recovered from colitis on the days following DSS induction.

When performing the sonographic examination on the small animals, sedation with isoflurane gas had to be achieved in order to immobilize them. The examinations took place on the 1st, 3rd, 7th, 14th and 21st day after ligating the IVC. Sonography is not the only possible method to analyze thrombus. Another approach would have been to sacrifice a calculated amount of mice at specific points in time, such as day 1, 3, 7, 14, 21, and perform histological analysis of thrombi. However, due to the non-invasiveness of sonography fewer animals are needed as the clot progress can be tracked over time in all animals and the optimal time for euthanasia can be selected. On the other hand, euthanizing a small group of mice at each time point may lead to more histological data but would need a larger number of animals, which is against the 3R rule (Replacement, Reduction, Refinement) in animal ethics: “Reduction” means that animal research should employ methods that enable researchers to obtain similar information with fewer animals. We believe that this is the case with sonography. In fact, the results obtained at the last sonography exam resulted in similar thrombus size as the histological exam.

The first sonography dates (day 1/3/7 post ligation) exposed easily identifiable and measurable thrombi. Furthermore, clear boundaries were distinguished to surrounding blood flow. I personally observed that all thrombi were better visible in the first week (day 1/3/7 post ligation) because the clotting with fibrin resulted in a difference in tissue densities. The clots were easily identifiable. The last two imaging dates (day 14/21 post ligation) were harder to image because of thrombus composition at similar densities as the surrounding blood flow. Surprisingly, the WT clots were mainly affected by this feature, whereas the thrombi of IL-10 KO mice remained highly echogenic even on the last dates of ultrasound guided examination. The final sonographic examination 21 days following surgery showed that the thrombi of the IL-10 KO group remained clearly identifiable with an average volume

of 0.81 [0.28 - 1.37] mm^3, whereas the WT group presented almost resolved clots (volume: 0.31 [0.20 - 0.42] mm^3).

It should be noted that this imaging method was flawed by examiner subjectivity, visibility of the clot, and size (0.1 mm^2). Nevertheless, thrombi of IL-10 KO animals appeared to be identifiable on all examination dates even though their size was minimal. This was due to high echogenicity. For further analysis, the introduction of a 28th time point is important in future experiments.

The blood findings from the cardiac puncture 21 days after the surgical ligation revealed anemia and reduced levels of thrombocytes in WT animals while the platelet count of the IL-10 KO mice was in range of the reference parameters. The low levels of platelets in the WT mice may correlate with the complete resolution of the thrombi. As delineated in a previous chapter, thrombocytes and their tendency to aggravate are known to be increased in IBD (107-109). Cardiac puncture and blood cell counts are variable and unlikely to be highly reproducible. Larger animal counts may be needed to show differences between groups.

Histological observation showed that the IL-10 KO appeared to have impaired resolution as seen in the consistent thrombi. Clots were not identified in the preparations of the WT animals, which is expected. It can be discussed that the similar values of thrombus areas in IL-10 KO animals using sonography and histology may also be applicable to the WT animals. Looking at the cross-sectional area of thrombi in the control group, the computed average size using sonography amounted to 0.12 [0.10 - 0.14] mm^2 depicting almost half the value compared to the IL-10 KO group (area: 0.20 [0.12 - 0.32] mm^2). Thus, the clot in the WT may have been too small to preserve after excising the vein from the animals.

In order to investigate the extent of intestinal inflammation and link it with the thrombus size, a histological analysis of the intestinal samples was needed. The grading was achieved by taking use of two practical scoring systems as specified in the methods section. Due to the reason that Berg et al (83) didn’t take mice with DSS induced colitis into account, we included the pathological scoring system of Cooper et al (136). When investigating the intestines, the IL-10 KO distinctively exposed inflammatory processes occurring in the intestinal mucosa with invasion of immune cells. The gut of the WT group was nearly normal

in spite of the severe clinical manifestations. As expected, the two WT mice were able to recover from acute DSS colitis, whereas the IL-10 KO group developed chronic intestinal inflammation with only minimal clinical symptoms. The result outlines that prolonged intestinal inflammation may affect thrombus resolution. Alternatively, the loss of IL-10 itself may have caused this phenotype. Nevertheless, in order to acquire a significant and comparable outcome larger numbers of animals are needed, including a group of IL-10 KO mice without DSS treatment.

In summary, the aim of this diploma thesis was to establish an animal model to study thrombus formation and resolution under conditions of chronic intestinal inflammation. We performed IVC ligation in a model of chronic colitis in mice. The result of this pilot study demonstrates that such model is feasible with bigger and sustainable thrombi. Better timing, use of sonography, and more generous animal numbers will provide a distinct answer.

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