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Inflammatory Bowel Disease and Haemostasis

Clinical and Biochemical Studies

A.A. Vrij

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Inflammatory Bowel Disease and Haemostasis

Clinical and Biochemical Studies

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Chapter 1

General introduction

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General introduction

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Inflammatory bowel disease (IBD) is characterized by a chronic relapsing intestinal inflammation. IBD is divided into two main clinical phenotypes: Ulcerative colitis (UC) and Crohn's disease (CD). In approximately 10% of proctitis and/or colitis cases, a definitive diagnosis of CD and UC cannot be made, which is classified as indeterminate colitis. IBD symptoms include diarrhea, body wasting, abdominal pain, anemia or rectal bleeding.

CD may involve any part of the gastrointestinal tract, but characteristically the terminal ileum and colon are most commonly macroscopically involved. Bowel inflammation is transmural and discontinuous; it may contain granulomas or be associated with intestinal or perianal fistulas.

In contrast, in UC, the inflammation is continuous and limited to rectal and colonic mucosal layers, and fistulas and granulomas are rarely observed. Extra-intestinal manifestations are common in both diseases. These predominantly include inflammation of the skin, eyes, or joints.

Recently, in a multicenter epidemiological study of 20 centers across Europe, the mean annual incidence of UC was reported to be $10.4/10^5$ (95% CI: 7.6-13.1), and of CD it was $5.6/10^5$ (95% CI: 2.8-8.3). The highest incidence of CD was observed in Maastricht, the Netherlands ($9.2/10^5$), and the highest incidence for UC was found in Iceland ($24.5/10^5$)¹.

Vascular involvement and the system of haemostasis

Although the majority of research into IBD pathogenesis has focused on immune dysregulation, vascular involvement in IBD has also been recognized over the past four decades, as these disorders have been associated with hypercoagulability and vasculitis. Advances in vascular biology have delineated a central role for the microcirculation in the initiation and perpetuation of the inflammatory process.

Interaction between thrombosis and inflammation is increasingly recognized, and interest has arisen in the role of thrombosis in inflammatory conditions, including IBD.

Although the association between active IBD and thromboembolic complications has long been known, there has been resurgence in research into the role of thrombosis and the haemostatic system in the pathogenesis of both ulcerative colitis and Crohn's disease. An association between IBD and thrombosis has been recognized for more than 60 years. Not only are patients with IBD more likely to have thromboembolic complications, but it has also been suggested that thrombosis might be pathogenic in IBD.

Here we review the increased frequency of thromboembolic complications occurring in chronic inflammatory diseases, and more specific in patients with IBD; whether thrombosis might play a part in the initiation and maintenance of

inflammation in IBD; abnormalities of the coagulation system found in patients with IBD; platelet dysfunction in IBD; the mechanisms by which haemostatic processes might be proinflammatory in IBD; and how these interactions might impact not only on the prevention of complications, but also on the treatment of the underlying inflammation in IBD.

Thromboembolism as a complication of chronic inflammatory diseases

Inflammation and thrombosis are intimately connected. The cellular and humoral components of clotting and inflammation have common evolutionary origins and likely derived from multi-functional host defense mechanisms that both contained and destroyed invading microbes and sealed wounds². These ancient origins gave rise to modern molecular mechanisms that link inflammation and haemostasis. For example, specialized integrins on myeloid leukocytes and platelets recognize fibrinogen, which influences inflammation in addition to being a critical clotting component, and some signaling molecules, such as platelet activating factor (PAF), activate a common receptor that is expressed by PMNs, monocytes and platelets³⁻⁵.

An established index of inflammation is interleukin-6 (IL-6), which is a circulating cytokine produced by monocytes, macrophages, T lymphocytes and endothelial cells. IL-6 can induce a prothrombotic state by increasing expression of fibrinogen, tissue factor, factor VIII and von Willebrand factor, as well as by activating endothelial cells and increasing platelet production⁶. Indeed, inflammation leads to the adhesion, recruitment and trans-endothelial migration of leucocytes into the intima, which is mediated by adhesion molecules on the endothelial cell membrane; leading to the initial capture and rolling of leucocytes along the endothelium⁷⁻¹⁰. In particular, E-selectin is a specific endothelial cell product, which mediates contact between endothelial cells and neutrophils and monocytes. E-selectin expression is generally absent in normal tissues, and it has not been seen on cells other than the endothelium^{11,12}. Raised levels of soluble E-selectin have been reported in hypertension¹³, diabetes¹⁴, and in atherosclerotic vascular disease, such as stroke¹⁵.

Activated coagulation factors can also stimulate inflammation. E.d. thrombin, a central coagulation protease, is a potent proinflammatory mediator¹⁶. These and other molecular connections between inflammation and thrombosis are homeostatic under physiologic conditions but can be inappropriately triggered or become dysregulated, causing disease. Linked mechanisms of inflammation and thrombosis influence a host of clinical disorders, including atherosclerosis and its acute complications^{17,18}, and experimental models indicate that inflammatory effector responses contribute to the natural history and outcome of DVT^{18,19}. There are also many associations between DVT and clinical

syndromes with major inflammatory components. Again, this association occurs in atherosclerosis²⁰.

In Rheumatoid arthritis (RA), an autoimmune disease characterized by a T helper-1 lymphocyte driven inflammation also found in Crohn's disease, activation of coagulation and fibrinolysis has been demonstrated both in the joints, and in the circulation. Also, in RA, a small vessel vasculitis may occur²¹. Raised levels of TF (tissue factor), tissue factor pathway inhibitor (TFPI) activities, thrombin-antithrombin III (TAT) complexes, and prothrombin fragment 1+2, fibrin d-dimer and thrombin-activated fibrinolysis inhibitor (TAFI) antigenic levels were found both in plasma and synovial fluid in patients with active RA²². Especially higher levels of TAT in the synovial fluid and plasma as well as TAFI in the joints were found in RA compared with other joint diseases. This could explain why fibrin formation was so prominent in RA related arthritis²². In a multiple logistic regression model controlling for several conventional cardiovascular risk factors and pharmacological treatment at sampling, raised levels of PAI-1 and tPA were significantly associated with cardiovascular disease progression, a common complication in patients with RA²³.

In patients with giant cell arteritis (GCA), characterized by a large-vessel vasculitis, plasma levels of three factor VIII-von Willebrand factor components (von Willebrand antigen, ristocetin cofactor, and factor VIII coagulant) were higher than those in patients with either inactive giant cell arteritis or active polymyalgia rheumatica²⁴. Levels of factor VIII-von Willebrand factor components tended to decline gradually after disease activity had been suppressed by corticosteroid therapy and could be indicators of vascular damage. In patients with GCA, a high prevalence of anti-phospholipid antibodies was found in several studies, a phenomenon generally associated with thromboembolic disease. Although inflammatory conditions such as GCA predispose to stroke²⁵, the level of anti-phospholipid antibodies in GCA was not related to ischemic manifestations like stroke or ischemic cardiac disease. Moreover, GCA-related or GCA-unrelated ischemic manifestations did not appear to be due to congenital thrombophilic risk factors²⁶.

Thromboembolism as a complication of Inflammatory Bowel Disease

Thromboembolic complications of IBD are well recognized and were first described in UC in 1936²⁷. Although pulmonary embolism (PE) and lower limb deep vein thrombosis (DVT) are the most common thromboembolic phenomena seen in IBD, case reports of thrombosis of most other veins and arteries can be found in the literature. E.d. thrombophlebitis migrans, a cutaneous problem thought to be due to a prothrombotic state and more

usually associated with pancreatic malignancy, has been described in association with UC^{28,29}.

The quoted frequency of thromboembolic events in patients with IBD varies widely (Table 1.1). In clinical studies, thromboembolic events are said to occur in 1%–8% of patients, whereas in postmortem studies, rates reach 41%. Many of these studies, however, are from an era in which the management of IBD was more invasive than it is now; in one study, more than half of the group surveyed (patients with UC) had undergone surgery³⁰.

Moreover, the introduction of prophylactic measures against thromboembolism, such as heparin, antithrombotic stockings, and early postoperative mobilization, postdates some of the earlier reports. Although postmortem data overcome the problem of failing to diagnose subclinical thromboembolic events, it is difficult to interpret the results of these as well as other studies without an appropriate control group.

Two recent studies have addressed these difficulties and provide more robust evidence of the increased risk of thromboembolism in IBD; in these, patients with IBD had a 3-fold increased risk of either DVT or PE compared with the general population^{31,32}. Bernstein et al. performed a population-based cohort study and calculated incidence rates of DVT or PE of 40/10,000 person years for CD and 50/10,000 person years for UC³¹.

Moreover, in the other study, an inflammatory control group (RA) and a gastroenterologic disease control group (celiac disease) both had fewer episodes of thromboembolism than the IBD patients, even after adjustment for confounding variables such as operations, oral contraceptive pill (OCP) use, smoking status, and body mass index³².

However, not all studies have shown an increased risk of thromboembolism in IBD, although one negative study showed that patients with IBD who do have thromboembolic events have them at a younger age than the general population³³. Further evaluations of patients with IBD who have experienced thromboembolic events have revealed disease characteristics associated with an increased risk of thromboembolism.

In a review of 98 IBD patients with thrombo-embolism, most of the patients with CD had colonic disease, whereas those with UC had extensive disease, suggesting that the risk of thromboembolism might correlate with the extent of colonic involvement³⁴. Others have noted this observation previously, in patients with UC^{35,36}.

Disease activity is also probably a risk factor for thromboembolism, a fact supported by the relationship between thrombocytosis, coagulopathy, and disease activity (see below)^{34,37}. However, it is of note that a third of thromboembolic complications occur while the disease is quiescent³⁷.

Thrombosis as a pathogenic mechanism in Inflammatory Bowel Disease

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It has been suggested that thrombosis and ischemia might be involved in the pathogenesis of both UC and CD. Indeed, ischemic conditions of the bowel can present in a similar fashion to, and hence be mistaken for, both UC and CD^{38,39}. Although interest has recently moved away from the proposal that ischemia is a primary cause of IBD, it has become increasingly clear that inflammatory and thrombotic processes are linked, and current evidence suggests that thrombosis has some involvement, if not in the initiation, at least in the maintenance of the inflammatory process in IBD.

A vascular component to the pathogenesis of CD was first proposed only a year after Crohn et al. described the condition⁴⁰. Subsequently, in 1989, a series of changes comprising vascular injury, focal arteritis, fibrin deposition, arterial occlusion, and then microinfarction or neovascularization was proposed as a possible pathogenetic sequence in CD⁴¹. In this study, resin casts of the intestinal vasculature showed changes ranging from intravascular fibrin deposition to complete thrombotic occlusion. Furthermore, the early vascular changes appeared to precede mucosal changes, suggesting that they were more likely to cause rather than result from the pathologic features of CD.

Subsequent studies showed that intravascular fibrin deposition occurred at the site of granulomatous destruction of mesenteric blood vessels⁴², and positive immunostaining for platelet glycoprotein IIIa occurred in fibrinoid plugs of mucosal capillaries in CD⁴³. In addition, intracapillary thrombus has been identified in biopsies from inflamed rectal mucosa from patients with CD⁴⁴. When combined with evidence of ongoing intravascular coagulation in both active and quiescent CD, the above data point toward a thrombotic element contributing to the pathogenesis of CD^{45,46}.

The case for a thrombotic component in the pathogenesis of UC is less clear. In the 1960s, a study of the early histologic changes in UC identified intravascular platelet aggregates⁴⁷, a finding supported by the discovery of intracapillary thrombus in rectal biopsies from patients with UC⁴⁴.

In addition, changes in the former study included thickening of the subepithelial capillaries, with occlusion of their feeding vessels and intravascular fibrin deposition in association with platelet thromboses⁴⁷.

Microvascular dysfunction might also contribute to ischemic changes in IBD. Loss of vasodilatory ability, decreased microvascular volume (in ileal CD), and the presence of elevated levels of potent vasoconstrictors, such as endothelin-1, in both tissue and plasma could contribute to the reduction in blood flow seen in chronic UC and CD⁴⁸⁻⁵⁴. Likewise, microvascular injury, as suggested by increased levels of circulating von Willebrand's factor and anti-endothelial cell antibodies, might contribute to localized thrombosis and, in the case of von

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Willebrand's factor, which is a potent mediator of platelet adhesion and aggregation, to systemic thromboembolism additionally^{53,54}.

Smoking not only predisposes to CD but also has an adverse effect on disease progression⁵⁵⁻⁵⁸. Smoking is itself a risk factor for thrombosis⁵⁹. It can cause platelet activation⁶⁰ and platelet-leukocyte aggregate (PLA) formation⁶¹, both of which might be involved in the pathogenesis of IBD. One argument used against thrombosis being pathogenic in UC is the seemingly protective effect of smoking. It is possible, however, that any of the myriad components of tobacco, such as nicotine, a drug that has been used in the treatment of UC, might outweigh any disadvantageous prothrombotic effect^{62,63}.

Furthermore, a weak epidemiologic association between CD and OCP usage has been demonstrated, although conflicting data exist⁶⁴⁻⁶⁷. Although OCP usage is known to predispose to thromboembolism, it does not appear to have an adverse effect on disease progression, and it is possible that confounding factors, such as smoking, account for the positive findings^{68,69}. Furthermore, the introduction in recent years of low dose OCPs might have decreased the risk of OCP usage; the estrogen-related alteration in blood coagulation might be related to a threshold dose⁷⁰.

Conversely, patients with inherited bleeding diatheses appear less likely to have IBD⁷¹. In a survey of hemophilia center directors, the observed number of cases of IBD in patients with hemophilia A, hemophilia B, or von Willebrand's disease was about a third of the expected number on the basis of population studies. The authors concluded that bleeding diatheses might therefore be protective against IBD. However, because the study relied on the hemophilia center directors' ability to recall whether any of their patients had coexisting IBD, it is unsafe to draw too firm a conclusion from these data.

Hence, as the interaction between thrombosis and inflammation has become clearer over the years, it has become more widely accepted that thrombosis might play some part in the pathogenic processes involved in IBD. Further evidence for the complicated interplay between thrombosis and inflammation is provided by the coagulation abnormalities found in IBD, an issue that we will address next.

The Coagulation and Anticoagulation Systems

Before examining abnormalities of the coagulation system associated with IBD, we shall briefly outline the mechanisms by which thrombosis occurs.

Endothelial injury results in exposure of collagen and expression of tissue factor (TF), a 47 kDa transmembrane protein, which might also be expressed by (stimulated) monocytes. This initiates platelet adhesion and aggregation and activation of coagulation with, ultimately, deposition of fibrin.

Although the coagulation system has traditionally been divided into the extrinsic and intrinsic pathways, in vivo it is a naturally integrated system as outlined hereafter (Figure 1.1).

Coagulation is initiated by tissue damage exposing tissue factor. Factor VII binds TF and the TF-factor VII complex, via VIIa, activates factor X to factor Xa, and some factor IX to factor IXa. In the presence of factor Xa, tissue factor pathway inhibitor (TFPI) inhibits further generation of factor Xa and factor IXa.

After inhibition by TFPI the amount of factor Xa produced is insufficient to maintain coagulation. Further factor Xa, to allow haemostasis to progress to completion, can only be generated by the factor IX/factor VIII pathway. Enough thrombin, produced by the initial activation of factor X, exists at this point to activate factor VIII, and together with factor IXa (generated by TF-factor VIIa), further activation of factor X can proceed. This amplification system allows the continued production of thrombin, which in turn, in addition to activating platelets, factors V, VIII, and XI, leukocytes, and endothelial cells, induces the production of fibrin from fibrinogen. Thrombin also activates factor XIII, which causes polymerization of fibrin monomers.

Clot formation in vivo is counterbalanced by various anticoagulant mechanisms to maintain homeostasis. As shown above, the TF-factor VIIa complex is inactivated by the TFPI; proteins C and S form a complex that inactivates factors V and VIII and also induces plasmin formation by inactivation of tissue plasminogen activator inhibitor, and antithrombin inactivates the active forms of the serine protease type clotting factors IX, X, XI, and XII, a process that is accelerated dramatically by heparin.

Both tissue-type and urokinase-type plasminogen activators induce formation of plasmin from plasminogen, resulting in fibrinolysis and the production of fibrin degradation products, which are themselves anticoagulant through their inhibition of fibrinogen to fibrin conversion by thrombin. Also, endothelial cells have an anticoagulant role. This is predominantly mediated by activated protein C. This anticoagulation occurs on the endothelial cell surface via thrombomodulin, which, on binding thrombin, activates protein C and, in addition, via endothelial protein C receptor (EPCR), which also facilitates protein C activation. Endothelial cells also express plasminogen receptors that activate plasminogen. The above is, of course, a simplification of what is a very complex system.

More recently, in contrast to the traditional cascade model, a cell-based model of haemostasis has been proposed involving initiation, amplification, and propagation phases⁷². Further examination of this model is beyond the scope of this review. Suffice it to say that an intricate balance exists that facilitates haemostasis when endothelial injury occurs but prevents spontaneous intravascular thrombosis. In IBD, disturbance of this balance can occur.

Prothrombotic state and Inflammatory Bowel Disease

Bearing in mind the complexity of thrombosis, alterations in the homeostatic mechanism can take place at many points as a result of inherited or acquired abnormalities. Many such abnormalities have been reported in association with IBD (although much conflicting evidence exists), and a recent study suggested that in at least a third of patients with IBD who incur a thromboembolic complication, there is a definable prothrombotic abnormality³⁴.

Moreover, a study of 116 patients with IBD showed that they were more likely to have not only single but also combined inheritable and acquired defects predisposing to thrombosis than a group of 141 asymptomatic blood donors⁷³. In addition to any increased risk of thromboembolism from definable thrombophilic tendencies, patients with IBD often have other risk factors such as fluid depletion, immobilization, and intra-abdominal inflammatory masses and abscesses with the need to undergo surgery. In 1968, the first study to examine hypercoagulability in patients with active UC demonstrated increased factor VIII activity, elevated fibrinogen levels, and accelerated thrombin generation⁷⁴. Since then, many other abnormalities have been described that either predispose to a hypercoagulable state or are evidence of ongoing coagulation (for example, elevated D-dimer and prothrombin 1+2 fragment levels and decreased factor XIII A2B2 levels) (Table 1.2)⁷⁵. Some, although not all, of these abnormalities are related to disease activity.

Not only are many different prothrombotic changes described in association with IBD, but they can also have multiple causes.

Hyperhomocysteinemia, for example, is known to predispose to thrombosis, and patients with IBD are more likely to have hyperhomocysteinemia than control subjects⁷⁶. Hyperhomocysteinemia in IBD might be due to multiple possible causes, such as deficiencies of vitamin B12 as a result of terminal ileal disease or resection; lack of vitamin B6, which is commonly reduced in IBD⁷⁷; and/or lack of folate, e.d. as a result of therapy with sulfasalazine or methotrexate^{78,79}.

Moreover, some of the IBD-related prothrombotic risk might result from inflammation itself; as some of the proteins involved in haemostasis form part of the acute phase response.

Because of the association between IBD and thromboembolism, several investigators have looked for an association between inherited prothrombotic abnormalities and IBD. Recently, a review of studies of the 3 most common inherited risk factors for thromboembolism (factor V Leiden mutation, G20210A mutation in the prothrombin gene, and homozygous C677T mutation in the methylenetetrahydrofolate reductase gene) found that these mutations were no more common in patients with IBD than in the general population⁸⁰. Although some studies have found positive associations between these and other

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mutations and IBD, data thus far suggest that inherited prothrombotic changes are unlikely to play a major role in IBD. Disturbance of the endothelium's anticoagulant role has also been investigated in IBD. Increased serum levels and decreased endothelial expression of thrombomodulin and the endothelial cell protein C receptor (EPCR) have been demonstrated in both UC and CD^{81,82}. These changes would be expected to decrease protein C activation and, therefore, increase the likelihood of thrombogenesis. In the latter of these two studies, mRNA levels of EPCR were reported to be reduced in uninvolved as well as involved tissue from patients with IBD compared with control subjects. Whether this endothelial dysfunction is a primary alteration, rather than a consequence of the inflammatory response, is unclear.

Cytokines play a pivotal role in the pathogenesis of IBD, and, more recently, their manipulation has led to new developments in the treatment of both UC and CD^{83,84}. They also have important interactions with the coagulation cascade⁸⁵ and hence might contribute to the prothrombotic state associated with IBD. Tumor necrosis factor- α (TNF α), for example, affects the coagulation cascade at a number of points. It decreases protein C activation and induces TF expression on endothelial cells⁸⁶. In addition, release of von Willebrand's factor and levels of plasminogen activator inhibitor-1, tissue plasminogen activator, and platelet-activating factor are all increased by tumor necrosis factor- α ^{85,87-89}. Interleukin-1 also increases TF expression and decreases proteins C and S activity, whereas the regulatory cytokine interleukin-10 inhibits TF release, and interleukin-5 decreases thrombomodulin expression^{90,91}. However, the interplay between inflammation and haemostasis involves not only the coagulation system, but also platelet function.

Platelets and platelet activation in Inflammatory Bowel Disease

Platelets are small (1–3 μ m), disc-shaped anuclear cells whose main function was conventionally thought to be the formation of haemostatic plugs at sites of vascular injury and, consequently, to aid in the initiation of thrombosis. On activation, platelets become spherical and develop pseudopodia. As a consequence of activation, they express cell surface glycoproteins (such as P-selectin and CD63) and release a range of potent proinflammatory mediators. As such, they are not only a vital component of the haemostatic mechanism, but they also interact with many immunoregulatory cells.

The realization that platelets are not only prothrombotic but also proinflammatory has stimulated interest in their role in both the pathogenesis and complications of IBD⁹². The association between thrombocytosis and active IBD was first described more than 30 years ago in 5 patients with CD and 1 with UC⁹³. This was subsequently confirmed by a larger study of 159 patients with IBD, which also highlighted the relationship between disease

activity and platelet count⁹⁴. This and another more recent study showed decreased or normal platelet survival, suggesting that IBD-related thrombocytosis is due to increased thrombopoiesis⁹⁵. This in turn is probably driven by an interleukin-6-induced increase in thrombopoietin synthesis in the liver⁹⁶.

Initial studies of platelet function from patients with active IBD showed decreased aggregation in response to adenosine diphosphate, adrenaline, and collagen⁹⁷. The authors concluded that, paradoxically, this might provide evidence of increased platelet activation in active IBD; the platelets, they suggested, might have been rendered refractory *in vivo* by prolonged exposure to ADP produced from hemolyzed erythrocytes. Indeed, after clinical improvement with treatment, these patients' "refractory platelets" returned to function normal. It is possible, however, that in these experiments the most reactive platelets were removed during the preparation of the platelet-rich plasma used. Indeed, since then, several other authors, by using different methods of preparing platelet-rich plasma, have produced evidence of increased *in vivo* and *in vitro* platelet aggregation in active IBD. Spontaneous *in vitro* platelet aggregation occurs in platelets isolated from 30% of patients with IBD but not in platelets from control subjects⁹⁶. Moreover, collagen, arachidonic acid, ristocetin, and ADP-induced platelet activation are more marked in platelets from patients with active IBD than in those from healthy control subjects^{95,98}. These results provide evidence of increased platelet activation in patients with IBD.

With formaldehyde-fixed peripheral blood samples from 85 patients with IBD, Collins et al.⁹⁹ demonstrated increased *in vivo* platelet aggregation compared with both healthy and inflammatory control subjects (the latter being a group of patients with inflammatory arthropathies).

A subsequent study, in which mesenteric arterial and venous blood samples were compared, showed that, in CD, these aggregates form in the mesenteric microvasculature¹⁰⁰. Although platelet aggregation might be a marker for platelet activation, more recent studies in IBD patients have concentrated on serum and surface markers of platelet activation. Serum levels of platelet factor 4, and β -thromboglobulin are increased in active IBD^{95,99,101-103}.

Flowcytometric evaluation of P-selectin expression has shown that this platelet surface protein, which is expressed on platelet activation¹⁰⁴, is upregulated in patients with UC and CD^{99,103-108}. Moreover, in a study in which platelet P-selectin levels were measured in paired capillary and venous blood samples, platelets from patients with IBD were more readily activated *in vivo* than platelets from healthy subjects¹⁰⁷. No correlation between platelet activation and disease activity has been demonstrated; P-selectin expression is increased whether the disease is active or not, implying that platelet activation is not simply a marker of inflammation or of thrombocytosis but is an

underlying, possibly pathogenic, abnormality in IBD. Platelet activation might be pathogenic in IBD in several ways (Figure 1.2). Platelet activation might increase platelet aggregation, hence increasing the likelihood of thrombus formation at sites of vascular injury, for example, within the mesenteric circulation. P-selectin is the major ligand for leukocyte-endothelial interaction and is responsible for the rolling of platelets, leukocytes, and PLAs on vascular endothelium^{109,110}.

Moreover, platelets adherent to injured vascular endothelium support leukocyte adhesion via P-selectin, an effect that could contribute to leukocyte emigration from the vasculature into the lamina propria in patients with IBD^{111,112}. In addition, P-selectin is the major platelet ligand for platelet-leukocyte interaction, which in turn causes both leukocyte activation and further platelet activation¹¹³. More recently it has become apparent that platelets from patients with IBD express higher levels of CD40 ligand (CD40L, another marker of platelet activation) than do platelets from healthy control subjects¹¹⁴. Human intestinal microvascular endothelial cells express CD40, expression of which is up regulated in inflamed IBD mucosa¹¹⁵.

Activated platelets, acting through CD40-CD40L interaction, cause human intestinal microvascular endothelial cells to increase expression of intercellular adhesion molecule 1 and vascular cell adhesion molecule 1, and to produce interleukin-8¹¹⁶. CD40L has prothrombotic properties and is involved in the stabilization of thrombus¹¹⁷. Elevated soluble CD40L levels are a risk factor for vascular events and have recently been demonstrated in IBD^{114,118}.

Platelet-leukocyte aggregation in Inflammatory Bowel Disease

Recently, studies showing that platelets and leukocytes that circulate together in aggregates (PLA) are more activated than those that circulate alone have generated interest in the role of these PLA in various inflammatory and thrombotic conditions. PLA numbers are increased in patients with ischemic heart disease, systemic lupus erythematosus and rheumatoid arthritis, myeloproliferative disorders, and sepsis as well as by smoking^{61,119-124}. It was recently shown that patients with IBD have more PLAs than both healthy and inflammatory control subjects (patients with inflammatory arthritides)¹⁰⁴. As with platelet activation, there was no correlation with disease activity, suggesting that increased PLA formation might be an underlying abnormality^{99,105,125}.

PLAs could contribute to the pathogenesis of IBD in a number of ways (Figure 1.2). As previously mentioned, TF is key to the initiation of thrombus formation. TF has recently been demonstrated on the surface of activated platelets and in platelet-derived microvesicles. Interaction between neutrophils and activated platelets, or microvesicles, vastly increases the activity of "intravascular" TF¹²⁶. Moreover, platelet-monocyte interaction increases monocyte-produced TF¹²⁷⁻¹²⁹. Thus, enhanced TF production is one way in which PLA formation

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increases the likelihood of thrombus formation, whether it is within the mesenteric microvasculature or within the rest of the systemic circulation.

PLAs are involved in thrombus formation, and it is therefore possible that they could contribute to intestinal microinfarction¹³⁰. It has also been observed that leukocytes forming PLAs have a more highly activated adhesion molecule profile than those that circulate alone, and that platelet-monocyte aggregates are more likely to adhere to activated vascular endothelium than monocytes alone^{131,132}. In addition, because platelet aggregation with leukocytes causes platelet activation, PLAs could enhance the role that activated platelets play in the pathogenesis of IBD. Finally, it is possible that PLAs might contribute to the thromboembolic phenomena seen in IBD by directly exacerbating thrombus formation within the systemic circulation and by enhancing the production of TF by monocytes.

Thrombosis and the treatment of Inflammatory Bowel Disease

For some years there has been interest in the role of heparin as a specific treatment for active UC and, to a lesser extent, CD. Initial reports from Russia claimed marked benefit from the use of unfractionated heparin in the treatment of UC. Subsequently, uncontrolled studies appeared to support these findings¹³³⁻¹³⁵.

Only two randomized controlled studies have been published in full, however, and these generated conflicting results.

In a study of 20 patients with colitis (17 UC, 3 CD), intravenous heparin followed by subcutaneous unfractionated heparin appeared to be as effective as intravenous steroids followed by oral steroids¹³⁶.

However, in the same year, a similar study was published comparing intravenous unfractionated heparin and intravenous steroids in 25 patients with moderate to severe UC¹³⁷. In the latter study, heparin was not only ineffective, but it was also associated with significant bleeding; the trial was terminated prematurely. A possible explanation for the discrepancy between the two trials is that concomitant mesalamine usage was permitted in the former but not the latter. Since then, no further randomized studies with unfractionated heparin have been published; hence it is difficult to draw conclusions regarding its efficacy.

A limited number of studies have been performed with low molecular weight heparin (LMWH), which in theory has the advantage of lesser bleeding complications than unfractionated heparin. However, in two recent randomized, placebo-controlled studies, a benefit with LMWH was not shown, this in the context of a high placebo response^{138,139}. If heparin should be beneficial in UC, its anticoagulant properties are only one possible mode of action; heparin also has anti-inflammatory effects and might interfere with activation of leukocytes, their adhesion to vascular endothelium, and their migration into the lamina

propria¹⁴⁰. Alternatively, or in addition, it has been suggested that heparin replenishes binding sites for fibroblast growth factors, such as syndecan-1, the epithelial expression of which is decreased in active IBD. In this way heparin could improve healing of ulcerated mucosa¹⁴¹.

No other antithrombotic therapy has been effective in randomized controlled trials in IBD. There have been no studies with aspirin, either as a therapeutic agent or as a prophylactic against systemic thrombosis, perhaps because of concerns about the risks of exacerbation of IBD by NSAIDs¹⁴².

Ridogrel, a thromboxane synthase inhibitor, was shown to be ineffective in the treatment of CD and UC, and SR27417A, a platelet-activating factor antagonist, was ineffective in the treatment of active UC¹⁴³⁻¹⁴⁵.

Although picotamide, a thromboxane antagonist, improved symptoms in a small open trial in patients with active CD, the findings have not been reproduced in a controlled study; the drug is no longer marketed¹⁴⁶.

Recent work, however, suggest that further investigation in this field remains worthwhile: an antibody to P-selectin glycoprotein-1 (the leukocyte ligand for P-selectin) has been shown to attenuate established dextran sodium sulfate colitis in mice¹⁴⁷.

There is some evidence that drugs used in the treatment of IBD might work, at least in part, by decreasing the prothrombotic state; demonstrating causality however is difficult. Mesalamine decreases platelet activation in vivo and in vitro¹⁰⁸. Patients taking thiopurines have fewer PLAs than those who are not, and treatment with monoclonal antibodies to TNF α reduces thrombin generation in CD^{106,147}.

Despite the inconclusive data regarding the use of antithrombotic medication in the treatment of IBD, the use of prophylactic heparin to decrease the risk of thromboembolic complications in hospitalized patients with IBD is recommended, albeit on a limited evidence base. Indeed, the ethical and practical difficulties of performing a controlled trial probably preclude the production of data to prove that such therapy reduces the incidence of systemic thromboembolism in IBD¹⁴⁹⁻¹⁵¹.

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First author (reference)	Year	Number of cases examined	Frequency of thromboembolic disease %
Clinical studies			
Bergen (27)	1936	1500	1.2
Ricketts (152)	1949	206	1.9
Dennis (30)	1952	261	7.5
Edwards (153)	1964	624	6.7
Talbot (37)	1986	7199	1.3
Webberley (95)	1993	104	7.7
Jankelson (154)	1997	145	2.8
Jackson (155)	1997	11402	0.7 ^a
Miehsler (32)	2004	618	6.2
Postmortem studies			
Bergen (27)	1936	43	31
Warren (156)	1949	180	6.7
Sloan (157)	1950	99	41
Graef (158)	1966	100	39

Table 1.1 Frequency of thromboembolic complications in patients with IBD

^a Review of hospital admissions with first or second diagnosis of IBD, of which 0.7% had a thrombotic episode documented as a concurrent diagnosis.

References	
Clotting abnormality	
↑ Fibrinogen	74, 97, 98, 159–166
↑ Factor V	97, 160
↑ Factor VIII	74, 97, 159, 160
↑ Factor IX	160
↓ Factor XIII or factor XIII A subunit	43, 75, 165, 167–169
↓ Protein S levels	170–172
↓ Antithrombin	97, 160, 165, 173
↑ Tissue factor activity	45, 174
↓ Tissue, ↑serum thrombomodulin	81, 166
Abnormality of fibrinolysis	
↓ Tissue-type plasminogen activator	171, 175–178
↑ Urokinase-type plasminogen activator	161, 175, 176
↑ Plasminogen activator inhibitor	161, 166, 176–178
Possible nutritional factors	
Hyperhomocysteinemia	36, 76, 179
↑ Lipoprotein (a)	164, 180
Platelet abnormalities	
Thrombocytosis	93, 94
Increased activation and aggregation	95, 99, 105–108, 114
Increased PLA	106
Endothelial abnormalities	
Circulating von Willebrand's factor	53
↑ Anti-endothelial cell antibodies	54

Table 1.2 Haemostatic abnormalities in patients with IBD

Figure 1.1 The coagulation system. Endothelial injury exposes TF, which forms a complex with factor VII. This complex activates factors X and, to a lesser extent, IX. TFPI prevents this activation progressing further; for coagulation to progress, factor Xa must be produced via factors IX and VIII. Thrombin, generated by the initial production of factor Xa, activates factor VIII and, through factor XI, factor IX, resulting in further activation of factor X. This positive feedback loop allows coagulation to proceed. Fibrin polymers are stabilized by factor XIIIa. Activated proteins C+S (APC+S) together inhibit factors VIIIa and Va, whereas antithrombin (AT) inhibits factors VIIa, IXa, Xa, and XIa. Fibrinolysis balances this system through the action of plasmin on fibrin. Plasminogen activator inhibitor controls the plasminogen activator-induced conversion of plasminogen to plasmin.

Figure 1.2 The roles of activated platelets and PLAs in mucosal inflammation. Activated platelets can interact with other cells involved in the inflammatory response either through direct contact or through the release of soluble mediators. Activated platelets interact directly with activated vascular endothelium, causing the latter to express adhesion molecules (like ICAM-1 and VCAM-1) and release inflammatory and chemotactic cytokines. Activated platelets can also aggregate with leukocytes, further activating the constituent platelets and leukocytes, e.d. by the production of platelet activating factor (PAF). The resultant PLAs are more likely to adhere to vascular endothelium than leukocytes that circulate alone. Platelets also release a host of soluble mediators that have a broad range of effects on intravascular and extravascular cells; for example, they attract and activate leukocytes, activate vascular endothelium, and cause fibroblast proliferation, in which platelet derived growth factor (PDGF) may play a role. Leucocytes might add to the activation of haemostasis by the production of tissue factor (TF), which can be transferred to platelets.

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Aim of the thesis

The aim of the thesis is to investigate the long-term course of blood coagulation, fibrinolysis and platelet factors in patients with Crohn's disease and ulcerative colitis, both during active and inactive disease, as well as to determine the influence of low molecular weight heparin treatment on disease activity, haemostasis factors, and the occurrence of microvascular thrombi, in patients with ulcerative colitis.

Outline of the thesis

This thesis is divided in three parts.

In the **first part** we present a case report of a patient with active ulcerative colitis and pancreatitis, who was treated with low molecular weight heparin (*chapter 2*).

The **second part** is an observational study on the long-term course of serologic markers of coagulation and fibrinolysis (*chapter 3*), coagulation factor XIII (*chapter 4*), as well as platelet factors (*chapter 5*), in relation to clinical disease activity in IBD patients. To demarcate the specificity of our findings, the markers were determined in patients with IBD, as well as in patients with giant cell arteritis, a biopsy-proven vasculitis with characteristics of a granulomatous disease similar to Crohn's disease. Also, age- and sex-matched controls without signs of inflammation were investigated.

The **third part** involves therapeutic interventions, describing the influence of anticoagulant treatment with low molecular weight heparin (LMWH) on disease activity, as well as markers of coagulation and fibrinolysis, in patients with ulcerative colitis.

In an open study, we evaluated the tolerability and safety of self-administered LMWH (nadroparin) and its influence on clinical disease activity, as well as the laboratory, endoscopy and histology outcome in patients with moderate-to-severe ulcerative colitis, which did not respond to glucocorticosteroids.

In this study, we additionally investigated the presence of microvascular thrombi in colonic mucosal biopsies to address the influence of LMWH treatment (*chapter 6*).

In a second, double blind, placebo-controlled trial, we investigated the influence of LMWH (reviparin) in patients with light-to-moderately active ulcerative colitis on clinical, laboratory, endoscopy, and histology characteristics (*chapter 7*). In these patients, we also studied the influence of LMWH versus placebo therapy on thrombin generation, as well as well-known parameters of coagulation and fibrinolysis (*chapter 8*).

Finally, the results of the thesis are discussed in *chapter 9*, and the summary and conclusions are described in *chapter 10*.

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Chapter 2

Low molecular weight heparin treatment in
steroid resistant ulcerative colitis

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*A case report, presented at the EC-IBD meeting on October the 23d 1998 in
Maastricht, The Netherlands*

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The patient was a 25-year-old woman with a 12-year-history of ulcerative colitis (UC), restricted to the proctosigmoid colon. At the time of diagnosis in 1996, she was using a sub-50 estrogen contraceptive; she did not smoke or use alcohol. Treatment started with mesalazine 3 grams a day orally, because she disliked enema therapy. After 3 months she had a moderate flare-up of the colitis, as well as mesalazine induced nausea. Prednisolone (25 mg a day) was started and clinically she improved. After 8 months, the patient was admitted to the hospital because of vomiting and a sudden attack of upper abdominal pain, radiating to the back, together with bloody stools and 3 kg of weight loss in the past month. At that time she took 10 mg of prednisone daily, as well as 2 grams of mesalazine.

On physical examination, she was moderately ill with normal vital signs. Her weight was 64.2 kg at a length of 169 cm. No abnormalities were found except for reduced peristaltic sounds and slight upper abdominal pain on pressure. Abnormal laboratory findings were: ESR 17 (norm values: 0-13) mm/hr, CRP 99 (0-8) mg/l, serum amylase 202 (upper limit 150) IU/l and lipase 1242 (upper limit 300) IU/l, alkaline phosphatase 264 (25-100) IU/l and gamma-glutamyl-transpeptidase 148 (15-40) IU/l.

Because of the suspicion of acute pancreatitis, an abdominal ultrasound was made to exclude biliary disease.

It showed distended bile ducts (slightly intra-hepatic, and 10 mm extra-hepatic), and an oedematous pancreatic body, consistent with an acute pancreatitis. An abdominal CT scan showed pancreatic oedema without peri-pancreatic fat infiltration, necrosis or ductular abnormalities. On ERCP, the cholangiogram showed calibre changes of the intrahepatic ducts, possibly resulting from a primary sclerosing cholangitis. The major pancreatic duct had an irregular appearance.

On colonoscopy, a pancolitis was diagnosed, characterized by superficial ulcers, but no signs of ileitis.

Histology confirmed the diagnosis of UC, characterized by superficial inflammatory and crypt-architectural changes, and without signs of cytomegalovirus infection. Faecal cultures and examinations for dysenteric bacteria and amoebas were negative.

A liver biopsy was done to confirm the presence of a primary sclerosing cholangitis. However, only slight sinusoidal dilatation was found, without signs of an overt triaditis or cholangiolitis. These findings were possibly due to the use of oral contraceptive medication.

To summarize, the patient had a prednisolone resistant colitis, complicated by a pancreatitis of unknown origin. The medication used (mesalazine, prednisone, oral anti-conceptives) and - less probably - a pancreatic involvement of a primary sclerosing cholangitis could have played a role in the

genesis of the pancreatitis. At the time, pancreatic autoantibodies were not measured, but the IgG1-4 status as well as p-ANCA was normal.

Treatment of the pancreatitis consisted of total parenteral nutrition, followed by elementary enteric feeding. However, rectal bleeding persisted and the pancolitis seemed difficult to treat, as immunosuppressant medication with high doses of prednisolone, azathioprine or cyclosporine could theoretically worsen the course of the pancreatitis.

In view of a recent publication on heparin therapy in UC patients (1), we reinvestigated the biopsies of our patient, and an ELISA staining against early cross-linked fibrin was done on paraffin embedded biopsy material, comparable with the MSB staining done by Gaffney and co-workers¹.

In our patient, microvascular thrombi were found in submucosal vessels of the inflamed sigmoid colon, with cellular elements - like red blood cells and platelets - within the fibrin matrix. (Figure 2.1)

In addition to the continued 20 mg of prednisolone iv, we decided to give a heparin derivative for the treatment of the colitis. To reduce the risk of bleeding, we chose for a Low Molecular Weight Heparin (LMWH), i.e. nadroparin (Fraxiparine®), 5.700 IE anti-Xa/0.6 ml (or 15.000 AXa IC-E/0.6 ml) twice daily, similar to the treatment of deep vein thrombosis.

After 2 weeks of treatment with this combination, a clinical and biochemical remission of both the UC and the pancreatitis was achieved.

After a follow-up of 1.5 years, the patient did not have flare-ups of UC, while taking 5 mg of oral prednisolone and self-administered nadroparin bid sc. Side effects of the LMWH in our patient were small bruises at the site of injection; she had no liver test abnormalities.

Other complications consisted of a temporary amenorrhea for half a year. A Dual Energy X-ray Absorption (DEXA)-scan suggested lumbar spine osteopenia, probably due to the use of steroids, although (unfractionated) heparin can induce osteopenia. A follow-up DEXA-scan after 1 year showed stabilized values under calcium-vitamin D suppletion without bisphosphonate therapy.

Steroids were withdrawn, and patient agreed to continue nadroparin once daily. After 2 years of quiet disease, an attempt was made to stop nadroparin, but within 3 days patient developed the clinical picture of erythema nodosum on her legs, a well-known extra-intestinal complication of UC. Notably, at that time the colitis activity index was low.

It was decided to reintroduce nadroparin without steroid therapy and, within 1 week, the erythema nodosum disappeared. Thereafter, we were able to taper and stop nadroparin therapy without complications.

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In conclusion, we have presented a case of steroid resistant ulcerative colitis, complicated by an idiopathic pancreatitis, in which the pancolitis could be safely treated with additional LMWH therapy, and steroids could be withdrawn in time. We asked ourselves whether the presence of microvascular thrombi could predict that patients would benefit from this additional treatment. We felt that controlled studies on the subject of LMWH treatment in inflammatory bowel disease should be performed to assess efficacy as well as side effects, before widespread use of LMWH in UC could be advocated.

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submucosal layer

mucosal layer

Figure 2.1 Early cross-linked fibrin staining (brown material) in a patient with active proctosigmoiditis. Microthrombi are detected in the smaller vessels of the submucosal layer of the sigmoid colon.

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Chapter 3

Coagulation and fibrinolysis in inflammatory
bowel disease and in giant cell arteritis

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Interaction of studied coagulation and fibrinolysis markers.

Abstract

Background

In inflammatory bowel disease (IBD), gut microvascular thrombosis as well as thrombo-embolic complications has repeatedly been observed. We examined the long-term course of markers of coagulation and fibrinolysis in relation to clinical disease activity.

Materials and methods

In a prospective study, prothrombin fragment 1 and 2 (F1.2), thrombin-anti-thrombin complex (TAT), antithrombin (AT), D-dimer, plasmin-alpha2-antiplasmin complex (PAP) and plasminogen-activator-inhibitor-1 (PAI-1) were measured in 20 patients with Crohn's disease (CD), 18 with ulcerative colitis (UC), and 19 with giant cell arteritis during active and inactive disease, as well as in 51 controls without inflammation.

Results

Levels of F1.2, TAT, D-dimer, PAP and PAI-1 were significantly higher in active versus inactive CD and UC. However, even after 12 months of follow-up, in CD and UC the mean levels of F1.2, D-dimer and PAP were significantly higher than the levels of the controls.

Conclusions

Levels of F1.2, D-dimer and PAP were markedly raised for a long time in clinically inactive IBD, underlining a chronic state of hypercoagulation and enhanced fibrinolysis.

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Introduction

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In patients with inflammatory bowel disease (IBD), clinical assessment of disease activity as well as the response to treatment may be difficult, and biochemical markers reflecting these processes would be helpful¹. Most widely used are acute phase proteins, like C-reactive protein (CRP), synthesized in the liver as a response to mediators such as interleukin-6, probably liberated from the inflammation in the gut. In general, a close link exists between coagulation and inflammation, and fibrin degradation products are capable of inducing an inflammatory response². The observation of intestinal microvascular damage in Crohn's disease (CD)³⁻⁶ as well as fibrinoid microvascular thrombi in the gut mucosa of patients with ulcerative colitis (UC)⁷ could be of etiologic importance.

Recurrent thromboembolic complications have been reported in 10–13% of patients with IBD^{8,9} with a 3-fold increased risk of developing deep venous thrombosis and pulmonary embolism in an age- and sex-matched population-based study¹⁰. Also, inherited disorders of hypocoagulation appear to protect against IBD¹¹. However, no single inherited procoagulant disorder, like e.g. factor V Leiden¹²⁻¹⁵, was found to account for the risk of venous thrombosis in IBD¹⁶. Although alterations in blood markers of coagulation and fibrinolysis have been found in patients with IBD¹⁶⁻²⁶, the duration of follow-up was relatively short. In a prospective manner and during a longer time of follow-up than was studied previously, we investigated the course of markers for coagulation activation prothrombin fragment 1 and 2 (F1.2), thrombin-antithrombin complex (TAT) and coagulation inhibition (antithrombin, AT), as well as fibrinolysis activation (Ddimer) and fibrinolysis inhibition [plasmin-2-antiplasmin complex (PAP), plasminogen activator inhibitor-1 (PAI-1)].

These protein complexes or end products with relatively long plasma half-lives were chosen to reliably characterize coagulation and fibrinolysis processes, as well as the relationship with disease activity over a longer time span. The markers were determined in patients with IBD, as well as in patients with a well-known vasculitis (giant cell arteritis, GCA), to demarcate the specificity of our findings. GCA has characteristics of a granulomatous disease similar to CD, although in GCA, medium to large arteries are generally involved²⁷. However, GCA is well diagnosed with a temporal artery biopsy, while in patients with a small-vessel vasculitis like in Henoch-Schönlein purpura or (microscopic) polyangiitis, a uniform histology diagnosis is more difficult to obtain.

Our hypothesis was that in active but also in inactive IBD and GCA, levels of coagulation and fibrinolysis were raised. Furthermore, to assess the possible role of coagulation and fibrinolysis factors as markers of disease activity, a

correlation with indices of clinical activity as well as biochemical activity like the erythrocyte sedimentation rate (ESR) or CRP was investigated.

Materials and methods

Patients

In a prospective study, performed at the Department of internal medicine of the Atrium Medical Centre Heerlen, The Netherlands, 38 newly diagnosed in- or outpatients with IBD were enrolled from December 1991 to December 1994 and followed in an active and inactive phase of disease. The Ethics Committee of the Atrium Medical Centre Heerlen approved the study and patients provided written informed consent before entry. Twenty patients with CD and 18 patients with UC were included with a median duration of follow-up of 12 (range 6–21) months. In CD and UC, the diagnosis was documented according to the criteria of Lennard-Jones²⁸. Although it was not an exclusion criterion in this study, no patients were categorized as having indeterminate colitis.

In CD, the severity of disease was classified according to the Crohn's Disease Activity Index (CDAI)²⁹. Active disease was defined as a CDAI score >150. At entry, 6 patients had a CDAI >450, 11 patients had a CDAI score between 300 and 450 and in 3 patients, the CDAI ranged from 150 to 299.

In patients with UC, severity of disease was classified according to the Truelove-Witts criteria³⁰. For statistical purposes, the classification was quantitatively modified³¹. The score in severe disease was between 15 and 18, in moderate disease between 9 and 14, in mild disease between 4 and 8 and in inactive disease it was 3 or less. At entry, disease activity was severe in 12 patients, moderate in 6 patients and none had mild or inactive disease. In 19 patients with a histologically proven and newly diagnosed GCA, activity was assessed according to the criteria of the ARA²⁷, as well as by the ESR and CRP levels. Since the GCA group was considerably older than the group of IBD patients, separate groups of controls were included.

Twenty-five age- and sex-matched orthopaedic patients, seen before elective minor surgery, served as a control group for patients with IBD. They had no signs of vasculitis or gastrointestinal disease. Also, 26 age- and sex-matched controls, consisting of 19 healthy females of an older age and 7 male urology patients seen in the outpatient clinic because of benign prostate hypertrophy, were all without signs of inflammation and served as a control group for patients with GCA.

None of the patients was on oral anticoagulants or aspirin. Patients with pre-existing liver disease, with coexisting malignancy, or who were pregnant and patients who refused consent were excluded from the study.

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Depending on the severity of disease, therapy in IBD consisted of oral 5-aminosalicylic acid (5ASA) and/or prednisone (starting dose varying between 0.5 and 1 mg/kg/day), azathioprine (2.5 mg/kg), and was sometimes combined with enema therapy. Prednisone was started in case of insufficient clinical response to 5ASA treatment in active CD (CDAI >150) and moderate to severe UC (Ulcerative Colitis Activity Index, UCAI)¹⁸. In active GCA, oral prednisone (absolute dose 40 mg/day) was started, and according to the clinical and acute phase response, dosage was gradually lowered³². None of the patients received plasma derivatives other than packed cells, and none of the patients with IBD had surgery during follow-up.

Plasma samples were collected during active UC, CD and GCA before conventional therapy, and – after intervals of 3 months – in inactive disease while on low-dose prednisone (less than 15 mg/day), azathioprine and/or mesalazine (oral or enema) in the IBD group. In the noninflammatory controls, blood samples were taken twice within a month before surgery. All patients with UC underwent a sigmoidoscopy and/or colonoscopy at entry, and in CD (an additional) selective small bowel enteroclysis was performed.

However, unlike the clinical activity and laboratory investigations, follow-up endoscopies were not performed in a standardized manner.

The characteristics of patients and controls are shown in **table 3.1**.

Methods

All blood samples were drawn fasting between 8 and 10 a.m. to avoid diurnal variation³³. After a resting period of 20 min, trained operators performed non-traumatic venipuncture in a standardized manner. After discarding the first 2 ml of blood, 4.5 ml of blood was drawn into test tubes with 0.5 ml citrate and immediately kept on ice. Within 1 h from sample collection, all test tubes were centrifuged for 30 min (3,000 g) at 4°C. Platelet poor plasma was aspirated from the central part of the plasma, without disturbing either the top layer or the buffy coat. The plasma was snap-frozen in aliquots, stored at –70°C in plastic tubes and thawed in a water bath at 37°C for 5 min, immediately prior to serial analysis. Plasma concentrations of F1.2, TAT, D-dimer, PAP and PAI-1 were determined by sandwich-type enzyme immunoassays, and the activity of AT was measured by a chromogenic substrate (Boehringer Mannheim). The intra-assay coefficient of variation was between 5-10% for the assays. CRP was measured by an enzyme immunoassay (Behring, Germany; normal values 0-5 mg/l), and the ESR was measured according to the Westergren method (normal values 5-15 mm/hr).

Statistical analysis

All patients were included in the follow-up data. In UC, CD and GCA, median values with the minimum-maximum range (**table 3.1**) or mean values with the

95% confidence intervals (CI) for the mean (table 3.2) were calculated in case of a skewed or even distribution of data, respectively. To test differences for significance, the Mann-Whitney signed rank test was performed for the longitudinal patient follow-up as well as the Mann-Whitney U test to compare the three patient groups with the age- and sex-matched control groups (table 3.2). Furthermore, the Spearman rank correlation coefficient and the two-tailed significance of the correlation were calculated between coagulation, fibrinolysis, and other biochemical markers and disease activity indices. A correction for multiple comparisons was performed using the Bonferroni-adjusted level of significance. Calculations were done with CD and UC as separate groups, not with IBD as a whole. For statistical analysis, SPSS 10.0 software was used (SPSS, USA).

Results

Markers of coagulation activation and inhibition in active and inactive disease (table 3.2)

F1.2 was significantly higher in active and inactive CD, UC and GCA compared to the controls ($p < 0.0001$). Also, F1.2 values were higher in active than in inactive CD or UC ($p = 0.005$), unlike in active versus inactive GCA ($p = 0.297$). TAT was higher in active CD, UC and GCA compared to the controls ($p < 0.0001$), and higher in active versus inactive CD or UC ($p < 0.001$) unlike GCA ($p = 0.402$). AT activity was normal in active and inactive IBD, but rose in inactive compared to active GCA ($p < 0.0001$).

In the non-inflammatory control groups, laboratory values were within normal limits.

Markers of fibrinolysis activation and inhibition in active and inactive disease (table 3.2)

D-dimer was significantly higher in active and inactive CD, UC and GCA compared to the controls ($p < 0.0001$), and higher in active versus inactive CD, UC or GCA ($p < 0.0001$). PAP was higher in active CD, UC and GCA, compared to the controls ($p < 0.0001$), as well as in active versus inactive CD ($p = 0.002$), UC ($p < 0.0001$) or GCA ($p < 0.0001$). PAI-1 was higher in active UC and GCA, but not in active CD compared to the controls ($p = 0.003$; $p = 0.001$; $p = 0.582$, respectively). PAI-1 levels were reduced in inactive compared to active CD ($p = 0.011$), UC ($p < 0.0001$) and GCA ($p = 0.006$).

PAP and PAI-1 values were normal in the controls, but D-dimer was higher in the elderly (GCA) controls ($p = 0.001$).

Correlations between coagulation and fibrinolysis markers

In CD and UC, F1.2 correlated with AT, D-dimer, PAP (all $p < 0.0001$), and PAI-1 ($p = 0.012$). TAT correlated with D-dimer, PAP and PAI-1 ($p < 0.0001$). AT did not correlate with the other coagulation and fibrinolysis markers in CD and UC, except for TAT in UC ($p = 0.009$). D-dimer correlated with PAP ($p < 0.0001$) in CD and UC, as well as PAI-1 in CD ($p = 0.002$), but not in UC. PAP correlated with PAI-1 in UC ($p = 0.007$) unlike in CD.

In GCA, the markers F1.2 and TAT did not correlate with the other coagulation and fibrinolysis markers. D-dimer correlated with PAP and PAI-1 ($p < 0.0001$ and $p = 0.006$), and PAP correlated with AT ($p = 0.002$) and PAI-1 ($p = 0.003$).

Correlations of coagulation and fibrinolysis markers with clinical and biochemical indices of disease activity (table 3.3)

In CD, the CDAI, as well as the ESR and CRP, correlated with F1.2, TAT, D-dimer, and PAP, but not with AT. Also, PAI-1 correlated with the CDAI and ESR, but not with CRP.

In UC, the UCAI, as well as the ESR and CRP, correlated with F1.2, TAT, D-dimer, PAP and PAI-1, but not AT.

In GCA, the ESR did not correlate with F1.2 or TAT, but inversely correlated with AT ($r = -0.428$; $p = 0.005$). The ESR however correlated with D-dimer ($r = 0.726$; $p < 0.0001$), PAP ($r = 0.710$; $p < 0.0001$), and PAI-1 levels ($r = 0.425$; $p = 0.005$).

Correlation of coagulation and fibrinolysis markers with the extent of bowel involvement at entry

The endoscopic extension of bowel involvement in patients with active CD or UC was investigated as well as its relationship to the coagulation and fibrinolysis markers.

Patients with CD involving only the small bowel ($n = 10$) or only the colon ($n = 4$), and CD of both small and large bowel segments ($n = 6$) were distinguished. Levels of F1.2, TAT and AT, as well as D-dimer, PAP and PAI-1 were not significantly different according to disease localization and extension. However, the number of patients does not allow us to make definite statements in this respect.

Patients with UC involving only the rectum and/or sigmoid colon ($n = 9$), and UC extended to the more proximal colon segments (left-sided colitis: $n = 3$; pan colitis: $n = 6$) were distinguished. Levels of TAT ($p = 0.022$), as well as D-dimer ($p = 0.031$), PAP ($p = 0.002$) and PAI-1 ($p = 0.049$) were significantly higher in more extensive UC, and a correlation with the extent of bowel involvement at entry was found with TAT ($r = 0.534$; $p < 0.0001$), D-dimer ($r = 0.477$; $p = 0.002$),

PAP ($r=0.446$; $p=0.004$), PAI-1 ($r=0.463$, $p=0.003$), as well as CRP ($r=0.618$; $p<0.0001$).

The course of coagulation and fibrinolysis markers in time in CD and UC

In CD, after 12 months of follow-up, the mean F1.2 was higher by a factor of 1.5 than the control value (0.86 and 0.56 mmol/l, respectively; $p<0.0001$), and in 3 of 18 patients tested it was above the upper reference value.

As a contrast, TAT equalized the mean control value between 3 ($p=0.048$) and 6 months ($p=0.892$). The AT activity in CD was stable and not different from the controls during 12 months of follow-up (**figure 3.1**).

The mean D-dimer level in CD was higher by a factor of 1.9 than in the controls (524 and 283 $\mu\text{g/l}$; $p<0.0001$; above upper reference in 11 of 18), and the mean PAP level was higher by a factor of 1.7 after 12 months (486 and 282 $\mu\text{g/l}$; $p<0.0001$; above upper reference in 8 of 18).

The initially higher mean values of PAI-1 became lower by a factor of 1.7 as compared to the controls (16.0 and 26.8 $\mu\text{g/l}$; $p=0.005$; in 1 of 18 below the lower reference value) (**figure 3.2**).

In UC, after 12 months the mean F1.2 count was higher by a factor of 1.5 than in the controls (0.89 and 0.56 mmol/l respectively; $p=0.009$; above upper reference in 6 of 17). However, TAT equalized the controls between 3 ($p=0.024$) and 6 months ($p=0.225$). As in CD, the AT activity course in UC did not differ from the controls (**figure 3.3**).

The mean D-dimer level was higher by a factor of 2.0 in UC than in the controls (555 and 283 $\mu\text{g/l}$; $p<0.0001$; above upper reference in all 17 patients tested), and the mean PAP level was higher by a factor of 1.6 than in the controls (461 and 282 $\mu\text{g/l}$; $p<0.0001$; above upper reference in 11 of 17) after 12 months. Like in CD, initially higher mean values of PAI-1 became lower by a factor of 1.7 as compared to the controls (15.9 and 26.8 $\mu\text{g/l}$; $p=0.005$; no scores below the lower reference value) (**figure 3.4**).

Discussion

Not only in the active, but also in the inactive phase of IBD we found a significantly higher level of coagulation and fibrinolysis as compared to the age- and sex-matched controls. Second, long-term patterns of coagulation and fibrinolysis were similar in GCA and IBD. This suggests that common pathways, e.g. microvascular thrombosis or vasculitis, may play a role in both IBD and GCA. Although we did not examine coagulation activity in biopsies in the present study, we and others have shown that microthrombi are present in gut mucosa even when histology shows no inflammation^{7,34}. We therefore

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hypothesize that hypercoagulation and disturbed fibrinolysis might add to the chronicity of these diseases.

Unlike the study of Henegan et al.³⁵, but analogous to the study of van Bodegraven et al.³⁶, we did not find a significantly low AT level in IBD as compared to the controls. In GCA however, levels of AT were significantly lower in active versus inactive GCA. Also, TAT levels were raised both in active and inactive GCA, and this might point to a disseminated intravascular coagulation with more consumption of AT during active disease as compared to IBD³⁷. In the two noninflammatory control groups, the only difference was a higher D-dimer in the older patients, also found by others³⁸. Although we cannot find a clear explanation, it might be a feature of more background atherosclerosis occurring in the elderly. However, we would have expected that the other markers would be raised to some degree as well^{39,40}. In this study, a clinical remission was not documented with follow-up endoscopy and histology in IBD. Therefore, it is possible that at least part of the raised hemostasis activity was due to ongoing inflammation. At entry, levels of coagulation (TAT) as well as fibrinolysis factors (D-dimer, PAP, PAI-1) were lower in proctosigmoiditis than in more extensive UC.

Although this suggests a relationship between the size of the mucosal defect and the magnitude of coagulation activation, these markers also correlated with CRP, fitting in with the concept of a more generalized systemic inflammatory and hemostatic response. After treatment, CRP levels rapidly declined but our treatment regimen with mesalazine, prednisone and azathioprine did not alter the pattern of hypercoagulation and fibrinolysis during long-term follow-up. Although patients were clinically well, our data suggest that their disease was still subclinically active. In theory, the medication could have promoted this disturbed pattern of hemostasis.

Corticosteroids can induce neutrophilia⁴¹, and apart from fibrinolysis, high levels of D-dimer could result from neutrophilia in IBD and GCA, due to enhanced elastase release by these polymorph nuclear cells^{42,43}. However, in CD and UC, the use of steroids resulted in a moderate decrease in D-dimer, F1.2 and PAP, and no significant difference in D-dimer was found between patients with or without steroid use. Corticosteroids might have reduced PAI-1 levels as in Henoch-Schönlein purpura⁴⁴, and in our study, levels of PAI-1 became low during follow-up. However, in IBD, steroids were generally withdrawn within 3–6 months, while mean PAI-1 levels did not change significantly between 3 and 12 months of follow-up.

To our knowledge, no direct effect of azathioprine on coagulation has been described. The use of azathioprine could have led to (opportunistic) infections, like e.g. cytomegalovirus reactivation, resulting in a disturbed coagulation⁴⁵. However, no clinical signs of chronic (systemic) infections were encountered in our follow-up in vitro platelet activation has been described in patients with

IBD⁴⁶. However, no secondary rise in coagulation and fibrinolysis factors has been documented.

It is unknown what exactly happens early in the course of IBD. Indirect evidence has come from recent studies that the activation of hemostasis and fibrinolysis is most likely a result of stimulation by certain cytokines from the inflammatory process in the gut, such as tumor necrosis factor- α ⁴⁷. Anti-tumor necrosis factor- α antibody (Remicade[®]) treatment was accompanied by a clear reduction in coagulation and fibrinolysis activity as studied in 8 patients with CD⁴⁸, suggesting a secondary role for the coagulation and fibrinolysis system in CD.

Are the markers tested also clinically useful? A positive correlation was found between the CDAI as well as the UCAI with F1.2, TAT, D-dimer, PAP and PAI-1. Also, TAT, D-dimer, PAP and PAI-1 correlated with the extent of bowel involvement at entry. However, the persistently higher than normal levels of F1.2, D-dimer and PAP preclude their use as rapid diagnostics for measuring the influence of therapy, unlike e.g. an acute phase reactant like CRP^{24,49}.

Nevertheless, the measurement of plasma concentrations of F1.2, TAT, D-dimer, PAP and PAI-1 could be useful as an additional tool. They might reflect inadequate control of the inflammatory process in the gut, and possibly a systemic tendency towards thrombo-embolic complications⁵⁰. Future therapeutic intervention studies with drugs which could influence both inflammation and microvascular thrombosis⁵¹, should therefore be welcomed.

	CD	UC	GCA	C1	C2
Patients	20	18	19	25	26
Female	14	11	14	16	19
Male	6	7	5	9	7
Median age, years	34 (20-71)	47 (17-86)	72 (65-88)	41 (22-74)	71 (67-84)
Median follow-up, months	12 (6-21)	12 (6-18)	10 (6-22)	1 (0.5-1)	1 (0.5-1)
Smoking	8	4	6	6	7
Oral anticonceptive use	5	4	0	8	0

Table 3.1 Characteristics of the patients and control groups (C1 for CD and UC and C2 for GCA).
Figures in parentheses represent range.

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Laboratory Tests	CD, CDAI >150	CD, CDAI <150	UC, UCAI >4	UC, UCAI <4	GCA, ARA active	GCA, ARA inactive	C1	C2
F1.2 (0.26-1.14 mmol/l)								
mean	1.70	0.87	1.3	1.04	2.37	1.52	0.56	0.70
95 % CI	0.99-2.41	0.73-1.01	1.24-1.97	0.74-1.34	1.11-3.64	1.04-1.99	0.46-0.67	0.56-0.84
signif	***	**	***	**	***	***		
TAT (0.7-4.2 µg/l)								
mean	5.55	2.34	5.35	2.85	5.08	3.77	2.34	2.24
95 % CI	3.61-7.49	1.50-3.18	3.93-6.77	1.85-3.85	3.14-7.02	3.09-4.43	1.94-2.75	1.81-2.68
signif	***	ns	***	ns	***	**		
AT (80-120 %)								
mean	96.5	98.8	105.4	102.8	97.0	116.3	98.9	100.0
95 % CI	89.7-103.2	93.6-103.9	95.7-115.1	99.3-106.2	89.5-104.5	109.9-122.6	94.8-103.0	95.1-105.0
signif	ns	ns	ns	ns	ns	***		
D-dimer (0-400 µg/l)								
mean	1469	583	1509	566	1780	853	283	414
95 % CI	1139-1799	373-794	1126-1891	389-744	1151-2410	188-1518	244-321	352-476
signif	***	***	***	***	***	***		
PAP (80-470 µg/l)								
mean	948	550	1059	540	1162	735	282	259
95 % CI	745-1151	373-727	844-1274	456-624	1017-1306	617-853	242-322	215-302
signif	***	***	***	***	***	***		
PAI-1 (4.0-43.0 µg/l)								
mean	31.0	16.3	47.6	16.4	49.2	25.4	26.8	30.0
95 % CI	21.5-40.5	12.5-20.2	35.7-59.5	13.0-19.7	37.1-61.3	14.6-36.3	21.9-31.7	25.7-34.2
signif	ns	**	**	*	**	*		
ESR (5-15 mm/hr)								
mean	51	10	42	11	82	14	15	17
95 % CI	34-68	6-13	32-51	9-13	69-95	11-17	7-18	10-20
signif	***	*	***	ns	***	ns		
CRP (0-5 mg/l)								
mean	122	6	78	7	108	6	7	8
95 % CI	83-161	5-7	54-102	6-9	72-143	5-7	5-9	6-10
signif	***	ns	***	ns	***	ns		

Table 3.2 F1.2, TAT, AT, D-dimer, PAP, PAI-1, ESR and CRP in active and inactive CD (n=20), UC (n=18) and GCA (n=19). Mean and 95% CI of the mean (lower - upper bounds) are shown.

The significance of the differences between the means of the separate groups of activity in IBD and GCA, and their respective group of controls (C1 for IBD, C2 for GCA) was calculated by means of the Mann-Whitney U-test.

* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.0001$, ns = not significant

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Laboratory tests	CD	UC				
	CDAI	ESR	CRP	UCAI	ESR	CRP
F1.2 Significance	0.376 ***	0.453 ***	0.382 ***	0.383 ***	0.483 ***	0.390 ***
TAT Significance	0.442 ***	0.369 ***	0.450 ***	0.508 ***	0.541 ***	0.448 ***
AT Significance	0.051 ns	0.037 ns	- ns	0.085 ns	0.244 ns	0.047 ns
D-dimer Significance	0.514 ***	0.502 ***	0.475 ***	0.433 ***	0.522 ***	0.462 ***
PAP Significance	0.412 ***	0.265 **	0.269 **	0.502 ***	0.553 ***	0.449 ***
PAI-1 Significance	0.265 **	0.317 **	0.213 ns	0.455 ***	0.465 ***	0.354 **
ESR Significance	0.633 ***			0.703 ***		
CRP Significance	0.806 ***			0.736 ***		

Table 3.3 Spearman-rank correlation coefficient values and the two-tailed significance of the correlation are shown between the coagulation and fibrinolysis laboratory tests, and clinical (CDAI, UCAI) and biochemical (ESR, CRP) indices of activity in CD and UC.
** = p<0.015 (Bonferroni-adjusted significance);
*** = p<0.0001; ns=not significant

Figure 3.1 Mean levels of F1.2, TAT and AT on entry and during 12 months of follow-up in 20 patients with CD. Sample sizes of F1.2, TAT and AT at 0, 3, 6, 9 and 12 months were 20-20-18-20 and 18. The interrupted line shows the course of the CDAI. AT levels were in the normal range during 12 months of follow-up. Within 6 months, the mean TAT level was equal to the control group. However, even after 12 months of follow-up, the mean F1.2 levels were 1.5 times higher than the levels of the control group.

Figure 3.2 Mean levels of D-dimer, PAP and PAI-1 on entry and during 12 months of follow-up in 20 patients with CD. Sample sizes of D-dimer, PAP and PAI-1 at 0, 3, 6, 9 and 12 months were 20-20-18-20 and 18. The interrupted line shows the course of the CDAI. After 12 months of follow-up, the mean D-dimer and PAP levels were 1.9 and 1.7 times higher than the levels of the control group. However, PAI-1 levels were 1.7 times lower in CD as compared to the control group after 12 months of follow-up.

Figure 3.3 Mean levels of F1.2, TAT and AT on entry and during 12 months of follow-up in 18 patients with UC. Sample sizes of F1.2, TAT and AT at 0, 3, 6, 9 and 12 months were 18-16-18-17 and 17. The interrupted line shows the course of the UCAI. AT levels were in the normal range during 12 months of follow-up. Within 6 months, the mean TAT level was equal to the control group. However, even after 12 months of follow-up, the mean F1.2 levels were 1.5 times higher than the levels of the control group.

Figure 3.4 Mean levels of D-dimer, PAP and PAI-1 on entry and during 12 months of follow-up in 18 patients with UC. Sample sizes of D-dimer, PAP and PAI-1 at 0, 3, 6, 9 and 12 months were 18-16-18-17 and 17. The interrupted line shows the course of the UCAI. After 12 months of follow-up, the mean D-dimer and PAP levels were 2.0 and 1.6 times higher than the levels of the control group. However, PAI-1 levels were 1.7 times lower in UC as compared to the control group after 12 months of follow-up.

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Chapter 4

Differential behavior of coagulation Factor XIII in patients with inflammatory bowel disease and in patients with giant cell arteritis

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Interaction of studied coagulation markers.

Abstract

Background

The aim of this prospective study was to examine the role of coagulation factor XIII (FXIII) in relation to disease activity in inflammatory bowel disease (IBD) and in giant cell arteritis.

Materials and methods

Plasma FXIII activity was studied during active and inactive disease in newly diagnosed patients with Crohn's disease (CD; n=20), ulcerative colitis (UC; n=18) and giant cell arteritis (GCA; n=19), in 3-month intervals (median follow-up 12 months). FXIII was also measured in two non-inflammatory control groups, age and sex matched for IBD (n=25) and GCA (n=26).

Results

FXIII activity was significantly lower in active CD or UC than in active GCA or the non-inflammatory controls. Both in CD and UC, FXIII activity correlated inversely with indices of clinical disease activity, the erythrocyte sedimentation rate, and fibrinogen and C-reactive protein levels.

Conclusions

Low FXIII activity is a characteristic feature of active IBD, and serial measurements may be useful to assess IBD activity.

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Introduction

In Crohn's disease (CD) and ulcerative colitis (UC), vasculitis and microvascular thrombosis have been described as possible etiopathogenic mechanisms^{1,2}. In general, vascular damage can stimulate the system of haemostasis and fibrinolysis, with subsequent maintenance of the inflammatory response³. Indeed, in active CD as well as in active UC, evidence of hypercoagulability^{4,5} and disturbed fibrinolysis^{6,7} as well as an important role of platelets⁸ has been described. This is also the case in an active vasculitis like giant cell arteritis (GCA)⁹⁻¹¹.

More recently, a pronounced deficiency in FXIII has been found in patients with UC and CD¹²⁻¹⁶. Interestingly, especially in CD, FXIII could be demonstrated in 'fibrinoid' capillary plugs, and in perivascular-located dendritic macrophages of both the small and large bowel submucosa¹⁷. FXIII activity leads to the cross-linking of fibrin¹⁸⁻²¹, which stimulates fibroblast migration into the extravascular matrix. This might contribute to the occurrence of submucosal fibrosis in inflammatory bowel disease (IBD). As FXIII also attenuates the enzyme release from phagocytes²² in IBD, inflammation and wound healing could be modulated by FXIII.

The reduction in FXIII in IBD has been correlated with a reduced FXIII-A concentration (active subunit), while FXIII-B levels (carrier protein) were in the lower range of normal values^{15,16,23}. In a group of patients with an active small vessel vasculitis like Henoch-Schönlein purpura, presenting with intestinal bleeding²⁴, plasma FXIII levels were reduced. In the present study, active FXIII was measured in the plasma of patients with IBD and in patients with a medium to large vessel vasculitis (GCA). The latter disorder has characteristics of granulomatous disease similar to CD but differs in the size of arteries involved. Because of the known state of hypercoagulation in GCA⁹⁻¹¹, we hypothesized that in both active IBD and GCA, FXIII would be diminished as a result of its consumption. Furthermore, to assess the relevance of FXIII as a marker of disease activity, a possible correlation with well-known indices of clinical activity as well as of biochemical activity (erythrocyte sedimentation rate, ESR, C-reactive protein, CRP and fibrinogen) was investigated.

Materials and methods

Patients

In a prospective study, performed at the Department of Internal Medicine of the Atrium Hospital Heerlen, The Netherlands, 38 consecutive and newly diagnosed in- or outpatients with chronic IBD were enrolled from December 1991 to December 1994 and followed in active and inactive phases of disease.

The Ethics Committee of the Atrium Hospital Heeren approved the study, and patients provided written informed consent before entry. Twenty patients with CD and 18 patients with UC were followed up for a median of 12 months (range 6–21 months). In CD and UC, the diagnosis was made according to the criteria of Lennard-Jones²⁵.

In CD, the severity of disease was classified according to the CD Activity Index (CDAI)²⁶. The CDAI was worked out from a diary card completed by the patient every day for 1 week. Active disease was defined as a CDAI score >150. On entry, in 10 patients with CD only the terminal ileum, in 6 patients both small and large bowel, and in 4 patients only the colon were involved.

In UC, the severity of disease was classified according to the Truelove-Witts and Witts criteria²⁷. For statistical purposes, the classification was quantitatively modified. Following the original classification as close as possible, 6 subjects were scored individually from 0 to 3: (1) diarrhoea frequency (0, 1–3 times daily, 4–5 and 6 times or more), (2) percent bloody stools (0, 1–24, 25–75 or 175%), (3) fever (<37.5, 37.5–38.0, 38.1–38.5 or 138.5°C), (4) pulse rate (<70, 70–80, 81–90 or 190 b.p.m.), (5) anaemia (in males: 18.5, 7.5–8.5, 6.5–7.4 or <6.5 mmol/l, and in females: 17.5, 7.0–7.5, 6.5–6.9 or <6.5 mmol/l), and (6) the ESR (in males: 0–15, 16–25, 26–30 or 130 mm/h, and in females: 0–20, 21–25, 26–30 or 130 mm/h).

The total score in severe disease was between 15 and 18, in moderate disease between 9 and 14, in mild disease between 4 and 8, and in inactive disease it was 3 or less. On entry, 6 UC patients had a pancolitis, 3 patients had a left-sided colitis and 9 patients had a proctosigmoiditis diagnosed endoscopically.

Twenty patients with CD (14 females, 6 males) with a median age of 34 years (range 20–71) had a median follow-up of 12 months (range 6–21).

In 18 patients with UC (11 females, 7 males; median age 47 years, range 17–86), the median duration of follow-up was 12 months (range 6–18).

In 19 patients with a histological proven and newly diagnosed GCA (14 females, 5 males), median follow-up was 10 months (range 6–22). Here, activity was assessed according to the criteria of the American Rheumatism Association²⁸, as well as by conventional laboratory parameters (ESR, CRP). Since the GCA group was considerably older than the group of IBD patients (median age: 72 years; range 65–88), separate control groups were included.

Twenty-five age- and sex-matched orthopaedic patients (female/male ratio 16:9; median age 41 years, range 22–74), seen before elective minor surgery, served as a control group for patients with IBD.

They had no signs of vasculitis or gastrointestinal disease. Also, 26 age- and sex-matched controls, consisting of 19 healthy females of older age and 7 male urologic patients – seen at the outpatient clinic because of benign prostate hypertrophy – were all without signs of inflammation and served as a control group for patients with GCA. Their median age was 71 years (range 67–84). In

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the non-inflammatory control groups, blood samples were taken twice within 1 month and before surgery.

None of the patients and controls was on oral anticoagulants or low-dose salicylate. Patients with pre-existing liver disease compromising coagulation factor synthesis, with coexisting malignancy, or with pregnancy, and patients who refused consent were excluded from the study. Depending on the severity of disease, therapy in IBD consisted of oral 5-aminosalicylic acid (5ASA) and/or prednisone (starting dose varying between 0.5 and 1 mg/kg/day), sometimes combined with enema therapy. Although the patients were not treated in a standardized manner, prednisone was generally started in case of insufficient clinical response to 5-ASA treatment in active CD (CDAI >150) and in moderate to severe UC (UCAI >8). In active GCA, oral prednisone (dose: 40 mg a day) was started, and, according to the clinical and acute phase response, dosage was gradually lowered²⁹. None of the patients received FXIII concentrate or substitution of plasma derivatives other than red packed cells.

Upon study entry, history, physical examination and venous blood samples for determination of plasma FXIII, fibrinogen, CRP, ESR and red and white blood cell counts were obtained from all patients. Plasma samples were collected during active UC, CD and GCA before conventional therapy, and – after intervals of 3 months – in inactive disease while on low-dose prednisone (<15 mg a day) and/or mesalazine (oral or enema) in the IBD group.

Sampling technique

In a standardized manner venous blood was drawn between 09.00 and 11.00 a.m. by an experienced technician employed at the coagulation laboratory, after a resting period of 20 min and after applying minimal stasis. As an anticoagulant, trisodium citrate (0.106 M) was used. Plasma was collected after centrifugation at 1,600 *g* for 20 min at room temperature, and immediately thereafter it was deep-frozen. The plasma was stored at –70°C in plastic tubes and thawed in a water bath at 37°C for 5 min before serial analysis.

Analytical methods

FXIII activity was measured by photometry (Behring, Germany; normal values between 70 and 120% for both men and women)³⁰. Tests were conducted on the Cobas Bio[®] centrifugal analyzer. The intra-assay coefficients of variation were between 0.83 and 2.68%, and inter-assay coefficients of variation were between 3.4 and 4.5%. Plasma fibrinogen was determined with a clotting assay (Merz and Dade) according to the method of Clauss, measured by means of a Schnittger and Gross coagulometer. For cell counts, venous blood was collected in plastic vials containing 1.6 g/dl EDTA-K2 (Sarstedt, Nümbrecht, Germany). The white blood cell count and differentiation, haemoglobin (Hb) and haematocrit levels were determined with a Sysmex E-4000 (Kobe, Japan)

cell counter. CRP was measured by an enzyme immunoassay (Behring), and the ESR was measured according to the Westergren method.

Statistical Analysis

In UC, CD and GCA, mean and the 95% confidence intervals (CI) for the mean of the above-described parameters were calculated.

To test differences for significance, the Mann-Whitney-Wilcoxon rank sum test was performed. Furthermore, the Spearman rank correlation coefficient and the two-tailed significance of the correlation were calculated. As a measure of the linearity, the equation of the regression line as well as the square of the correlation coefficient (R^2) was calculated. Statistical analysis was carried out using SPSS 8.0 software (SPSS, USA).

Results

Correlation of FXIII with disease activity

In CD, 6 patients had a CDAI >450, 11 patients had a CDAI score between 300 and 450 and in 3 patients the CDAI was within 150–299 on entry. After 6 months of treatment, 3 patients had a CDAI between 150–299 and 17 patients had a CDAI <150.

In UC, disease activity on entry was severe in 12 patients and moderate in 6 patients; after 6 months of treatment, 2 patients had moderate, 10 patients had mild and 6 patients had inactive disease.

In **table 4.1**, mean and 95% CI of the FXIII activity and fibrinogen levels in active and inactive disease states are shown.

FXIII activity was significantly lower in active CD (mean 68; 95% CI: 52–84%) and active UC (61; 51–71%) than in active GCA (115; 104–126%) or the non-inflammatory controls (C1: 110; 103–117% with $p<0.0001$, and C2: 110; 102–119% with $p<0.0001$).

In active as compared to inactive disease, the mean FXIII level was significantly lower in both CD and UC ($p<0.0001$). FXIII levels did not significantly differ between active UC or active CD ($p=0.593$). However, in inactive UC, the mean FXIII level was significantly lower than in inactive CD ($p=0.019$) or in the controls ($p=0.002$). In inactive GCA, FXIII levels were significantly higher than in CD, UC or the controls (all $p<0.01$).

In CD, FXIII correlated inversely with the CDAI ($r=-0.562$; $p<0.0001$). The regression equation was $y=-0.147x + 121.527$ with $R^2=0.158$ on entry, and $y=-0.130x + 115.922$ with $R^2=0.208$ after 12 months of follow-up. Also, fibrinogen levels correlated with the CDAI ($r=0.597$; $p<0.0001$).

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In UC, FXIII correlated inversely with the UCAI ($r=-0.518$; $p<0.0001$). The regression equation was $y=-1.259x + 94.325$ with a calculated R^2 of 0.099 on entry, and $y=-1.125x + 96.992$ with $R^2=0.011$ after 12 months of follow-up. Also, fibrinogen levels correlated with the UCAI ($r=0.501$; $p<0.0001$).

As shown in **figures 4.1** and **4.2**, in CD the mean FXIII level increases and equalized that of the controls after 12 months of follow-up. However, in UC the mean FXIII level was still lower than the mean FXIII level of the controls after 12 months.

In CD and UC, mean fibrinogen levels were raised even after 12 months compared to the controls ($p=0.013$ and $p=0.002$, respectively).

As is shown in **figure 4.3**, in GCA mean FXIII levels were normal at entry ($p=0.346$) and higher after 12 months ($p=0.01$) compared to the controls. Like in CD and UC, the mean fibrinogen level in GCA was raised at entry and decreased slowly, but was still higher than the control value after 12 months of follow-up ($p=0.031$).

Correlation of FXIII with other laboratory parameters

In CD, FXIII correlated inversely with CRP ($r=-0.561$; $p<0.0001$), ESR ($r=-0.204$; $p=0.037$), and with fibrinogen ($r=-0.272$; $p=0.005$). In UC, FXIII also correlated inversely with CRP ($r=-0.679$; $p<0.0001$), ESR ($r=-0.661$; $p<0.0001$) and fibrinogen ($r=-0.329$; $p<0.001$). In GCA however, FXIII was not correlated with disease activity, as represented by the ESR ($r=-0.144$; $p=0.545$), CRP ($r=-0.376$; $p=0.102$) or fibrinogen ($r=-0.178$; $p=0.254$).

Except for FXIII, for CRP, ESR and fibrinogen a similar profile was found in active CD, UC and GCA (**table 4.1**). In the non-inflammatory control groups, laboratory values of FXIII, fibrinogen, CRP and ESR were within normal limits.

Correlation of FXIII with the extent of bowel involvement

A division was made between patients with CD involving only the small intestine or only the colon, and CD of both small and large bowel segments.

Although mean FXIII levels were lower in combined disease, differences between FXIII values in extensive and less extensive bowel involvement were not significant (ileum involvement versus colon and ileum involvement: $p=0.282$; colon versus colon and ileum: $p=0.806$; ileum versus colon only: $p=0.602$).

In UC, the difference between FXIII values in distal colitis, confined to the procto-sigmoid area, and proximal colitis extending from rectum at least to the descending colon was not significant either ($p=0.365$).

Discussion

In this study it is shown that FXIII activity inversely correlated with clinical and biochemical indices of disease activity in UC and CD. However, this was not the case in GCA. This could suggest a specific significance of FXIII in active IBD.

The inverse correlation of FXIII with CDAI, the UC activity index, ESR, CRP and fibrinogen on the one hand confirms the results of other groups^{17,23,31}, on the other hand it conflicts in part with the results of Hudson et al.¹⁷ who did not find a significant correlation with CRP in patients with CD. Although we cannot provide a clear explanation for this disagreement, differences in sample sizes, in severity and duration of disease and follow-up may play a role. Despite the inverse correlation between FXIII activity and indices of IBD activity, it is noteworthy that 10.0% (2 of 20) of the patients with CD and 16.7% (3 of 18) of the patients with UC had subnormal activity of FXIII even during inactive disease. Therefore, it is likely that serial rather than single measurements would be required if FXIII was used as an index of activity in CD or UC.

A relationship between FXIII activity and the endoscopic extent of bowel inflammation might also have been expected. Surprisingly, on entry no significant correlation was found, in contrast with the results of the study of Seitz et al.³¹. In the present study, as in others, no systematic endoscopic follow-up was performed during the follow-up of FXIII activity.

How is the lowered activity of FXIII in active IBD to be explained? In the plasma of patients with CD and UC low levels of active FXIII could point to the consumption of FXIII as a result of active coagulation^{12,13,17}. However, a reduction in FXIII in active IBD due to increased thrombin generation in general seems uncertain, particularly as in GCA high levels of fibrinogen – similar to fibrinogen levels in IBD – were not accompanied by a FXIII activity reduction.

In comparison to GCA one could argue for the effect of age on FXIII. However, we did not find any differences in the content of plasma FXIII between younger and older patients and controls in this study. Otherwise, it could be possible that CD or UC might behave more like a small vessel vasculitis with fibrinoid deposits, whereas a larger vessel vasculitis like GCA might be characterized by a similar inflammatory state but without active clot formation. Like in CD or UC, however, high levels of coagulation factors (fibrinogen, FVIII and von Willebrand Factor) as well as fibrinolysis products like D-dimer have been found in GCA⁹⁻¹¹. The difference in FXIII activity might support the idea of a difference in the pathophysiology of IBD and GCA.

In quiescent CD, we have found normal to raised FXIII activity, as in agreement with Seitz et al.³¹. The low plasma FXIII activity in active CD could be the result of greatly enhanced turnover, reflecting increased intestinal consumption or leakage of plasma proteins. The latter condition, comparable to the intestinal

faecal α 1-antitrypsin loss³², is frequently encountered in active CD, although we are not aware of studies performed to measure the intestinal clearance of FXIII. Subunit analysis could have helped to discriminate between a fall in FXIII-A as a result of consumption in the process of coagulation and a protein loss of both FXIII-A and FXIII-B in the gut. In active UC, loss of FXIII activity could also be related to persistent intestinal bleeding, leading to consumption and loss of coagulation factors like FXIII.

The question arises whether FXIII has a role in the maintenance of normal intestinal integrity as well as intestinal repair mechanisms. Factor XIII could be involved in the stabilization of the microvasculature itself, leading to a reduced leakage at the site of intestinal inflammation. This sealing effect of FXIII on the capillary permeability has been shown in patients with scleroderma³³, and it might also play a role in IBD.

In our study, levels of FXIII remained low for a longer time in UC than in CD, and this could motivate FXIII application in UC.

In pilot studies, treatment with FXIII has been reported to be beneficial in both UC and CD^{23,34}, although a recent study on FXIII supplementation in addition to steroids in active UC showed no clear benefit³⁵. The potential therapeutic benefit of FXIII-A administration is to stabilize clot formation and to prevent its breakdown into fibrin degradation products and the subsequent perpetuation of intravascular coagulation. However, FXIII-A has the potential to stimulate an excessive fibroblast response, which might result in scarring instead of regeneration in prolonged treatment, a non-desirable effect especially in CD. Further controlled studies are therefore needed to elucidate the therapeutic role of FXIII in IBD, and its possible adverse effects, before it can be accepted as a therapy beyond the level of clinical experiments.

In summary, the decrease in FXIII in active IBD is an interesting phenomenon with pathophysiological and potentially diagnostic and therapeutic implications. The finding supports the idea that further research should be directed towards the role of coagulation in IBD.

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Laboratory Tests	CD, CDAI	UC, CDAI	UC, CDAI	GCA, ARA	C1	C2
	>150	<150	>4	<4	active	inactive

FXIII (70-120 %)

mean	68	105	61	92	115	128	110	110
95 % CI	52-84	100-110	51-71	85-99	104-126	119-136	103-117	101-119
p value	***	*	***	ns	ns	*		

Fibrinogen (1.7-4.0 g/l)

mean	5.4	3.7	5.4	3.5	6.5	3.4	2.6	2.8
95 % CI	4.8-6.0	3.2-4.1	4.7-6.0	3.0-3.9	5.4-7.6	3.1-3.7	2.4-2.9	2.4-3.2
p value	***	*	***	**	***	*		

ESR (5-15 mm/hr)

mean	51	12	42	11	82	14	8	11
95 % CI	34-68	9-14	32-51	9-13	69-95	11-17	7-9	9-12
p value	***	*	***	ns	***	ns		

CRP (0-5 mg/l)

mean	122	7	78	7	108	6	7	8
95 % CI	83-161	6-7	54-102	6-9	72-143	5-7	6-8	7-8
p value	***	ns	***	ns	***	ns		

Table 4.1 FXIII, fibrinogen, CRP and ESR at various stages of disease activity in CD (n=20), UC (n=18) and GCA (n=19). Mean values and 95% CI of the mean (upper and lower bounds) are shown. The significance of the differences between the means of the separate groups of activity in IBD and GCA, and their respective group of controls (C1 for IBD, C2 for GCA) was calculated by means of the Mann-Whitney-Wilcoxon test.

* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.0001$, ns = not significant

Figure 4.1 FXIII compared to fibrinogen (mean and 95% CI) as well as the CDAI (means presented as dotted line) on entry and 3, 6, 9 and 12 months later in 20 patients with CD.

Figure 4.2 FXIII compared to fibrinogen (mean and 95% CI) as well as the UCAI (means presented as dotted line) on entry and 3, 6, 9 and 12 months later in 18 patients with UC.

Figure 4.3 FXIII compared to fibrinogen (mean and 95% CI) as well as the ESR (means presented as dotted line) on entry and 3, 6, 9 and 12 months later in 19 patients with GCA.

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Chapter 5

Platelet factor 4 and β -thromboglobulin in
inflammatory bowel disease and in giant cell
arteritis

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Interaction of studied platelet factors.

Abstract

Background

As platelet factors are important in the inflammatory response, we examined the course of platelet factor 4 and β -thromboglobulin in relation to disease activity in inflammatory bowel disease and in giant cell arteritis.

Patients and methods

In a prospective study, the platelet count, platelet factor 4 and β -thromboglobulin were measured in 20 patients with Crohn's disease, 18 with ulcerative colitis, and 19 with giant cell arteritis during active and inactive disease, as well as in 51 controls without inflammation.

Results

Platelet counts were significantly higher in active vs. inactive Crohn's disease, ulcerative colitis and giant cell arteritis. Levels of platelet factor 4 and β -thromboglobulin were significantly higher in active inflammatory bowel disease and giant cell arteritis, as well as in inactive inflammatory bowel disease and giant cell arteritis, than in the non-inflammatory controls.

A positive correlation was found between the Crohn's disease activity index and the platelet count, platelet factor 4 and β -thromboglobulin. Also, a positive correlation was found between the ulcerative colitis activity index and β -thromboglobulin.

However, even after 12 months of follow-up, in Crohn's disease and ulcerative colitis the mean levels of platelet factor 4 and β -thromboglobulin were significantly higher than the levels of the controls.

Conclusions

Platelet factors were correlated with inflammatory bowel disease activity. Levels of platelet factor 4 and β -thromboglobulin, however, were markedly raised for a long time in clinically inactive inflammatory bowel disease, which might point to a pre-thrombotic state of disease.

Introduction

Interest in the role of platelets in inflammatory bowel disease (IBD) was formerly confined to an awareness of thrombocytosis as a marker of disease activity¹, and as a possible predisposing factor to systemic thromboembolism². More recently however, an important role of platelets in the pathogenesis of IBD has been suggested by the histological finding of gut microvascular thrombosis in these patients³⁻⁵, as well as the identification of circulating platelet aggregates in venous blood in active Crohn's disease (CD) and ulcerative colitis (UC)⁶. Interestingly, the abnormal function of platelets was found to be independent of disease activity, leading to the assumption that a platelet abnormality could underlie the procoagulant state observed in patients with IBD^{2,7-13}.

Only a few studies have reported raised plasma levels of the platelet-derived factors β -thromboglobulin (BTG) and platelet factor 4, PF4 in active IBD^{2,7,14}. PF4 is rapidly taken up by endothelial cells, through a reversible binding of PF4 to heparan sulphates at the endothelial surface, and may be important in directing neutrophils to endothelium prior to diapedesis^{15,16}. BTG, however, has only a low anti-heparin activity¹⁷. As the activation of platelets also attenuates the enzyme release from phagocytes¹⁸⁻²⁰, in IBD, haemostasis, inflammation and wound healing could be modulated by the degree and endurance of platelet activation.

We investigated the course of plasma levels of PF4 and BTG in patients with IBD, as well as in patients with a medium to large vessel vasculitis (giant cell arteritis; GCA), during a longer period of follow-up than has been done previously^{2,7,14}. The latter disorder has characteristics of granulomatous disease similar to CD but differs in the size of arteries involved. In GCA it is relatively easy to diagnose patients with the help of a temporal artery biopsy, while in patients with a small vessel vasculitis like in Henoch-Schönlein purpura or (microscopic) periarteritis nodosa, a uniform diagnosis is more difficult to obtain. Our hypothesis was that not only in active but also in inactive IBD and GCA, levels of PF4 and BTG would be raised as a result of enhanced platelet activity.

Furthermore, to assess the possible role of PF4 and BTG as markers of disease activity, a correlation with well-known indices of clinical activity as well as biochemical activity like the Erythrocyte Sedimentation Rate (ESR) or C-reactive protein (CRP) was investigated.

Materials and methods

Patients

In a prospective study, performed at the Department of Internal Medicine of the Atrium Hospital Heerlen, The Netherlands, 38 newly diagnosed in- or out-patients with chronic inflammatory bowel disease were enrolled from December 1991 to December 1994 and followed in an active and inactive phase of disease. The study was approved by the Ethics Committee of the Atrium Hospital Heerlen and patients provided written informed consent before entry. Twenty patients with CD and 18 patients with UC were included with a median duration of follow-up of 12 months (range 6 - 21 months). In CD and UC, the diagnosis was documented according to the criteria of Lennard Jones²⁴.

In CD, the severity of disease was classified according to the Crohn's Disease Activity Index (CDAI)²⁵. Active disease was defined as a CDAI score >150. At entry, six patients had a CDAI score >450, 11 patients had a CDAI score between 300 and 450 and in 3 patients the CDAI was between 150 and 299.

In patients with UC, severity of disease was classified according to the Truelove-Witts criteria^{26,27}. For statistical purposes, the classification was modified quantitatively. Following the original classification as close as possible, the diarrhea frequency and bloody stools, fever, tachycardia, anaemia and the ESR were scored individually (**table 5.1**). The Ulcerative Colitis Activity Index (UCAI) score for severe disease was between 15 and 18, for moderate disease between 9 and 14, for mild disease between 4 and 8, and for inactive disease 3 or less. At entry, disease activity was severe in 12 patients, moderate in 6 patients and none had mild or inactive disease. In nineteen patients with a histologically proven and newly diagnosed GCA, activity was assessed according to the criteria of the American Rheumatism Association (ARA)²⁸, as well as by the ESR and CRP levels. As the GCA group was considerably older than the group of IBD patients, separate groups of controls were included.

Twenty-five age- and sex-matched orthopaedic patients, seen before elective minor surgery, served as a control group for patients with IBD. They had no signs of vasculitis or gastrointestinal disease.

Twenty-six age- and sex-matched patients, consisting of 19 healthy females of older age and seven male urology patients (seen because of benign prostate hypertrophy) all without signs of inflammation, served as a control group for patients with GCA.

None of the patients was on oral anticoagulants or low-dose aspirin. Patients with pre-existing liver disease compromising coagulation factor synthesis, with coexisting malignancy, or with pregnancy, and patients, who refused consent, were excluded from the study. Depending on the severity of disease, therapy in IBD consisted of oral 5-aminosalicylic acid (5ASA) and/or prednisone (starting

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dose varying between 0.5 and 1 mg/kg o.d.), sometimes combined with enema therapy. Although the patients were not treated in a standardized manner, prednisone was generally started in case of insufficient clinical response to 5ASA treatment in active CD (CDAI >150) and moderate to severe UC (UCAI >8). In active GCA, oral prednisone (dose 40 mg o.d.) was started and, according to the clinical and acute phase response, dosage was gradually lowered²⁹. None of the patients received plasma derivatives other than red packed cells.

Plasma samples were collected during active UC, CD and GCA before conventional therapy, and, after intervals of 3 months, in inactive disease while on low dose prednisone (less than 15 mg o.d.) and/or mesalazine (oral or enema) in the IBD group. In the non-inflammatory control groups, blood samples were taken twice within a month before surgery. The characteristics of patients and controls are shown in **table 5.2**.

Methods

All blood samples were drawn in the morning (08.00 - 10.00 h), after a resting period of 20 min, in a standardized manner to avoid diurnal variation^{30,31}. Non-traumatic venipuncture was performed by trained operators.

After discarding the first 2 ml of blood, 4.5 ml of blood was drawn into test tubes containing citric acid, theophylline, adenosine and dipyridamol (CTAD, Boehringer Mannheim Corp., Mannheim, Germany) to prevent *in vitro* platelet activation, and immediately kept on ice. Within 1 h of sample collection, all test tubes were centrifuged for 30 minutes (3000 *g*) at 4°C.

Platelet-poor plasma was aspirated from the central part of the plasma, without disturbing either the top layer or the buffy coat. The plasma was snap-frozen in aliquots, stored at -70°C in plastic tubes, and thawed in a water bath at 37°C for 5 min, immediately prior to serial analysis.

Plasma PF4 and BTG concentrations were determined by an enzyme-linked immunosorbent assay (ELISA) method (Boehringer Mannheim Corp.).

The normal value of PF4 was 0-15 IU/ml and in BTG it was 10-100 IU/ml; values were standardized against the First National Standards 83/505 and 83/501 respectively. The intra-assay coefficient of variation was between 5% and 10% for both assays.

For the cell count, venous blood was collected in plastic vials containing 1.6 g/l EDTA-K2 (Sarstedt, Nümbrecht, Germany). The white blood cell count and differentiation, haemoglobin (Hb) and hematocrit, as well as the platelet count (normal values 150-300 x 10⁹/l) were determined with a Sysmex E-4000 (Kobe, Japan) cell counter. CRP was measured by an enzyme immunoassay (Behring, Germany; normal values 0-8 mg/l), and the ESR was measured according to the Westergren method (normal values 0-12 mm/hr).

Statistical analysis

In UC, CD and GCA, mean, median values and the 95% confidence intervals (CI) for the mean of the above described parameters were calculated. To test differences for significance, the Mann-Whitney-Wilcoxon rank-sum test was performed. Furthermore, the Spearman rank correlation coefficient and the 2-tailed significance of the correlation were calculated.

As a measure of the linearity, the equation of the regression line as well as the root square of the correlation coefficient (Rs_q) was calculated. The statistical analysis was carried out using SPSS 8.0 software (SPSS Inc., Chicago, USA).

Results

Platelet count, PF4 and BTG in active and inactive disease

In **table 5.3** and **figure 5.1**, platelet counts and levels of PF4 and BTG in the patient groups in active and inactive disease are shown. Laboratory values were within normal limits in the non-inflammatory control groups (**table 5.3**).

Platelet counts were significantly higher in active CD, UC and GCA than in inactive CD, UC, GCA or the controls ($p < 0.0001$). Also, platelet counts were higher in active GCA than in active CD ($p = 0.002$) or UC ($p = 0.036$), but not significantly different between active CD and active UC.

PF4 was significantly higher in active CD, UC and GCA, as well as in inactive CD, UC and GCA, than in the non-inflammatory controls ($p < 0.0001$). No significant differences in the PF4 level were found between active and inactive CD, UC or GCA. Also, PF4 levels were not significantly different between active CD and UC, or quiescent CD and UC.

BTG was significantly higher in active CD, UC and GCA, as well as in inactive CD, UC and GCA, than in the non-inflammatory controls ($p < 0.0001$). In contrast with PF4, BTG levels were significantly higher in active versus inactive CD ($p < 0.0001$), UC ($p < 0.0001$) and GCA ($p = 0.002$). Also, in active CD, BTG levels were significantly higher than in active UC ($p = 0.022$).

Correlations of the platelet count, PF4 and BTG with CDAI and UCAI

In CD, the CDAI correlated with the platelet count ($r = 0.319$; $p = 0.001$), PF4 ($r = 0.304$; $p = 0.002$) and BTG ($r = 0.505$; $p < 0.0001$).

The linear regression equation between platelets and CDAI was $y = 0.310x + 265.499$, with $R_{sq} = 0.145$; between PF4 and the CDAI it was $y = 0.040x + 84.152$ with $R_{sq} = 0.103$, and between BTG and the CDAI $y = 0.773x + 381.577$ with $R_{sq} = 0.187$.

In UC, the UCAI correlated with BTG ($r=0.395$; $p<0.0001$), however, no significant correlation was found between the UCAI and the platelet count or PF4.

The linear regression equation between BTG and UCAI was $y=15.394x + 284.591$, with $Rsq=0.172$.

Correlations of platelet count, PF4 and BTG with biochemical indices of disease activity

In CD, the ESR correlated with platelets ($r=0.329$; $p=0.001$) and PF4 ($r=0.231$; $p=0.018$). Also, CRP correlated with platelets ($r=0.244$; $p=0.012$), PF4 ($r=0.203$; $p=0.038$) and BTG ($r=0.371$; $p<0.0001$).

In UC, the ESR correlated with platelets ($r=0.353$; $p=0.001$), PF4 ($r=0.328$; $p=0.002$), and BTG ($r=0.467$; $p<0.0001$). Also, CRP correlated with platelets ($r=0.354$; $p=0.001$), PF4 ($r=0.282$; $p=0.007$) and BTG ($r=0.418$; $p<0.0001$).

In GCA, the ESR correlated with platelets ($r=0.648$; $p<0.0001$) and BTG ($r=0.411$; $p=0.006$), and CRP correlated with the platelet count ($r=0.596$; $p<0.0001$).

The time-course of the platelet count, PF4 and BTG in UC, CD and GCA

As is shown in **figure 5.2**, the mean platelet count in CD equalized the mean value of the controls (**table 5.3**) within 9 months of follow-up. However, even after 12 months of follow-up, the mean PF4 and BTG levels were 6 and 7 times higher than the levels of the control group. As is shown in **figure 5.3**, the mean platelet count in UC equalized the mean value of the controls (**table 5.3**) within 9 months of follow-up. However, even after 12 months, the mean PF4 and BTG levels were five times higher than the control group levels. As is shown in **figure 5.4**, the mean platelet count in GCA equalized the mean value of the controls (**table 5.3**) within 9 months of follow-up. However, even after 12 months, the mean PF4 and BTG levels were seven and six times higher than the control group levels.

Correlation of the platelet count, PF4 and BTG with the extent of bowel involvement at entry

The endoscopic extension of bowel involvement in patients with active CD or UC was investigated, as well as its relationship to the platelet factor levels.

Patients with CD involving only the small intestine ($n=10$) or only the colon ($n=4$), and CD of both small and large bowel segments ($n=6$) were distinguished; however the platelet count, PF4 and BTG levels were not significantly different according to disease localization.

Patients with UC involving only the rectum and/or sigmoid colon (n=9), and UC extended to the more proximal colon segments (left-sided colitis: n=3; pancolitis: n=6) were distinguished.

Although mean levels of the platelet count, PF4, and BTG were higher in more extensive UC, platelet factors did not correlate with the extent of bowel involvement at entry.

Discussion

In this study, PF4 and BTG were markedly raised in CD and UC, compared with the controls. Our findings confirm the study reports on raised levels of BTG² and PF4⁷ in IBD patients, although others did not find a correlation with disease activity² or a significant difference in quiescent disease¹⁴. PF4 and BTG levels were also raised during a long time in GCA, pointing to a non-IBD specific mechanism. The increase of PF4 and BTG in the inactive phase of both IBD and GCA could reflect common underlying pathophysiological mechanisms, for example a vasculitis or diffuse intravascular thrombosis^{3,4}, contributing to the chronicity of these disease entities. Endothelial cell damage might expose basement membrane collagen, to which platelets are very sensitive, and this could lead to platelet activation. The increase in plasma Von Willebrand factor (a marker of endothelial damage) found in IBD and GCA is consistent with this possibility^{22,33}.

The sustained rise of PF4 and BTG levels preclude their use as short-term indices of activity in IBD and GCA. The question whether or not a normalization of the values of PF4 and BTG in IBD or GCA reflect a complete remission of disease remains unanswered. If platelet dysfunction should play a major role in the pathophysiology of IBD then agents who reduce platelet activity could prove useful in its treatment. Indeed, selective anti-thromboxane agents (whose effects *in vivo* include inhibition of platelet aggregation) suppress inflammation associated with the trinitrobenzene-sulphonic acid model of colitis, endotoxin-induced intestinal damage and nonsteroidal anti-inflammatory drugs (NSAID)-induced small bowel ulceration in rats³⁴⁻³⁶. Sulphasalazine, a drug that is widely prescribed for colon involvement in IBD, inhibits platelet aggregation, at least *in vitro*³⁷, however, the high concentration required for this action (50% inhibitory concentration 2 mM) and its many other pharmacological effects³⁸, preclude the conclusion that sulphasalazine *in vivo* primarily works by this mechanism.

Specific dual thromboxane synthase inhibition with diazinyphenyl-ketoximen (Ridogrel®, Janssen, Beerse, Belgium) has been applied in a small group of patients and seemed to be of benefit in active UC³⁹. Also, platelet activating factor (PAF) antagonists are currently under investigation in chronic

inflammatory disease, although in 176 patients with moderately active UC the use of the PAF antagonist SF274177 at 10 mg a day did not improve the remission rate during 28 days of follow-up, as compared with placebo⁴⁰.

As yet, no dose-effect studies have been performed in patients with UC, and the influence on the platelet degranulation activity was not measured in these patients. More controlled studies are therefore needed to elucidate the influence of platelet activity-inhibiting drugs on IBD and GCA, as well as the possible adverse effects. In such studies, PF4 and BTG could have an important role as markers of disease activity.

Criteria		Disease activity score			
		0	1	2	3
Diarrhoea frequency		no	1 - 3	4 or 5	6 or more
Bloody stools (%)		no	1 - 24	25 - 75	> 75
Temperature (°C)		< 37.5	37.5 - 38.0	38.1-38.5	> 38.5
Heartrate (beats per min.)		< 70	70 - 80	81 - 90	> 90
Hb (mmol/l)	Male	> 8.5	7.5 - 8.5	6.5 - 7.4	< 6.5
	Female	> 7.5	7.0 - 7.5	6.5 - 6.9	< 6.5
ESR (mm/hr)	Male	0 - 15	16 - 25	26 - 30	> 30
	Female	0 - 20	21 - 25	26 - 30	> 30
Maximum		0	6	12	18

Table 5.1 The modified (quantified) Truelove-Witts classification of ulcerative colitis. Six criteria were used and individually graded from 0 to 3. The activity score was arbitrarily defined as the sum of 6 scores

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		Age (years)				Follow-up (months)	
Disease	<i>n</i>	Female	Male	Median	Range	Median	Range
CD	20	14	6	34	20-71	12	6-21
UC	18	11	7	47	17-86	12	6-18
GCA	19	14	5	72	65-88	10	6-22
Controls (1)	25	16	9	41	22-74	1	0.5-1
Controls (2)	26	19	7	71	67-84	1	0.5-1

Table 5.2 Characteristics of the patients and control groups; controls (1) for Crohn’s disease (CD) and ulcerative colitis (UC), and controls (2) for giant cell arteritis (GCA).

Laboratory tests	CD		UC		GCA		C1	C2
	CDAI	UCAI	UCAI	ARA	Active	Inactive		
	> 150	< 150	> 4	< 4	Active	Inactive		

Platelets(150-300x10⁹/l)

mean	372	294	427	311	507	304	235	268
median	342	282	450	258	511	291	226	268
95 % CI	321-424	257-332	365-489	247-374	447-568	279-330	213-257	244-292
significance	***	**	***	ns	***	ns		

PF4 (0-15 IU/ml)

mean	97	92	88	80	81	81	14	13
median	100	90	93	88	75	80	14	13
95 % CI	88-107	86-97	77-99	71-90	74-89	78-83	12-16	11-15
significance	***	***	***	***	***	***		

BTG (10-100 IU/ml)

mean	685	443	534	299	650	439	62	65
median	673	375	490	316	700	430	68	68
95 % CI	588-781	337-550	417-650	257-341	507-792	374-504	52-73	53-77
significance	***	***	***	***	***	***		

ESR 0-12 (mm/hr)

mean	51	10	42	11	82	14	15	17
median	45	8	39	11	79	14	4	8
95 % CI	34-68	6-13	32-51	9-13	69-95	11-17	7-18	10-20
significance	***	*	***	ns	***	ns		

CRP (0-8 mg/l)

mean	122	6	78	7	108	6	7	8
median	129	5	82	7	89	5	6	7
95 % CI	83-161	5-7	54-102	6-9	72-143	5-7	5-9	6-10
significance	***	ns	***	ns	***	ns		

Table 5.3 Platelets, platelet factor 4 (PF4), β -thromboglobulin (BTG), erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) in active and inactive Crohn's disease (CD; n=20), ulcerative colitis (UC; n=18) and Giant cell arteritis (GCA; n=19). Mean, median and 95% confidence intervals (CI) of the mean (lower - upper bounds) are shown. The significance of the differences between the means of the separate groups of activity in inflammatory bowel disease (IBD) and GCA, and their respective group of controls (C1 for IBD, C2 for GCA) was calculated by means of the Mann-Whitney-Wilcoxon test.

* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.0001$; ns, not significant..

Figure 5.1 Platelet count, platelet factor 4 (PF4) and β -thromboglobulin (BTG) levels in Crohn's Disease (CD), ulcerative colitis (UC) and giant cell arteritis (GCA), in active (a) or inactive (i) disease, and in controls (C1 for CD and UC, C2 for GCA). Bars show mean values, error bars show 95% CI of the means.

Figure 5.2 Mean platelet count ($10^9 L^{-1}$), PF4 and BTG (IU mL^{-1}) on entry and during 12 months of follow-up in 20 patients with Crohn's disease. The interrupted line shows the course of the Crohn's disease activity index (CDAI). The mean platelet count equaled the control group within 9 months. However, even after 12 months of follow-up, the mean PF4 and BTG levels were 6 and 7 times higher than the levels of the control group.

Figure 5.3 Mean platelet count ($10^9 L^{-1}$), PF4 and BTG ($IU mL^{-1}$) on entry and after 12 months of follow-up, in 18 patients with ulcerative colitis. The interrupted line shows the course of the UCAI (values multiplied by 10). The mean platelet count equaled the control group within 9 months. However, even after 12 months, the mean PF4 and BTG levels were 5 times higher than the control group levels.

Figure 5.4 Mean platelet count ($10^9 L^{-1}$), PF4 and BTG ($10^9 mL^{-1}$) on entry and after 12 months of follow-up, in 19 patients with giant cell arteritis. The interrupted line shows the course of the ESR (values multiplied by 10). The mean platelet count equaled the control group within 9 months. However, even after 12 months, the mean PF4 and BTG levels were 7 and 6 times higher than the control group levels.

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Chapter 6

Low molecular weight heparin treatment in
steroid refractory ulcerative colitis:
clinical outcome and influence on mucosal
capillary thrombi

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Abstract

Background

In ulcerative colitis, a state of hypercoagulation has frequently been observed. Unfractionated heparin has shown beneficial effects as an adjuvant treatment of steroid refractory ulcerative colitis in open trials and in one placebo-controlled trial. Low molecular weight heparin (LMWH) offers advantages in the method of administration, but it has not been evaluated in severe ulcerative colitis. We therefore assessed the tolerability, safety and potential therapeutic effects of LMWH in hospitalized patients with steroid refractory ulcerative colitis.

Patients and methods

Twenty-five patients with severely active ulcerative colitis were included in an open-labelled trial. All patients had a flare-up of disease under glucocorticosteroid treatment. Nadroparin calcium 5.700 IE anti-Xa/0.6 ml s.c. was self-administered twice daily for 8 weeks. Patients were monitored for possible adverse events, and changes in clinical symptoms and in laboratory, endoscopical and histological results were analysed.

Results

Tolerability and compliance were excellent and no serious adverse events occurred. In 20 of 25 patients, a good clinical and laboratory response was observed. Also, the endoscopic and histological signs of inflammation significantly improved. However, this was not accompanied by a significant reduction in the number of mucosal microvascular thrombi after 8 weeks of LMWH treatment.

Conclusions

LMWH may be a safe adjuvant therapy for patients with active, glucocorticosteroid refractory ulcerative colitis.

Introduction

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In inflammatory bowel disease (IBD), histologic and systemic signs of enhanced coagulation are documented by several independent investigations¹⁻⁹, and an increased risk of thromboembolic complications in patients with ulcerative colitis (UC) and Crohn's disease (CD) is well known¹⁰⁻¹². The use of anticoagulants seems paradoxical as a therapy for a disorder characterized by bleeding. However, 'fibrinoid' mucosal capillary thrombi have been detected in rectal biopsies of patients with UC¹³. As the microthrombi were found without a definite relationship to the degree of surrounding inflammation, they might represent an underlying pathophysiological mechanism. Also, thromboembolic complications and a state of hypercoagulability associating flares of both UC and CD suggest that heparin may produce a benefit by affecting microthrombi in the intestinal circulation¹⁴. Also, heparin has immunomodulatory effects¹⁵. Administration of heparin may therefore be rational in patients with UC or CD resistant to conventional forms of treatment.

Unfractionated heparin (UFH) has been reported to be beneficial in the treatment of highly active UC, with improvement of clinical and endoscopic disease activity in several uncontrolled trials¹⁶⁻¹⁹, and one recently reported placebo-controlled study²⁰. Low molecular weight heparin (LMWH) has been demonstrated to be at least as effective as UFH in thromboembolic diseases²¹ and has replaced UFH as prophylaxis against thromboembolic complications in many clinical situations. However, there are only a few reports in the literature on the potential role of LMWH in inflammatory conditions²²⁻²⁴, and it remains to be documented whether LMWH can be applied as an effective and safe adjuvant in the treatment of patients with UC.

The aim of this study was to evaluate the tolerability and safety of self-administered LMWH as well as to provide more data to clarify whether LMWH improves clinical disease activity, laboratory values, and the endoscopic or histologic appearance of the colonic mucosa in patients with highly active UC, who did not respond to glucocorticosteroids. We additionally investigated the occurrence of microvascular thrombi in colonic mucosal biopsies to address the influence of LMWH treatment.

Materials and methods

Patients

In a prospective open-labelled study, 25 patients with moderately to severely active steroid refractory UC were seen at the Academic Hospital Maastricht, The Netherlands, and enrolled in this study from March 1996 to March 2000.

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Fifteen men and 10 women with a median age of 50 years (range: 8-85 years) were included because of reactivation of disease under tapering off corticosteroid treatment, and the median dose of prednisolone was 20 mg¹⁰⁻³⁰, at the moment the LMWH treatment was started. Also, five patients had used azathioprin for more than 3 months (dose 2.5 mg/kg). Most of the patients were on long-term concomitant treatment with 5-aminosalicylic acid. Four patients with moderate-to-severe UC were not enrolled for various reasons: two patients had to undergo colectomy before study entry, and two patients preferred to continue conventional therapy.

All patients had an active UC under near maximum treatment. However, the study group was made up heterogeneously. Four patients with active UC were suffering from a deep venous thrombosis, necessitating anticoagulant treatment. Two patients had severe anaemia and received red packed cells before the use of LMWH. Two patients had an acute pancreatitis at the time of flare-up of the UC. One patient had active disease in the third trimester of her pregnancy, and, as LMWH does not cross the placental barrier²¹, was willing to join the study. One patient used 80 mg of acetylsalicylic acid daily because of intermittent claudication.

Study design

The study was conducted according to a protocol approved by the medical ethical committee of the Academic Hospital Maastricht. We intended to treat patients for 8 weeks with nadroparin calcium (Fraxiparine[®], Sanofi Winthrop, Maassluis, The Netherlands) 5.700 IE anti-Xa/0.6 ml (or 15.000 AXa IC-E/0.6 ml) bid, s.c., as a supplement to the (continued) immunosuppressive medication. During the first week of hospitalization, patients were instructed to self-administer nadroparin.

Clinical disease activity was determined by the Colitis Activity Index²⁶ or CAI (from 0 to 21; severe disease defined as >12, inactive disease <4), as well as a patient diary.

The endoscopic severity of disease was scored by means of the Endoscopic Grading System²⁷ or EGS (from 0 to 26; severe disease >18) within 2 weeks prior to LMWH treatment (**table 6.1**).

Also, a grading of histologic disease activity (HDA, from 0 to 12; severe inflammation >10) was performed²⁷ (**table 6.2**). Duplicate biopsies (3 mm) were taken from the most severely affected area, at 25 cm in the sigmoid and at 10 cm from the anus in the rectum. The recording of endoscopic and histological scores was done with reference to the visually most inflamed site in the colon or rectum. The EGS and HDA score were repeated after 8 weeks of treatment, and one experienced pathologist assessed all biopsies in a blinded manner.

The presence of microvascular thrombi was investigated in paraffine-embedded biopsies with the help of a peroxidase-linked immunosorbing assay

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against early cross-linked fibrin (Dako Ltd., UK). A vessel was considered to have a microthrombus if more than half of its diameter was occluded and stained positive. Before counting the microvascular thrombi, patient data were blinded and a physician not involved in the study coded biopsies. A medical student, a gastroenterologist, and an experienced pathologist studied biopsies in random order. The number of microvascular thrombi was counted per 3 mm biopsy specimen. The EGS, HDA and microvascular thrombi score were repeated after 8 weeks of treatment.

At regular intervals (week 0 or just before treatment, and after 1, 2, 4, 6 and 8 weeks of treatment), laboratory tests like the erythrocyte sedimentation rate, red and white blood cell counts, platelet counts (normal values: $130-350 \times 10^9/l$), mean platelet volume (MPV 8.6-9.7 fL), serum levels of C-reactive protein (CRP 0-9 mg/l), alkaline phosphatase (AP 30-125 IU/l), gamma-glutamyl-transpeptidase (GGT 10-50 IU/l), lactate dehydrogenase (LDH 100-250 IU/l), and serum levels of aspartate aminotransferase (AST 5-40 IU/l) and alanine aminotransferase (ALT; 5-40 IU/l) and creatinin were performed. Different markers of coagulation like the APTT (24-35 s), prothrombin time or Quick test (11.0-14.0 s), thrombin-antithrombin complex, prothrombin fragments 1 and 2, and of fibrinolysis (anti-thrombin III, D-dimers), as well as anti-IIa activity, anti-Xa activity and the thrombin potential were also determined. However, in the present report we will focus on the course of CRP, AST, ALT, MPV, and the platelet count.

Inclusion and exclusion criteria

Before inclusion, the diagnosis of UC was made according to the Lennard-Jones criteria²⁸, and included proctitis, proctosigmoiditis, left-sided or pancolitis. Disease activity had to be moderate to severe according to the Truelove classification²⁹. Also, patients had to be investigated endoscopically no more than 2 weeks prior to the start of the treatment.

Patients with proven Crohn's disease, infectious colitis, ischaemic colitis or irradiation colitis were excluded, as well as patients with a previously known systemic coagulation disorder.

Also, the regular use of non-steroidal anti-inflammatory drugs, a known active (peptic) ulcer disease, a history of adverse effects on heparin, severe hepatic disease (with AST or ALT levels >3 times the normal values), or renal insufficiency (creatinin levels >300 $\mu\text{mol/l}$) were considered as contraindications for additional LMWH treatment.

Patients were withdrawn from the study if no clinical improvement (CAI) was noted within 4 weeks of nadroparin treatment, if a progression of disease occurred, or if major side effects (clinical and/or biochemical, like the rise of AST/ALT) were encountered.

Ethical considerations

All patients gave their informed consent to participation in the study, which was approved by the local Ethics Committee at the Academic Hospital Maastricht.

Statistical analysis

Mean, median values and the 95 % confidence intervals (CI) for the mean of the above described parameters were calculated. To test differences for significance, the Mann-Whitney-Wilcoxon rank-sum test was performed. Furthermore, the Spearman rank correlation coefficient and the 2-tailed significance of the correlation was calculated. The statistical analysis was carried out using SPSS 9.0 software (SPSS Inc., USA).

Results

Adverse events

Among the 25 patients included, no major complications due to nadroparin treatment were observed, apart from some small haematomas at the injection sites. However, of 4 patients withdrawn from the study after two weeks of nadroparin treatment, 2 suffered from persistent rectal blood loss and needed repetitive transfusions before surgery was performed. In the other two patients who still had active disease, levels of AST and ALT rose to a 3-fold value after 2 weeks of nadroparin use, but normalized within 1 week after withdrawal. Of the remaining 21 patients followed for 8 weeks, the patient who used acetylsalicylic acid had a temporary increase in rectal blood loss, possibly due to a thrombopathy, but without a decrease in the platelet count after 5 weeks of nadroparin treatment. Three other patients suffered from temporary muscle and joint pains, without the presence of synovitis, and before the withdrawal of prednisolone was started after the 8 weeks period. In two other patients, AST and ALT levels were temporary raised at 4 weeks, and normalized after 6 weeks of follow-up. The two patients with acute pancreatitis made a good recovery, and LMWH treatment was not associated with a rise in serum amylase and/or lipase in these patients. The use of nadroparin during the 3rd trimester of pregnancy was not associated with complications in the child.

Influence of additional LMWH treatment on the colitis activity index

A significant reduction in the CAI was observed within 4 weeks of additional LMWH treatment in 20 of 25 patients (80%; $p < 0.0001$). Mean and 95% CI values of the CAI were 12.2 (11.2-13.2) at entry, 5.6 (4.8-6.4) after 4 weeks

and 5.3 (4.4-6.2) after 8 weeks. Thus, following definitions, disease activity changed from severe to mild disease.

Frequency of blood loss, in particular, but also the diarrhoea frequency was significantly lower within 4 weeks ($p=0.007$ and $p=0.041$ respectively). Although the mean CAI further reduced between 4 and 8 weeks, this change was not significant ($p=0.356$). (figure 6.1) Only in 1 of 21 patients followed for 8 weeks of LMWH treatment, did the CAI value remain unchanged compared to the pretreatment value.

Influence of additional LMWH treatment on the acute phase response, and other laboratory findings

A significant reduction in the CRP value after 4 weeks of additional LMWH therapy was found ($p=0.003$), but CRP levels did not further improve between 4 and 8 weeks of follow-up ($p=0.750$). CRP levels correlated positively with the CAI ($r=0.441$; $p<0.0001$).

Although the mean platelet counts were normal-to-raised, as compared to the hospital normal values, significantly lowered MPV levels were found during 8 weeks of follow-up. LMWH did not significantly influence the platelet count and MPV levels after 8 weeks ($p=0.757$ and 0.593 respectively). Mean (95% CI) levels of platelets and MPV were $315 \times 10^9 / l$ (267-362) and 7.5 fL (7.2-7.8) at entry, $320 \times 10^9 / l$ (262-377) and 7.6 fL (7.1-8.0) after 4 weeks and $322 \times 10^9 / l$ (280-364) and 7.4 fL (7.0-7.8) after 8 weeks. Although the MPV levels did not significantly correlate with CAI, EGS and HDA scores, an inverse correlation was found with CRP ($r=-0.243$; $p=0.034$) and ESR ($r=-0.304$; $p=0.008$).

APTT levels were significantly higher with LMWH treatment ($p=0.012$), and in 10 of 21 patients, LMWH treatment led to a small but persistent rise in APTT levels above the norm, as long as LMWH treatment was continued (follow-up after termination).

Influence of LMWH treatment on the endoscopic findings

Of the 25 patients investigated by endoscopy at entry, 8 patients had a pancolitis, 16 patients had a left-sided colitis (proctosigmoiditis at least), and 1 patient had an ulcerative proctitis. Of the 21 patients followed after 8 weeks, in 15 (71%) the range of endoscopic bowel involvement was unchanged, in 1 (5%) it was worse and in 5 (24%) patients it was less extensive. After 8 weeks, 7 patients had a pancolitis, 12 had a left-sided colitis and 2 of 21 patients had a proctitis. In 2 patients (9%), the endoscopic appearance was unchanged, and in 1 (5%) it worsened.

According to the semi-quantitative EGS score, 18 of 21 patients (86%) improved significantly ($p<0.0001$), with mean and 95% CI values of 12.5 (11.1-14.0) before and 6.4 (4.3-8.5) after 8 weeks (figure 6.2) The EGS score

correlated significantly with the CAI ($r=0.703$; $p<0.0001$) and CRP levels ($r=0.576$; $p<0.0001$).

Influence of LMWH treatment on histological inflammation and microvascular thrombi

Of 21 patients followed endoscopically, 17 (80%) showed a histological improvement of inflammation. In 2 patients histology seemed unchanged, and in 2 others histology worsened after 8 weeks of follow-up. The mean (95% CI) HDA score was reduced from 6.8 (5.7-7.9) to 3.7 (2.4-5.1) ($p=0.001$) (**figure 6.3**). The HDA score correlated significantly with the CAI ($r=0.537$; $p<0.0001$), CRP levels ($r=0.475$; $p=0.001$), and EGS score ($r=0.705$; $p<0.0001$).

In 20 of 21 patients, it was possible to stain the biopsies for occlusive microvascular thrombi (**figure 6.4**, photograph), and in all of these patients they could be detected before LMWH treatment. After treatment, apart from the occlusive thrombi that were still present, we regularly saw a smaller (circular) layer of fibrin against the vessel wall. In 14 patients the number reduced, in 6 it increased, and only in 1 patient no microthrombi could be detected.

Although the mean (95% CI) number of fibrin-thrombi per 3 mm biopsy reduced from 25.4 (12.4-38.4) to 14.1 (10.6-17.5), this difference was not significant ($p=0.338$). (**figure 6.5**) The presence of microvascular thrombi did not correlate with the CAI ($p=0.499$), the EGS ($p=0.146$) or HDA scores ($p=0.399$), the platelet count ($p=0.122$) or MPV levels ($p=0.267$).

Follow-up after 8 weeks of LMWH treatment

Following the 8 weeks' LMWH treatment period, we were able to reduce and terminate steroid treatment in 18 of 21 (86%) patients within another 8 weeks (median; min-max: 3-14). Azathioprin was initiated to maintain remission in all patients, except in the cases of the 8-year-old child, the patients recently suffering from pancreatitis, and the pregnant patient who underwent surgery after childbirth. Three other patients were operated on within 6 months of follow-up. In one patient, symptoms recurred after 9 weeks of nadroparin treatment, and after an unsatisfactory trial with intravenous cyclosporin and corticosteroids, surgery was performed. Two patients had recurrent complaints shortly after the withdrawal of nadroparin, and were also in need of surgery. Nadroparin use was continued in a small group of six patients during at least 6 months; 5 were not initially reacting to azathioprin. No major side effects (bleeding, liver test disturbances) occurred in these patients.

In one patient, local abdominal skin induration and pain after repeated injections brought nadroparin treatment to a halt after 6 months.

Interestingly, in 2 patients, within one week after stopping LMWH treatment, a sudden and first onset of erythema nodosum of the legs was observed. In another patient, a deep venous thrombosis of one leg occurred within 2 months

after the LMWH was withdrawn. Both the erythema nodosum and the venous thrombosis reacted promptly to re-administration of LMWH.

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Discussion

The present study demonstrates that LMWH may be beneficial in the treatment of active ulcerative colitis. We found that 5.700 IE anti-Xa/0.6 ml bid. s.c. of nadroparin for a period of 8 weeks did not cause major side effects. Clinical symptoms, inflammatory laboratory parameters like the CRP, endoscopic as well as histopathological scores all improved in the majority of patients, and a decrease in the infiltration of inflammatory cells into the affected colorectal segments was demonstrated histologically. It has been reported that UFH may improve the efficacy of conventional therapy for UC^{15,17-20}. Our findings demonstrate that LMWH induces a similar improvement with regard to clinical, endoscopic and histopathological healing. As all patients had already been treated with corticosteroids before study entry, a carry-over effect of steroids cannot be totally excluded. However, as this group had been treated with high doses of corticosteroids for more than 2 weeks without improvement, such influence on the study result appears unlikely. Similarly, oral azathioprin was probably not involved in the improvement, as it was already used for more than 3 months in the patients involved.

Despite the fact that the dose of UFH in previous studies and the dose of LMWH in our study were similar with regard to anticoagulative potency, the frequency of rectal bleeding initially increased in 20% of the patients receiving UFH¹⁷, and one serious bleeding has been reported¹⁹. With LMWH we observed no serious bleeding complications. However, although rectal bleeding could be stopped in the majority of patients, in 2 patients nadroparin supplementation did not overcome this problem. In one patient, also treated with low dose acetylsalicylic acid, bleeding recurred temporarily, but in none of the patients a thrombocytopaenia secondary to LMWH treatment was observed.

Raised levels of AST and ALT were found within 5 weeks after initiating nadroparin in 5 of 25 patients with UC (20%).

This is a known complication of UFH and LMWH treatment³⁰, and is possibly caused by a direct toxic effect of heparins on the hepatocytes³¹⁻³². In 2 patients who continued LMWH treatment, they normalized spontaneously. We did not find a rise in AP, GGT, LDH or creatine kinase levels in these patients. Although it seems to be a temporary phenomenon, it should be monitored regularly, as until now we don't know if the underlying mechanism is dosage dependent, or if patients with UC are more prone to develop liver toxicity due to LMWH therapy.

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With LMWH treatment, tolerability and compliance were excellent. Following the 8 weeks observation period, we were able to reduce and to terminate steroid treatment in 18 of 21 (86%) patients within 2 months. This effect of LMWH may be important over time, when one considers that glucocorticosteroids can have numerous adverse effects, including an impact on bone mineral density. LMWH seems to have much less impact on bone turnover compared to UFH^{21,33}, indicating another potential advantage in inflammatory bowel disease conditions. In certain circumstances, such as an acute pancreatitis, a relative contraindication exists against the use of high dose steroids, 5-ASA, azathioprin, 6-mercaptopurine or cyclosporin, and LMWH could have a beneficial role, as was shown in 2 of our patients. The group of patients studied was heterogeneous with respect to age, co-morbidity, disease extent and treatment before study entry or concomitant therapy, and further investigations are required to evaluate the effectiveness of LMWH as monotherapy in UC. As clinical experience with LMWH therapy in IBD is limited, its use as maintenance therapy must be critically followed. Hence, azathioprin was initiated to maintain remission in all patients who were regarded as refractory to any other form of treatment.

That UC is associated with an increased incidence of thrombo-embolism was in fact demonstrated in four of our patients before the start nadroparin treatment, and observed in one patient after the withdrawal of nadroparin therapy. Like UFH, LMWH may have a role in preventing the development of vascular complications in this patient group, and some authors have speculated that the observed effect of UFH was related to its anticoagulative properties.

The detection of microvascular thrombi in all of our patients with active steroid-refractory UC might support the hypothesis of a primary state of hypercoagulability as a pathophysiological mechanism in UC, although this phenomenon has been observed in other causes of bowel inflammation^{13,34,35}.

More important however, the appearance of occlusive fibrinoid lesions was present during clinically inactive disease and it was not related to the degree of mucosal inflammation, as was already found by Dhillon and co-workers¹³. Therefore, we have no arguments with the notion of a state of hypercoagulation due to the inflammation itself.

To our surprise, treatment with nadroparin did not significantly influence the presence of the microvascular thrombi.

As nadroparin preferentially inhibits coagulation factor X activity, the possibility of ongoing platelet activation⁹, a more directly activation of thrombin, or a disturbed fibrinolysis in UC¹⁵, could be important factors in the persistence of these microthrombi.

Indeed, the presence of activated platelets as represented by a smaller MPV but normal-to-raised number of the platelet count found in this study, as well as by others^{8,36}, could fit with an important role of platelets in the pathophysiology

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of UC. In this study however, neither the platelet count nor the MPV levels correlated with the presence of these microvascular thrombi. Another explanation is the presence of an endothelitis or small-vessel vasculitis¹⁵, and in this perspective future ultrastructural investigations could be of interest to characterize these vessels.

Obviously, LMWH treatment has an anti-inflammatory effect in UC, in addition to corticosteroid treatment. It has been shown that mucosal recruitment of leucocytes has a role in the pathophysiology of UC¹³, and the endothelial transmigration of leucocytes can be inhibited by LMWH treatment^{24,25}. UFH may have a possible protective effect on the endothelium, as was suggested by Korzenik et al.¹⁵. However, if microthrombi are in some way connected to endothelial inflammation, in the case of LMWH no clear protective effect after 8 weeks of treatment has shown in our study.

Taken together, in steroid refractory UC our findings indicate that treatment with nadroparin for a period of 8 weeks improves the clinical symptoms, and decreases macroscopic and microscopic inflammation. LMWH therapy seems to be well tolerated and not associated with significant side effects.

However, the results underline the presence of a chronic hypercoagulative disease state in UC, as 8 weeks of LMWH treatment did not significantly reduce the number of mucosal microvascular thrombi. We conclude that LMWH may be of value as an adjuvant therapy in ulcerative colitis. Based on these promising results, a multicentre and randomized controlled trial is now being undertaken.

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Parameter / grade		1	2
Erythema	Normal	Red	Deep red
Vascular pattern	Visible	Partially visible	Not visible
Friability	Normal	Slight	Severe
Granularity	Normal	Fine	Coarse
Spontaneous bleeding	Absent	Discrete	Severe
Occurrence of ulcers	Absent	Few	Multiple
Severity of ulcers	Absent	Superficial	Deep
Surface ulcerated	Absent	< 30%	> 30%
Mucopurulent exudate	Absent	Little	Much
factor	x 0	x 1	x 2
Maximum score	0	9	18

Table 6.1 Endoscopic grading system (EGS score).

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Parameter / grade	0	1	2	3
Ulceration	Absent	Mild	Moderate	Severe
Erosion	Absent	Mild	Moderate	Severe
Crypt abcesses	Absent	Sporadic	Moderate	Extensive
Cryptitis	Absent	Mild	Moderate	Severe
factor	x0	x1	x2	x3
Maximum score	0	4	8	12

Table 6.2 Histological disease activity (HDA score).

Figure 6.1 Influence of LMWH on the course of the colitis activity index (CAI) in 21 patients with steroid refractory UC before LMWH treatment, and in 21 patients after 4 and 8 weeks of LMWH treatment. Data of the CAI (■), the daily stool frequency (□) and the number of bloody stools per day (●) are presented as error bars (mean and 95% CI of the means).

Figure 6.2 Influence of LMWH on the course of the endoscopic grading system (EGS) in 21 patients with steroid refractory UC. Data are shown at 0 and 8 weeks after LMWH treatment, and presented as error bars (mean and 95% CI of the means).

Figure 6.3 Influence of LMWH on the course of the histological disease activity (HDA) in 21 patients with steroid refractory UC. Data are shown at 0 and 8 weeks after LMWH treatment, and presented as error bars (mean and 95% CI of the means).

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Figure 6.4 The presence of a microvascular 'fibrinoid' thrombus in the mucosa of a patient with ulcerative colitis, before LMWH treatment. In the peroxidase staining with anti-early-cross-linked fibrin antibodies, these microvascular thrombi are brown coloured.

Figure 6.5 Influence of LMWH on the course of the mucosal microvascular thrombi in 20 patients with steroid refractory UC. Data are shown at 0 and 8 weeks after LMWH treatment, and presented as error bars (mean number per 3 mm biopsy, and 95% CI of the means).

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Chapter 7

A randomised, placebo-controlled trial of low molecular weight heparin in active ulcerative colitis

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Abstract

Background

In several open and one controlled trial unfractionated heparin was effective in the treatment of active Ulcerative Colitis (UC). Low Molecular Weight Heparin (LMWH) had a similar effect in several open studies.

Study design

We studied the efficacy, safety and tolerability of LMWH in mild to moderately active UC in a randomized, double blind, placebo-controlled trial. Twenty-nine patients with a mild or moderate recurrence of UC during salicylate treatment were randomised to receive either reviparin 3.436 IU (n=14) s.c. twice daily or placebo (n=15). The study period was 8 weeks. Treatment was discontinued if there was no improvement at 4 weeks or at any disease progression. Primary outcome measure was clinical improvement at 8 weeks measured by Clinical Symptoms Grading (CSG, based on the colitis activity index (CAI)). Endoscopic and histologic grading and IBDQ were secondary outcome measures. Patients were closely monitored for adverse events.

Results

Twenty of 29 patients finished the 8 week treatment period (reviparin vs placebo: 11 vs 9; p=0.70). There was no difference in CSG, CAI, endoscopic and histologic grading or IBDQ. Treatment was well tolerated and no serious adverse events occurred.

Conclusions

In this study treatment with LMWH showed no significant clinical advantage compared to placebo in mild to moderately active UC.

Introduction

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In the majority of cases the clinical course of Ulcerative Colitis (UC) is characterized by exacerbations with abdominal pain and frequent bloody stools, alternated by periods of remission. The current treatment strategy is aimed at inducing remission with 5-aminosalicylates (5-ASA) and/or corticosteroids or - in refractory cases - with cyclosporin or anti-TNF α , and maintaining remission with 5-ASA and/or azathioprin. All these drugs can have significant side effects; also, a proportion of patients is refractory to them and might require colectomy. In 1952, in the Netherlands, the first paper was published describing the use of heparin as an anti-inflammatory drug in cases of acute polyarthritis¹. This was followed by a series of studies from Russia showing some success with heparin treatment in rheumatoid arthritis^{2,3}. These results led to the assumption that there might be a place for the clinical application of the anti-inflammatory properties of heparin. In 1982 a Russian study reported clinical benefit with unfractionated heparin (UFH) in patients with active UC⁴. In 1991 Gaffney et al. described three patients with active UC who showed a good response to treatment with UFH⁵; the first of these three patients had reported a remission of his colitis during treatment for a deep vein thrombosis. Successful treatment in further 6 of 7 patients was reported by the same group in 1995⁶. Subsequently, several studies have been published describing the treatment of active UC with heparin, mostly in the UFH form, two of which were controlled, comparing heparin to corticosteroids^{7,8}. The other studies were performed in an open, uncontrolled manner^{9,10}. The results varied, with an overall tendency towards a beneficial effect of UFH. Very few side effects have been reported.

Three studies performed with LMWH also showed benefit, with clinical improvement rates ranging from 58 to 91%¹¹⁻¹³. The treatment was well tolerated. None of these three studies, however, was controlled. In view of these results we designed a study to test the hypothesis that LMWH is an effective drug in the treatment of UC.

Materials and methods

Study Design

We performed a randomized, double blind two-centre trial comparing reviparin (Clivarin®, Knoll, Ludwigshafen, Germany) 3,436 IU twice daily to placebo in patients with mild to moderately active UC.

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Patient Population

Between August 1996 and February 2000 a total of 29 patients were enrolled at the Departments of Gastroenterology of the University Hospitals of Maastricht and Groningen, The Netherlands. Patients with mild to moderately active UC (diagnosis based on Lennard-Jones criteria¹⁴), with a severity score 4-14 according to the Truelove classification¹⁵ were eligible. The active colitis could either be the first manifestation or an exacerbation of known disease. Sigmoidoscopy had to be performed less than two weeks before start of treatment. Excluded from the trial were patients with proven Crohn's disease, infectious colitis (excluded through stool cultures), ischaemic colitis or irradiation colitis. Use of oral or rectal corticosteroids or other immunosuppressive drugs was prohibited within 4 weeks before study entry. Also excluded were patients with known thromboembolic disposition or current use of anticoagulants, patients with known or suspected bleeding tendency or with regular use of Non Steroidal Anti Inflammatory Drugs (NSAIDs), including low dose aspirin. Previous adverse events to heparin therapy, known active ulcer disease, serious hepatic disease (ASAT >3x upper limit) or renal failure (serum creatinin >300 µmol/l) as well as pregnancy or breast feeding in female patients were other exclusion criteria. Written informed consent was obtained from all patients.

The protocol was approved by the Ethical Committees of both participating hospitals.

Treatment

After randomization (random allocation), the patients received either reviparin (Clivarin[®], MW 3.900 dalton) 3,436 IU Pharm Eur/0.6 ml (corresponding to 10.000 U of unfractionated calcium heparin) subcutaneously or placebo twice daily. The drug and placebo were made available in individually packed disposable syringes. Drugs were administered through self-injection.

All patients were on stable treatment with either salazopyrine (n=4) or mesalazine (n=20) 1 gram 2-3 times daily or olsalazine (n=5) in a comparable dose.

Treatment was intended to last 8 weeks. Control visits were planned at 1, 2, 4, 6 and 8 weeks. Treatment was discontinued if there was no improvement after 4 weeks, according to the Clinical Symptom Grading (CSG) as used in the "Asacol Probe Trial" protocol [Tytgat G. Study protocol on the comparison of two different oral Mesalazine formulations in preventing relapses of ulcerative colitis: A dose-ranging, prospective randomised open blinded end-point (PROBE) study, 1993]¹⁶, and/or the Clinical Activity Index (CAI)¹⁷, or in any patient with progression of disease activity at any control visit. Improvement was defined as a reduction of the CSG and CAI score of more than 4 points and 6 points respectively (table 7.1 and 7.2).

Other reasons for discontinuation were Heparin Induced Thrombocytopenia (HIT) type 2^{18,19} (thrombocytes below $100 \times 10^9/l$) or severe bleeding (defined as Hb < 5.0 mmol/l, Hb > 2.0 mmol/l below baseline value, blood loss with bloodpressure $< 80/50$ mmHg and/or need for blood transfusion).

In patients in whom the study treatment was discontinued, conventional treatment with corticosteroids was initiated.

Outcome parameters

Primary end point of the study was clinical improvement after 8 weeks of treatment. Disease activity was assessed at entry and at every visit by means of the CAI (scale 0-21) and CSG (scale 0-16).

Secondary endpoints included an Endoscopic Grading System²⁴ (EGS; scale 0-18) recorded at sigmoidoscopy performed before entry and after 8 weeks. Biopsies were taken at 10 cm from the anus and from the mid-sigmoid and assessed according to a Histological Disease Activity²⁴ (HDA; scale 0-12) by two separate pathologists.

Quality of life was assessed at week 1, 4 and 8 by means of the Inflammatory Bowel Disease Questionnaire (IBDQ)²⁸.

Safety parameters measured at every visit included hemoglobin (Hb), hematocrit (Ht), white blood count (WBC), platelets, creatinin, alkaline phosphatase (ALP), aspartate transaminase (AST) and alanine transaminase (ALT).

Statistical methods

The sample size was based on categorical data ('did the patient improve?'). For the expected proportion with specified outcome was chosen: p_1 =improved on heparin 0.80, p_2 =improved on placebo 0.20. The common SD was 0.68.

Taking the power to be 0.85 and a two-sided significance level of 0.05, the sample size was calculated to be 24 for each of the two groups. Analysis was performed on an intention-to-treat basis with last value carried forward in case of premature discontinuation. Software used was SAS for Windows (version 6.12; SAS Institute Inc, Cary, N.C.). For qualitative parameters (categorical or ordered), frequency counts and percentages of each category were calculated by treatment group.

The significance of differences between placebo and LMWH treated patients was analysed with the Pearson Chi-square test with asymptotic 2-sided significance. For 2×2 tables, Fisher's exact test was computed. Group mean differences were calculated using unpaired t-tests for normally distributed variables or the Mann-Whitney Wilcoxon's test for skewed distributed variables.

Results

Patients

Fifteen patients were randomized to receive riviparin and fourteen to receive placebo (19 patients in the University Hospital Maastricht and 10 in the University Hospital Groningen). Demographic data and clinical characteristics of patients randomized to treatment are shown in **table 7.1**. There was no difference between the two groups with regard to age, gender, or smoking habits. Mean duration and extent of disease, previous steroid treatment and individual or family history of thrombosis or bleeding tendency were similar in both groups.

Primary efficacy endpoint

In the riviparin group 11/15 (73.3%) patients completed the 8 weeks of treatment, in the placebo group 9/14 (64.3%) ($p=0.70$). One patient in the placebo group was lost to follow-up after two weeks. In all other patients, reason for discontinuation was either lack of efficacy or exacerbation.

At baseline, the mean CAI and CSG levels were not significantly different between the riviparin and placebo treated patient groups. (**table 7.3**)

At 4 weeks, the mean CAI was 7 (95% CI 5-9) in both the riviparin and placebo group ($p=0.547$), and at 8 weeks the mean CAI was 5 (95% CI 3-7) in the riviparin group and 6 (95% CI 3-8) in the patients treated with placebo ($p=0.490$) (**figure 7.1**).

At 4 weeks, the mean CSG was 5 (95% CI 3-7) in both the riviparin and placebo group ($p=0.693$), and at 8 weeks the mean CSG was 4 (95% CI 1-6) in the riviparin group and 4 (95% CI 1-7) in the patients treated with placebo ($p=0.759$) (**figure 7.2**).

Secondary efficacy endpoints

The results of the secondary outcome measures are summarized in **table 7.4**. There were no significant differences in ESG, HDA or IBDQ between both groups.

Adverse events

There were no serious adverse events in either study group. There was no significant difference in adverse events between the two study groups (**table 7.5**).

Discussion

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Heparin is a member of the group of glycosaminoglycans. Presently it is mainly used in the treatment and prevention of thromboembolic disorders. Its anti-thrombotic action is achieved through enhancing the activity of anti-thrombin and thus inhibiting haemostasis. However, several other actions of heparin have been discovered. In vitro, there is stimulation of several growth factors, including basic fibroblast growth factor and insulin-like growth factors²¹⁻²⁴. Additionally, heparin has been shown to interfere with recruitment, adhesion and migration of leucocytes^{25,26}. Until recently, heparin therapy has mainly consisted of intravenous application of mixed molecular, unfractionated heparin (UFH). For most indications, this has now been replaced by subcutaneous low molecular weight heparin (LMWH). Only one recent paper has been published describing the effect of LMWH in the treatment of mild to moderately active UC in a randomized, placebo-controlled manner²⁷ and could not detect any significant advantage. Regarding this matter, two earlier controlled studies have been reported, both comparing UFH to corticosteroids. The study by Ang et al.⁸ demonstrated a similar response rate in both groups, with little side effects in the UFH group; in contrast, the study performed by Panés et al.⁷ showed no response in the heparin group and a significantly higher rate of rectal bleeding. The disappointing results of the latter study have been attributed to several factors, including the lack of concomitant 5-ASA therapy and the relatively short treatment period of ten days^{30,31}. Previous studies with LMWH¹¹⁻¹³ had treatment periods of 8-12 weeks. The administered dose, however, ranged from a low dose of enoxaparin (5 mg weekly)¹¹ to conventional therapeutic doses of dalteparin and nadroparin^{12,13}. All three studies were uncontrolled.

Our study was designed in order to maximize the possible effect of the LMWH by continuing the 5-ASA treatment and assuring an adequate duration of treatment. The anticoagulant potency of the dose administered was comparable to that used by Gaffney in his initial publications^{5,6}. Our results show that the treatment was excellently tolerated but demonstrate no beneficial effect of LMWH in UC. An important observation in this study is the unexpectedly high response in the placebo group of 54-85% (CSG-CAI) as compared to 9-48% in other placebo-controlled studies in UC^{28,29}. Possibly this is due to the relatively high proportion of patients with low disease activity at baseline. The demographic and clinical characteristics of the patients in both groups do not offer any other plausible explanation for this outcome as they were very similar at baseline. Several explanations have been suggested for the possible beneficial effects of heparin in UC. The finding of microthrombi in rectal biopsies of patients with UC³⁰ combined with the thrombotic tendency in these patients³¹ and the negative correlation between inherited coagulopathies

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and Inflammatory Bowel Disease³² has led to the theory that the anticoagulant property of heparin might be the most important factor. However, Vrij et al. found a high rate of clinical and histologic improvement of inflammation, but no significant change in microvascular thrombi in patients on LMWH therapy¹³. Thus other anti-inflammatory mechanisms may be involved, such as inhibition of leucocyte adhesion to the vascular endothelium³³, interference with transendothelial migration of leucocytes through inhibition of neutrophil elastase³⁴ or stimulation of basic fibroblastic growth factor leading to improved mucosal repair²¹. The current study (as well as the previously mentioned study by Bloom et al.²⁷) has not conclusively shown a major efficacy of LMWH in the treatment of UC.

Most experimental data are from studies with UFH, which contains a mix of molecules with a range in molecular weight from 3-30 kD. It is possible that the anti-inflammatory effect of heparin is mainly achieved by another fraction than the low molecular one (3-6 kD), in contrast to the anticoagulant effect. Both UFH and LMWH do not appear to be sufficiently effective to be used as monotherapy. Possibly there might be a role for heparin as adjuvant therapy to corticosteroids with the intention to delay or even to avoid the need for cyclosporin, anti- TNF α and/or colectomy.

Symptom	Score
Diarrhoea during the day	
0-2	0
3-4	1
5-6	2
7-9	3
>10	4
Diarrhoea at night	
No	0
Yes	1
Faecal blood loss (%)	
0	0
< 50	1
> 50	2
100	3
Incontinence for faeces	
No	0
Yes	1
Abdominal pain	
None	0
Mild	1
Moderate	2
Severe	3
General well being	
Perfect	0
Very good	1
Good	2
Moderate	3
Bad	4
Terrible	5
Abdominal tenderness	
None	0
Mild, localised	1
Mild to moderate, diffuse	2
Severe or rebound tenderness	3
Anti-diarrhoea medication	
No	0
Yes	1
Total score: (max = 21):	

Table 7.1 Colitis Activity Index (CAI).

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Parameter/Grade	0	1	2
Blood loss	none	sometimes	frequent
Mucus discharge	none	sometimes	frequent
Frequency of defaecation	<3/day	3-6/day	>6/day
Consistency of faeces	normal	semi-liquid	liquid
Tenesmus	absent	mild	severe
Abdominal pain	absent	mild	severe
Rectal pain	absent	mild	severe
Nausea/ vomiting	absent	sometimes	frequent
Accumulated score:	x 0	x 1	x 2
	↓	↓	↓
Total score:			
Maximum score:	0	8	16 = 24

Table 7.2 Clinical symptom grading (CSG).

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	Reviparin	Placebo	Significance
Number of subjects	15	14	ns
Age (mean)	38	42	ns
Sex (M/F)	9/6	7/7	ns
Smoking (previous/never)	3 (8/4)	1 (11/2)	ns
Previous steroid use	9	9	ns
CAI (min-max)	10 (5-16)	9 (3-13)	ns
CSG (min-max)	8 (4-14)	6 (2-10)	0.06 (ns)
Disease years (range)	6 (0-15)	7 (0-26)	ns

Table 7.3 Demographic and clinical data (CAI, CSG) at baseline.
ns= not significant.

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	Reviparin	Placebo	Significance
EGS (mean)			
day 0 (n)	10 (14)	9 (13)	ns
day 57 (n)	7 (11)	8 (9)	ns
patients improved (%)	9 (64)	8 (67)	ns
HDA (mean)			
day 0 (n)	3 (15)	4 (14)	ns
day 57(n)	2 (11)	3 (9)	ns
patients improved (%)	10 (67)	7 (53)	ns
IBDQ (mean)			
day 0	132	141	ns
day 57	162	173	ns

Table 7.4 Endoscopic (EGS), histological (HDA) and quality of life (IBDQ) outcomes.
ns= not significant.

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	Reviparin	Placebo	Significance
Patients (n)	15	14	ns
Adverse events (n)			
Haematoma on injection site	3 (20%)	2 (14.3%)	ns
Liver enzyme elevation	1 (6.7%)	0 (0 %)	ns
Headache	3 (20%)	2 (14.3%)	ns
Arthralgia	2 (1.3%)	1 (7.1%)	ns
Nausea	3 (20%)	2 (14.3%)	ns
Epistaxis	1 (6.7%)	2 (14.3%)	ns

Table 7.5 Adverse events. ns= not significant.

Figure 7.1 Box-Whisker plots of the Colitis Activity Index (CAI) in reviparin and placebo treated patients, at different time intervals, with outliers (°) and extremes (*). No significant differences were observed between the two groups.

Figure 7.2 Box-Whisker plots of the Clinical Symptom Grading (CSG) in reviparin and placebo treated patients, at different time intervals, with outliers (^o) and extremes (*). No significant differences were observed between the two groups.

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Chapter 8

Thrombin generation in mesalazine refractory
ulcerative colitis and the influence of low
molecular weight heparin

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J Thromb Thrombolysis, in press

Studied markers of thrombin generation and the influence of LMWH.

Abstract

Background

In ulcerative colitis, a state of hypercoagulation has frequently been observed. Low molecular weight heparin (LMWH) has shown beneficial effects as an adjuvant treatment of steroid refractory ulcerative colitis in open trials. We assessed potential therapeutic effects of the LMWH reviparin in hospitalised patients with mesalazine refractory ulcerative colitis, as well as its influence on haemostasis factors.

Methods

Twenty-nine patients with mild-to-moderately active ulcerative colitis were included in a double-blind placebo controlled trial. All patients had a flare-up of disease under mesalazine treatment. Reviparin (Clivarin®) 3.436 IU anti-Xa/0.6 ml or placebo s.c. was added, and self-administered twice daily for 8 weeks. Patients were monitored for possible adverse events and changes in clinical symptoms. Endoscopical, histological, biochemical and haemostasis parameters were analysed.

Results

Tolerability and compliance were excellent and no serious adverse events occurred. No significant differences were observed on the clinical, endoscopical and histological outcome, as compared to placebo. A high intrinsic and extrinsic thrombin potential was found before LMWH therapy. However, the significant reduction in the thrombin generation by LMWH was not related to the reduction in disease activity.

Conclusions

The LMWH reviparine reduces thrombin generation in patients with mild to moderately active, mesalazine refractory ulcerative colitis, but is not associated with a reduction in disease activity.

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Introduction

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In inflammatory bowel disease (IBD), histologic and systemic signs of enhanced coagulation are well documented¹⁻⁹, and an increased risk of thrombo-embolic complications is well recognised in patients with ulcerative colitis (UC) and Crohn's disease (CD)¹⁰⁻¹³. The use of anticoagulants like Low Molecular Weight Heparin (LMWH) seems paradoxical as a therapy for a disorder characterized by bleeding. However, 'fibrinoid' mucosal capillary thrombi have been detected in rectal biopsies of patients with UC¹⁴. As the micro-vascular thrombi were found without a clear relationship to the degree of surrounding inflammation, they might represent an association with underlying pathophysiological mechanisms^{14,15}. The finding of raised levels of plasma coagulation and fibrinolysis end products indirectly reflects a state of hypercoagulation in patients with IBD¹⁶.

More direct thrombin activity is difficult to measure, as it is a transient phenomenon during the clotting process. Currently, thrombin activity can be quantified by measuring the amount of product that is produced from an artificial thrombin substrate during coagulation. The time course of the thrombin generation curve reflects the function of the ensemble of plasmatic pro- and anticoagulant factors in platelet-poor plasma (PPP), as well as the procoagulant role of platelets in platelet-rich plasma (PRP)¹⁷. Both the extrinsic- and intrinsic thrombin potential (ETP and ITP) seems to be increased in other thrombosis-prone states investigated^{18,19}, but in IBD patients, the level of thrombin generation has not been reported before. As LMWH therapy may produce a benefit by affecting micro-vascular thrombi in the gut^{20,21}, its administration could be rational in patients with UC or CD resistant to conventional forms of treatment.

The aim of this study was to clarify whether an improvement of clinical disease activity after LMWH therapy was associated with a reduced thrombin generation in patients with mild-to-moderately active UC, unresponsive to mesalazine. We additionally investigated the relationship of thrombin generation with other haemostasis factors in these patients.

Methods

Patients

In a prospective randomized double-blind placebo controlled trial, 29 patients with mild-to-moderately active mesalazine refractory UC were seen at the University Hospitals of Maastricht and Groningen, The Netherlands, and enrolled in this study from August 1996 to February 2000. The diagnosis UC was based on the Lennard-Jones criteria²², and patients with a severity score

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of 4 to 14 according to the modified Truelove classification²³, were eligible. The active colitis could either be the first manifestation or an exacerbation of the disease. Sigmoidoscopy had to be performed less than two weeks before start of treatment. Excluded from the trial were patients with proven Crohn's disease, infectious colitis, ischaemic colitis or irradiation colitis. Use of oral or rectal corticosteroids or other immunosuppressive drugs was prohibited within 4 weeks before study entry. Also excluded were patients with known thromboembolic disposition or current use of anticoagulants, patients with known or suspected general bleeding tendency, and patients with regular use of Non Steroidal Anti- Inflammatory Drugs (NSAID's) or aminosalicylates. Previous adverse events to heparin therapy, known active ulcer disease, serious hepatic disease (ASAT >3x upper limit) or renal failure (serum creatinin >300 µmol/l), as well as pregnancy or breast-feeding in female patients, were other exclusion criteria. Written informed consent was obtained from all patients. The Ethical Committees of both participating hospitals approved the protocol.

Study design

After randomization (random allocation), the patients received either reviparin (Clivarin[®]) 3,436 IU Pharm Eur/ 0.6 ml (corresponding to 10.000 U of unfractionated calcium heparin) or placebo subcutaneously twice daily.

The drug and placebo were made available in individually packed disposable syringes. Both were administered through self-injection. All patients were on stable treatment with either salazopyrine (n=4) or mesalazine (n=20) 1 gram 2-3 times daily or olsalazine (n=5) in a comparable dose.

Clinical disease activity was determined by the Colitis Activity Index (CAI)²⁴ (from 0 to 21; severe disease defined as >12, inactive disease <4) on week 0 or just before treatment, and after 1, 2, 4, 6 and 8 weeks of treatment. Quality of life was assessed at week 1, 4 and 8 by means of the Inflammatory Bowel Disease Questionnaire (IBDQ)²⁵.

The endoscopic severity of disease was scored by means of the Endoscopic Grading System²⁶ or EGS (from 0 to 26; severe disease >18) within 2 weeks prior to LMWH treatment. Also, a grading of histologic disease activity (HDA, from 0 to 12; severe inflammation >10) was performed²⁶. Duplicate biopsies (3 mm) were taken from the most severely affected area, at 10 cm from the anus in the rectum and at 25 cm in the sigmoid. The recording of endoscopic and histological scores was done with reference to the most inflamed site in the colon or rectum. The EGS and HDA score were repeated after 8 weeks of treatment, and one experienced pathologist assessed all biopsies in a blinded manner.

At regular intervals (week 0 or just before treatment, and after 1, 2, 4, 6 and 8 weeks of treatment), laboratory tests were performed: the erythrocyte

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sedimentation rate, red and white blood cell counts, platelet counts (normal values: $130\text{--}350 \times 10^9/\text{l}$), mean platelet volume (MPV; $8.6\text{--}9.7 \text{ fl}$), platelet distribution width (PDW; $15\text{--}18 \%$), serum levels of C-reactive protein (CRP; $0\text{--}9 \text{ mg/l}$), alkaline phosphatase (AP; $30\text{--}125 \text{ IU/l}$), gamma-glutamyl-transpeptidase (GGT; $10\text{--}50 \text{ IU/l}$), lactate dehydrogenase (LDH; $100\text{--}250 \text{ IU/l}$), and serum levels of aspartate aminotransferase (AST; $5\text{--}40 \text{ IU/l}$) and alanine aminotransferase (ALT; $5\text{--}40 \text{ IU/l}$) and creatinin (creat; $40\text{--}90 \mu\text{mol/l}$).

Different markers of coagulation like the APTT ($24\text{--}35 \text{ sec}$), thrombin-antithrombin complex (TAT; $0.00\text{--}1.90 \mu\text{g/ml}$), prothrombin fragment 1 and 2 (F1.2; $0.40\text{--}1.45 \text{ nmol/l}$), as well as anti-IIa activity ($0.0\text{--}0.07 \text{ U/ml}$), anti-Xa activity ($0.0\text{--}0.02 \text{ U/ml}$) and the intrinsic and extrinsic thrombin potential (ITP and ETP) were determined. Treatment was intended to last 8 weeks. Control visits were planned at 1, 2, 4, 6 and 8 weeks.

Treatment was discontinued if there was no improvement after 4 weeks according to the CAI²⁴, or in any patient with progression of disease activity at any control visit. Other predefined reasons for discontinuation were Heparin Induced Thrombocytopenia (HIT) type 2^{27,28} and/or severe bleeding (defined as Hb $<5.0 \text{ mmol/l}$, Hb $>2.0 \text{ mmol/l}$ below baseline value, blood loss with blood pressure $<80/50 \text{ mmHg}$ and/or need for blood transfusion). In patients in whom the study treatment was discontinued, a treatment with corticosteroids was initiated.

Preparation of Plasma

The collection of blood was strictly timed, i.e. 30 minutes before the next dose of LMWH in the morning, and 30 minutes before laboratory analysis started.

Fresh citrated blood (9 parts of blood to 1 part of 0.13 mol/l trisodiumcitrate) was centrifuged at $250 g$, 15°C for 10 min. The platelet count was adjusted to $3 \times 10^8/\text{ml}$ using autologous Platelet Poor Plasma (PPP), made by double centrifugation of platelet rich plasma at $1000g$, 15°C for 10 min. Before storage at -80°C , PPP was centrifuged twice at $1,000g$ for 10 min. Plasma was defibrinated by adding $1/50$ volume of Ancrod and clot dissolution. After the thrombin generation test had been performed, the remaining serum was put on ice and centrifuged at $15,000 g$ for 2 min. The supernatant was stored at -80°C . Normal pool plasma was pooled PPP from at least 10 apparently healthy male and female donors and stored at -80°C for less than 4 months.

Measurement of Thrombin Generation

Thrombin generation was measured with a subsampling technique, as previously described in detail¹⁷⁻¹⁹, either triggered by tissue factor (extrinsically) or by contact activation (intrinsically). In short, thrombin was determined by monitoring optical density of the pNA at 405 nm , released from a slow-reacting chromogenic thrombin substrate (DEMZ-Gly-Arg-pNA) which was added to the

defibrinated plasma upon recalcification. The reacting mixture for the measurement of thrombin generation consisted of one part of buffer containing phospholipid vesicles (20 mol-% phosphatidylserine and 80 mol-% phosphatidylcholine).

For the extrinsic system, 4 μM phospholipid with 15 pM recombinant human tissue factor was added, and for the intrinsic system 4 μM phospholipid and 1/6 volume of Actin-FSL (Dade Behring®). The ETP and ITP level is expressed as a percentage of the ETP and ITP of normal pool plasma, measured simultaneously. The mean value of ETP in PPP in healthy controls was 737 nmol/l * min (range 720-790), the mean ITP level in PPP in healthy controls was 798 nmol/l * min (range 780-850)^{29,30}.

The normal values of ETP, ITP, as well as TAT and F1.2, were obtained from 10 healthy controls (5 male, 5 female), and were compared with the values of the patients.

Statistical analysis

The sample size of the clinical trial was based on categorical data ('did the patient improve?'). For the expected proportion with specified outcome was chosen: p_1 =improved on LMWH 0.80, p_2 =improved on placebo 0.20. The calculated SD was 1.2. Taking the power to be 0.85 and a one-sided significance level of 0.05, the sample size was calculated to be 24 for each group. Analysis was performed on an intention-to-treat basis with last value carried forward in case of premature discontinuation. To test differences for significance, the Mann-Whitney-Wilcoxon rank-sum test was performed. A Bonferroni adjustment was used for multiple comparisons, and an alpha level of 0.017 was considered significant with tests performed at 0, 4 and 8 weeks³¹. Also, the Spearman rank correlation coefficient and the 2-tailed significance of the correlation were calculated between the coagulation factors and other biochemical and disease parameters. SPSS 13.0 software (SPSS Inc., USA) was used for statistical analysis.

Results

Patients

Fifteen patients were randomized to receive the LMWH reviparin and fourteen to receive placebo (19 patients were studied in the University Hospital Maastricht and 10 in the University Hospital Groningen). Demographic data and clinical characteristics of patients randomized to treatment are shown in **Table 8.1**. There was no difference between the two groups with regard to age, gender, smoking habits or disease severity as measured by CAI.

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Mean duration and extent of disease, previous steroid treatment and individual or family history of thrombosis or bleeding tendency were similar in both groups.

Influence of additional LMWH treatment on the CAI, IBDQ, EGS and HDA

In the reviparin group 11/15 (73,3%) patients completed the 8 weeks of treatment, in the placebo group 9/14 (64.3%) ($p=0.70$). One patient in the placebo group was lost to follow-up after two weeks. In all other patients, reason for discontinuation was either lack of efficacy or exacerbation. At 8 weeks the improvement in CAI was 3.80 points in the reviparin group and 2.08 points in the patients treated with placebo ($p=0.20$). In the reviparin group 12/15 (80%) patients had improved, compared to 11/13 (85%) in the placebo group ($p=1.0$).

There were no significant differences in IBDQ, ESG, or HDA between both groups. (**Table 8.2**).

Haemostasis characteristics and the influence of LMWH treatment on thrombin generation, haemostasis and fibrinolysis factors at baseline, at 4 and at 8 weeks

At baseline, patients in the LMWH group had above normal levels of ETP (777; 171) and ITP (824; 188) ($p=0.02$). Also, patients in the placebo group had higher levels of ETP (784; SD 87) and ITP (833; SD 100), than the normal control values of ETP (737; 19 with $p<0.001$), and ITP (798; 19 with $p<0.01$).

At baseline, TAT and F1.2 were significantly raised in the LMWH and placebo group, compared to the healthy controls ($p<0.0001$ and $p<0.0001$ respectively). The APTT, the anti-IIa and the anti-Xa activity was within normal limits in the LMWH and placebo group.

At 4 weeks, the ETP and ITP values were significantly lower in the LMWH treated group, as compared to the placebo group ($p=0.014$ and 0.015), as well as the healthy controls ($p=0.001$ and 0.001). However, TAT and F1.2 levels were higher, both in the LMWH group ($p<0.0001$ and $p=0.004$) and placebo group ($p<0.0001$ and $p=0.004$), as compared to the healthy controls. Also, anti-IIa ($p<0.0001$) and anti-Xa activity ($p<0.0001$), as well as APTT ($p=0.007$) and ALT levels ($p=0.008$), were significantly higher in the LMWH treated patients, as compared to placebo.

At 8 weeks, the ETP and ITP values were not significantly lower in the LMWH treated group, as compared to the placebo group ($p=0.536$ and 0.887), or the healthy controls ($p=0.238$ and $p=0.892$). However, TAT ($p<0.0001$) and F1.2 ($p=0.039$) were higher in the placebo group, as well as was TAT in the LMWH group ($p<0.0001$), as compared to the healthy controls.

Also, the anti-Xa activity, but not the anti-IIa, APTT or ALAT levels, was significantly higher at 8 weeks in the LMWH treated patients, as compared to placebo ($p=0.002$).

Table 8.3 summarizes the differences of laboratory data between the reviparin and placebo treated groups. The time course of ETP and ITP is shown in **figure 8.1** (reviparin) and **figure 8.2** (placebo). The time course of TAT and F1.2 is shown in **figure 3** (reviparin) and **figure 4** (placebo). The Box-whisker plots represent the median (line between the 25th and 75th percentile box) and min-max values (whisker lines extended from the box as smallest and largest values), as well as outliers (⁰) and extremes (*).

Relationship between haemostasis parameters and CAI, CRP, endoscopic and histologic parameters

In the placebo treated patients, the clinical activity score CAI was positively correlated with the ITP ($r=0.267$; $p=0.038$), as well as platelet count ($r=0.506$; $p<0.0001$) and APTT ($r=0.336$; $p=0.007$), and inversely correlated to PDW ($r=-0.552$; Spearman's rho, 2-tailed significance $p<0.0001$). CRP correlated positively with ETP ($r=0.531$; $p<0.0001$), ITP ($r=0.664$; $p<0.0001$) and anti-Xa ($r=0.336$; $p=0.007$), and inversely with MPV ($r=-0.361$; $p=0.002$) and PDW ($r=-0.382$; $p=0.018$). The endoscopy and histology score EGS and HDA did not correlate with the haemostasis parameters in the placebo group.

In the LMWH treated group, CAI correlated inversely with MPV ($r=-0.293$; $p=0.008$), and inversely with the APTT ($r=-0.271$; $p=0.016$). CRP correlated with platelet count ($r=0.344$; $p=0.001$), ETP ($r=0.472$; $p<0.0001$), ITP ($r=0.491$; $p<0.0001$), TAT ($r=0.283$; $p=0.014$), and APTT ($r=0.315$; $p=0.004$). The EGS did not correlate with ETP, and the HDA correlated inversely with ETP ($r=-0.456$; $p=0.029$).

Discussion

To our knowledge, this is the first study to present data on direct thrombin generation in patients with UC. The study demonstrates that the thrombin potential, both intrinsic and extrinsic, is above the norm in patients with active UC, confirming more indirect data on hypercoagulation in these patients¹⁶. The administration of LMWH diminished the intrinsic and extrinsic thrombin potential in patients with UC, comparative to the effect of LMWH on patients with active thrombo-embolic diseases^{18,30,32}.

In the reviparin treated group, the anti-IIa activity and APTT were raised after 4 weeks of treatment. However, both normalised after 8 weeks, while the anti-Xa activity was still raised. Although we did not find a clear explanation for the

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gradual loss of anti-IIa activity in our patients, a more selective LMWH- anti-Xa activity was expected from literature²⁰. To our surprise, the administration of a therapeutic dose of reviparin was accompanied by a rise in the thrombin potential at week 8, not different from baseline levels.

On the one hand, this questions the effectiveness of selective anti-Xa blocking agents on reducing the thrombin potential over time in patients with UC. The size of the effect on thrombin generation might depend on the type of activation used to initiate the clotting, which could be dependent of higher amounts of tissue factor³³.

On the other hand, the scheduled dose of reviparin (3436 IU /12h) corresponds to 10.000 IU/12h of unfractionated heparin, and this might be considered as an intermediate dose. We do not know if a higher dose (e.d. 15.000 IU/12h) would be effective in improving the clinical outcomes of UC, as the reached anti-Xa plasma level at 8 weeks was below the generally accepted therapeutic range of 0.4 to 1.0 aXa IU/ml.

No correlation was found between the (level of) reduction of the thrombin potential and both clinical (CAI; IBDQ), endoscopy and histology scores. However, a positive correlation was found between ETP and ITP with CRP, suggesting a pattern of acute phase reactivity in thrombin generation. The more stable end-products F1.2 and TAT did not correlate with CAI and CRP in the placebo-treated patients and this might favour the use of ITP or ETP as indices of UC disease activity over that of F1.2 and TAT.

No additional benefit of reviparin was found compared to the placebo treated patients, on CAI, IBDQ, EGS and HDA. This is in contrast with the positive results found in a previous but open study done with nadroparin, in corticosteroid resistant UC patients¹⁵. It suggests that LMWH, in combination with aminosalicylates but in the absence of corticosteroids, does not have any therapeutic advantage above a placebo, as was recently also shown by other investigators³⁴.

A masking effect due to the use of aminosalicylate therapy remains possible, although 8 weeks of therapy should have led to induction of remission. Also, the 8-week observation period following LMWH and placebo might have been too short to evaluate if a reduced thrombin generation has any influence with respect to the HDA score. However, the combination of LMWH and corticosteroids did reduce the HDA score significantly within 8 weeks of therapy in a previous study¹⁵.

Regrettably, the number of subjects needed to reach statistical power was not met in this study, because of slow recruitment.

Also, the placebo response in this patient group was above 60%, and was much higher than the 20% anticipated. These factors may have contributed to

lacking evidence for benefit of LMWH treatment on clinical, endoscopy and histology outcomes in patients with mild to moderately active UC.

Also, patients with known arterial or venous thrombo-embolic complications were excluded to overcome the bias of having an underlying tendency of hypercoagulation, unrelated to UC. However, data from open trials showed that LMWH therapy can reduce colitis activity in patients with active thrombosis, and these could have been patients to most benefit from LMWH treatment.

To prevent thrombo-embolic complications frequently seen in patients with UC, the use of an LMWH seems logical, as it reduces the state of hypercoagulation as was present in our patients at baseline. A higher dose of reviparin could be necessary to effectively reduce the thrombin potential in these patients over time periods longer than 4 weeks. This phenomenon of therapeutic "resistance", or possibly tachyphylaxis, needs further investigation.

With the reviparin doses used we observed a temporary rise in the liver ALT level, a known side effect of heparin treatment³⁵, also reported in a previous study on LMWH in UC¹⁵.

Fortunately, as in other studies^{15,34}, no serious bleeding complications were encountered, suggesting that LMWH is a relatively safe drug in this patient group with tendency for mucosal bleeding.

In conclusion, in patients with mild to moderate ulcerative colitis, the addition of the LMWH reviparin to mesalazine did not change clinical outcome; it reduces the thrombin potential and might therefore prevent thrombo-embolic complications in these patients.

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	Placebo	Reviparin	Significance
Number of patients	14	15	ns
Age (mean)	42	38	ns
Sex (M/F)	7/7	9/6	ns
Smoking (previous/never)	1 (11/2)	3 (8/4)	ns
Previous steroid use	9	9	ns
CAI (min-max)	9 (3-13)	10 (5-16)	ns
Duration of disease in years (range)	7 (0-26)	6 (0-15)	ns

Table 8.1 Baseline characteristics of placebo and reviparin treated patients.

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	Placebo			Reviparin		
	Week 0	Week 4	Week 8	Week 0	Week 4	Week 8
CAI	9.1 [3.1]	7.3 [3.2]	5.8 [3.1]	9.9 [2.9]	6.7 [3.5]	5.1 [3.5]
IBDQ	147 [24]	154 [25]	167 [23]	133 [30]	159 [38]	169 [44]
EGS	9.5 [3.3]	Not done	7.3 [5.3]	9.6 [3.2]	Not done	7.4 [4.3]
HDA	3.9 [2.1]	Not done	3.1 [2.5]	2.9 [1.8]	Not done	2.0 [2.2]

Table 8.2 Clinical (CAI, IBDQ), endoscopic (EGS) and histological (HDA) activity indices, before and after reviparin therapy or placebo, are shown. Means and standard deviations ([]) between the groups with reviparin and those with placebo are presented at week 0, 4 and 8. No significant differences were found between these groups.

	Placebo			Reviparin		
	Week 0	Week 4	Week 8	Week 0	Week 4	Week 8
Biochemistry						
CRP (mg/l)	11.4 [16.2]	11.2 [12.0]	18.9 [19.5]	16.0 [18.2]	10.4 [13.0]	20.8 [36.8]
Albumin (g/l)	40.3 [5.4]	40.5 [4.7]	40.7 [4.2]	40.3 [4.6]	41.3 [6.5]	41.4 [3.6]
ALT (IU/l)	18.6 [6.2]	20.0 [6.4]	18.0 [6.5]	26.1 [26.2]	35.3 [18.9] *	24.8 [13.2]
Platelets						
Number (10⁹/l)	276 [60]	280 [81]	285 [77]	275 [66]	274 [65]	286 [71]
MPV (fl)	8.0 [0.9]	7.9 [0.7]	7.8 [1.1]	7.7 [1.0]	7.8 [0.6]	7.8 [0.6]
PDW (%)	16.4 [0.7]	16.5 [0.3]	16.6 [0.3]	16.5 [0.5]	16.3 [0.6]	16.5 [0.3]
Coagulation						
APTT (sec)	31.4 [3.9]	30.6 [3.0]	31.7 [4.4]	32.7 [4.5]	35.3 [4.7] *	36.3 [5.2]
TAT (µg/ml)	3.61 [2.19]	5.23 [8.29]	2.63 [0.65]	6.73 [9.09]	7.44 [17.13]	2.47 [1.48]
F1.2 (nmol/l)	1.29 [0.45]	1.53 [0.62]	1.40 [0.45]	2.26 [2.40]	1.92 [2.12]	1.29 [0.52]
ETP (nmol/l*min)	784 [87]	743 [115]	767 [73]	777 [171]	598 [172] *	722 [183]
ITP (nmol/l*min)	833 [100]	815 [99]	782 [79]	824 [188]	613 [211] *	793 [256]
Anti-IIa (U/ml)	0.043 [0.034]	0.053 [0.038]	0.064 [0.025]	0.038 [0.023]	0.135 [0.057]*	0.095 [0.052]
Anti-Xa (U/ml)	0.006 [0.009]	0.008 [0.006]	0.007 [0.005]	0.013 [0.019]	0.552 [0.173]*	0.334 [0.271]*

Table 8.3 Laboratory data with means, standard deviations ([]), and significant differences (*=p<0.017) between the groups treated with reviparin or placebo, at week 0, 4 and 8.

Figure 8.1 Box-whisker plots of ETP and ITP (nmol/l * min; normal mean ETP value was 737 and ITP mean value 798), with outliers (⁰), during 8 weeks of follow-up in patients with UC, treated with mesalazine and reviparin.

Figure 8.2 Box-whisker plots of ETP and ITP (nmol/l * min; normal mean ETP value was 737 and ITP mean value 798), with outliers (⁰), during 8 weeks of follow-up in patients with UC, treated with mesalazine and placebo.

Figure 8.3 Box-whisker plots of TAT ($\mu\text{g/ml}$; normal upper limit $1.90 \mu\text{g/ml}$) and F1.2 (nmol/l ; normal upper limit 1.45 nmol/l), with outliers ($^{\circ}$) and extremes (*), during 8 weeks of follow-up in patients with UC, treated with mesalazine and reviparin.

Figure 8.4 Box-whisker plots of TAT ($\mu\text{g/ml}$; normal upper limit $1.90 \mu\text{g/ml}$) and F1.2 (nmol/l ; normal upper limit 1.45 nmol/l), with outliers ($^{\circ}$) and extremes (*), during 8 weeks of follow-up in patients with UC, treated with mesalazine and reviparin.

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Chapter 9

General discussion

Significance of haemostasis in IBD

Bloody stools characterize IBD, occurring especially in UC, but also in CD^{1,2}. However, major or intractable intestinal blood loss, leading to surgical intervention, is an infrequent finding in IBD^{1,2}. An explanation for this paradox is lacking; although mucosal damage in IBD may be superficial, complete mucosal denudation or deep ulcerations are regularly found. Apparently, larger blood vessels are occluded before arriving at the surface, probably due to local inflammatory responses, or as a result of primary occlusion of supplying vessels³. The prothrombotic – and probably haemostatic – tendency in IBD (**Chapter 1**) might contribute to (micro-)vascular occlusions.

The hypothesis of a thrombotic etiology for IBD³⁻⁶ has been reinforced by the association with thrombotic risk factors like hyperhomocysteinemia⁷, smoking⁸ and oral contraceptives⁹⁻¹¹, and the frequently encountered thrombo-embolic complications in UC and CD^{12,13}. Also, the response of certain UC or CD patients to tranexaminic acid¹⁴, FXIII addition^{15,16} and heparin¹⁷ might support this hypothesis. Although the etiology of IBD remains elusive and thrombosis might be an epiphenomenon secondary to chronic inflammation, it could explain certain histological findings in IBD, like damage of endothelial capillary walls as early signs of postoperative recurring CD¹⁸, and perhaps also typical localizations, such as segmental disease which follows the end-arterial distribution of the inferior mesenteric artery in UC^{19,20}.

Overall, the thrombotic tendency in IBD potentially has severe clinical consequences²¹. At short term, spontaneous venous thrombo-embolic complications, such as pulmonary embolism²², but also postoperative superior mesenteric vein and portal vein thrombosis, may occur²³. At the long term, the risk of arterial thrombosis, especially in smoking patients with CD²⁴, but also in UC²⁵, may be increased. Studies of the haemostatic balance of IBD patients are therefore warranted.

Difficulties in the interpretation of IBD-associated haemostatic alterations

In general, comparisons of the numerous studies concerning haemostatic variables in IBD patients are hampered by several factors.

Haemostatic variables are determined by using many types of tests and these tests have various intra-assay and inter-assay variations.

In addition, pre-analytical variables that are very important in tests of the haemostatic-thrombotic system, may vary greatly^{26,27}. Also, the relation between plasma study findings and local vascular circumstances of the inflamed intestine remains to be established. Moreover, clinical descriptions of the

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patient groups vary almost per study regarding sub-classification and assessment of disease severity, so that it becomes difficult to link clinical and laboratory findings.

In our studies, IBD was classified by means of the Lennard-Jones criteria²⁸. As indices of clinical disease activity, in Crohn's disease the CDAI²⁹ and in UC the modified Truelove-Witts score was used (**Chapters 3-5**)^{30,31}. In the LMWH intervention studies in UC patients (**Chapters 6-8**), the clinical disease activity scores CAI³² and CSG³³ were used. Laboratory handling and characteristics of haemostasis factor analysis have been described in detail in the aforementioned chapters.

Coagulation markers in IBD and in GCA

We found no significant differences in the coagulation activation and inhibition patterns between patients with active CD and UC, and neither between patients with clinically inactive CD and UC (**Chapter 3, table 3.2**).

In CD and UC patients, mean AT levels were in the normal range during 12 months of follow-up. TAT levels were raised in active disease, but normalised within 6 months in clinically inactive disease; however, even after 12 months of follow-up, the mean F1.2 levels were 1.5 times higher than the levels of the healthy control group.

No significant differences were found between IBD patients and patients with GCA, with respect to TAT and F1.2. However, levels of AT were significantly lower in active versus inactive GCA. Also, TAT levels were raised, in active more than inactive GCA, and this might point to relative more consumption of AT, i.e. binding of thrombin during active GCA, as compared to IBD patients.

Fibrinolysis markers in IBD and in GCA

We found no significant differences in the fibrinolysis activation markers D-dimer and PAP between patients with CD and UC; neither in clinically active nor in inactive disease (**Chapter 3**).

During 12 months of follow-up in CD and UC, the mean D-dimer levels were 1.9 and 2.0 times higher than the levels of the control group, and the mean levels of PAP were 1.7 and 1.6 times higher than the controls.

However, the course of the fibrinolysis inhibition marker PAI-1 was somewhat different between patients with CD and patients with UC or GCA (**Chapter 3, table 3.2**). In active CD, PAI-1 levels were equal but in inactive CD, PAI-1 levels were 1.7 times lower as compared to the control group after 12 months of follow-up.

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In active UC and active GCA, PAI-1 was 1.5 and 1.6 times higher as compared to patients with CD, and 1.8 and 1.6 times higher than the non-inflammatory controls; but after 12 months of follow-up, in both UC and GCA, PAI-1 levels were significantly lowered in inactive disease, as compared to the controls. To summarize, in inactive disease, CD, UC as well as GCA patients have subnormal PAI-1 levels, which might indicate more fibrinolysis, as also witnessed by D-dimer and PAP. However, in active disease, PAI-1 might behave like an acute phase reactant in patients with UC and GCA, as it positively correlates with both CRP and ESR (**Chapter 3**)

Can these coagulation and fibrinolysis markers be used to judge the clinical situation?

A positive significant correlation was found between the clinical activity scores CDAI as well as the UCAI, with F1.2, TAT, D-dimer, PAP and PAI-1. Also, a positive and significant correlation of the extent of bowel involvement (procto-sigmoiditis versus more extensive disease) in patients with UC was found with TAT, D-dimer, PAP, PAI-1 as well as CRP.

In CD, the small numbers of patients in the subgroups (colon, small bowel, or both), did not allow us to make definite statements in this respect. The persistently higher than normal levels of F1.2, D-dimer and PAP preclude their use as rapid diagnostics for measuring the influence of therapy, unlike e.g. an acute phase reactant like CRP³⁴⁻³⁷.

To conclude, clinical interpretation of these findings may be difficult because some of the markers reflect an expected acute phase response.

However, the persistently-raised markers might reflect ongoing or chronic inflammation, and could in theory be candidates for predicting the risk of thrombo-embolism in further studies.

FXIII in IBD and GCA

In **Chapter 4** we have shown that coagulation FXIII activity inversely correlated with clinical and biochemical indices of disease activity in UC and CD. However, this was not the case in GCA, which might suggest a specific significance of FXIII in active IBD.

A reduction in FXIII in active IBD due to increased thrombin generation in general seems improbable, because in GCA, similarly high levels of fibrinogen were not accompanied by a reduction of FXIII activity (**Chapter 4**). Like in CD or UC, high levels of coagulation factors (fibrinogen, FVIII and von Willebrand Factor) and fibrinolysis factors like D-dimer have been found in GCA³⁸. The low

plasma FXIII activity in active CD and in active and inactive UC could be the result of greatly enhanced turnover, reflecting increased intestinal consumption or leakage of plasma proteins.

However, we are not aware of studies performed to measure the intestinal clearance of FXIII. Subunit analysis could have helped to discriminate between a fall in FXIII-A as a result of consumption in the process of coagulation and a protein loss of both FXIII-A and FXIII-B in the gut. A comparative state of intestinal protein losing is found in IBD patients, as demonstrated by faecal alpha1-antitrypsin, as well as the loss of faecal calprotectin, a leukocyte protein^{39,40}. In active UC, loss of FXIII activity could also be related to persistent intestinal bleeding, leading to consumption and loss of coagulation factors like FXIII.

We found an inverse correlation of FXIII with the clinical disease activity scores CDAI and UCAI, as well as the ESR, CRP and fibrinogen level. Despite the inverse correlation between FXIII activity and indices of IBD activity, it is noteworthy that 10.0% (2 of 20) of the patients with CD and 16.7% of the (3 of 18) patients with UC had subnormal activity of FXIII even during inactive disease. Therefore, it is likely that serial rather than single measurements would be required if FXIII was used as an index of activity in CD or UC.

Platelet factors in IBD and GCA

Interest in the role of platelets in IBD was formerly confined to an awareness of thrombocytosis as a marker of disease activity. Besides their classical haemostatic task, platelets are now recognized as novel cells deeply involved in inflammation. In IBD, platelets contribute to microvascular endothelial activation and recruitment of inflammatory cells, thus fostering and amplifying mucosal inflammation.

In **Chapter 5**, platelet counts and the platelet derived factors beta-thromboglobulin (BTG) and platelet factor 4 (PF4) were studied in IBD and GCA patients, both in active and inactive disease. PF4 and BTG were markedly raised in CD and UC, compared with the controls. Our findings confirm the study reports on raised levels of BTG and PF4 in IBD patients^{41,42} although others did not find a correlation with disease activity⁴³ or a significant difference in quiescent disease⁴⁴. PF4 and BTG levels were also raised during a long time in GCA, pointing to a non-IBD specific mechanism. The increase of PF4 and BTG in the inactive phase of both IBD and GCA could reflect common underlying pathophysiological mechanisms, for example a vasculitis^{45,46}, and possibly contributes to disease chronicity. Endothelial cell damage might expose basement membrane collagen, to which platelets are very sensitive, and this could lead to platelet activation.

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The increase in plasma Von Willebrand factor (a marker of endothelial damage) found in IBD and GCA is consistent with this possibility⁴⁷. However, in patients, suffering from (pre-)eclampsia and documented disseminated intravascular thrombosis⁴⁸, PF4 and BTG levels were not elevated. The sustained rise of PF4 and BTG levels, as found in patients with IBD and GCA, preclude their use as short-term indices of activity. The question of whether or not a normalization of the values of PF4 and BTG in IBD or GCA reflects a complete remission of disease remains unanswered.

Influence of medication on coagulation and fibrinolysis in IBD and GCA

In theory, the medication used to control disease, could itself have promoted the disturbed pattern of haemostasis found in our patients with IBD and GCA (**Chapters 3-5**).

Corticosteroids can induce neutrophilia⁴⁹, and apart from fibrinolysis, high levels of D-dimer could result from neutrophilia in IBD and GCA, due to enhanced elastase release by these polymorph nuclear cells⁵⁰.

However, in our patients with CD and UC, the use of steroids did not result in a significant decrease of D-dimer, F1.2 and PAP during 12 months of follow-up, and no significant difference in D-dimer was found between patients with or without steroid use. Corticosteroids might have reduced PAI-1 levels as in Henoch-Schönlein purpura⁵¹, and in our study, levels of PAI-1 became low during follow-up. However, in IBD, steroids were generally withdrawn within 3-6 months, but mean PAI-1 levels did not significantly change between 3 and 12 months of follow-up.

To our knowledge, no direct effect of azathioprin on coagulation has been described. The use of azathioprin could have led to (opportunistic) infections, like e.g. a cytomegalovirus (CMV) reactivation, resulting in a disturbed coagulation⁵²⁻⁵⁴. However, no clinical signs of chronic (systemic) infections like CMV were encountered in our follow-up group.

With oral mesalazine, a lower spontaneous ex vivo and in vitro platelet activation has been described in patients with IBD⁵⁵. However, no secondary rise in coagulation and fibrinolysis factors has been documented under mesalazine therapy.

Influence of surgery on haemostasis in IBD

In a study in CD patients, terminal ileum resection was associated with higher plasma homocysteine levels⁵⁶, which in theory might add to the risk of future

thrombo-embolic complications. Another study, performed at the Mayo clinic by Solem and co-workers⁵⁷, describes the outcome of patients with confirmed IBD and deep venous thrombosis (DVT) or pulmonary embolism (PE). Of the 16 UC patients who underwent proctocolectomy following DVT/PE, there were two recurrences of thrombo-embolic events (13%), similar to the three recurrences (10%) seen among the 29 patients who had intact colons. The mortality rate from thrombo-embolism was 22% after a median follow-up of 1.8 yrs. In their study, thrombophilia evaluations had a relatively high diagnostic yield overall, although specific genetic abnormalities were individually uncommon, and other DVT/PE risk factors were usually present. They conclude that proctocolectomy is not protective against recurrent DVT/PE.

To test the hypothesis that in IBD patients, haemostasis is activated during clinically inactive disease, it would also be interesting to perform haemostasis studies in UC patients without colonic disease, i.e. patients who have undergone an ileal pouch anastomosis and are without signs of chronic pouchitis.

However, we are not aware of any other haemostasis studies done in postoperative UC patients, and during the study period described in **Chapters 3-5**, no patients underwent surgery.

Thrombin generation in mesalazine refractory UC

In **Chapter 8**, we have demonstrated that the thrombin potential, both intrinsic and extrinsic, is above the norm in patients with mild-to-moderately active UC. This confirms more indirect data on hypercoagulation in patients with active UC, as demonstrated in **Chapters 3-5**.

The thrombin potential has been shown to be increased in other thrombosis-prone states⁵⁸, but in IBD patients, the level of thrombin generation has not been reported before.

No correlation was found between the thrombin potential and clinical (CAI; IBDQ), endoscopy and histology scores of IBD. However, in the patient group treated with mesalazine only, a positive correlation was found between ETP ($r=0.531$, $p<0.0001$) and ITP ($r=0.664$, $p<0.0001$) with CRP, suggesting a pattern of acute phase reactivity in thrombin generation, i.e. without the influence of LMWH (reviparin) therapy. During 8 weeks of follow-up, F1.2 and TAT did not correlate with CAI and CRP in this patient group. This might favour the use of ITP or ETP, as indices of UC disease activity, over that of F1.2 and TAT. It confirms our findings on the course of F1.2 and TAT, reported in **Chapter 3**.

Microvascular thrombosis as a possible sign of disseminated intravascular coagulation

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Activated coagulation and the formation of microvascular thrombi is a part of a disease state known as disseminated intravascular coagulation (DIC), where fibrin is formed in the circulation as a result of the generation of thrombin triggered by soluble active tissue factor. In DIC, all three physiologic delimiters of coagulation — antithrombin (AT), protein C, and tissue factor–pathway inhibitor (TFPI) — are simultaneously impaired^{59,60}, and high plasma levels of PAI-1 suppress endogenous fibrinolysis. This results in intravascular formation of fibrin that is not balanced by adequate removal.

In clinical practice the disorder can be diagnosed on the basis of the following findings: an underlying disease known to be associated with DIC; an initial platelet count of less than 100,000 per cubic millimetre or a rapid decline in the platelet count; prolongation of clotting times, such as the prothrombin time (PTT) and the activated partial-thromboplastin time (APTT), due to a deficiency of all **coagulation** factors; the presence of fibrin-degradation products in plasma; and low plasma levels of coagulation inhibitors, such as AT. In patients with DIC, the blood smear may contain schistocytes, and histological analysis of organ-biopsy tissue may reveal the deposition of fibrin in small or mid-size vessels. Interestingly, in a murine model of sepsis induced DIC, LMWH treatment could reduce the number of microthrombi and attenuate multiple organ failure⁶¹.

In our studies in IBD patients (**Chapters 3-5**), the haemostasis findings were not compatible with generalized DIC: we neither found low platelet counts and prolongation of the clotting times (PTT/APTT), nor lowered levels of AT, during long time follow-up.

We are not aware of studies reporting the presence of blood schistocytes, as part of the fibrin-microthrombi complex, in patients with IBD.

Microvascular thrombosis as a pathogenetic mechanism, and the influence of LMWH intervention

The observation of a connection between thrombo-embolic events and IBD raises the question whether IBD is a risk factor for thrombo-embolic disease or whether derangements of a thrombo-embolic nature are part of the pathogenetic mechanism. Although these two aspects are often discussed together, they represent two different issues.

Macrovascular involvement, like venous thrombo-embolism and arterial thrombosis of medium to large sized vessels, are two conditions associated with acquired and - for venous - inherited risk factors that have been thoroughly

investigated. The reasons for an increased prevalence of these events in IBD are unclear but a number of studies have addressed these issues, as reported in **Chapter 1**. It is uncertain whether these thrombo-embolic events contribute to disease progression or prognosis in IBD patients. Deep venous thrombosis without such risk factors was in fact demonstrated in four of our patients as reported in **Chapter 6**, i.e. before the start of nadroparin treatment, and observed in 1 patient after the withdrawal of nadroparin therapy.

Microvascular (mucosal) thrombosis on the other hand recognizes different pathogenesis mechanisms, still unclear at present.

By similarity with other situations in which it occurs, such as for example in sepsis⁶², one can propose that it derives from a local imbalance of pro-coagulant versus anti-coagulant mechanisms triggered by inflammation. In IBD, whatever is the temporal sequence of these events (clotting or inflammation), it is more or less agreed that microvascular thrombosis contributes to mucosal ischemia and sloughing, thereby affecting disease progression.

On the basis of the assumption that microthrombosis is a factor in the etiology of IBD, it is a relevant question whether heparin administration might be beneficial through its anticoagulant action.

A positive outcome would not settle the question of pathogenesis, however, because in-vitro investigations have discovered that heparin not only acts as an anticoagulant, but also has a number of anti-inflammatory effects^{63,64}. Among these, heparin causes an enhancement of the endothelial barrier function by substituting glycosaminoglycans, and by inhibiting heparinase; an inhibition of adhesion molecules P- and L-selectins; a reduction of stable leukocyte attachment by reducing endothelial intercellular adhesion molecule-1 (ICAM-1); the prevention of neutrophils diapedesis by inhibiting neutrophil elastase; a reduction of pro-inflammatory cytokines; and a reduction in production of reactive oxygen metabolites^{17,65,66}.

In the study described in **Chapter 6**, in 20 of 21 patients with UC, it was possible to stain the biopsies for occlusive microvascular thrombi (**Chapter 6, figure 6.4**), and in all of these patients they could be detected in active disease, before LMWH (nadroparin) treatment. After 8 weeks of treatment, apart from the occlusive thrombi that were still present, we regularly saw a smaller (circular) layer of fibrin against the vessel wall. In 14 patients the number reduced, in 6 the number increased, and only in 1 patient we could not detect microthrombi after 8 weeks of LMWH treatment.

Although the mean (95% CI) number of fibrin-thrombi per 3 mm biopsy was reduced from 25.4 (12.4–38.4) to 14.1 (10.6–17.5), this difference was not significant ($p=0.338$). The presence of microvascular thrombi did not correlate with the platelet counts/mean platelet volume, clinical, endoscopy, or histology scores in our study.

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The detection of microvascular thrombi in all of our patients with active steroid-refractory UC might support the hypothesis of a primary state of hypercoagulability as a pathophysiological mechanism in UC, although this phenomenon has also been observed in CD and in other causes of bowel inflammation, such as in gut related sepsis⁶⁷.

More important, however, the appearance of occlusive fibrinoid lesions was present during clinically inactive disease and it was not related to the degree of mucosal inflammation, as already found by Dhillon and co-workers with different techniques³. Nadroparin treatment did not significantly diminish the number of microscopic, fibrin containing thrombi, although a trend in reduction was observed. Nadroparin inhibits coagulation factor X and AT activity, and other factors may have by-passed its anti-coagulant properties: ongoing activation of platelets and generation of platelet-derived micro particles, as well as gut microvascular PLA formation^{68,69}; a more direct activation of thrombin by enhanced TF expression in the gut microvasculature⁷⁰, and a disturbed fibrinolysis in UC⁷¹, all potentially important factors in the persistence of these microthrombi.

Our findings indicate that in steroid resistant patients, additional therapy with nadroparin improves clinical symptoms, as well as it decreases macroscopic and microscopic inflammation, i.e. in an open study, and during 8 weeks of follow-up. This was also shown by Torkvist and co-workers with the LMWH dalteparin, given for 12 weeks⁷². However, the histology results in our patient group underline the presence of a chronic hypercoagulative disease state in UC.

As shown in the case described in **Chapter 2**, long-term LMWH therapy was well tolerated. This was also found during LMWH therapy and follow-up of paediatric UC patients with cerebral sinovenous thrombosis, even in combination with warfarin and salicylic acid, as described by Kao and co-workers⁷³.

LMWH therapy in mesalazine refractory UC

In **Chapter 7**, we reported on a double blind, randomized controlled trial on the clinical and biological effects of the LMWH reviparin in moderately active UC. Patients were monitored for adverse events, change of clinical symptoms, and histological outcome as study endpoints. In addition, thrombin generation was measured. No significant differences in the clinical outcome were observed between reviparin treated and placebo patients.

As shown in **Chapter 8**, thrombin generation was significantly reduced in patients with LMWH, however, no influence was observed with respect to the clinical course of the disease. Although a reduction in clinical disease activity

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was observed, the response of patients with mesalazine alone was high, confirming the fact that more severely diseased UC patients were excluded in this study. Disease activity was accompanied by relatively low CRP levels, as well as the absence of extensive (pan-colitic) endoscopic disease, and these factors may have contributed to a high placebo response, as found in other interventional studies in IBD as well⁷⁴. Also, the fact that the study is underpowered is difficult to overcome in the evaluation.

The histology score on inflammation tended to improve with the use of LMWH, and it might have reached statistical significance if the number of enrolled subjects was adequate. Regrettably, we did not have the opportunity to co-investigate the appearance of micro-vascular thrombi in this study.

The question arising from the case report in **Chapter 2**, whether a higher number of microthrombi represents more severe disease, remains unanswered.

Unfractionated heparin and LMWH therapy in UC

A reduction of the thrombin potential by the LMWH reviparin did not change the clinical outcome during 8 weeks of follow-up in patients with mesalazine alone, as shown in the placebo-controlled study described in **Chapter 7**. The addition of LMWH to steroids, however, did change the clinical outcome in UC patients in our open study, described in **Chapter 6**. Heparin is a heterogeneous polysaccharide, with separate activities like predominantly anticoagulant properties of the smaller pentasaccharides, as well as more anti-inflammatory properties linked to the larger tetradecasaccharides⁷⁵.

The difference in clinical outcome between the studies described in **Chapters 6 and 7** could thus be medication-specific, i.e. reviparin (mean molecular weight 3900 Dalton)⁷⁶ might not have additional anti-inflammatory effects like nadroparin (4500 Dalton)⁷⁷. However, to our knowledge, this has never been studied head to head. Also, a different dosage of LMWH might have different anti-inflammatory effects; a hypothesis which has yet to be investigated in the treatment of IBD patients.

A third possibility is a lack of efficacy of LMWH monotherapy, as compared to unfractionated heparin monotherapy in the treatment of UC⁷⁸. However, Panes and co-workers demonstrated that unfractionated heparin administered for 10 days was ineffective as monotherapy in the treatment of moderate or severe UC, and was associated with significant bleeding complications⁷⁹. Recent data suggest that the predominant anti-inflammatory action of heparins is mediated by blocking P- and L-selectin dependent cell adhesion and that sulphation of the 6-O position of glucosamine is critical for this effect⁸⁰. In-vitro experiments have shown that, whereas unfractionated heparin completely inhibits P- and

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L-selectin binding at low concentration, LMWH (enoxaparin and dalteparin) achieved this only at concentrations above those recommended for anticoagulation⁸¹. Moreover, chemical production of LMWH may critically alter end groups, sulphation and charge density, which are important in the anti-inflammatory activity of unfractionated heparins.

Finally, it is possible that other proposed mechanisms of the anti-inflammatory actions of unfractionated heparin, such as alterations in cell adhesion⁸², and release of chemokines from intestinal epithelial cells⁸³, may be lost with fractionation. Still, unfractionated heparin poses more bleeding risks than LMWH therapy in UC patients, who may need prolonged heparin therapy to achieve clinical effects⁸⁴.

Unfractionated heparin and LMWH therapy in CD

Folwaczny and co-workers found no clinical and laboratory effect of unfractionated heparin in 4 patients with active CD, while seven patients with UC achieved complete remission after an average of 4 weeks of heparin treatment⁸⁴.

To date, no (blinded) placebo-controlled trials have been published concerning the use of LMWH in CD, although in isolated cases, clinical remission has been documented⁸⁵.

Heparin-steroid interaction: synergistic effect, steroid sensitizer, or other relationships

The observation that LMWH to date has only shown significant clinical effects in conjunction with immunosuppressive treatment like corticosteroids, in patients with UC (**Chapter 6**)⁷², might be due to a synergistic effect of heparin on steroid therapy itself.

Although glucocorticosteroids exert a potent anti-inflammatory effect, they may induce a hypofibrinolytic state, more directly⁸⁶, or indirectly via hyperlipidemia⁸⁷. It is possible that anticoagulation eliminates the adverse effect of corticosteroids on the intravascular coagulation balance. However, as shown in **Chapters 3-5**, we did not find a state of hypofibrinolysis in the steroid treated UC patients.

Another synergistic effect of heparin is the possible reduction of neutrophil recruitment to the inflamed epithelium, while steroids are still essential to inhibit tissue neutrophil function^{88,89}. LMWH could additionally bind to the important cytokine IL-2, as shown in in-vitro studies⁹⁰; IL-2 is a lymphocyte activating

cofactor, and an up-regulated cytokine in IBA patients with steroid refractory disease⁹¹, as well as in UC⁹². Also, in a Chinese study, the oral LMWH sulodexide was capable of reducing the tissue expression of CD64 (ICAM-1), a membrane glycoprotein up-regulated in steroid refractory UC^{93,94}. As a consequence, this might result in a reduced tissue infiltration of lymphocytes and monocytes.

In-vitro and in-vivo animal studies have shown that low-dose heparin suppresses the expression of T-lymphocyte heparinase activity and concurrently inhibits T-cell migration⁹⁴. The latter phenomenon is normally part of the effect of steroid therapy, which could be reduced in steroid refractory UC⁹⁵.

Interestingly, in a study on patients with interstitial pulmonary fibrosis (IPF), the addition of LMWH to steroids was associated with a reduction in mortality⁹⁶; Hiroshi and co-workers found that patients with IPF had less tissue fibrosis, possibly due to reduced fibrin deposition in the sub-endothelial space.

As the outcome was less pronounced in patients on warfarin and corticosteroids, the effect seems linked to the additional use of LMWH. It has been shown that heparin-steroid conjugates can inhibit the proliferation of vascular endothelial cells, which potentially reduces fibrosis, at least in vitro⁹⁷.

Parallel to the pulmonary changes in IPF patients, an increased production of vascular endothelial growth factor in intestinal mucosa has been found in patients with IBD, which might enhance fibrosis⁹⁸.

Finally, to test the hypothesis of a steroid-sensitizing effect of LMWH, additional studies measuring in-vitro steroid sensitivity of proliferating peripheral blood lymphocytes, before and after an additional LMWH dosage in IBD patients treated with prednisolone, could be performed⁹⁹.

Suggestions to minimize the risk of thrombo-embolism in patients with IBD

As has been shown in the previous chapters, thrombo-embolic complications are frequently encountered in IBD patients, on micro- and macrovascular level. The use of LMWH, perhaps in a therapeutic dose, and/or support stockings should be considered for hospitalized patients. Early mobilization and avoidance of parenteral nutrition, except when the enteral route cannot be used, is desirable. Especially patients with Crohn's disease should be informed that smoking and oral contraceptives are risk factors for this complication¹¹. Patients with small bowel disease in particular should be screened for folate deficiency as a cause for hyperhomocysteinemia^{7,56,100}. Optimal treatment of mucosal inflammation may be the most reliable method of reducing the hypercoagulable state⁷.

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Anti-TNF therapy with infliximab has been reported to not only induce clinical remission and mucosal healing but also has a direct inhibitory effect on the hypercoagulable state^{101,102}. Until more data are available, a search for inherited thrombophilias in patients with IBD and thromboses is recommended only for patients with a personal or family history of thrombo-embolism.

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Chapter 10

Summary and conclusions

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This thesis describes the course of haemostasis factors in patients with Crohn's disease (CD) and ulcerative colitis (UC), both during active and inactive disease, as well as the influence of low molecular weight heparin (LMWH) on clinical, biochemical, endoscopic and histological disease activity, in patients with UC.

In the introduction in **Chapter 1**, the potential role of haemostasis in patients with inflammatory bowel disease (IBD) is described, as well as the aims and outline of our studies. Patients with CD or UC appear to have a 3-4-fold risk of developing thrombo-embolism^{1,2}. The actual frequency of thrombo-embolism is uncertain with different methodological approaches reporting in the range of 2-10%^{1,3-7}. Estimates from autopsy reports are much higher⁵, suggesting that many episodes of this serious complication go undiagnosed. It is noteworthy that thrombo-embolism in IBD occurs in a younger age group than in those without IBD^{8,9}. Inherited defects of coagulation appear to protect against the risk of developing IBD^{10,11}. However, in most studies, the thrombotic events in IBD were not associated with inherited causes of thrombophilia, like factor V Leiden or prothrombin G20210A gene mutation¹¹. Thus far, no single laboratory marker has emerged with sufficient predictive value to identify patients at particular risk for thrombo-embolic complications. Most, but not all studies, have suggested that the activity of the inflammatory disease is related to the thrombo-embolic risk. It is becoming increasingly apparent that thrombosis and inflammation are intrinsically linked. Hence the involvement of pro-thrombotic processes in the pathogenesis of IBD, although perhaps not as the primary event, seems likely, and motivated our studies.

In **Chapter 2**, a case is described of a patient with active corticosteroid resistant colitis and pancreatitis; she had histological signs of gut microvascular thrombosis, and LMWH was successfully added to corticosteroid therapy.

In **Chapters 3, 4 and 5**, the course of haemostasis factors in IBD and - as controls with a proven vasculitis - in patients with giant cell arteritis (GCA), is described. Plasma concentrations of several coagulation- (FXIII, fibrinogen, F1.2, AT and TAT), fibrinolysis- (D-dimer, PAP and PAI-1) and platelet factors (PF4 and BTG) were studied.

Not only in the active, but also in the inactive phase of IBD and GCA, we found a significantly higher level of coagulation and fibrinolysis factors, as compared to the age- and sex-matched controls without signs of vasculitis or inflammation.

The course of the haemostasis parameters investigated was quite similar during 12 months of follow-up in IBD and GCA patients, with a few exceptions described in detail in the general discussion in chapter 9. It suggests that common pathways, e.g. microvascular thrombosis and/or small vessel vasculitis, may play a role in both IBD and GCA. To conclude, the haemostasis factors studied could reflect disease activity, and possibly inadequate

therapeutic control of the inflammatory process in the gut, as well as a systemic tendency towards thrombo-embolic complications.

In **Chapter 6**, an open study on the influence of LMWH (nadroparin) on steroid refractory UC is presented. A therapeutic dose of nadroparin, in addition to steroids, proved to be safe and induced clinical remission in 80% of the patients, with early cessation of rectal bleeding. Although after 8 weeks of nadroparin therapy, a significant reduction in histological inflammation was achieved, the number of microthrombi was not significantly reduced.

The microthrombi might represent a protective (i.e. less mucosal bleeding) phenomenon, or represent ongoing enhanced coagulation and disturbed fibrinolysis, possibly adding to the chronicity of UC. This, as well as the potential anti-inflammatory effects of LMWH, is further discussed in chapter 9.

In **Chapter 7**, a double-blind placebo controlled trial on the use of another LMWH, reviparin, is described in patients with mild-to-moderately active mesalazine refractory UC. Regrettably, a 60% placebo response was accompanied by a non-significant difference in clinical, biochemical, endoscopical, and histological improvement in both groups. However, a therapeutic dose of reviparin proved safe without bleeding complications, and was well tolerated in UC patients.

In **Chapter 8**, the influence of reviparin on thrombin generation in patients with mild-to-moderately active UC is described. Both in the reviparin and placebo group, patients started with higher levels of extrinsic (ETP) and intrinsic (ITP) thrombin potential. A therapeutic dose of reviparin reduced the ETP and ITP levels; however, after 8 weeks, a rise in the thrombin generation to baseline levels was noted. This phenomenon of "therapeutic resistance" needs further investigation.

In **Chapter 9**, our study results are discussed. That IBD promotes thrombosis is a more widely accepted principle and, with the associated morbidity and mortality of thrombosis complications, is important to remember when treating patients with IBD. Although anti-thrombotic compounds have not shown to be therapeutically effective in IBD to date, manipulation of the pathways involved might yet yield novel agents to add to the range of medications currently available. Future therapeutic intervention studies with drugs, which could influence both inflammation and microvascular thrombosis, should therefore be welcomed.

IBD patients with active disease are at high risk of thrombo-embolic events and, accordingly, intervention with subcutaneous LMWH to prevent thrombo-embolic complications is recommended while they are in hospital¹². The anticoagulation effect of the dosage of LMWH in these patients could be monitored by anti-Xa/IIa activity and/or the measurement of the thrombin potential.

In conclusion, gut microvascular thrombosis, as well as thrombo-embolism as an extraintestinal manifestation, are common phenomena in IBD, and associated to a state of plasmatic hypercoagulation. Low molecular weight heparin therapy can be given to patients as prophylaxis against thrombosis, but not as an established therapy against IBD.

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Chapter 11

Nederlandse samenvatting en conclusies

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Dit proefschrift beschrijft het beloop van hemostase factoren bij patiënten met de ziekte van Crohn (CD) en colitis ulcerosa (UC), zowel tijdens actieve als rustige ziekte, evenals de invloed van laag molecuul gewicht heparine (LMWH) op de klinische, biochemische, endoscopische en histologische ziekte activiteit, bij patiënten met UC.

In de inleiding in **Hoofdstuk 1** wordt de potentiële rol van hemostase bij patiënten met inflammatoir darmlijden (IBD) beschreven, evenals de doelen en omschrijving van onze studies. Patiënten met CD of UC hebben een 3 tot 4-voudig verhoogd risico op het ontwikkelen van trombo-embolische complicaties t.o.v. leeftijdgenoten^{1,2}. De feitelijke incidentie van trombo-embolie is onzeker door verschillende methodologische benaderingen waarin een spreiding van 2 tot 10% wordt gerapporteerd^{1,3-7}.

Schattingen van autopsie rapporten zijn veel hoger⁵, hetgeen suggereert dat vele episoden van deze serieuze complicatie niet worden gediagnosticeerd.

Het is opmerkelijk dat trombo-embolieën bij patiënten met IBD op een jongere leeftijd optreden dan bij patiënten zonder IBD^{8,9}. Erfelijke stollingsdefecten lijken te beschermen tegen de ontwikkeling van IBD^{9,10}. Echter, in de meeste studies was het optreden van trombose bij IBD niet geassocieerd met erfelijke oorzaken van trombofilie, zoals factor V Leiden of prothrombine G20210A gen mutatie¹¹.

Tot dusverre heeft een enkelvoudige laboratorium bepaling nog onvoldoende voorspellende waarde om patiënten met een bepaald risico voor trombo-embolische complicaties te identificeren. In de meeste, maar niet in alle studies, werd gesuggerereerd dat de activiteit van de inflammatoire ziekte gerelateerd was aan het trombo-embolische risico. Het is in toenemende mate duidelijk dat trombose en ontsteking intrinsiek met elkaar verbonden zijn.

In **Hoofdstuk 2** wordt een casus beschreven van een patiënte met een actieve, voor corticosteroïden resistente colitis, evenals een pancreatitis; zij had histologisch tekenen van microvasculaire trombose in de darm, en aan de corticosteroid behandeling werd met succes LMWH toegevoegd.

In de **Hoofdstukken 3, 4 en 5** wordt het beloop van hemostase factoren bij patiënten met IBD, en - als controle groep met een bewezen vasculitis - bij patiënten met een reuscel arteritis (GCA) beschreven. Plasma concentraties van verscheidene stollings- (FXIII, fibrinogeen, F1.2, AT en TAT), fibrinolyse- (D-dimeer, PAP, PAI-1) en bloedplaatjes-factoren (PF4 en BTG) werden onderzocht.

Niet alleen in de actieve, maar ook in de inactieve fase van IBD en GCA vonden wij een significant hogere concentratie van stollings- en fibrinolyse factoren, in vergelijking met de naar leeftijd- en geslacht- overeenkomende controle populatie zonder tekenen van vasculitis of ontsteking.

Het beloop van de onderzochte hemostase parameters kwam sterk overeen bij patiënten met IBD en GCA gedurende een controle periode van 12 maanden,

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met enkele uitzonderingen welke in detail zijn beschreven in de algemene discussie in hoofdstuk 2. Het suggereert dat gemeenschappelijke mechanismen zoals microvasculaire trombose en/of vasculitis van kleinere vaten, een rol spelen bij zowel IBD als GCA. Concluderend zouden de bestudeerde hemostase factoren een weerspiegeling kunnen zijn van ziekte activiteit, en daarmee mogelijk een inadequate therapeutische controle van het inflammatoire proces in de darm, evenals een meer systemische neiging tot trombo-embolische complicaties.

In **Hoofdstuk 6** wordt een open studie naar de invloed van LMWH (nadroparine) op steroïde refractaire UC beschreven. Een therapeutische dosering van nadroparine, toegevoegd aan corticosteroïden, bleek veilig en induceerde een klinische remissie bij 80% van de patiënten, met vroegtijdig stoppen van rectaal bloedverlies.

Hoewel er 8 weken na nadroparine therapie een significante daling optrad in histologische ontstekingsactiviteit, was het aantal microtrombi niet significant afgenomen. De microtrombi vertegenwoordigen mogelijk een beschermend fenomeen (d.w.z. minder mucosale bloeding), of juist een aanhoudende stolling en gestoorde fibrinolyse, welke mogelijk bijdraagt aan de chroniciteit van UC. Deze overwegingen, evenals de potentiële anti-inflammatoire effecten van LMWH, worden verder bediscussieerd in hoofdstuk 9.

In **Hoofdstuk 7** wordt een dubbel-blinde, placebo gecontroleerde studie beschreven met een ander LMWH, reviparine, bij patiënten met milde tot matig actieve, voor mesalazine refractaire UC. Helaas bleek sprake van een 60% placebo respons, en een niet-significant verschil in klinische, biochemische, endoscopische, en histologische verbetering in beide groepen. Een therapeutische dosering van reviparine bleek echter veilig en zonder bloedingcomplicaties, en werd goed getolereerd door UC patiënten.

In **Hoofdstuk 8** wordt de invloed van reviparine op het genereren van thrombine bij patiënten met milde tot matig actieve UC beschreven. Zowel in de reviparine als in de placebo groep startten patiënten met een hogere waarde van de extrinsieke (ETP) en intrinsieke (ITP) thrombine potentiaal.

Een therapeutische dosering van reviparine reduceerde de ETP en ITP waarden; echter, na 8 weken behandeling werd een stijging in het genereren van thrombine tot het uitgangsniveau waargenomen. Dit fenomeen van "therapie resistentie" verlangt verder onderzoek.

In **Hoofdstuk 9** worden onze studie resultaten bediscussieerd.

Het principe dat er bij IBD een versterkte neiging tot trombose bestaat wordt in het algemeen vaker geaccepteerd, en, door de morbiditeit en mortaliteit geassocieerd aan trombotische complicaties, is het belangrijk om te memoreren wanneer patiënten met IBD worden behandeld. Hoewel anti-trombotische middelen tot op heden niet therapeutisch effectief waren bij IBD, zou beïnvloeding van de betrokken paden van stolling- en ontsteking, nieuwe

middelen kunnen opleveren en toevoegen aan het huidige therapeutische arsenaal. Toekomstige interventie studies met medicamenten, welke zowel ontsteking als microvasculaire trombose zouden kunnen beïnvloeden, worden verwelkomd.

IBD patiënten met actieve ziekte hebben een sterk verhoogd risico op trombo-embolie en het is aan te bevelen om, gedurende de opname in een ziekenhuis, een LMWH te verstrekken ter preventie van trombo-embolische complicaties¹². Het antistollingseffect van de LMWH dosis bij deze patiënten zou gecontroleerd kunnen worden door meting van anti-Xa/IIa activiteit en/of de meting van de thrombine potentiaal.

Concluderend zijn microvasculaire trombose in de darm, evenals trombo-embolie als extra-intestinale manifestatie, frequent voorkomende fenomenen bij IBD, en geassocieerd met een verhoogde staat van bloedplasma stolling. Laag molecuul gewicht heparine kan als trombose profylaxe worden verstrekt aan deze patiënten, maar niet als een gevestigde therapie tegen IBD.

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Chapter 12

Abbreviations

Abbreviations

5ASA	5-aminosalicylic acid
ALT	alanine aminotransferase
AP	alkaline phosphatase
APC+S	activated proteins C+S
APTT	activated partial thromboplastin time
ARA	American Rheumatism Association
AST	aspartate aminotransferase
AT	antithrombin
BTG	β -thromboglobulin
CAI	Colitis Activity Index
CD	Crohn's disease
CD40L	CD40 ligand
CDAI	Crohn's Disease Activity Index
CI	confidence intervals
CMV	cytomegalovirus
CRP	C-reactive protein
CSG	Clinical Symptoms Grading
CTAD	citric acid, theophylline, adenosine and dipyridamol
DEXA	Dual Energy X-ray Absorption
DIC	disseminated intravascular coagulation
DVT	deep vein thrombosis
EGS	Endoscopic Grading System
ELISA	enzyme-linked immunosorbent assay
EPCR	endothelial protein C receptor
ESR	erythrocyte sedimentation rate
ETP	extrinsic thrombin potential
FXIII	factor XIII
GCA	giant cell arteritis
GGT	gamma-glutamyl transpeptidase
Hb	haemoglobin
HDA	histologic disease activity
HIT	heparin induced thrombocytopenia
Ht	hematocrit
IBD	inflammatory bowel disease
IBDQ	Inflammatory Bowel Disease Questionnaire
ICAM-1	intercellular adhesion molecule-1
IL-6	interleukin-6
IPF	interstitial pulmonary fibrosis
ITP	intrinsic thrombin potential
LDH	lactate dehydrogenase

LMWH	low molecular weight heparin
MPV	mean platelet volume
NSAID	non-steroidal anti-inflammatory drugs
OCP	oral contraceptive pill
PAF	platelet activating factor
PAI-1	plasminogen activator inhibitor-1
PAP	plasmin-alpha2-antiplasmin
PDW	platelet distribution width
PE	pulmonary embolism
PF4	platelet factor 4
PLA	platelet-leukocyte aggregate
PPP	platelet-poor plasma
PROBE	prospective randomised open blinded end-point
PRP	platelet-rich plasma
PTT	prothrombin time
RA	rheumatoid arthritis
Rs ²	Regression squared coefficient
TAFI	thrombin-activated fibrinolysis inhibitor
TAT	thrombin-antithrombin
TF	tissue factor
TFPI	tissue factor pathway inhibitor
TNF α	tumor necrosis factor- α
UC	ulcerative colitis
UCAI	ulcerative colitis activity index
UFH	unfractionated heparin
WBC	white blood cell

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Tele visie

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Elfriede Henriette Resida

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Chapter 13

Dankwoord

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Het vervaardigen van een proefschrift is mij geen sinecure gebleken, maar dat dit uiteindelijk ruim 10 jaar tijd heeft gekost, mag helaas toch wel opmerkelijk worden genoemd. Het is geen bewuste poging geweest om het “Guinness book of records” te halen, hoewel dit met deze tijdsspanne en de huidige halfwaardetijd van medische onderzoeksresultaten, toch een close finish moet zijn geweest.

Met name ook de periode dat ik Maastricht verruild heb voor het perifere werk als internist- MDL arts in Almelo trok een wissel op het tijdschema. Bovenal hebben zich echter de afgelopen 8 jaar vele “life events” in ons gezin voltrokken, die mede debet zijn geweest aan deze vertraging.

De promotoren en co-promotor moet ik complimenteren met hun enorme geduld, en ik waardeer het vertrouwen, dat zij in de uiteindelijke afloop hadden, des te meer. Bovenal waardeer ik het dat zij op inmiddels pensioengerechtigde leeftijd nog aan deze hersenkraker wilden meedoen.

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Het in dit proefschrift beschreven onderzoek werd verricht op 2 locaties.

Het begon in Heerlen, in het De Wever Ziekenhuis, later onderdeel van het Atrium Medisch Centrum, op de afdeling Interne Geneeskunde en op het Hematologisch Laboratorium. Aldaar zijn de gegevens verzameld van de nieuw gediagnosticeerde patiënten in een actieve en rustige ziektefase van de ziekte van Crohn en colitis ulcerosa, gedurende een langere periode.

Later is in het Academisch Ziekenhuis Maastricht, op de afdeling Maag- Darm- en Leverziekten, de invloed van laag molecuul gewicht heparine behandeling onderzocht bij patiënten met colitis ulcerosa.

Op beide locaties was de welwillende medewerking van de patiënten van cruciaal belang.

Een ieder die een bijdrage geleverd heeft aan de totstandkoming van dit proefschrift, op welke wijze dan ook, wil ik bij deze dan ook hartelijk danken.

Dit geldt in het bijzonder voor de volgende personen. **Prof. dr. R.W. Stockbrügger**, mijn promotor, globetrotter, heeft op kritische en tegelijkertijd geduldige wijze de totstandkoming van het proefschrift begeleid.

Alle manuscripten werden minutieus van opbouwende kritiek voorzien. Beste Reinhold, jouw interesse en kennis op het gebied van Gastroenterologie en verwante kennisgebieden is enorm. Maar ook de filosofische ondertoon, waarbij ik denk aan je opmerking tijdens de DDW in Orlando “kan dit proefschrift nog tijdens dit leven worden afgerond?” werkte stimulerend op mijn geloof ... in een goede afloop. Vooral tijdens de laatste 'schrijffase', wanneer ik dacht 'dat de inhoud van het artikel goed was uitgewerkt', waren daar altijd weer de waardevolle opmerkingen die het artikel compleet

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Petra Hawinkels was destijds als secretaresse in Heerlen een enthousiaste spil bij de verwerking van de Heerlense gegevens in een database. De dames van de polikliniek Interne Geneeskunde hebben door hun secretariële ondersteuning en instructie van de patiënten een belangrijke bijdrage aan het onderzoek in Heerlen geleverd. De coördinatie van het laboratoriumonderzoek in Heerlen was in goede handen bij de heren J. Schröder en W. Hanssen. De medewerkers van het **Haematologisch Laboratorium** hebben vele bepalingen voor mijn onderzoek verricht, waarvan het leeuwendeel op consciëntieuze wijze is afgenomen door **John Smeets**.

In het azM vond de verwerking van de data uit Heerlen plaats, en ving “deel 2” aan van het proefschrift. Van groot belang was het feit dat er reeds een studie- en behandel protocol lag dat oorspronkelijk was opgesteld door Dr. E. Schoon, Prof. dr. R.W. Stockbrugger en Prof. dr. H. C. Hemker.

En met dit protocol ben ik aanvankelijk samen met collega Schoon, en later met collega A. Oberndorff-Klein-Woolthuis, aan de slag gegaan. Erik, mede dankzij jouw protocol is er een meer praktische, therapeutische stap aan de materie toegevoegd.

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In latere instantie is ook het Academisch Ziekenhuis Groningen, thans UMCG, betrokken geraakt, in de personen Dr. G. Dijkstra en mevr. A.E. de Jong. Beste Gerard, bedankt voor je opbouwende kritiek en bijdrage aan de studie!

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Mijn **vakgroepgenoten in Almelo**, en met name mijn 'post' maatje Dr. L. van Bergeijk, dank ik voor het gestelde vertrouwen, en de niet aflatende blijken van interesse in de vorderingen van mijn proefschrift. Beste Leo, het idee om met een totaal gepromoveerde vakgroep uit eten te gaan, komt nu toch niet meer over als een utopie.

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Tot slot, lieve Henriette, wil ik jou danken voor je steun, je inzicht en het begrip, dat het werk zich niet afspeelde binnen kantooruren. Laten we wederom gaan genieten van ons gezin!

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Chapter 14

Publications, oral presentations and
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Articles

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7. Stolling en fibrinolyse bij polymyalgia rheumatica en arteriitis temporalis. Vrij A.A., Mazee H.A., Lustermans F.A.Th., Wersch van J.W.J. In: Trombose, Atherosclerose en Ontsteking: diagnostiek, preventie en behandeling anno 1994: 139-142. Onder redactie van: Cate ten J.W., Bas B.M., Briët E., Deventer van S.J.H., Kastelein J.J.P., Pannekoek H. De Bink BV juli 1995
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10. Flow cytometry patterns specifying celiac disease in lymphocytic enteritis (Marsh I) enteropathy: a pilot study. A.A. Vrij, A. Martens. Gut 2005; 54 (Suppl VII) A132

Oral presentations

20-03-1987: Voorjaarsvergadering Nederlandse Vereniging voor Gastro-enterologie te Koningshof, Veldhoven: A.A. Vrij et al. Endoscopic pancreatic drainage in chronic pancreatitis.

06-10-1995: Najaarsvergadering Nederlandse Vereniging voor Gastro-enterologie te Koningshof, Veldhoven: A.A. Vrij et al. Coagulation factor XIII in ulcerative colitis and Crohn's disease

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19-09-1996: 51. Tagung der Deutschen Gesellschaft für Verdauungs- und Stoffwechselkrankheiten Aachen: A.A. Vrij et al. Posterbegehung und diskussion: factor XIII in inflammatory bowel disease and giant cell arteritis

07-03-1997: Belgian IBD Research Group: "The immunological basis of IBD" Universitair Hospital Gent: A.A. Vrij et al. Reduced serum coagulation factor XIII: is it a characteristic phenomenon of active inflammatory bowel disease?

18-03-1999: Voorjaarsvergadering Nederlandse Vereniging voor Gastro-enterologie te Koningshof, Veldhoven: A.A. Vrij et al. Low Molecular Weight Heparin treatment in steroid refractory ulcerative colitis

16-03-2000: Voorjaarsvergadering Nederlandse Vereniging voor Gastro-enterologie te Koningshof, Veldhoven: A.A. Vrij et al. Mucosal capillary thrombi in steroid refractory ulcerative colitis: influence of low molecular weight heparin (LMWH) treatment

21-03-2001: Voorjaarsvergadering Nederlandse Vereniging voor Gastro-enterologie te Koningshof, Veldhoven: A.A. Vrij et al. Longterm follow-up of coagulation and fibrinolysis factors in ulcerative colitis and Crohn's disease

07-02-2004: South Carolina USA Clinic "Frontiers in Endoscopy" (Prof. Cotton) A.A. Vrij. Benign broncho-gastric fistula following transhiatal esophagectomy for cancer: treatment with a covered "gastric" stent

07-10-2005: Najaarsvergadering Nederlandse Vereniging voor Gastro-enterologie te Koningshof, Veldhoven: A.A. Vrij et al. Flowcytometry patterns specifying celiac disease in lymphocytic enteritis (Marsh I enteropathy): a pilot study

Curriculum Vitae

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De auteur van dit proefschrift werd geboren op 5 september 1964 in Amsterdam. In 1982 behaalde hij zijn VWO diploma aan het Ichthus College in Enschede. In 1982 begon hij met de studie geneeskunde aan de Universiteit van Amsterdam. In 1986 haalde hij het doctoraal examen, en in 1989 werd het artsexamen afgelegd. Na een korte periode als agnoscisch werkzaam te zijn geweest op de afdeling interne geneeskunde in het Bergwegziekenhuis in Rotterdam, respectievelijk 1 jaar agnoscisch in het De Wever Ziekenhuis te Heerlen, startte hij in april 1991 in Heerlen met de specialisatie interne geneeskunde (opleider: Dr. F.A.Th.W.B.H. Lustermans). In 1995 werd deze opleiding in het academisch ziekenhuis te Maastricht vervolgd (opleiders: Prof.dr. J.A. Flendrig, Prof.dr. H.F.P. Hillen, Prof. dr. R.W. Stockbrügger). De registratie tot internist volgde op 1 april 1997, waarna direct aangevangen werd met de opleiding tot gastroenteroloog in het academisch ziekenhuis te Maastricht (opleider: Prof.dr. R.W. Stockbrügger) en registratie volgde op 1 april 2000. Tijdens de opleidingen interne en gastroenterologie werd gestart met de voorbereidingen van het wetenschappelijk onderzoek beschreven in dit proefschrift. In april 2000 werd hij aangesteld als stafid bij de werkgroep maag/darm- en leverziekten van het academisch ziekenhuis te Maastricht (hoofd: Prof.dr. R.W. Stockbrügger). Vanaf januari 2001 werd de loopbaan vervolgd als maag/darm- en leverarts en internist in het Twenteborg Ziekenhuis te Almelo. In 1995 is hij gehuwd met Henriette Mazee. Samen hebben ze drie kinderen: Casper (1998), Marjon (1998) en Julian (2001).