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New Insights into the Management of Bleeding Disorders

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Pier Mannuccio Mannucci and Fernando Hernández

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Ancient Greek

αιμα [aima] = blood;
αιματος [aimatos] = of blood,
λογος [logos]= reasoning

Scientific Latin

haematologicus (adjective) = related to blood

Scientific Latin

haematologica (adjective, plural and neuter, used as a noun) = hematological subjects

Modern English

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New Insights into the Management of Bleeding Disorders

Guest Editors:
Pier Mannuccio Mannucci
Fernando Hernández

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paper

New insights into the management of bleeding disorders

PIER MANNUCCIO MANNUCCI
FERNANDO HERNANDEZ

In the past few years haemophilia has switched from the status of a handicapping and life-endangering ailment to a fully defined group of molecular-pathological entities for which safe and effective treatment is available. However, much remains unknown about optimal management of haemophilia as well as other coagulation disorders, and the complications that develop during treatment — such as inhibitor development.

The first Novo Nordisk European Symposium on bleeding disorders was an attempt to share new information about haemophilia and other coagulation disorders. The presentations were grouped into the following sessions: acquired haemophilia A; current activated recombinant factor VII (rFVIIa; NovoSeven®, Novo Nordisk A/S, Bagsvaerd, Denmark) clinical trials; control of bleeding in coagulation factor deficiencies; insights into congenital haemophilia therapy; and, the control of bleeding in Glanzmann's thrombasthenia (GT). This supplement provides manuscripts of these interesting sessions.

Acquired haemophilia A (AH) is the subject of the first two manuscripts. In the first, Peter Collins provides an informative review of the literature on the natural history of AH. While it seems that up to 30% of patients with AH do not require therapy with haemostatic agents, the data are mainly from small, referral centre case collections and larger retrospective surveys. Thus, it is likely that a patient selection and reporting bias is present. Dr. Collins is hopeful that the UK Haemophilia Centres Doctors' Organisation survey, which has collected data on all patients presenting with AH in the UK over a 2-year period, will provide more robust data on the true incidence of spontaneous remission in AH.

In the second manuscript, Mario von Depka presents data on the use of the chimeric anti-CD20 monoclonal antibody rituximab for immunotherapy in AH. Rituximab — primarily indicated for use in the treatment of non-Hodgkin's lymphoma — has been used in several small series of patients with AH. Long-term response rates seem to be around 50% with remissions occurring a few weeks up to several months after completion of the treatment course. Although relapses have been reported, the results are promising.

Ulla Hedner opens the section on Novo Nordisk clinical trials by providing a background rationale for current rFVIIa clinical trials within the area of haemophilia. Based on the results of home treatment studies, a single injection dose of 270 µg/kg will be investigated as a potential haemostasis enhancing therapy. Additional research will take place in younger patients who appear to have increased rFVIIa clearance. A clinical trial to elucidate any preventive effect of regular rFVIIa administration on bleeding pattern is also planned.

The paper by Karsten Lollike reviews Novo Nordisk rFVIIa clinical trials outside of haemophilia. Since the European registration of rFVIIa in 1996, many case studies have reported its successful use as a haemostatic agent in non-haemophilia patients. Based on these reports, Novo Nordisk initiated a broad clinical trial program investigating the haemostatic effects of rFVIIa in different bleeding episode indications, such as rescue therapy in severe life-threatening bleeding or during surgery to improve haemostasis.

Guglielmo Mariani moves onto the topic of bleeding control in coagulation factor deficiencies with his manuscript on the clinical picture and management of factor VII (FVII) congenital deficiency. Factor VII is the most common of the rare, inherited coagulation disorders with a heterogeneity ranging from lethal to mild, or asymptomatic forms. However, treatment for this condition is now available in the form of rFVIIa.

The paper by Waander van Heerde presents data on the mechanism of action from three patients receiving rFVIIa prophylaxis. Results from his study suggest that platelets are presensitised by rFVIIa. The

hypothesis presented is that FVII deficiency is a coagulopathy that might in part be explained by impaired platelet function, providing further insight at to why rFVIIa is also applicable in thrombocytopathy.

In the final paper of this session, Flora Peyvandi provides a comprehensive overview of rare coagulation disorders. The rare disorders are generally milder than the haemophilias because life- and limb-threatening symptoms are less frequent. Although they only have a global prevalence of around 3-5%, this increases by 10-20 times in countries with increase rates of consanguineous marriages. An international registry of rare bleeding disorders is being established which hopes to foster the development of orphan drugs for deficiencies with no available therapeutic concentrate.

Marijke van den Berg offers an interesting review of her centre's experience of inhibitor development. Data from a prospectively followed cohort who were registered at birth show that inhibitor development appears to be linked to earlier usage of factor VIII (FVIII). Thus the older the patients are when they receive their first dose of FVIII, the less chance there is of developing an inhibitor. Dr. van den Berg cautions us that we need more data from large randomized clinical trials to confirm this link.

In the paper by Ton Lisman the effects of rFVIIa-mediated thrombin generation on adhesion and aggregation of platelets from patients with GT were investigated under flow conditions. The results showed that rFVIIa-mediated thrombin generation substantially enhances adhesion of GT platelets to subendothelial proteins. Furthermore, GT platelets, which do not normally aggregate, were shown to interact with each other, and give a normal aggregation response via rFVIIa-mediated fibrin formation. Dr Lisman concluded that these mechanisms might explain the efficacy of rFVIIa in GT patients.

In the second paper on GT, Roseline d'Oiron reports on the results of an international survey conducted to evaluate the efficacy and safety of rFVIIa in GT patients. The treatment was effective in the majority of patients with a significantly higher success rate observed in severe bleeding episodes. The data would appear to suggest that rFVIIa is a potential alternative to platelet transfusion in GT patients, particularly in those with anti-platelet antibodies and/or platelet refractoriness.

The symposium was closed by Pier Mannuccio Mannucci with a state of the art lecture titled *The Future of Haemophilia Treatment*. In the manuscript, the point is made that haemophilia is one of the most promising fields among monogenic disorders for gene transfer therapy, however, several obstacles must still be tackled and overcome before results of this approach will be seen in the clinic. In the meantime alternative options are in the pipeline. One major boost may the development of factors in transgenic farmyard animals, providing less expensive and easily available replacement material. Another approach will be the improved engineering of factors to make them more biologically active and more stable with reduced immunogenicity and antigenicity. All these improvements will help us treat our patients with better results.

All this makes for compelling reading, and we would like to thank the authors for their excellent contributions to both this supplement and the meeting at which they presented their research and findings. A word of thanks must also go to Novo Nordisk for organizing an open and stimulating meeting attended by haematologists from around Europe. We hope that the results of the meeting will help clinicians and researchers reassess their approach to treating both haemophilia and rare coagulation disorders with a view to improving patients' prognoses.

Acquired haemophilia A: immune suppression

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A B S T R A C T

The natural history of acquired haemophilia is of spontaneous remission in 31% of patients but severe or fatal bleeding in 38%. The severity of the haemostatic defect varies between life-threatening and very mild, with about 30% of patients requiring no treatment with haemostatic agents. Immunosuppression to eradicate the inhibitor is usually attempted with either steroids or a combination of steroids and cytotoxic agents. The literature on acquired haemophilia, however, consists of small, referral centre case collections (4-34 patients) and larger retrospective referral centres surveys. This is likely to have introduced bias into patient selection and reporting, with a preponderance of young and severely affected patients, and good outcomes being reported. A single prospective randomized study comparing prednisolone and cyclophosphamide did not demonstrate a statistical difference between treatment arms. Numerous small non-randomized retrospective studies have shown variable outcomes and a meta-analysis of 20 of these studies suggested that a combination of steroids and cyclophosphamide may be better than steroids alone. Studies also highlight the risk of complications of immunosuppression such as diabetes, neutropaenia and sepsis. To address the issue of reporting bias the UK Haemophilia Centres Doctors' Organisation has collected data on all patients presenting with acquired haemophilia in the UK over a 2-year period. It is unlikely that substantial progress will be made in the management of acquired haemophilia without prospective randomized studies of immunosuppression, investigating both efficacy and adverse events.

Key words: Acquired haemophilia, cyclophosphamide, immune suppression, meta-analysis, steroids.

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Acquired haemophilia A (AH) is a rare bleeding disorder in which individuals develop autoantibodies against clotting factor VIII (FVIII); it is therefore an autoimmune disease. The underlying cause of the disease is unknown. The risk is increased in pregnancy, malignancy or in the presence of certain other autoimmune diseases (e.g. rheumatoid arthritis, systemic lupus erythematosus), but the majority of cases are observed in elderly individuals without other risk factors.

The disease has a high incidence of morbidity and mortality, with many individuals developing severe bleeds that are difficult to control and can be fatal. A typical clinical presentation of AH usually involves severe subcutaneous and soft tissue bleeding, with extensive superficial bruising; in contrast, joint bleeding, typical in congenital haemophilia, is relatively uncommon.

Treatment of AH therefore has two main aims: control of the bleeding, and eradication of the FVIII inhibitor by immunosuppression. This paper will focus on immunosuppression for AH. When examining the efficacy of various types of immune suppression in the literature of AH, a number of factors need to be considered before any real judgments can be made.

Current literature on acquired haemophilia

The studies

Descriptions and conclusions regarding AH have primarily been based on a number of referral centre cohort studies, in particular one large retrospective cohort of 215 referral centre patients by Green and Lechner,[1] in which information was obtained by ques-

tionnaire. A prospective, randomized controlled trial, evaluating the safety and efficacy of prednisolone and cyclophosphamide in 31 patients, has also been published.[2] Both retrospective and prospective data from numerous small referral centre cohort studies are also available.

An important milestone in the understanding and treatment of AH has been the publication of a review and meta-analysis by Delgado *et al.*,[3] which is the most comprehensive overview on the literature of the disease so far. This analysis pooled together data from many different referral centre cohort trials into a coherent whole.

However, one of the potential problems associated with this analysis is the introduction of bias into the data sets. For example, only tertiary referral centre patients are reported in the literature; any patients not referred to major haemophilia centres are therefore not represented. Some form of treatment bias is also likely, since larger centres may have different treatment procedures for their patients than other, smaller centres. A reporting bias is also likely, since the focus in the literature is often on the reporting of good outcomes and special patients, such as younger patients or those more severely affected.

UK surveillance of acquired haemophilia

In order to try to address the issues outlined above, and to obtain a comprehensive overview of the disease, the UK Haemophilia Centre Doctors' Organisation (UKHCDO) has collected surveillance data for AH in the UK over a 2-year period, from May 2001 to April 2003. The study collected data from every haematology department in the UK every 6 months on all their patients with AH. Thus, data have been obtained from virtually all individuals with AH in the UK. This study has several strengths in its favour, compared to similar studies in the literature: it represents a consecutive cohort of all AH patients in the UK; there is no referral bias; there is no reporting bias; and, the population denominators are known. It is therefore possible to accurately establish the incidence of AH, at least in the UK, for the first time. The full analysis of all the data from this study is still pending, and the results will be published soon.

Natural history of acquired haemophilia

When considering eradication of FVIII inhibitor in AH, it is important to consider the natural history of the disease. A study by Lottenberg *et al.*,[4] followed 16 patients with no immunosuppression for 4-120 months. Five patients died during the course of the study, including two deaths from fatal haemorrhage. Another five patients underwent spontaneous remission, but in two cases this was associated with neurological damage. Life- or limb-threatening bleeding was experienced in 10 patients, while four patients (25% of the cohort) required no haemostatic treatment. The authors concluded that the bleeding phenotype is variable and that some patients undergo spontaneous remission.

Options for FVIII inhibitor eradication

A number of options are potentially available for eradication of FVIII inhibitor, including: steroids alone; steroids plus a cytotoxic agent (e.g. cyclophosphamide, azathioprine, CVP [cyclophosphamide, vincristine and prednisolone] or 6-mercaptopurine); immunosuppression plus FVIII priming; cyclosporin A; rituximab; and, plasmapheresis or immunoabsorption.

Steroids

Steroids, such as prednisolone, have been used in AH mainly in standard doses of approximately 1 mg/kg. A complete response (CR) rate of about 30% is often quoted for steroid treatment,[2] whereas the recent review and meta-analysis by Delgado *et al.*[3] gave a much higher CR rate of approximately 70% in 57 patients receiving steroids alone. In order to ascertain a potentially more accurate figure, some of the literature was reviewed again, including some studies not included in the Delgado meta-analysis, and excluding some studies from the meta-analysis. The excluded studies were those of Lian,[5] and Nemes and Pitlik,[6] which address the issue of immunosuppression and factor VIII priming, the study of Jansen *et al.*[7] which focuses on immunoabsorption and the studies of Green and Lechner[1] and Solymoss[8] which do not report CR. The number of patients administered steroids in these studies ranged from one to 14, with response rates ranging from 0% to 100%. As shown in Table 1, the average CR rate was approximately 66%, substantially higher than the CR rate of 30% often quoted from current literature. One reason for this may be that, in the Green *et al.* study from which the 30% CR rate comes,[2] 31 patients were treated for 3 weeks with prednisolone, after which time 10 patients had gone into remission; hence, the CR rate of 30%. However, in many of the other studies reviewed here, steroid treatment was over a longer period. Therefore, in the prospective randomized study quoted,[2] the duration of steroid treatment was probably not long enough to obtain a clear idea of what the true CR rate would be.

Steroids plus cytotoxic agents

For the use of cytotoxic agents with or without steroids in AH, the CR rate from current literature is often quoted as 60% to 100%. The Delgado meta-analysis, however, gives a CR rate of 89%. The literature review used here excludes the studies of Lian and Nemes as their main aim was to investigate the role of

Table 1. Complete response rates from steroids or steroids plus cytotoxic agents, used in literature review.

	Steroids		Steroids + Cytotoxic Agent	
Study	*N*	*CR (%)*	*N*	*CR (%)*
Bayer, 1999[17]			7	100
Bossi, 1998[18]	8	88	18	83
Burnet, 2002[19]			5	80
Di Bona, 1997[20]	9	55	5	20
Dykes, 2001[14]	7 (+IVIG)	57		
Godreuil, 2001[21]	3	66	2	0
Grunewald, 2001[22]	3	33	6	100
Ji, 1998[23]	8	88		
Lian, 2002[24]			6	83
Mazzucconi, 2001[25]	4	75		
Sallah, 2001[26]	9	77	16	50
Sallah, 1998[27]			3	33
Saxena, 2000[28]	1	100	5	80
Shaffer, 1997[29]			9	100
Sohngen, 1997[30]	1	0	7	100
Spero, 1981[31]	14	50		
Schwartz, 1995[13]	2 (+IVIG)	50	1	0
Yee, 2000[32]	4	75	11	82
Total	73	66	101	77

IVIG = intravenous immunoglobulin.

factor VIII priming. This makes an important impact on the results as they have high CR rates. The results of the literature review gave an average CR rate of 77%, similar to the CR rate achieved with steroids alone.

Different results are observed if we examine the prospective randomized trial by Green *et al.*[2] In this trial, 20 of the 21 patients who did not achieve remission after 3 weeks were randomized to one of three arms: prednisolone 1 mg/kg; cyclophosphamide 2 mg/kg; or, a combination of both.[2] In those that continued with steroids alone, there was a remission rate of 75%, while in the cyclophosphamide arms there was 50% remission in each arm. However, these results are difficult to interpret as the study was not adequately powered for a three-way randomization. The study is also biased against cyclophosphamide, since the 10 patients who achieved early remission with steroids alone were removed after 3 weeks. Cyclophosphamide was therefore used only in patients who did not achieve early remission with steroids. However, the benefit in response rate with cyclophosphamide is suggested by the results of the Delgado meta-analysis.[3]

Factor VIII plus immunosuppression

The rationale for the use of FVIII therapy in immunosuppressive regimens is that the FVIII will stimulate the autoantibody-producing cells into division, making them more susceptible to cytotoxic agents. It was first reported over 30 years ago, in a 54-year-old woman who had no response to treatment with methotrexate, cyclophosphamide and azathioprine after 6 weeks.[9] However, 8 months later, the patient showed a rapid response to a high dose of FVIII (10,000 IU) plus cyclophosphamide.

A later study by Lian *et al.* treated 12 patients with 3-weekly cycles of CVP, with FVIII priming (50-100 IU/kg) given at the start of each cycle.[10] A CR rate of 92% was observed. However, because there were no controls in the study, it is possible that the more intensive immunosuppression, rather than the FVIII priming, accounts for the high response rate. This may possibly be reflected by the finding that three patients required hospital admission for neutropaenic sepsis.

A similar approach was taken in a more recent study by Nemes *et al.*,[11] where intravenous cyclophosphamide and prednisolone were used in combination with a daily FVIII regimen. The CR rate was 95%, with a median time to remission of 4.7 weeks. These results compared well with the historical controls used, who were treated with steroids, with or without cyclophosphamide; here the CR rate was 66%, with a much longer time to remission of 28 weeks.

Immunoglobulin

Initial successful treatments were reported by Sultan *et al.*,[12] and a later prospective study by Schwartz *et al.*[13] demonstrated a CR rate of 19% in patients treated with immunoglobulin, although some also received other immunosuppressive agents. All patients who responded in this study had very low initial Bethesda titres. In a more recent study, a CR of 57% was achieved in patients receiving immunoglobulin in combination with steroids.[14] These results are similar to the CR reported in the literature for steroids alone. Immunoglobulin may play a role in the management of patients with AH in patients with low initial Bethesda titres.

Cyclosporin A

Analysis of a collection of single case reports shows that responses have been achieved with cyclosporin after various other treatment regimens have failed. Cyclosporin may therefore have a role in patients not responding to first-line therapy.

Plasmapheresis/immunoabsorption

Most studies in the literature have used plasmapheresis/immunoabsorption to decrease the inhibitor titre when treating bleeding episodes. However, immunoabsorption has been used in combination with immunosuppression with or without FVIII.[7] In this study, the CR rate was 75%, similar to other immunosuppression studies, but the median time to remission was shorter (18 days).

Establishing the incidence of pregnancy-related acquired haemophilia

Whether pregnancy-related AH should be treated as a separate entity remains an open question. Pregnancy-related AH has obvious implications for the obstetric management of the patient and of the foetus, as the neonate will have AH at birth. The condition may present *antepartum*, but more often presents from 1-12 months *postpartum*, and often presents with episodes of bleeding. Factor VIII inhibitors may recur in subsequent pregnancies. The largest study published in the literature used case reports from 51 patients, with no differences between the CR rates with treatment using steroids alone or in combination with cytotoxic agents.[15] However, the median times to remission were longer than in patients in other studies (8 months for patients taking steroids and cytotoxic agents, and 12 months for those on steroids alone), suggesting that pregnancy-related AH may respond more slowly.

In a later review of 14 patients receiving immunosuppression, the median time to inhibitor eradication was 27 months, but it was not possible to assess the CR rates.[8]

In contrast, data from a retrospective Italian registry study, which reported 20 cases of pregnancy-related AH over 15 years, showed that first CR was achieved in 78% of patients, with a median time to remission of 2.7 months.[16] Interestingly, 45% of the patients did not require haemostatic treatment.

Survival and mortality

The Delgado meta-analysis[3] provides information on overall survival of patients with AH according to treatment regimen. In this review, up to a period of about 120 months, there appeared to be no obvious difference in overall survival rates between patients treated with steroids, those treated with cyclophosphamide, or untreated patients.

Conclusions from the meta-analysis

Several conclusions can be drawn from the Delgado meta-analysis,[3] which is one of the most accurate, well-balanced reviews of AH in the literature to date. Firstly, eradication of the FVIII inhibitor is essential to prevent further bleeding episodes. Cyclophosphamide appears to have advantages over steroids for eradication of the inhibitor, but not for overall survival. The authors also suggested that some patients may be dying of complications associated with the intensive immunosuppressive regimen, particularly with cyclophosphamide. Data regarding the use of immunoglobulin appear to be conflicting, and the role of immunoglobulin therapy in AH has not yet been fully clarified. The authors also emphasized that consideration of the potential complications of therapy for AH is as important as eradication of the FVIII inhibitor. Potential immunosuppressive regimens should therefore be evaluated in terms of efficacy and adverse events. It may be important to consider risk-tailored protocols, i.e. tailored to the risk of bleeding in individual patients.

Overall conclusions

The incidence of AH appears to be approximately 1.5 cases/million/year, with a highly variable phenotypic presentation, ranging from fatal, due to extensive spontaneous bleeding, to very mild, requiring no haemostatic treatment. The most common treatments, e.g. steroids alone or steroids in combination with cytotoxic agents, appear to be roughly equivalent in terms of survival, and the addition of immunoglobulin appears to have little or no effect. The mortality of the disease, as one would expect is high, but not all deaths are due to bleeding; it may well be that some of the deaths in AH are related to the immunosuppressive regimen.

It seems likely that as much data as possible have been gleaned from cohort patient studies from individual centres. Further progress in the management of AH will therefore only be made through the use of prospective randomized trials, but it must be recognized that studies like this are very difficult to perform in these patients, due to the rarity of the disease, and are hampered further by new legislation.

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Rituximab for immune tolerance in acquired haemophilia

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A B S T R A C T

Acquired haemophilia is a rare disorder with an estimated annual incidence of 0.2-1 cases per million individuals. The aetiology of the disorder remains obscure, although approximately half of all cases are associated with other underlying conditions. In acquired haemophilia, the severe hemorrhagic diathesis is caused by the development of autoantibodies directed against a clotting factor, most commonly factor VIII. Bleeding can be life-threatening in a high percentage of cases. Immune tolerance therapy has been shown to successfully eradicate or suppress inhibitors in patients with congenital haemophilia A. However, in patients with acquired haemophilia immunosuppressive therapy involving corticosteroids alone or in combination with other cytotoxic drugs or immunoadsorption are employed in the majority of cases. Recently, rituximab, a chimeric anti-CD20 monoclonal antibody causing selective B-cell depletion has been used in several small series of patients with acquired haemophilia and in single patients with congenital haemophilia complicated by inhibitory alloantibodies. Reports suggest that rituximab has a role in the management of haemophilia resulting from autoantibody formation and warrant large scale multicentre studies. Furthermore, it needs to be elucidated whether rituximab has a role in congenital haemophilia complicated by inhibitory autoantibodies, too, as first-line treatment alone or in combination with immune tolerance treatment.

Key words: rituximab, immune tolerance, hemophilia.

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Acquired haemophilia (AH) is a rare disease, with an estimated annual incidence of 0.2-1 cases per million individuals.[1-4] However, the condition is associated with a mortality rate of approximately 20%.[3] Acquired haemophilia is caused by the spontaneous development of autoantibodies which target a clotting factor, most commonly factor VIII (FVIII). Although the aetiology of the disorder remains unclear, approximately half of all cases are associated with other conditions, such as malignancies, autoimmune diseases and pregnancy (Figure 1).

Rituximab is a monoclonal antibody directed against CD-20 which is present on B-cells. Its initial development was for the treatment of B-cell non-Hodgkin's lymphoma (NHL). However, there are some reports of usage of rituximab in patients with autoimmune diseases, such as rheumatoid arthritis, prompting its use in AH.

Immunobiology of AH

Little is known about the immunobiology and pathomechanisms of AH. Each person is normally tolerant to his/her own clotting factors, tolerance being defined as a state of un- or hyporesponsiveness as a consequence of prior exposure to an antigen e.g. FVIII. This is typically induced pre- and perinatally, but may be achieved by different mechanisms such as deletion, anergy, suppression by regulatory cells or by the interaction of anti-idiotypic antibodies.

A naïve T-cell may develop into an activated T-cell following exposure and signalling from an antigen presenting cell (APC). The subsequent development of a proliferating T-cell and further development depends on the presence or absence of survival signals or co-stimulatory signals. In the absence of these signals apoptosis occurs. If prolonged activation of signals

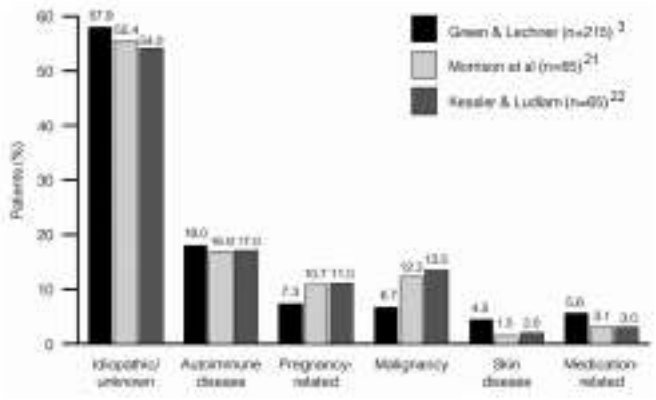

Figure 1. Aetiology of AH. From: von Depka M. Immune tolerance therapy in patients with acquired hemophilia. Hematology 2004, in press. Copyright American Society of Hematology, used with permission.

occurs, T-cells develop into memory cells which can be reactivated upon exposure to the antigen.

Loss of tolerance may involve both the humoral immune system and cellular components (B and T-cells). However, as little is known about the immunobiology of AH, it is still unknown whether it is a T or B-cell disease, or whether both cell compartments are involved. B-cells pose an interesting target in the treatment of autoimmune diseases, for B-cells as precursors of plasma cells produce the antibodies. B-cells also form the pool of APCs which, as mentioned previously, initiate the development of a naïve T-cell into an activated one. Hence, targeting B-cells may influence both cellular compartments. Several diseases have been treated with monoclonal antibodies such as rituximab, and therefore may play a role in the further understanding of the immunobiology of AH.

Rituximab in autoimmune diseases

Rituximab is a chimeric anti-CD20 monoclonal antibody consisting of a mouse fragment antigen-binding area, along with a human framework. Although rituximab targets the clonally differentiated antigen CD20, present on B-cells, CD20 is not present on B stem cells within bone marrow or on plasma cells. Therefore rituximab alone may not be an ideal therapeutic agent for AH, underlining the need for a new agent which can target plasma cells. Only with the eradication of specific memory B-cells, will long-term remission occur.

Rituximab has shown efficacy in the treatment of idiopathic and autoimmune thrombocytopenic purpura, and autoimmune haemolytic anemia.[5-7] There have also been reports of effective use of rituximab in multiple sclerosis, systemic lupus erythematosus, rheumatoid arthritis and in essential cryoglobulinaemia, including patients suffering from hepatitis C.[8-12] These observations along with the current knowledge of immunological background, form the rationale of immune tolerance therapy of AH with rituximab.

Rituximab in acquired haemophilia

Wiestner *et al.* published the first full paper on the treatment of acquired FVIII inhibitors with rituximab.[13] This was a study of four haemophiliac patients treated with rituximab, although one of these patients had congenital haemophilia A. The remaining patients with acquired haemophilia included a 69-year old man suffering from chronic renal failure, a 38-year old man with ascites and lymphadenopathy of unknown origin, and a 79-year old woman with polymyalgia on immunosuppressive treatment with prednisone. All three patients had prolonged activated partial thromboplastin time (APTT) and low FVIII activity, with two of the three patients having low inhibitor titres.

The patients in this study were treated with a single weekly dose of 375 mg/m^2 rituximab for four consecutive weeks, the same regimen as that utilized in the treatment of B-cell NHL. All three patients with AH received concomitant immunosuppressive treatment involving prednisone alone or together with cyclophosphamide.

All three patients had an immediate drop in their inhibitor titres following the first dose of rituximab along with an increase in FVIII activity. Factor VIII activity normalized over a period ranging from 3 to 12 weeks after the first administration of rituximab, demonstrating efficacy of this agent over the observed period of time.

Similar results were observed in a study by Stasi *et al.*[14] Ten patients with AH were included in this uncontrolled, monocentre study who were treated with the

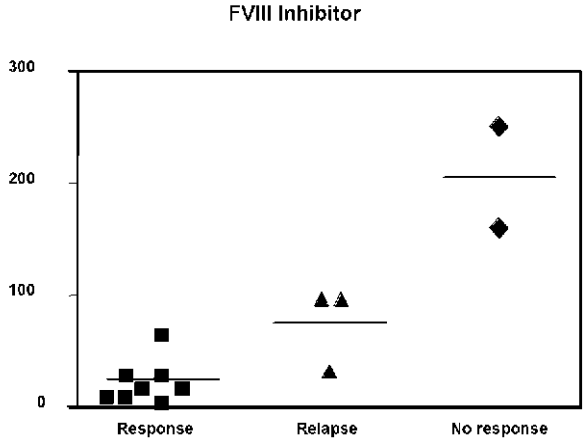

Figure 2. Response to immunosuppressive treatment as measured by FVIII inhibitor titres (data from Wiestner A *et al.*[13] and Stasi R *et al.*[14]).

same rituximab regimen as that recommended for B-cell NHL, and also used in the previously described study. Complete remission was defined as a clinical improvement based on bleeding episodes and tendencies, FVIII activity within the normal range, and undetectable inhibitor levels < 0.6 BU.

The age range of the 10 cases was 27-78 years, with an equal divide between the sexes. All but two of the patients presented clinically with ecchymoses along with various other bleeding sites. Four patients had concomitant diseases, but all had a residual FVIII activity level below the detection limit suggesting the presence of type 1 inhibitor. Six of the 10 patients had received no previous treatment for their AH, whilst the others had previous immunosuppressive therapies.

Half of the patients responded, with marked clinical improvement within the first few days after the first dose of rituximab, a response also reported by Wiestner *et al.*[13] Subsequent bleeding tendencies were evidently reduced. Three patients showed an initial response to rituximab but relapsed, developing inhibitors again. The time to relapse ranged from 10-23 weeks, suggesting that inhibitors may reoccur up to 6 months after treatment and moreover, after the initial disappearance of inhibitors. Therefore, it is necessary and important to follow-up on patients' conditions, and to re-evaluate their management for a long period of time.

The remaining two of the 10 patients did not respond to treatment, and interestingly had the highest FVIII inhibitor titres in the cohort. In both studies there was a correlation between response and inhibitor titres, with responders having low Bethesda units, and non-responders, having the highest titres (Figure 2). In the Stasi *et al.* study, patients with inhibitor titres < 50 BU seemed to benefit most from treatment with rituximab[14] suggesting a correlation between inhibitor levels and treatment outcomes.

Overall in the Stasi *et al.* study, eight out of 10 patients responded, in which five had long-term remission and three patients relapsed but responded once again to another cycle of rituximab, with a normalization of FVIII activity and a disappearance of the inhibitor. Two of the 10 patients were refractory to treatment but responded with respect to a change in FVIII activity and inhibitor titre. The median follow-up time was 28.5 months, with a range from 12-42 months. Statistically, the immunosuppressive treatment of these 10 patients resulted in a 20% failure rate and an 80% response rate, with a 50% long-term remission and a 30% relapse rate.

Important observations of this study include a clinical response seen within a few days after the first dose. Time until remission ranged from 2-12 weeks, again a similar observation to Wiestner *et al.*,[13] and time until remission from 10-23 weeks. Prior to immunosuppressive treatment, some of the patients had antinuclear antibodies (ANA) and lupus anticoagulant (LAC) antibodies. These disappeared following treatment with rituximab, although the total immunoglobulin G level remained stable. Two of the three patients who relapsed and one of two patients in whom treatment failed had LAC. Due to the low number of patients in this study, it is difficult to state any inferences between the presence of ANA and LAC to patients' response to rituximab. However, when the authors reported relapses of reduction in FVIII activity and an increase in inhibitor titres, they did not report whether they were associated with clinical symptoms.

Adverse events with rituximab

Stasi *et al.* reported adverse events in three patients, all in which were mild.[14] These included nausea, fever

and chills, and occurred after the first dose of rituximab. Nevertheless these did not result in cessation of treatment, with the patients becoming more tolerant to subsequent treatments. However, use of rituximab has also been reported to be associated with more severe adverse events compared with the mild cases reported in this study. Severe adverse events observed from our own as well as other patients treated with rituximab include vomiting, rectal abscesses, aspergillus pneumonia and sepsis, although side-effects are often observed to be related to total dose and amount of drug administered.[15]

Alternative immunosuppressive treatments in AH

Spontaneous remission can occur in up to one third of patients with AH, however, remission usually occurs late during the course of the disease.[1] Prednisone monotherapy has an observed response rate of 30%, although longer treatment regimens of prednisone alone can increase the response rate up to 70%.[16] Combination immunosuppressive agents (e.g. prednisone and cyclophosphamide) demonstrate an even higher response rate compared with rituximab monotherapy, with rates ranging from 70-100% within 3-37 weeks.[17]

Rituximab in pregnancy

Only few data exist for the usage of rituximab in pregnancy. In a single case in which rituximab was used in a pregnant patient with B-cell lymphoma,[18] a single dose of rituximab was administered together with a cycle of chemotherapy (doxorubicin, vincristine, prednisone), with the patient delivering a healthy baby girl who has shown normal development and normal B-cells at 4 months. One further recent report suggests that rituximab during pregnancy seems to be safe.[19] However, as there are only two reported cases of rituximab in a pregnant patient, further data are required before conclusions can be made regarding the safety and efficacy of rituximab in this patient population.

Discussion

Many questions remain open regarding the safety and efficacy of rituximab reported in AH. Both Wiestner *et al.* and Stasi *et al.* reported a sudden onset in clinical remission. However, rituximab is directed against B-cells and not the antibodies that cause the disease. Although the half-life of antibodies is approximately a couple of weeks, this does not fully explain the mechanism of action and efficacy demonstrated by rituximab in order to achieve the sudden onset of remission.

The true response rate of rituximab is also questionable, taking into account that spontaneous remission occurs in AH, and that many of the patients in these studies received concomitant immunosuppressive treatment; only two patients who responded received rituximab monotherapy. The authors reported B-cell recovery from 12-26 weeks, the time when relapses occurred, however, no correlation was noted between the recovery of the B-cell and the reoccurrence of the inhibitors. This is important for the monitoring of inhibitor titres, with the Bethesda assay not being the most sensitive measure of response in AH. Assessment of B-cells may be more appropriate, however, examination of circulating B-cells alone may not be justifiable without measurement of B-cells in lymph nodes – this may be difficult to measure, especially in patients with increased bleeding tendencies.

The three patients in the Stasi *et al.* study in whom were reported to have received a further cycle of rituximab following relapse were subsequently followed-up for 8-18 weeks, despite an earlier observed relapse period of up to 23 weeks.[14] Hence all three of these patients remain at risk of developing a subsequent relapse outside the observed follow-up period.

It remains questionable as to whether rituximab can be regarded as a good first-line treatment in AH and whether monotherapy is more efficacious compared with combination regimen. If rituximab is more effective in combination treatment, which combination would provide the best result? The current dose, dosing intervals and regimen used in these studies are developed for the treatment of NHL, a malignant condition which may require different doses to target tumour cells, compared with an autoimmune condition such as AH. Data is comparable but the question remains as to whether these are the correct doses and regimen for AH. The effectiveness of rituximab in patients with high inhibitor titres remains unclarified, including the safety of repeated administrations in relapsing patients and whether it is justifiable to continue treating resistant patients with rituximab.

In summary, rituximab has been shown to have role in the treatment of AH despite the many unanswered questions. It may shorten the time to response. However, this reduction is similar to that seen with other immunosuppressive agents such as prednisone. These studies demonstrate that rituximab is safe and efficacious, though the many open and unanswered questions highlighted should be taken into account. Rituximab may be a first choice treatment agent in patients with severe bleeding, an inference from the marked reduction in bleeding tendencies following a single

dose of rituximab. Rituximab may be considered in patients with bleeding tendencies refractory to the alternative immunosuppressive agents, and may be considered for repeated administration in patients who are resistant to treatment.

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Background rationale for current clinical trials on NovoSeven® within haemophilia

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A B S T R A C T

In a home treatment study in which activated recombinant coagulation factor VII (rFVIIa) was administered at first sign of bleeding to prevent the development of a joint or soft tissue bleeding, 2.2 injections (90 µg/kg per injection) were required to achieve haemostasis. Ideally, an optimal dose should give full haemostatic effect when given as one single injection. *In vitro* studies of the effect of rFVIIa on enhancing thrombin generation on activated platelets indicated that a dose corresponding to 2-3 times of 90 µg/kg may be required to reach an effect closer to a *normal* haemostasis. Based on this finding, the efficacy of a dose of 270 µg/kg given as one single injection will be explored. Furthermore, a pharmacokinetic study has confirmed that rFVIIa clearance in children younger than 15 years old is increased as compared to adults, indicating that higher doses may be required for full haemostasis in younger individuals. The ideal haemophilia care includes so-called prophylaxis- regular administration of FVIII/FIX concentrates several times per week aiming to minimise the number of joint bleeds. Chronic haemophilia arthropathy is correlated to the number of joint bleeds. The use of exogenous rFVIIa at high dose to achieve haemostasis is a new treatment concept, and currently there is no measurement capable of directly linking haemostasis effectiveness with the dose of rFVIIa. Nevertheless, regular administration of rFVIIa once or twice per day has been shown to diminish the number of bleedings and even to break a vicious circle of bleeding in a target joint. Although the plasma half-life of rFVIIa is between 2 and 3 hours, plasma levels of FVII 24 hours post-injection may still be higher than baseline, following a dose of around 200 µg/kg. The planned clinical trial on regular administration of rFVIIa aims to elucidate any preventive effect of regular rFVIIa administration on bleeding pattern.

Key words: haemophilia, recombinant factor VIIa, clinical trial, inhibitors.

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Current concept of haemostasis and mode of action of rFVIIa in haemostasis

Haemostasis is initiated by the exposure of tissue factor (TF) which is located in the deeper layers of the vessel wall. Under normal conditions, TF is not in contact with circulating blood. Upon vascular injury, the exposed TF forms a complex with FVIIa in the circulation. Approximately 1 percent of total FVII protein mass is present in an activated form in the circulation; however, FVIIa is haemostatically inactive until it forms a complex with TF. The TF-FVIIa complex generates low amounts of thrombin which is insufficient to provide a firm haemostatic plug but it activates FVIII, FV and FXI, as well as platelets. Subsequently, activated platelets expose negatively-charged phospholipids on their surface providing a template for the propagation phase of coagulation which resulting in a burst of thrombin. Full thrombin generation is necessary for full haemostasis.

Thrombin converts fibrinogen into fibrin via the formation of soluble fibrin monomers which are then cross-linked with FXIIIa to form a stable haemostatic plug. Factor XIII is also activated by thrombin. Furthermore, thrombin activates thrombin-activatable fibrinolysis inhibitor (TAFI) which protects the fibrin haemostatic plug from premature fibrinolysis.

In an *in vitro* model, the addition of activated recombinant coagulation factor VII (rFVIIa; NovoSeven®, Novo Nordisk A/S, Bagsvaerd, Denmark) at a concentration of 50 nM (equivalent to a dose of 90-100 µg/kg) to FVIII-deficient plasma substantially increases thrombin generation but not

to the levels seen in normal plasma.[1] Therefore, higher concentrations of rFVIIa may be required to increase the levels of thrombin generated.

Another *in vitro* model has shown that the addition of rFVIIa at increasing concentrations to thrombocytopenic samples (50,000 platelets/μL) shortens the lag phase of thrombin generation in a dose-dependent manner.[2] This indicates that rFVIIa also acts on the initiation of thrombin generation. The rapidity of thrombin generation is important for the structure of the fibrin plug.

Using a confocal electron microscopy, it has been shown that the structure of fibrin clot in haemophilia plasma in the absence of rFVIIa is loose and porous. In contrast, in the presence of rFVIIa, the structure of fibrin clot is much tighter.[3]

In summary, rFVIIa enhances thrombin generation on already activated platelets thereby providing tight, less porous, stable haemostatic plugs less prone to be dissolved by fibrinolysis.

Patients with haemophilia(s) lack FVIII or FIX. In these patients, small amounts of thrombin are generated but cannot progress into the propagation phase. Bleeding in these patients may stop initially but the bleeding recurs as firm haemostatic plugs cannot be formed. Recombinant FVIIa binds to activated platelets and directly activates FX to FXa which leads to the formation of thrombin compensating for the lack of FVIII or FIX.

Current treatments for patients with haemophilia

Current haemostatic treatments for patients with haemophilia include substitution therapy with FVIII or FIX concentrates for haemophilia A and haemophilia B, respectively; and pharmacological therapy with rFVIIa which aims to compensate for the impaired thrombin generation independently of FVIII or FIX. The exact relation between the FVII:C level in plasma and a full thrombin burst on thrombin-activated platelets in the absence of FVIII or FIX is not known. The current recommended dose for rFVIIa was based on a study using haemophilia plasma which shows that rFVIIa at concentrations of 1.0 and 3.8 μg/mL substantially shortens and normalises the APTT time, respectively. As a result, a dose between these two concentrations, 2 μg/mL which is equivalent to 90-100 μg/kg, was chosen. This dose provides 90-100% efficacy in major surgery, 83-95% in other serious bleedings and 92% in home treatment. Nevertheless, with an increasing number of patients treated with rFVIIa, the variation in individual response has become increasingly apparent. This phenomenon is common for all haemophilia treatments available in the market.

Rationale for higher doses of rFVIIa

The current recommended dose of rFVIIa at 90-120 μg/kg may be, in some patients, in the lower range to generate sufficient thrombin to compensate for the lack of FVIII or FIX. This could be due to low recovery. The recovery of rFVIIa varies between 40-80% between patients. The difference in recovery may significantly influence the efficacy of FVIIa. Furthermore, children (less than 15 years old) have higher clearance as compared to adults.[4] In addition, the thrombin generation capacity on platelet surface may vary between patients. In a study using a modified thromboelastography based on low concentrations of TF and whole blood from a patient with haemophilia with inhibitors, it has been shown that rFVIIa 90 μg/kg totally normalises the thromboelastrogramme pattern. This effect abates two hours afterwards which is in accordance with the efficacy of rFVIIa in clinical settings. In another patient, the same dose of rFVIIa does not normalise the thromboelastrogramme pattern, but an increased dose of 436 μg/kg does so. These findings exemplify the variation of rFVIIa response between patients.

In a home treatment study including 566 bleeding episodes, rFVIIa 90 μg/kg every 2 hours for a maximum of 3 doses was administrated at first sign of an upcoming bleeding to prevent the development of a joint or soft tissue bleeding.[5] The efficacy was around 92% with a mean number of 2.2 injections required to achieve haemostasis. This indicates that the dose used might not be sufficient, as the optimal dose should give full haemostatic effect with one single injection.

Efficacy of higher doses of rFVIIa

Kenet et al has shown that for the treatment of haemarthrosis, a dose of 300 μg/kg per bolus injection gives a higher efficacy than protocols that use lower doses whereas the total dose required to achieve haemostasis does not increase.[6] Moreover, data from the Haemophilia Research Society Registry including 556 bleeding episodes demonstrates that the efficacy in the group treated with rFVIIa at doses higher than 200 μg/kg is higher than the group treated with doses between 100 to 200 μg/kg (efficacy 97% vs 84%).[7]

Cooper et al has reported a 14-year-old patient with haemophilia B with inhibitors who had poor response to rFVIIa even at 160 μg/kg per dose and the dose was increased to 300 μg/kg.[8] The latter dose allowed the patient to undergo passive mechanical traction, casting and serial wedging as treatments for the bilateral knee flexion contractions. During a period of 80 days, the patient received 240-300 μg/kg once or twice daily as a prophylaxis. There were only 3 minimal break-

through bleeds, all injury-related. No signs of any thrombotic events were seen.

Prophylactic use of rFVIIa

Saxon *et al.* has reported a 5-year-old patient with haemophilia A with inhibitors who had 6 left ankle bleeds in 7 weeks, each bleed required a mean of 4.7 doses of rFVIIa (90 μg/kg).[9] The patient had a further 10 ankle bleeds and 9 periarticular bleeds in 9 weeks, each bleed required a mean of 4.0 doses of rFVIIa (90 μg/kg). As the treatment appeared to be suboptimal and could not prevent further bleeding episodes, it was decided to give a daily prophylactic dose of 90 μg/kg. Over a period of 11 weeks, there were only 2 ankle bleeds and 4 periarticular bleeds, each bleed requiring a mean of 2.8 doses of rFVIIa. The number of doses per week in the prophylactic protocol (mean 8.5) was similar to that in the on-demand treatment (mean 8.4), whereas the prophylactic protocol decreased the frequency of bleeding.

Clinical trials within haemophilia

Novo Nordisk has completed a study on the pharmacokinetic of rFVIIa in children as compared to adults with haemophilia. The results will be published in Haemophilia. Furthermore, two studies on the safety and efficacy of a single high-dose of rFVIIa (270 μg/kg) are currently on-going. In addition, a study on the daily prophylactic use of rFVIIa in haemophilia has been initiated.

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Current Novo Nordisk sponsored clinical trials on NovoSeven® outside haemophilia

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A B S T R A C T

Activated recombinant coagulation factor VII (rFVIIa, NovoSeven®) was first registered in Europe in 1996, and is currently approved in most regions of the world for the treatment of bleeding episodes and prevention of bleeding during invasive procedures or surgery in patients with haemophilia with inhibitors to factor VIII or IX. Since rFVIIa has become available, many case stories have shown that rFVIIa is an effective haemostatic agent in non-haemophilia patients. This led Novo Nordisk to initiate a broad clinical trial programme in 2000, to investigate the haemostatic effects of rFVIIa in patients with many different types of bleeding episodes. Most of these phase II trials have been designed for the use of rFVIIa in critical bleeding in patient with the following underlying conditions: upper gastrointestinal bleeding, vitamin K antagonist treatment, stem cell transplantation, intracerebral bleeding, and trauma. In addition, there are trials in which rFVIIa has been used to improve haemostasis during surgery in settings known to result in major bleeding (orthotopic liver transplantation, liver resection, spinal fusion, and orthopaedic surgery of pelvic fractures). Overall, the trials aim to demonstrate that rFVIIa is effective and safe outside haemophilia. Most trials are monitored by independent safety monitoring boards, and so far there have been no safety concerns.

Key words: recombinant factor VIIa, clinical trial

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Currently, in Europe, activated recombinant coagulation factor VII (rFVIIa; NovoSeven®, Novo Nordisk A/S, Bagsvaerd, Denmark) is indicated for the treatment of bleeding and the prevention of bleeding in those undergoing surgery or invasive procedures in the following patient groups:

- in patients with congenital haemophilia with inhibitors to FVIII or FIX > 5 BU
- in patients with congenital haemophilia who are expected to have a high anamnestic response to FVIII or FIX administration
- in patients with acquired haemophilia
- in patients with congenital FVII deficiency
- in patients with Glanzmann's thrombasthenia with antibodies to GPIIb-IIIa and/or HLA, and with past or present refractoriness to platelet transfusions.

Since rFVIIa became available, several case stories have reported its successful use as a haemostatic agent in non-haemophilia patients; and the need for randomised controlled clinical trials has been mentioned. Novo Nordisk has recognised the need to establish clinical guidance and when possible, regulatory approvals for indications outside the current approved indications. Accordingly, since 2000 Novo Nordisk has undertaken a clinical development programme for rFVIIa covering a wide range of indications.

Overview of clinical trials outside haemophilia

Overall, there are 12 multinational trials including more than 1800 patients worldwide (Figure 1). In addition, there are a

Table 1. Worldwide trials on the use of rFVIIa outside haemophilia

Coagulation factor deficiency		Platelet disorders		General haemostasis	
Single factor	*Multiple factors*	*Low platelet count*	*Defective platelets*	*Surgical bleeding*	*Spontaneous bleeding*
	Upper gastrointestinal bleeding*°	Stem cell transplantation°		Liver resection°	Intracerebral haemorrhage°
	Orthotopic liver transplantation*°			Spinal surgery	Trauma°
	Liver resection*°				
	Severe bleeding in oral anticoagulant treatment				

**patients with cirrhosis. °Trial concluded.*

Table 2. Local trials on the use of rFVIIa outside haemophilia.

Coagulation factor deficiency		Platelet disorders		General haemostasis	
Single factor	*Multiple factors*	*Low platelet count*	*Defective platelets*	*Surgical bleeding*	*Spontaneous bleeding*
Factor XI deficiency (UK)°		Haemorrhagic cystitis (USA)		Prostathectomy° (The Netherlands)	
				Pelvic reconstruction (UK)°	
				Burns revision (Denmark)°	
				Cardiac surgery in neonates (Australia)	

°Trial concluded.

number of local trials (Figure 2). Many trials have been in areas where no previous haemostatic trials have been conducted and therefore there have been no validated haemostatic endpoints to be applied to rFVIIa. This has complicated the development programme. All trials are phase II trials and the safety data are being continuously monitored by external data safety monitoring boards. So far, no studies have been suspended or terminated due to safety concerns.

A number of trials have been completed. These include trials in cirrhotic patients with upper gastrointestinal bleeding (240 patients), in patients undergoing orthotopic liver transplantation (83 and 180 patients), in cirrhotic patients undergoing liver resection (235 patients), in patients undergoing liver resection (200 patients), in patients undergoing stem cell transplantation (100 patients), and in trauma patients (283 patients). In addition, two dose escalation safety trials in patients with intracerebral haemorrhage have been completed, and a phase II trial is currently on-going. Furthermore, a trial in patients undergoing spinal surgery is ready to start. There is only one trial, severe bleeding in oral anticoagulant treatment, which has been prematurely terminated due to poor recruitment.

Conclusions

Since 2000, several placebo-control trials on rFVIIa have been initiated in a variety of clinical settings. Many of these trials have now been completed and the publications of the results are on-going. Follow-up trials will be initiated in some areas and trials in new areas are under evaluation. So far, no trials have raised a safety concern.

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Congenital factor VII deficiency: clinical picture and management

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A B S T R A C T

Among the rare inherited coagulation disorders, factor VII (FVII) deficiency is the most common. Its bleeding manifestations are heterogeneous, ranging from asymptomatic to fatal. Life-threatening bleedings such as bleedings in the central nervous system (CNS) and gastrointestinal tract (GI) occur early in life; whereas haemarthrosis and muscle haematoma occur when infants start to walk. In female patients, menorrhagia requires particular attention in terms of prevention and treatment. There is a poor correlation between FVII:C levels and bleeding tendency, and FVII:C can hardly be used to predict the severity of bleeding. We therefore propose a classification of disease severity based on clinical manifestations. The severe form is characterised by life-threatening and crippling bleedings (CNS, GI and joint bleeds). The moderate form has three or more bleeding symptoms, and the mild form has one or two. Factor FVII has a short half-life and its concentration in the circulation is low (350-450 ng/mL or 10 nM). The low concentration makes the preparation of plasma-derived concentrates difficult, and also makes fresh frozen plasma (FFP) unsuitable for prolonged administrations. Conventional treatments for FVII deficiency include FFP, plasma-derived FVII concentrates and prothrombin complex concentrates. Recently, activated recombinant coagulation factor VII (rFVIIa) has been licensed in Europe for the treatment of FVII deficiency. In children and in case of prolonged administrations, rFVIIa (15-30 µg/kg body weight) should be used as first-line treatment. Factor VII deficiency does not offer protection against thrombosis. Our group has observed 11 cases of FVII deficiency who had experienced thrombotic episodes. Most were venous thromboses associated with surgical interventions or replacement therapy. The incidence of the development of FVII inhibitors is unknown because of the lack of a prospective screening. A web-based, prospective, observational study (*www.irf7therapy.org*) has been established to collect and evaluate efficacy and safety data from all types of treatment.

Key words: factor VII deficiency, recombinant factor VIIa, registry, bleeding, surgery, efficacy

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Congenital factor VII (FVII) deficiency is a rare coagulation disorder. Bleeding severity, even among homozygous cases, is heterogeneous ranging from asymptomatic to life-threatening. In most cases, however, the bleeding is mild involving mucous membrane. There is a poor correlation between bleeding severity and the levels of FVII:C.

In circulating blood, FVII is present in two forms, 99% (400 ng/mL or 10 nM) in a zymogen form and 1% (4ng/mL or 0.1 nM) in an activated form (FVIIa). In terms of coagulation, tissue factor (TF) which is the cofactor/receptor of FVIIa is the rate-limiting molecule. *In vitro* and *ex vivo* data show that a level of FVIIa at 0.01 nM may be sufficient to initiate coagulation.

The current routine assay of FVII:C is inaccurate, and insensitive to low levels (2-3%) of FVII zymogen and physiologic levels of FVIIa (2-10 ng/mL). This is due to the excess of TF used in the assay that causes an activation which is far from physiological. Consequently, the classification of disease severity based on FVII levels is not useful. We therefore propose a classification based on clinical symptoms. Most of the data reported in this publication derive from the International Factor VII Deficiency Study Group database. Moreover, clinical data on the use of activated recombinant coagulation factor VII (rFVIIa; NovoSeven®, Novo Nordisk A/S, Bagsvaerd, Denmark) in this group of patients will be discussed.

Prevalence of bleeding symptoms in patients with FVII deficiency

Based on 315 patients with clinical symptoms in the International Factor VII Defi-

ciency Study Group database, nose bleed, easy bruising and gum bleed are the most common, followed by joint bleed, muscle haematoma and GI bleed (Table 1). Furthermore, post-surgical bleed was seen in 23.8%. In female patients in the fertile age, menorrhagia occurred in 62.9%.

Table 1. Prevalence of bleeding symptoms in patients with congenital FVII deficiency (n=315).

Bleeding symptoms	*Prevalence (%)*
Epistaxis	60.9
Easy bruising	45.4
Gum bleeding	30.2
Haemarthrosis	18.4
Muscle haematoma	18.1
GI bleeding	14.1
Haematuria	8.2
CNS bleeding	5.7
Dental extraction	4.1
Cephalohaematoma	1.0
Rectal bleeding	1.0
Haemoptysis, post-partum, retroperitoneal, umbilical and wound bleeding	0.6
Post-surgical bleeding (among surgical patients)	23.8
Menorrhagia	62.9

Classification of severity

We have proposed a classification based on clinical symptoms listed in Table 1. The disease is classified as severe when bleeding symptoms consist of CNS, GI or joint bleed, with or without other bleeding symptoms. Moderate and mild forms are characterised by other bleeding symptoms apart from CNS, GI and joint bleeds; with moderate having three or more symptoms and mild having one or two. From the database, the majority (45%) of patients had a mild form, 25% had a moderate form, and 30% had a severe form.

Significant determinants of bleeding

Multivariate analysis of the database consisting of 514 patients shows that women are at higher risk of bleeding as compared to men and that FVII:C together with the zygosity status is a strong determinant of bleeding. In addition, homozygotes and double heterozygotes are virtually indistinguishable with reference to bleeding risk and severity.

Relationship between genetics and clinical manifestation

Overall, 340 patients were genotyped, and 104 diffeent mutations were found. Among these mutations, 81% were missense mutations, 12% were splicing site, 5% were nonsense, and 2% were small deletion or frameshift. Novel mutations were found in 24 patients.

From the database, patients homozygotes or double heterozygotes presented with bleeding symptoms at an earlier age than patients heterozygotes. Moreover, approximately 60% of patients heterozygotes (n=200) were asymptomatic and 75% of these patients were diagnosed during family studies or because a prolonged prothrombin time (PT) during pre-surgical screening was found.

Bleeding during surgical procedures

From the database, excessive bleeding could be seen in various minor and major surgical procedures (Figure 1). Without replacement therapy, bleeding was seen in 52% of patients; and even with replacement therapy, bleeding still occurred in 27% of patients (Table 2). This implies that the therapy used might not be adequate and a standardised therapy is required.

Treatment options for FVII deficiency

Currently, several products are available for the treatment of bleeding in FVII deficiency. These include FFP, viral-attenuated FFP, prothrombin complex concentrates (PCCs), plasma-derived FVII, and rFVIIa. Table

Table 2. Frequency of excessive bleeding during surgical procedures with and without replacement therapy.

Class of severity	*Without replacement therapy*	*With replacement therapy*	*Odds ratio (confidence interval)*
Severe and moderate	12/23 (52.2%)	24/89 (26.9%)	3 (1.2–7.6)
Mild	14/36 (38.9%)	12/44 (27.3%)	1.7 (0.7–4.3)

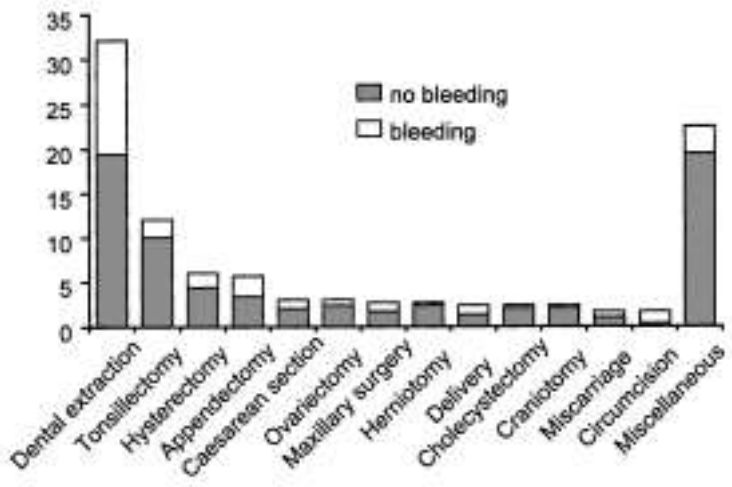

Figure 1. Bleeding during surgical procedures in patients with FVII deficiency.

Table 3. Treatment options for FVII deficiency.

Products	*Potency (IU/mL)*	*Advantages*	*Disadvantages*
FFP	1	Cost & availability	Risk of viral transmission Circulatory overload Unsuitable for surgery
FFP (viral attenuated	1	Reduced risk of viral transmission	Circulatory overload Unsuitable for surgery
PCCs	5-10	Reduced risk of viral transmission Suitable for surgery	Risk of thrombosis Other vit.K-dependent clotting factors
Plasma-derived FVII	20-30	Reduced risk of viral transmission Suitable for surgery	Other vit.K-dependent clotting factors
rFVIIa	> 25,000	Very effective (very low doses required)	Cost

3 summarises the potency, advantages and disadvantages of each product.

Recombinant FVIIa appears to be the treatment of choice as it contains only the missing factor in a concentrated form. As a recombinant product, rFVIIa does not have the risk of blood-borne infections. The average dose required to normalise PT INR is 20 µg/kg.[1] The current recommended dose is 15-30 µg/kg. Children and patients who have not been exposed to plasma-derived products should receive rFVIIa as first-line treatment.

Thrombotic events in patients with FVII deficiency

Patients with FVII deficiency are not protected from thrombosis. There are 11 such cases with venous or arterial thrombosis reported in the literature[2-13] In our database, there were 11 cases with thrombotic episodes, of which 9 cases were previously published.[14] Among these 9 thrombotic events, 7 cases were triggered by surgery and/or replacement therapy whereas the remaining 2 cases were apparently spontaneous. Of the 9 cases, 2 were arterial, 6 venous, and 1 was disseminated intravascular coagulation (DIC). There was no relationship between FVII gene mutations nor bleeding manifestations and thrombotic events.

The *Seven Treatment Evaluation Registry* (STER) programme

The STER programme is a prospective, observational study on the safety and efficacy of current treatment modalities for patients with congenital FVII deficiency. The STER programme is an on-line registry (*www.irf7therapy.org*) which collects data on any type of replacement therapy used in spontaneous bleeding episodes or surgical procedures. A stringent efficacy evaluation will be applied and all adverse events will be recorded. Moreover, the evaluation of *in vivo* recov-

ery of FVII and inhibitor assay will be centralised. Further information can be obtained from Professor Guglielmo Mariani at *gmariani@cc.univaq.it* or *assister@targetseven.org*.

Conclusions

A classification of the severity of congenital FVII deficiency has been proposed based on clinical symptoms. Several treatment options are currently available. However, the overall efficacy and safety of these treatments are unknown. The STER programme has been established with the aim of collecting and evaluating data from all treatment modalities. The data generated will help standardise the treatment in patients with congenital FVII deficiency.

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Prophylactic use of rFVIIa in congenital factor VII deficiency: mechanism of action

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A B S T R A C T

Three patients with congenital factor VII (FVII) deficiency were treated with activated recombinant coagulation factor VIIa (rFVIIa). Two patients with frequent acute severe bleedings received prophylactic treatment with 1.2 mg rFVIIa two to three times a week. This treatment decreased both the frequency and severity of bleedings as compared to previous treatment with plasma-derived FVII. The third patient with moderate bleedings received rFVIIa on-demand; and the efficacy was similar to that of plasma-derived FVII. In light of the short half-life of rFVIIa, we were interested in the mechanism of action of its prophylactic effect in these patients. We therefore studied the effect of rFVIIa on platelet activation and thrombin generation *in vivo*. Flow cytometric analysis showed that rFVIIa presensitised platelets. Unlike thrombin generation which reached a maximum level immediately after rFVIIa injection, platelet presensitisation occurred at a delayed phase. Further *in vitro* studies using washed platelets revealed that presensitisation was observed immediately after the addition of rFVIIa to the platelets implying that the process *in vivo* is more complex than *in vitro*. The presensitised platelets showed faster activation by thrombin as compared to control platelets. Moreover, the process was not inhibited by a selective thrombin inhibitor, hirudin. From these data, we propose that FVII deficiency is a coagulopathy that might be explained in part by impaired platelet function. The effect of rFVIIa on platelets may account for its efficacy when used prophylactically.

Key words: factor VII deficiency, prophylaxis, recombinant factor VIIa, bleeding

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In normal haemostasis, upon vascular injury tissue factor (TF) is exposed; and subsequently it binds to activated factor VII (FVIIa) in the circulation. The TF-FVIIa complex leads to an initial formation of thrombin and small amounts of fibrin. The initial thrombin formed also activates platelets. Thrombin generation is accelerated by the activation of FXI and the tenase complex resulting in higher amounts of thrombin formation. This so called *Bouma loop* results in increased thrombin levels, and only these high levels of thrombin activate thrombin-activatable fibrinolysis inhibitor (TAFI) which is an inhibitor of fibrinolysis. Activated recombinant coagulation factor VII (rFVIIa; NovoSeven®, Novo Nordisk A/S, Bagsvaerd, Denmark) is able to generate sufficient amounts of thrombin to directly regulate fibrin formation as well as activation of TAFI. So far, it is not known how rFVIIa regulates platelet activation. It might well be regulated via thrombin or via a direct activation.

We treated three adult patients with congenital FVII deficiency. Patient 1 displayed the most severe phenotype, having a homozygous insertion of a guanine at position Leu13/Gln14 in exon 2. The levels of FVII:Ag and FVII:C in this patient were undetectable (<1%). Patient 2 was a compound heterozygous for a Gln100Arg in exon 5 and an Arg290His transition in exon 8, rendering a FVII:Ag level of 8% and an undetectable level of FVII:C. The Gln100Arg mutation is located in the EGF2 domain that plays a dominant role in binding to tissue factor. The Arg290His mutation is located in the active site of factor VII. Patient 3 was homozygous for the Gln100Arg transition in the EGF2 domain. The levels of FVII:Ag and FVII:C in this

patient were similar to that in patient 2.

Recombinant FVIIa 20 μg/kg was given prophylactically to patients 1 and 2, twice and three times per week, respectively. In addition, patient 1 also received concomitant treatments with tranexamic acid and oral contraceptive pills; while patient 2 received only oral contraceptive pills. Patient 3 only received rFVIIa on-demand. During the period the patients were treated prophylactically with rFVIIa, the total numbers of bleeding episodes in all three patients decreased as compared to previous treatment with plasma-derived products (total number of bleeding episodes, plasma-derived products vs rFVIIa, patient 1: >160 vs 5, patient 2: 20 vs 12, patient 3: 15 vs 10). Patient 1 had been treated prophylactically with rVIIa for five years and patient 2 for two years. The results were published in detail elsewhere.[1]

Despite the short half-life of rFVIIa, data from these patients show that prophylactic use of rFVIIa is safe and efficacious. However, it is unresolved why a prophylactic treatment two to three times a week is efficacious as the documented half-life of rFVIIa is only two to three hours. We therefore investigated the mechanism of action of rFVIIa in these three patients with congenital FVII deficiency. The study was carried out both *in vivo* and *in vitro*. This manuscript summarises the key findings.

In vivo study

The half-lives of rFVIIa in each patient as measured by FVIIa:C and FVII:Ag were as follows: FVII:C and FVII:Ag, 35 and 82 min, 50 and 102 min, 54 and 103 min, for patients 1, 2, and 3 respectively). These half-lives appear to be much shorter than previously reported, due to the higher clearance in our patients. So far, the discrepancy between the activity and antigen levels is unresolved.[1]

Recombinant FVIIa shortened the time to peak thrombin generation. This effect abated within one to two hours, which was in accordance with the duration that coagulation time was corrected by rFVIIa.

We further studied the effect of rFVIIa on platelet activation. Using flow cytometry and enzyme-linked immunosorbent assay (ELISA), we measured activated αIIb/β3 integrin, CD63, P-selectin, and β-thromboglobulin (β-TG) and platelet factor 4 (PF4) release. The results indicated that rFVIIa induced platelet preactivation showing by the increased levels of activated αIIb/β3 integrin but not P-selectin and CD63. Furthermore, rFVIIa increased the levels of β-TG and PF 4; and this effect lasted for more than 24 hours except for patient 3 in which it lasted for only 4 hours.

From this *in vivo* study, we conclude that rFVIIa induces mild activation, so-called preactivation of platelets, and the effect on β-TG appears earlier than that on activated αIIb/β3 integrin. However, there is no effect on platelet number i.e. does not induce thrombocytopenia. We therefore carried out a further *in vitro* study to investigate whether rFVIIa leads not only to preactivation but also to presensitisation of the platelets resulting in an effective thrombin-dependent platelet-activation.

In vitro study

Washed platelets from normal volunteers were incubated with various combinations of rFVIIa, active site-inhibited rFVII (rFVIIai), thrombin, and hirudin. The changes in activated αIIb/β3 integrin levels were detected using flow cytometry. We found that rFVIIa alone increased the levels of activated αIIb/β3 integrin in a dose-dependent manner. This phenomenon was not inhibited by hirudin indicating that the process is thrombin independent. Moreover, the active site of FVIIa was essential for this process as replacing rFVIIa with rFVIIai in the test system eliminated the enhanced response. Furthermore, we found that platelet preactivation led to platelet sensitisation resulting in faster response to thrombin.

From this *in vitro* study, we conclude that preactivation of platelets by rFVIIa occurs very rapidly. The process is rFVIIa-dose dependent but thrombin independent. Moreover, the active site of rFVIIa is an absolute requirement. The preactivation sensitises platelets and may result in more rapid platelet activation.

Conclusions

Upon administration of rFVIIa to patients with congenital FVII deficiency, the coagulation process is enhanced immediately and lasts up to 2 hours. Furthermore, the levels of β-TG are also increased immediately lasting up to at least 7 hours. In contrast, platelet presensitisation begins 2 hours after rFVIIa injection and remains evident at 24 to 48 hours. We have found that platelet presensitisation occurs immediately after the addition of rFVIIa *in vitro.* This indicates that the process is more complex *in vivo* than *in vitro.* The duration of platelet presensitisation effect after rFVIIa administration *in vivo* is unknown and we are planning to investigate this issue. Moreover, the effect of other plasma-derived products will also be studied.

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Rare coagulation disorders

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A B S T R A C T

Rare coagulation disorders (RCD) are typically orphan diseases, relatively neglected until recently by health care providers, advocacy organizations and pharmaceutical companies. They include deficiencies of fibrinogen, prothrombin, factors V, combined V and VIII, VII, X, XI and XIII, and together have a global prevalence of around 3% to 5%. However, this prevalence rises greatly in countries where consanguineous marriages are more common. The clinical presentation of patients affected by RCD varies from mild-to-moderate bleeding episodes to potentially serious or life-threatening haemorrhages. However, they are usually less severe than haemophilia A and B, because life- and limb-threatening symptoms are less frequent. Due to the rarity of each factor deficiency, purified factor concentrates are not as readily available as they are for the haemophilias. Following 8 years of clinical and laboratory experience gained from treating RCD patients from around the world, an International RCD Registry has been set up. It aims to develop a registry of mutations to help standardize laboratory methods for phenotypic diagnosis of RCDs, thus fostering the development of orphan drugs for deficiencies with no available therapeutic concentrate.

Key words: rare coagulation disorder, RBDD, International RCD Registry, factor.

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Haemophilia A and B combined with Von Willebrand's disease represent the majority (95% to 97%) of inherited bleeding or coagulation deficiencies.[1] The remaining bleeding disorders are the group of deficiencies classed as rare coagulation disorders (RCD) with a global prevalence of 3% to 5%.[2,3] The RCD include fibrinogen, prothrombin, factor V (FV), combined factors V and VIII (FV/VIII), factor VII (FVII), factor X (FX), factor XI (FXI) and factor XIII (FXIII) deficiencies. These disorders are typically orphan diseases, and were until recently relatively neglected by health care providers, advocacy organizations and pharmaceutical companies.

The clinical presentation of patients affected by RCD varies from a mild to moderate bleeding tendency to potentially serious or life-threatening haemorrhages. Their prevalence varies greatly from 1:500,000 for FVII deficiency – one of the most common RCD – to one in 2-3 million for FXIII deficiency – one of the rarest.[1,4]

The transmission of RCD is autosomal recessive, therefore two heterozygous partners would produce normal children in 25% of cases, heterozygous children in 50% of cases and affected homozygous children in 25% of cases (Figure 1). As these RCD are inherited in a recessive autosomal manner, the practice of consanguineous marriages increases population prevalence.[3] Thus, a marked increase in occurrence of RCD is seen in the Middle East, Southern India and North Africa, where consanguineous marriages are more common. In some cases this increase in prevalence can be by as much as 10- or 20-fold.[3,5] Indeed, the prevalence of some RCD can rise to match that of haemophilia B in some areas, creating a real demand for an improvement in the diag-

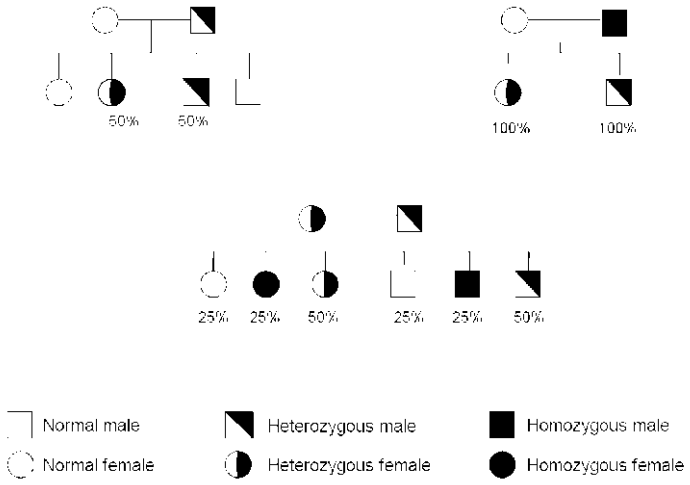

Figure 1. The genetic transmission of RCD.

nosis and treatment of some of these conditions.

The global patterns of prevalence are very much affected by the ethnic populations living in specific areas. Taking three countries, Iran, Italy and the United Kingdom, as an example, while there is a similar prevalence of haemophilia A and B in all three countries (because of the chromosome X-linked transmission), there are marked variations in the prevalence of specific RCD. Thus, in the UK there is a far greater prevalence of FXI deficiency as a result of the larger Jewish population than in either Italy or Iran. Likewise, the prevalence of other RCD is much greater in Iran than in either Italy or the UK.

Nevertheless, despite the increased prevalence in some countries, the type and severity of bleeding symptoms, the underlying molecular defects and the actual management of bleeding episodes are not as well established as for haemophilia A and B as a consequence of the relative rarity of these deficiencies in the West. Thus, one of the key drivers for studying RCD in more depth is to address these issues in order to improve prognosis for patients with these rare deficiencies.

The Rare Bleeding Disorders Database

Methods

With this setting as a background, the Milan Centre for Haemophilia chose to focus on the molecular, clinical and therapeutic aspects of RCD in a large-number patient series using data collected from different parts of the World over the past 8 years. Patient records were obtained from countries with increased prevalence in order to ensure a faster recruitment rate and to increase the chances of finding some founder gene effects. Inherited deficiencies of FXII, prekallikrein and high molecular weight kininogen were excluded as these are not associated with a bleeding tendency.[1]

Tailored questionnaires were completed by a country-specific physician who obtained the full medical records and the necessary samples to perform blood analyses. Phenotype analysis was conducted using prothrombin time and activated partial thromboplastin time to confirm the molecular biology of the RCD. Genotype analysis was also performed using polymerase chain reaction to amplify DNA samples from the coding regions of genes as well as 300-500 base pairs from the 3' and 5' tail regions. In RCD patients, genotype analysis usually reveals that patients are homozygotes or compound heterozygotes and symptomatic. Heterozygotes (parents and children of the probands) have approximately half-normal levels of coagulation factors and are usually asymptomatic; the reason for this is that these patients often have semi-conserved levels of coagulation factors.

Molecular defects in RCD usually stem from defects in the structure of genes that code for proteins involved in the relevant coagulation factors themselves. There are two notable exceptions. The first relates to combined FV/VIII deficiency, where a genetic coding error in the proteins involved in the intracellular processing of FV and FVIII exist[6,7] — potentially explaining why the combined FV/VIII deficiency is not as severe as either deficiency alone which have a different aetiology. In the second case, the combined deficiency of vitamin K dependent factors are the result of mutations in two separate genes (VKOCR1 and γ-glutamyl carboxylase gene) that are both involved in the carboxylase reaction.[8,9]

Table 1. Recruitment of patient records for the analysis.

Country	*Patient Records*	*Country*	*Patients Records*
Iran	95	Pakistan	2
Italy	70	Hong Kong	1
India	18	Saudi Arabia	1
Turkey	15	Sweden	1
United Kingdom	4	Thailand	1
Slovenia	3	Lebanon	1

Baseline demographics

Data for 212 RCD cases from Europe, the Middle East and Asia were obtained (Table 1). The majority of patients, 47%, were recruited from the Middle East, with 43% from Europe and 9% from India and Asia. All the RCD under study were represented by the patient group. Severe defects, classified as having less than 2% of a factor's activity, were present in nearly 75% of all specific factor deficiencies.

Clinical manifestations

In haemophilia A the most severe bleeding symptoms are joint and muscle bleeding episodes, which are seen in 75% and 81% of patients, respectively. Post-operative bleeding also has a high prevalence (75%) along with oral cavity bleeds (90%), although these are not classed as fatal bleeding complications. Interestingly, nose bleeds have a low frequency (15%), and umbilical cord bleeding was not seen.[10]

In contrast, RCD appear very much less severe than haemophilia A and B, because the specific life- and limb-threatening symptoms such as joint, muscle, central nervous system (CNS) and gastro-intestinal tract (GI) haematomas are definitely less frequent. The most severe symptoms were found in patients with FX, FXIII, afibrinogenaemia, and FII deficiency, with a relatively high frequency of joint and muscle bleeding.

Among patients affected by RCD, FX deficiency patients had the highest reported rates of joint (69%) and muscle (66%) bleeding episodes. Conversely, the lowest frequencies of these two bleeding symptoms were, interestingly, in the combined FV/VIII deficiency with joint (25%) and muscle (7%) bleeding episodes. Severe manifestations such as CNS and GI bleeding were relatively rare for all defects, bar FX deficiency, with a high frequency of GI bleeding (38%), FVII and FXIII deficiency with 17% and 25% CNS bleeding, respectively. Central nervous system bleeding episodes, frequent miscarriages and umbilical cord bleeding were present in FXIII deficiency patients.

Of note, was the fact that the less severe bleeding symptoms — such as nose bleeds and umbilical cord bleeding — occurred with an increased frequency compared with haemophilia A patients. For example, nose bleeding — relatively uncommon in haemophiliacs — as a result of mucosal bleeding, were an unexplained common feature of the RCD with a prevalence range from 32% for FXIII to 77% for the combined FV/VIII deficiency. Umbilical cord bleeding, typical of afibrinogenaemia and FXIII deficiency, was also relatively frequent in cases of prothrombin, FV and FX deficiency.

It remains unclear why most patients with RCD present nose bleeding. Although an increased rate of nose bleeding might be expected in deficiencies such as FV and fibrinogen — where the factors are usually present on the platelets — it would not be expected in the FII, FVII and FX deficiencies.

Of the female patients, approximately 50% were menorrhagic. In these patients, menorrhagia occurred without any particular differences between the RCD studied, except for FXI where the frequency seemed to be lower. More and larger studies are required to establish these bleeding symptoms. Excessive bleeding was seen in patients undergoing surgery — e.g. 84% of cases with FXIII deficiency vs. 75% of cases with haemophilia. In these cases, the use of replacement blood was a mandatory requirement. Indeed in severe RCD cases — even those who may not be *bleeders* — the use of prophylactic therapy prior to surgery may be a wise precaution as a patient's reaction to surgical intervention may not be predictable, especially in those patients without any previous surgical history.

Symptoms of hypoprothrombinaemia were extremely different compared with the haemophilias, with no patients recording thrombin levels of less than 4% activity. To our knowledge, no case of aprothrombinaemia have been reported, suggesting that this deficiency is incompatible with life.[11]

As noted, the combined FV/VIII deficiency had one of the lowest rates of severe bleeding symptoms. Indeed, it would appear from the data gathered in this survey that this combined deficiency does not lead to a worsening of haemostasis compared with each deficiency alone.[12] For RCD in general, the usual relationship between factor activity plasma levels and severity and frequency of clinical manifestations seen in the haemophilias would appear not to be true, with the relationship between factor levels and bleeding tendency being poor — especially for FVII and FXI deficiencies.

Genotype analysis

Genotype analysis was carried out in most of the reported cases and revealed 108 mutations (Table 2). Of these, only 30 have been previously reported in the literature. In effect, 72% of the mutations discovered were previously unknown. Following complete geno-

Table 2. Results of the genotype analysis revealed 108 mutations of which 78 were new and thus previously unreported or unknown about.

Factor deficiency	*Families**	*Mutations found*		
		Already known	*New*	*Total*
Afibrinogenemia	33	3	14	17
Factor II	14	1	11	12
Factor V	31	3	12	15
Factor V/VIII	10	3	1	4
Factor VII	47	13	18	31
Factor X	31	5	14	19
Factor XI	6	-	2	2
Factor XIII	17	2	6	8

**10% of families are still in progress.*

type analysis, it was important to try and identify any founder mutations in this pool of genes, as this could have a significant impact on the diagnosis of patients and genetic counselling — especially in countries where there might be a high prevalence of a particular gene defect.

Preliminary analyses revealed that four of the gene mutations might be the result of a specific founder gene. The first is $Arg^{77}His$ found in FXIII deficiency. This mutation was present in 50% of the Iranian population enrolled in the study and was not seen in any other patients with FXIII deficiency.[4] The second was a mutation in the $Gln^{100}Arg$ amino acid in patients with FVII deficiency. This mutation was only found in European cases and corroborated data previously reported by other groups.[13,14] The third was a $Met^{1}Thr$ mutation in LMAN1 gene identified in Italian families with combined FV/VIII deficiency only. Mutations in this gene had also been reported by another group and matched these findings.[15,16] The final potential founding mutation was identified in four families with FX deficiency, two coming from Northern West Iran and two from Southern East Turkey. Interestingly one other group had reported this $Gly^{222}Asp$ mutation, although in this case it was in a family living in Germany.[17,18] However, further investigation revealed that this family was also of Turkish origin. An extension of data from the International Registry is required to extract and confirm the reported preliminary genetic analysis by detailed haplotype analysis.

Treatment of RCD

Following the identification of RCD and their possible causes, specific treatment becomes a more realistic possibility and increasing priority. At present there are no specific guidelines for the treatment of patients with RCD, specifically for prophylaxis. Thus, the mainstay of therapy consists of using the most readily available viral-inactivated and purified plasma blood product(s) containing the missing factor(s) in an attempt to maintain as normal a haemostasis as possible. This will usually be either purified concentrates, prothrombin complex (FII, FVII, FIX, FX) concentrates, cryoprecipitates, or fresh frozen plasma. Purified factor concentrates for RCD are not as readily available as they are for haemophilia A and B, furthermore, because RCD are rare, the demand for replacement factors is relatively low.

Dosages and frequency of treatment with replacement factors varies depending on the extent or severity of the particular deficiency, which is assessed by haemostasis levels of the deficient factor and the type of bleeding episode the patient displays. Under such conditions, it is imperative to be able to guarantee the safety of the replacement materials in order to protect patients from further possible harm through infection (e.g. HIV or hepatitis).

Table 3 summarizes the most commonly used therapies for the different RCD. Prophylaxis regimens often vary depending on the half-life of the replacement factor. For example, with a half-life of 10-14 days, FXIII could be administered every 2-3 weeks – i.e. twice monthly – while FVII deficiency would require once- or twice-daily dosing.

The availability of concentrates also plays an important role in the treatment of patients: a few single-factor, plasma-derived concentrates are available for fibrinogen, the prothrombin complexes, FVII, FXI and FXIII. However, at a recombinant level, only one replacement factor is available – activated recombinant coagulation factor VII (rFVIIa; NovoSeven®, Novo Nordisk A/S, Bagsvaerd, Denmark). Furthermore, this has only been registered in Europe and not yet in the USA.

Unfortunately no FV concentrates are available yet, making plasma transfusions the only possible treat-

Table 3. Recommended therapy for RCD.

Deficiency	*Target levels*	*Plasma half-life*	*Treatment*
Fibrinogen	50 mg/dL	4-5 days	Cryoprecipitate (5-10 bags) SD treated plasma (15-30 mL/kg) Fibrinogen concentrates (20-40 mg/kg)
Prothrombin	20-30%	3-4 days	SD treated plasma (15-20 mL/kg) FIX concentrates and PCC (20-30 U/kg)
Factor V	10-15%	36 hrs	SD treated plasma (15-20 mL/kg)
Combined Factor V/VIII	10-15%	36 hrs (FV); 10-14 hrs (FVIII)	As Factor V
Factor VII	10-15%	4-6 hrs	FVII concentrates (30-40 mL/kg) FIX concentrates and PCC rFVIIa (15-30 µg/kg every 4-6 hours)
Factor X	10-20%	10-40 hrs	SD treated plasma (10-20 mL/kg) FIX concentrates and PCC (20-30 U/kg)
Factor XI	15-20%	60-65 hrs	SD treated plasma (15-20 mL/kg) FXI concentrates (15-20 U/kg)
Factor XIII	2-5%	10-14 days	Cryoprecipitate (2-3 bags) SD treated plasma (3 mL/kg)

FXIII concentrates (10-20 U/kg every 5-6 weeks for prophylaxis and 50 U/kg for high hemorrhagic events). PCC = prothrombin complex concentrates; SD = solvent/detergent

ment option in these patients. In patients affected by combined FV/VIII deficiency, a mild deficiency of FVIII could be correct by desmopressin acetate (DDAVP) infusion. Another aspect that needs care when treating patients with prothrombin complex deficiencies is the risk of thromboembolism.[19] Thus when FIX levels are over half of normal levels, additional monitoring is required to prevent any unwanted thromboses.

Non-transfusional therapies also have a role to play. These treatments are antifibrinolytic amino acids — such as epsilon aminocaproic acid and tranexamic acid — which can be used alone or in combination with replacement therapies in less severe cases of mucosal tract haemorrhages. Oestrogen-progesterone preparations also offer treatment options for women who might suffer iron-deficiency anaemia as a result of increased blood loss during menorrhagia.

Future steps

In some countries the prevalence of RCD increases in non-Western populations to a rate equal to that of haemophilia B. This makes the correct identification and diagnosis of RCD important in the treatment of these conditions. Furthermore, the use of genotyping can help reduce RCD through genetic counselling, carrier detection and prenatal diagnosis on DNA samples obtained by chorionic villus sampling or amniocentesis.

In order to aid this process, the Rare Bleeding Disorders Database (RBDD) has been established. Hosted on the Angelo Bianchi Bonomi Centre's server, the RBDD has been set-up through the collaboration of several haemophilia and RCD centres around the globe as an internet tool to share patient data.

The database collects laboratory data for phenotype and genotype tests, as well as clinical data derived from medical reports — such as family history, concomitant disease and treatment and prenatal diagnosis. In making this information available to a wider audience of researchers, the RBDD aims to decrease the time between data production and the implementation of study results in clinical practice with the aim of improving our patients' outcomes.

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Age at first exposure is a risk factor for inhibitor development

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A B S T R A C T

The use of prophylactic therapy with factor VIII has enabled patients with haemophilia A to lead relatively normal lives. However in the presence of inhibitors, the increased frequency of bleeding episodes and the development of target joints complicates treatment. The use of factor VIII itself plays an important role in the development of inhibitors. More specifically, age at first exposure and frequency of exposures appear to affect whether or not patients develop an inhibitor. In a prospective study of a cohort of patients registered at birth the effects of age at first exposure were analyzed. Patients that developed an inhibitor did so after a mean of 22 exposures. There appeared to be no significant difference between patients with and without the intron 22 gene inversion. Mean age for inhibitor patients was significantly lower than for the patients without an inhibitor ($p < 0.04$). Because these observations might have an important impact on the treatment of small children it is very important to confirm the results in other studies. Likewise the effects of patient and product related factors on inhibitor development should be assessed in large prospective controlled studies to investigate possible treatment paradigms that might avoid or reduce the development of inhibitors.

Key words: age, development, exposure, PUP, prophylaxis, inhibitor.

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Through the use of prophylactic therapy, patients with severe haemophilia can now expect a normal life expectancy if they do not develop inhibitors. However, when high-titre inhibitors develop, there is an increase in bleeding episode frequency and development of target joints. In these patients, immune tolerance therapy is undertaken with the complication that a central line is needed for venous access.

A review of the development of inhibitors by Wight and Paisley,[1] reported a cumulative inhibitor incidence of 0% to 39% from several studies, stemming from a variety of patient, therapy and assay-related influences. However, this wide range of incidence may be a result of the data being derived from a number of small series with a maximum of only 100 patients, and a variable frequency of testing (between every 3 months and every 12 months). In addition, patients with moderate and mild cases of haemophilia were included. In recombinant studies with previously untreated patients (PUP) more transient and low-titre inhibitors were reported. This is probably due to a higher frequency of testing in these study populations. Some of the publications in the review also reported a low incidence of inhibitors in patients treated with low purity plasma products.

Other biases of PUP studies which affect the results are the inclusion of patients with short follow-up, by not using Kaplan-Meier, and that patient selection and inhibitor definition varies.

While much remains to be clarified about inhibitor development, investigations into the factors that affect their development continue. When looking at factors that might possibly influence inhibitor development, there is a need to differentiate

between those factors that are product related and those that are patient related.

Factors influencing inhibitor development

Product related factors

The majority of research has focused on product related factors. Differences in inhibitor development in recombinant versus plasma products still require further investigation. Likewise the purity of products, their protein contents, and factors relating to their purification process vary. As more results from research into these areas become available, the development of improved products and purification processes should help clarify the mechanisms of inhibitor development.

Patient factors

In the case of patient related factors, these can be divided into genetic and environmental factors. By-and-large, genetic risk factors are extensively addressed in ongoing studies, but little attention is paid to non-genetic risk factors.

Included under the mantle of genetic factors are both severity of disease and gene defects. In the case of the latter, data on intron 22 inversions, stop codons and nonsense mutations that cause complete absence of the factor VIII protein,[2,3] have been related to higher incidences of inhibitor development in haemophilic patients, although more information is needed. Likewise, other inherited factors, such as human leukocyte antigen, may also play a role.

Although a high incidence of inhibitors has been reported in African-Americans,[4,5] data from large prospective studies and retrospective registry analyses are missing to confirm this finding. Data from the Malmö International Brother Study (MIBS) reported a higher incidence of inhibitor development in haemophilic brothers of patients with a positive inhibitor history,[5] supporting the view that a genetic influence other than the haemophilia gene defect is implicated in inhibitor development.

In terms of environmental risk factors, the type of bleeding episode (e.g. location and severity) and cause (e.g. surgery or trauma) are suspected risk factors for inhibitor development. Immunological risk factors including infections, allergies, immunizations, breast-feeding and medication (antibiotics, corticosteroids) may also have an influence.

Combined product and patient factors

A third group of factors that are under increasing scrutiny are combination factors of such as age at first treatment, combined with start of prophylaxis as well as dose and frequency of treatment. One risk factor in particular, age at first exposure to factor VIII, has been shown to be related to the occurrence of inhibitors. Several retrospective reviews of single institution cohorts have found a inverse relationship of age at first exposure and the presence of inhibitor.[6,7]

Recombinant studies in PUPs

Data from three registration studies of recombinant therapy in PUPs have provided valuable information about inhibitor development.[8-10] Incidence of inhibitors in these trials ranged from 28% to 33%. In patients with a high-titre, incidence was lower, although in the ReFacto study, incidence reached nearly 16%. This however may have been driven by the lower titre definition of > 5 BU compared with 10 BU in the Kogenate and Recombinate studies.

Median exposure to medication prior to inhibitor development was 9-12 days across the three studies and median age at first exposure was 9 months (range 7.5 to 10 months). In the ReFacto study it was also noted that age at first exposure (7.5 months) was lower in those who developed inhibitor compared with those who did not.

Several studies have examined the effects of long-term prophylaxis in haemophiliac patients. Two European cohorts in particular, one in Sweden (established in 1992) and one in the Netherlands (van Creveldkliniek – established in 1997), have patient records dating back to very early in patients' lives, i.e. mostly from birth, providing a robust long-term database.

The data from the two groups are striking in their similarities. Records from 100 and 104 patients with severe haemophilia A are on file in the Swedish and Dutch registries, respectively, with inhibitor present in 19 (19%) and 19 (18%) patients, respectively. Of these, 11 and 12 patients, respectively, had high antibody titres of > 5 BU. These data suggest that incidence of inhibitor development is relatively low in these patients; an effect which may be the result of over 25 years of prophylaxis.

Age and the development of inhibitors

In 1994, Bray et al. posed the question of whether a given amount of recombinant factor VIII administered over different time intervals, might affect the development of inhibitors in haemophiliac patients.[9] In answer to this, Lorenzo and colleagues subsequently demonstrated a statistically significant difference in the incidence of inhibitors in children treated prior to 6 months of age (41%); between 6 and 12 months of age (29%); and later than 1 year (12%) ($p = 0.03$).[6]

Van Creveldkliniek cohort

The observations of Lorenzo *et al.*[6] were corroborated following a retrospective review of the Dutch van Creveldkliniek centre's experience.[7] The cohort consisted of 104 severe haemophilia A patients who were born between 1st January 1975 and 1st March 2003. Patients were only included if they were registered in the centre before their first exposure day, and if data on their first 50 exposures were available at the time of analysis.

Inhibitor screening was initially performed at least twice yearly in the first 4 years of the cohort's establishment. However, for the past 25 years the frequency was increased to at least four times a year. All inhibitor assays were performed in one laboratory using the Bethesda assay which was modified from > 1.0 BU to > 0.3 BU following the introduction of the Nijmegen modification in December 1996. The centre defined the presence of inhibitor as a patient having two sequential positive titres combined with a reduced recovery time in order to ensure true presence of inhibitor. Total cumulative exposures ranged from 35 to over 1,000. Of the 104 patients included in the analysis 19 patients (18%) developed an inhibitor to factor VIII.

Of the 19 patients who developed inhibitor, the mean number of exposures prior to inhibitor development was 22 (range 8-53). Inversion of intron 22 was not related to a higher chance of inhibitor development, with 63.2% and 54.4% of patients with and without the presence of inhibitor, respectively, displaying this mutation.

Mean age at first treatment was significantly lower in the patients with inhibitor, 245 days vs. 387 days, respectively (p = 0.04) (Figure 1). A joint analysis of the two European cohorts found that the probability of developing inhibitor was higher in patients treated at a younger age. At the same number of exposures, patients aged > 18 months did not develop inhibitor, in contrast to those who started therapy at a younger age (Figure 2).

No difference was seen in the rates of inhibitor development between patients who and had not been breast fed. Although, duration of breastfeeding was higher in patients with inhibitor, 2.2 months vs. 2.0 months, respectively, these differences did not reach statistical significance.

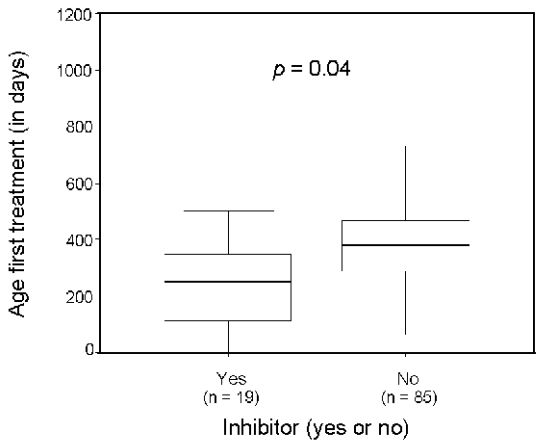

Figure 1. Mean age at first treatment (days) in the van Creveldkliniek cohort. Patients with inhibitor have a lower age at first treatment than those without.

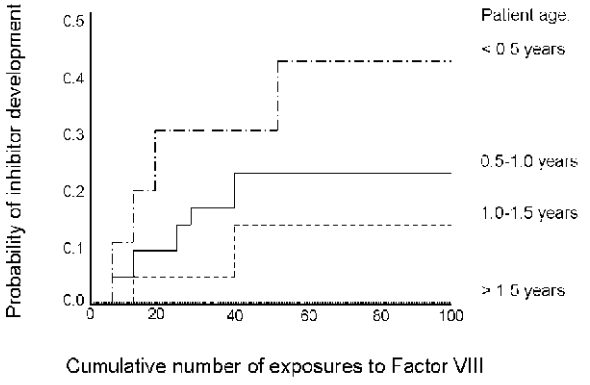

Figure 2. From van den Bom *et al.*[7]

Conclusions

In conclusion, age at first treatment with factor VIII is associated with higher inhibitor development. More specifically, younger age at first exposure increases the probability of developing inhibitor; in the van Creveldkliniek cohort, patients aged 18 months or more at first exposure did not develop factor VIII inhibitor.

In utero treatment is unlikely to prevent inhibitor development. However, while delaying inhibitor development may be possible through delaying first treatment, such an approach may raise ethical concerns. For example, one possible confounder is that younger patients respond better to immune tolerance therapy – thus the development of inhibitor at a later age may cause longer term problems.

Additional data are needed to establish the exact nature of the relationship between age and inhibitor development. Furthermore, more investigations of PUPs are needed in large prospective studies to generate data on factor VIII inhibitor development, with specific emphasis on the contribution of genetic factors, and clarifying the roles of both patient and product related factors and their possible confounding effects.

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Mechanism of action of recombinant factor VIIa in patients with Glanzmann's thrombasthenia

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A B S T R A C T

Activated recombinant coagulation factor VIIa (rFVIIa) was originally developed to treat patients with inhibitor-complicated haemophilia. It also appears to be a safe and effective haemostatic agent in a variety of haemostatic disorders including thrombocytopenia and thrombocytopathia such as Glanzmann's thrombasthenia (GT), a qualitative or quantitative defect of platelet integrin αIIb/β3. Nevertheless, the mechanism of action of rFVIIa in patients with GT is not completely known. It has been proposed that rFVIIa enhances thrombin generation compensating for the impaired haemostasis regardless of the nature of the bleeding disorders. To explain the mechanism of action of rFVIIa in patients with GT, we and others have studied the effect of rFVIIa-mediated thrombin generation on adhesion and aggregation of GT platelets under flow conditions. We have found that rFVIIa-mediated thrombin generation substantially enhances adhesion of GT platelets to subendothelial proteins. This thrombin is generated independently of tissue factor by a mechanism involving direct binding of rFVIIa to the already adhered platelets. In addition, GT platelets, which do not normally aggregate, appear to interact with each other, resulting in a normal aggregation response via rFVIIa-mediated fibrin formation. A fibrin receptor on the platelet presumably responsible for this process is yet to be identified. Enhancement of αIIb/β3-independent platelet adhesion and aggregation by rFVIIa and the increase in procoagulant platelet surface which facilitates further thrombin and fibrin generation via rFVIIa may, in part, compensate for the haemostatic defect in patients with GT.

Key words: Glanzmann's thrombasthenia, recombinant FVIIa, bleeding, platelet aggregation

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It is generally agreed that activated recombinant coagulation factor VII (rFVIIa; NovoSeven®, Novo Nordisk A/S, Bagsvaerd, Denmark) enhances thrombin generation locally at the site of vascular injury. Nevertheless, how rFVIIa enhances the thrombin generation remains an issue of discussion. Two pathways, tissue factor (TF)-dependent and TF-independent, have been proposed and it is possible that both of them account for the mechanism of action of rFVIIa. In the TF dependent mechanism, there is a competitive binding to TF between FVII and FVIIa, and rFVIIa increases the ratio of FVIIa/FVII. Based on the notion that the dose of rFVIIa required to induce haemostasis in patients with haemophilia is much higher than that required to saturate the available TF with rFVIIa, the TF independent mechanism has been proposed. In this mechanism, rFVIIa binds directly to activated platelets, although with low affinity, leading to the generation of FXa. This putative mechanism is further complicated by the finding that TF might be present on the surface of activated platelets.

The enhanced thrombin generation by rFVIIa does not directly induce haemostasis as haemostasis is achieved through the formation of a stable haemostatic plug. The latter requires balanced interactions between platelets, fibrin formation, and fibrinolysis. The enhanced generation of thrombin by rFVIIa leads to enhanced fibrin formation, changes in fibrin structure, enhanced activation of thrombin-activatable fibrinolysis inhibitor (TAFI) and FXIII, and accelerated platelet activation. Hence, the potential prohaemostatic effects of rFVIIa include not only procoagulant, but also platelet promoting and antifibrinolytic properties.

Effect of rFVIIa on platelet adhesion and aggregation

We designed a model to investigate how rFVIIa-mediated thrombin generation compensates for the lack of platelet aggregation seen in patients with Glanzmann's thrombasthenia (GT). In this model, washed platelets suspended in a 4% human albumin solution were mixed with washed red cells to obtain a final mixture with 200,000 platelets/μL and a haematocrit of 40%. The mixture was perfused over surfaces which were known to be adhesive to platelets, such as purified fibrinogen or collagen, or PMA-stimulated HUVEC extracellular matrix. Furthermore, we designed a thrombin generating system consisting of rFVIIa (60 U/mL), FX (10 μg/mL), prothrombin (20 ng/mL), and calcium chloride (3 mM).

The above combined models were used to study platelet adhesion to fibrinogen. We found that without the thrombin generating system, part of the surface was covered with both unactivated and activated (spread) single platelets. In contrast, when the thrombin generating system was added, more platelets adhered to the surface and these platelets appeared morphologically more activated (more spreading was seen). This effect was abolished in the presence of hirudin, a thrombin inhibitor, and was absent if rFVIIa was omitted, indicating that the effects could indeed be attributed to rFVIIa-mediated thrombin generation.

Furthermore, we studied platelet adhesion to a collagen-coated surface. As collagen is a more potent platelet agonist than fibrinogen, the interaction of platelets with collagen under flow not only results in platelet adhesion, but platelets also aggregate on the collagen surface. Recombinant FVIIa-mediated thrombin generation not only increased platelet adhesion to collagen, but also enhanced platelet aggregation. Moreover, using platelets at various concentrations, we found that these effects were platelet concentration dependent. Nevertheless, at all platelet concentrations tested (between 10,000 and 200,000/μL), rFVIIa-mediated thrombin generation enhanced the deposition of platelets to collagen and enhanced platelet aggregation. This may explain the mechanism of action of rFVIIa in patients with thrombocytopenia.

Effect of rFVIIa on platelet adhesion in patients with Glanzmann's thrombasthenia

The above model was modified to study the mechanism of action of rFVIIa in patients with GT. To mimic the abnormality seen in patients with GT, we added an RGD-containing peptide (dRGDW 200 μM) which blocks the ligand binding site of αIIb/β3 integrin.[1] In a system using PMA-stimulated endothelial cell matrix, dRGDW significantly decreased platelet adhesion and completely abolished platelet aggregation. The validity of the model was verified by the evidence that the same findings were seen when platelets from patients with type 1 GT were used. Furthermore, rFVIIa-mediated thrombin generation substantially increased platelet adhesion. The effect was inhibited by hirudin and annexin V, which blocks the binding site of rFVIIa on negatively-charged phospholipids exposed on activated platelets. However, an inhibitory antibody to TF did not inhibit this effect, indicating that the process required rFVIIa, and was thrombin dependent but TF independent. We were able to show that rFVIIa bound directly to platelets under conditions of flow and presumably thrombin was generated *in situ* by platelet-bound rFVIIa.

Effect of rFVIIa on platelet aggregation in patients with Glanzmann's thrombasthenia

As the defect in patients with GT is the lack of platelet aggregation, the ability of rFVIIa-mediated thrombin generation to increase platelet adhesion cannot explain the haemostatic effectiveness of rFVIIa in these patients. A recent publication has shown that αIIb/β3-inhibited platelets can aggregate through polymerising fibrin.[2] The process seems to be a true platelet-fibrin interaction and not just trapping of platelets in a fibrin network. We therefore investigated whether rFVIIa-mediated fibrin formation could restore the aggregation of αIIb/β3-inhibited platelets.

We added fibrinogen (0.5 mg/mL) to the thrombin generating system, and studied platelet aggregation in suspension. Platelet aggregation was induced by collagen or thrombin receptor activation peptide (TRAP), and aggregation was absent in GT platelets in the absence of fibrin generation. We found that fibrin generation via rFVIIa induced αIIb/β3-independent platelet aggregation.[3] Further electron microscopic studies confirmed that true platelet aggregates were formed. The same effect of rFVIIa on platelet aggregation was also confirmed using a flow model and whole blood. The same observation was reported by Galan *et al.* using denuded rabbit artery.[4]

Conclusions

The efficacy of rFVIIa in patients with GT might be explained by a combination of enhanced platelet deposition and induction or enhancement of αIIb/β3-

independent aggregation. This mechanism may also partly account for the mechanism of action of rFVIIa in other indications.

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Outcomes from the Glanzmann's thrombasthenia and recombinant factor VIIa data collection

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A B S T R A C T

Antibodies to glycoprotein (GP) IIb-IIIa and/or HLA may render platelet transfusions ineffective to stop bleeding or to cover surgery in patients with Glanzmann's thrombasthenia (GT). An international survey was conducted to evaluate the efficacy and safety of recombinant factor VIIa (rFVIIa) in GT patients. As the dosing regimens used varied considerably, an arbitrarily defined "optimal regimen" derived from a previous open-label study was used to compare with other regimens. The efficacy of this optimal regimen was significantly higher than other regimens. Furthermore, patients given maintenance doses had significantly fewer bleeding recurrences within 48 hours of bleeding cessation compared to those not given any. One thromboembolic event and one blood clot in the ureter occurring in surgical patients following prolonged use of continuous infusion of high dose rFVIIa and antifibrinolytic drug were reported. Recombinant FVIIa appears to be an alternative to platelet transfusion in GT patients with platelet refractoriness and/or antibodies to platelets.

Key words: Glanzmann's thrombasthenia, antibodies, glycoprotein IIb-IIIa, HLA, recombinant factor VIIa, platelet transfusion

Glanzmann's thrombasthenia (GT) is a rare autosomal recessive inherited platelet disorder characterised by impaired platelet aggregation. The incidence of GT is around 1/1,000,000 population. The defect in platelet membrane glycoprotein (GP) IIb-IIIa can be quantitative or qualitative. The genes coding for GPIIb and GPIIIa are both located on chromosome 17. The disease is diagnosed by normal platelet counts and morphology, prolonged bleeding time or closure time (PFA-100), decreased or absent clot retraction, and decreased platelet aggregation response to physiologic stimuli such as ADP, epinephrine, thrombin, and collagen but normal with ristocetin. The disease is classified into three types according to the levels of GPIIb-IIIa measured by flow cytometry: <5% in type 1, 5 to 15% in type 2, and normal levels but

functionally defective in type 3 (variant).

Clinical manifestations are variable with first manifestations usually appearing in early childhood. Bleeding manifestations range from easy bruising, purpura, epistaxis, gingival bleed, menorrhagia, gastrointestinal (GI) bleed, haematuria, haemarthrosis, haematoma, and central nervous system (CNS) bleed. Furthermore, bleeding may also be seen after dental extraction, surgery, and delivery.[1]

Conventional management of bleeding in GT

Local measures that may be effective include pressure/compression, nose packing for epistaxis, fibrin glue/topical thrombin, and Yag laser. Antifibrinolytic drugs can be helpful for mucosal bleeds, and oral contraceptives may limit menorrhagia. The standard treatment after conservative measures fail to stop bleeding is platelet transfusion. Preferentially, leucodepleted, single-donor apheresis, HLA-matched platelets should be used.

Platelet transfusions can lead to the formation of antibodies to GPIIb-IIIa and/or HLA. Furthermore, there is also a residual risk of transmission of blood-borne infections. A wide spectrum of methods have been used to detect antibodies to different epitopes on GPIIb-IIIa.[2] Apart from platelet refractoriness, a few cases of neonatal/foetal thrombocytopenia and intracerebral haemorrhage have been reported due to transferred antibodies from immunised GT mother.[3]

In case of platelet refractoriness, therapeutic options include massive transfusion with HLA-matched platelets. Moreover, plasmapheresis and immunoadsorption on protein-A sepharose columns may transiently restore platelets efficacy.[4] Curative treatment with allogeneic bone marrow transplantation has also been reported.[5] In addition, there is a successful report on gene therapy in animal models using transfection of the human β-3 subunit gene into bone marrow mononuclear cells from a β-3-KO mouse of haematopoietic stem cells.[6]

The use of recombinant FVIIa in GT

The first successful use of activated recombinant coagulation factor VII (rFVIIa; NovoSeven®, Novo Nordisk A/S, Bagsvaerd, Denmark) in GT was reported in 1996.[7] Subsequently, several publications have also shown the efficacy of rFVIIa for prophylaxis and treatment of bleedings in GT.[8-12] In an open-label study using rFVIIa 90 μg/kg every 2 hours in conjunction with antifibrinolytic drugs, the successful rate was found to be 92%.[13]

The *International Congenital Platelet Disorders Study Group* has collected data on the use of rFVIIa in GT, both retrospectively and prospectively. Data were collected anonymously consisting of the following information: type of GT, alloimmunisation status, previous platelet refractoriness, type and severity of bleed or procedure, rFVIIa dosing regimen (dosage, dosing interval, number of doses, continuous infusion or bolus), efficacy and safety. The detailed outcomes will be published in Journal of Thrombosis and Haemostasis.[14] Some key findings will be discussed in this manuscript.

Outcomes from the Glanzmann's thrombasthenia data collection

In total, 59 patients with GT were treated with rFVIIa for 108 bleeding episodes and 34 invasive procedures. The majority of patients were type 1 GT. Approximately 50% of the patients had antibodies to platelets. However, not all patients with antibodies experienced platelet refractoriness, and not all patients with refractoriness had detectable antibodies.

Although continuous infusion (CI) was used mainly in major surgery with the aim of decreasing the total amount of rFVIIa, it appeared that the total amount of rFVIIa required was higher in CI as compared to bolus injection. Regarding efficacy of CI, excellent results were seen in invasive procedures, whereas in bleeding episodes the failure rate was high.

Due to the wide variation in dosing regimens, an arbitrarily defined *optimal regimen* derived from an open-label study (≥80 μg/kg rFVIIa per injection, dosing interval 2.5 hours, ≥3 doses before failure declaration)[12] was used to compare with other regimens. In severe bleeds, the efficacy of this optimal regimen was significantly higher than other regimens, whereas in mild and moderate bleeds there was no difference. Furthermore, the majority of bleeding stopped in less than 6 hours. No difference in rFVIIa was seen between bleeding episodes treated with or without antifibrinolyic drugs. Maintenance dose(s) seems to be beneficial to limit recurrence of bleeding.

Concerning safety, there were two thrombotic events (1 pulmonary embolism and bilateral thrombophlebitis, 1 renal pelvis/ureter clot post-gynaecological surgery) considered possibly related to rFVIIa. Both cases occurred in patients treated with CI of high doses of rFVIIa concomitantly with antifibrinolytic drugs. In addition, there were 1 death (septicaemia) and 1 sepsis not considered related to rFVIIa.

Unresolved issues

The optimal platelet transfusion regimen for prophylaxis and treatment of bleedings in GT remains unknown. Antibodies to GPIIb-IIIa and platelet refractoriness are not always correlated. Furthermore, the techniques and monoclonal antibodies used for immunological detection of antibodies to GPIIb-IIIa are heterogeneous, and do not necessarily reflect the neutralising effect on aggregation of normal platelets. An optimal method and follow-up of inhibitors of platelet aggregation are required.

Conclusions

Recombinant FVIIa given as bolus injections appears to be a safe and mostly effective alternative to platelet transfusion for treatment and prevention of bleeding in GT patients with platelet refractoriness and/or antiplatelet antibodies. Currently, there is no definitive treatment guideline for treatment of bleeding. Nevertheless, the following should be taken into consideration: bolus injection at ≥80 μg/kg at ≤2.5 h interval; many of the bleeds may stop after 1–3 doses but some need more; 1 or more maintenance dose(s) might be useful to prevent bleeding recurrence. Antifibrinolytic drugs may be considered useful in mucosal bleeds.

The current approved indication in Europe is for prophylaxis and treatment of bleeding in GT patients with antibodies to GPIIb-IIIa and/or HLA, and past or present platelet refractoriness. Clinical studies or more data should help us define the place of rFVIIa for the prevention of platelet immunisation.

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The future of haemophilia treatment

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A B S T R A C T

In the last few years haemophilia has changed from a handicapping and life-endangering ailment to a fully defined group of molecular-pathological entities for which safe and effective treatment is available. Haemophilia is potentially one of the most promising fields among monogenic disorders for the implementation of gene transfer therapy. To cure the disease through gene therapy several obstacles must still be tackled and overcome, such as the limited degree of expression of the transgene, insufficient levels of clotting factors reached in plasma and maintained for too short a period of time, as well the occurrence of host immunological reactions that cause significant side-effects. Alternatives to gene transfer therapy are appearing on the horizon. Production of factor IX in the milk of transgenic farmyard animals may provide a source of less expensive and largely available replacement material. Attempts are also directed at engineering forms of factor VIII, factor IX and activated factor VII that may be more biologically active, with longer plasma half-lives and less immunogenicity and antigenicity. Hence, it can be confidently predicted that the new millennium will bring about several new options that will further improve the treatment of persons with haemophilia.

Key words: clotting factors, gene transfer, transgenic production.

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The future of the treatment in haemophilia is one of promises and expectations. It can be approached by tackling the present situations and challenges, as well as addressing current debatable issues. Hopes lie with the prospects of gene transfer and replacement therapy, the latter providing a possibility to improve currently available products and so dramatically change the lives of haemophilia patients.

The present as a prologue

Recombinant factors are efficacious and safe as both prophylactic and therapeutic agents, with little complications or problems associated with their use. However, in countries which have converted completely to recombinant products, measures must be taken to avoid shortages. Improvements have also been made with plasma-derived factors, becoming more efficacious and safer. Conversely, the costs of plasma-derived factors have increased, taking into account the additional tests and processes to increase the safety of these products. This poses difficulties for developing countries, which are already under-resourced. Despite the era of modern technology, only 10% of factor VIII (FVIII) is obtained from 1L of plasma. Attempts to increase this low yield have been made but efforts have not been greatly focused.

Continuous surveillance of treatment safety is mandatory and has proved beneficial. In collaboration with 140 haemophilia treatment centres, the Centers for Disease Control and Prevention carried out a monitoring process of 1,149 seroconversions for hepatitis viruses, identified among

Table 1. Gene transfer trials in haemophilia initiated from 1998 onwards.

Trial	*Vector*	*Method of gene transfer*	*Status to date*
Transkaryotic	Plasmid DNA	*Ex vivo*, autologous fibroblasts	On hold
Avigen 1	Adeno-associated virus	*In vivo*, intramuscular	Terminated
Chiron	Replication-deficient retrovirus	*In vivo*, intravenous	Terminated
Avigen 2	Adeno-associated, liver driven virus	*In vivo*, hepatic artery infusion	Terminated
Genstar	Gutted, liver-driven adenovirus	*In vivo*, intravenous	Terminated

patients with bleeding disorders, from May 1998 until June 2002.[1] None of these cases were attributable to blood-borne products, indicating the safety of the processes used in preparing the factors.

Immune tolerance has dramatically changed the treatment paradigm of haemophiliac patients who have developed inhibitors. Immune tolerance may effectively eliminate inhibitors in up to 70% of patients.[2] However, the issues of which dosages are better and which concentrates are more effective still remain unresolved, with variations existing between countries making it difficult to compare practices and to establish conventional procedures. The ongoing Immune Tolerance Induction Study may answer some of these questions.[3] This study compares the efficacy, morbidity and cost effectiveness of low versus high-dose immune tolerance in haemophilia A patients.

Possibilities still exist to improve the treatment of patients with inhibitors, through regular prophylaxis, FVIII inhibitor bypassing activity (Feiba VH®; Baxter AG, Austria) and activated recombinant coagulation factor VII (rFVIIa; NovoSeven®, Novo Nordisk A/S, Bagsvaerd, Denmark). Current ongoing trials show encouraging preliminary data and along with further trials which need to be conducted, good prospects exist to improve the treatment of patients with inhibitors or even to prevent them.

The remaining issues to be tackled include finding explanations for the anaphylactic reactions that develop in some haemophilia B patients. Current theories include the activation of the complement system but no evidence exists to support this. These patients are rare and hence definitive data difficult to obtain. Despite the risk of new blood-borne infections from viruses such as West Nile Virus and SARS, the successful viral inactivation process through which blood products are refined, indicates that this is not an issue.

The publication of the first transfusion-transmitted prion disease raises concern.[4] However, the fractionation procedures to obtain plasma-derived products have shown high levels of safety and should therefore be able to clear prions that enter the plasma pool.

Gene transfer

More than a decade ago, it was predicted that gene transfer therapy would play a large role in haemophilia therapy, even making haemophilia the first genetic disease to be cured in the millennium. On the basis of animal studies, promising data were obtained, however results proved better and more efficient compared with data from human studies, with respect to efficacy of transfer and duration of gene expression.

Gene transfer trials in haemophilia A and B patients (Table 1) have focused on various methods of gene transfer with a range of vectors. Despite the different approaches, most of these trials have either been terminated or are on hold due to preliminary unsuccessful results. The initial results were encouraging with respect to measurable levels of FVIII and factor IX (FIX) in the circulation. However, expression levels of the transgene were only transient and not sustained, and not sufficient to correct the phenotype to provide considerable benefit. The aim of gene transfer would be to transform severe haemophiliacs to moderate-mild disease status, requiring the occasional administration of recombinant products when needed. This is far from being achieved.

Reasons for current problems include poor and transient expression of the transgene and the need for multiple dose transfers. Repeated dosing is required to sustain levels of the transgene and may not be acceptable to the patient – single dose transfer is still a remote target. However, repeated gene transfer may not be an option due to safety issues. Two cases of T-cell leukaemia were observed after insertional mutagenesis in two children with severe combined immunodeficiencies.[5] This occurred during a trial of gene therapy based upon a Maloney retroviral vector. This has not been seen in haemophilia patients but raises concern over retroviral vectors. According to molecular biologists, this problem can be circumvented.

Transient positive vector DNA was found in semen samples of haemophilia A and B patients in the Chi-

ron and Avigen trials.[6,7] Germinal cells were not transfected, but these findings raise concerns over whether mature sperm are affected and the possibility of a sustained integration of vector DNA.

Other concerns have been raised with side-effects related to the host-immune reaction to the vector, especially adenoviruses. Abnormalities of transaminases, thrombocytopenia and inflammatory symptoms were observed in a haemophilia A patient in the Genstar trial, although these were reversible.[8] The high vector dose in the Avigen 2 trial may have resulted in the elevated transaminase levels in a patient with haemophilia B.[9] At present, haemophilia is a disease with good available treatment options. Therefore, strong considerations should be made before the use of gene transfer in patients with haemophilia, especially with respect to the potential associated risks.

The future of gene transfer therapy may yet show improvement. Progresses in plasmidology and vectorology have shown promising results. However, for greater advances to be made, a more enhanced understanding of tissue and vector-specific adaptive and innate immune responses are required, with intervention to modulate such responses if needed.

The future of replacement therapy

This provides the most promising alternative to gene therapy and may be achieved by improving recombinant products through the engineering of coagulation factors and the use of transgenic bioreactors. Current restrictions especially for recombinant FVIII include limited expression of this protein in mammalian cells, and the high cost associated with their production, purification and formulation. A high-efficiency production or expression of the recombinant factors would not only increase product availability, but also hopefully reduce the cost of the factor. Oral delivery of recombinant factors is a possibility and would be more acceptable to the patient, however, a large bulk of material would be required which may not be realistic.

The theoretical possibilities for the future of recombinant factors include increasing their expression, intracellular transport and secretion. Although increased expression has been achieved, this does not translate to an increase in secretion. Increase in activation of recombinant factors and reducing inactivation by physiological mechanisms, such as activated protein C and thrombin, provide promising targets.[10] Prolongation of FVIII and FIX half-lives could result in longer intervals between prophylactic treatments, and once again questions the need for gene transfer therapy. A product with decreasing immunogenicity and antigenicity is highly desirable. Molecular engineering can attempt to achieve all these goals.

PEGylation of molecules may improve the half-life of factors. Prolonged half-life may also be achieved by disrupting the receptors involved in the clearance of FVIII and FIX by genetic alteration/mutations of the proteins. Improved mutants may be more useful in gene transfer, especially those which interact with the two main clearance receptors, lipoprotein receptor-related protein, and heparan sulfate proteoglycan.[11,12] However, mutant molecules may be more immunogenic and surveillance is strongly recommended.

The use of bioreactors to produce recombinant factors may play a promising future role in the treatment of haemophilia. Transgenic pigs are able to produce milk containing FVIII and FIX, therefore providing an additional source of coagulation factors besides human donors.[13,14] The milk produced, as for human plasma, requires fractionation. Few data have been published on transgenic pigs, however, early reports show great potential of these bioreactors. Factor IX protein can be produced in high concentrations (100-1,000 U/mL) with specific activity (250-350 U/mg) and with good yields (> 75%).[15] More importantly, FIX is produced with proper post-translational modifications that are required for suitable recovery and clearance of the protein.

The advantages of pig bioreactors include a similar biochemistry to humans, with the protein produced being comparable to that produced by humans. Support is provided by a history of FDA approved products from transgenic bioreactors, with safety substantiated by the pathophysiology of the pigs which are resistant to bovine encephalopathies. Each pig can produce up to 3 litres of milk per day for 100 days a year, hence providing excellent yields. The productivity of the pigs themselves is also fairly substantial, with one pig having the ability to produce 21 offspring per year. Subsequently, it is easy to scale-up with minimal capital investment.

Preliminary data of the recombinant milk FIX show efficacy even when administered orally. Animal studies demonstrated that a single feed of FIX-containing pig milk was able to normalize the clotting time in haemophilia B mice for up to two days.[16] Additionally, administration of the protein induced immune tolerance; despite infusion with a heterologous factor, inhibitor development was not seen. These animal studies demonstrate the successful oral administration of recombinant factors and hence provide great potential for future treatment of haemophilia.

Conclusions

Despite the advances in haemophilia treatment and the future potentials on the horizon, 80% of haemophiliacs around the world do not receive any treatment at all. The World Federation of Haemophilia is attempting to tackle this problem, but face an enormous and complex challenge. Hope exists to improve the future of haemophilia treatment by improving the safety and efficacy of current recombinant factors, with focus on immune tolerance therapy to treat and prevent the development of inhibitors. Gene transfer therapy still provides the greatest potential for sustained effect and cure. However, safety and efficacy of the process still requires extensive research. Generation of further recombinant products through transgenic bioreactors have progressed farthest in the treatment of haemophilia, providing a new source of recombinant factors which may hopefully extend to benefit the lives of all haemophilia patients.

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