Coagulation factor VIIa: prohemostatic drug and biomarker for thrombosis

Schut, Anne Marieke

7

General discussion

(recombinant) Factor VIIa plays a central role in the studies described in this thesis, in which the aim of the research was two-fold. First, potential working mechanisms of rFVIIa when given prophylactically to inhibitor-complicated hemophilia patients were assessed. Second, the use of FVIIa as biomarker for venous thrombosis was examined. In this chapter, the contribution of our main findings to the current knowledge on the working mechanisms of rFVIIa prophylaxis as well as the initiating trigger of venous thrombosis will be discussed in a broader perspective.

Working mechanisms of rFVIIa

In chapter 2, we assessed potential other working mechanisms of prophylactically administered rFVIIa by administering a bolus doses of 90 μg/kg body weight rFVIIa to non-bleeding pigs [1]. During the time frame of prophylaxis, up to 48 hours, we collected plasma and platelets and determined FVIIa levels and associated hemostatic activity. Twenty-four hour post-administration, we found small but detectable levels of rFVIIa in the plasma. Low levels of FVIIa hours post injection have been reported before [2-5], although the authors of these previous published results did not recognize these low levels as being clinically significant. Furthermore, to our knowledge, we are the first to show *in vivo* uptake of rFVIIa by platelets. The FVIIa taken up by platelets is protected from clearance from the circulation and also appears to have a longer half-life compared to FVIIa in plasma. We hypothesize that these low, but hemostatically active levels of rFVIIa in both plasma and platelets at the end of the time frame of prophylaxis, may be responsible for the prevention of bleeding episodes in inhibitor-complicated hemophilia patients. The low levels of FVIIa in platelets may be in particular important at that point in time when local activation of these platelets with concomitant release of the FVIIa may result in a locally elevated FVIIa concentration.

Another working mechanism that may explain the prolonged efficacy of rFVIIa is the distribution of rFVIIa into the extravascular space, with accumulation in various tissues, bone and joints [6-8]. One study also suggested that rFVIIa was taken up by megakaryocytes within the bone marrow [8]. Megakaryocytes are the precursor cells of platelets and therefore we hypothesized that the uptake of rFVIIa in megakaryocytes may result in the production of rFVIIa-containing platelets. The experiments described in chapter 3 showed uptake of rFVIIa by cultured megakaryocytes, and after stimulation these cells produced platelet-like particles containing hemostatically active rFVIIa. Whether this mechanism acts *in vivo* remains to be elucidated, but delayed generation of rFVIIa-containing platelets appears a plausible mechanism to partly explain the efficacy of once-daily rFVIIa prophylaxis in hemophilia patients with inhibitors.

In both chapter 2 and 3 the experiments showed an important role of platelets in explaining the duration of rFVIIa prophylaxis, which substantially exceeds its circulating half-life of 2 hours.

Role of platelets

Platelets are traditionally known for their role in hemostasis, thrombosis, and wound healing. The role of platelets is, however, much more dynamic than that of a simple aggregate forming cell fragment as described in the explanation of the prophylactic efficacy of rFVIIa in chapter 2 and 3. Furthermore, it is known that platelets are involved in inflammation [9], host defense [10], liver regeneration [11,12], sepsis [13] and cancer [14].

Platelets contain three types of storage compartments; alpha-granules, dense bodies, and lysosomes. These storage compartments contain various constituents which are involved in processes that in part may contribute to these dynamic roles of platelets as described above [15,16]. Alpha-granules contain, among many other constituents, coagulation factors (e.g., FV and fibrinogen), membrane proteins (e.g., GPIIbIIIa, GPIb, and P-selectin), as well as growth factors involved in liver regeneration (e.g., platelet-derived growth factor (PDGF), hepatocyte growth factor (HGF), insulin-like growth factor (IGF), and vascular endothelial growth factor (VEGF) [17]). Dense bodies contain proteins such as serotonin (involved in vasoconstriction and liver regeneration) and ADP (weak platelet activator). Lysosomes are storage compartments containing various digestive enzymes. The proteins stored in these compartments are carried to the cell surface and released upon platelet activation.

It is well known that platelets are able to endocytose proteins stored in alpha granules (e.g., fibrinogen) [18]. In this thesis we showed that platelets are also capable of taking up rFVIIa as described in chapter 2 and the transfer of rFVIIa from MEG-01 cells to platelets as described in chapter 3. In these studies platelets were identified as a potential hiding place and/or storage unit, which may explain the prolonged prophylactic effect of a once-daily administered bolus injection of rFVIIa. Although we have not assessed the exact storage location of the rFVIIa taken up by platelets, the alpha-granules are a likely candidate considering the results of the staining pattern of the selectively transferred rFVIIa stored in platelet-like particles (chapter 3).

In the process of megakaryocyte maturation and biogenesis of platelets, selective transfer of agents from megakaryocytes to platelets has been described before [19]. Cecchetti et

al. showed that megakaryocytes selectively transferred some, but not all of the examined, messenger RNAs (mRNAs) to platelets. Platelets themselves, however, are upon stimulation also capable of transferring mRNA to other cell types such as monocytic and endothelial cells [20]. Recently, research from our laboratory demonstrated that platelets stimulate proliferation of HepG2 cells, which requires internalization of platelets in these liver cells and transfer of mRNA from platelets to HepG2 cells [21]. This may contribute to platelet-mediated liver regeneration, which is an example of the multi-dynamic role of platelets as previously mentioned.

Besides the transfer of mRNA from megakaryocytes to platelets, platelets may also take up mRNA from the plasma environment. Recently, it was demonstrated that platelets from patients with cancer contain tumor-derived RNA, which may be used as a biomarker for the presence of cancer [22]. Cancer cells can secrete membrane vesicles containing tumor-derived RNA, which is subsequently taken up by platelets. This means that these 'tumor-educated platelets' (TEP) contain an mRNA profile similar to that of a tumor. This phenomenon is an emerging concept for a blood-based cancer classification method. This method will include the isolation of blood platelets, which is a relatively fast and simple procedure, followed by the isolation and gene expression of the platelet RNA. A recently published study showed the ability to distinguish between mRNA profiles of healthy individuals and cancer patients [23]. Moreover, the platelet mRNA profile quite accurately identified the localization of the primary tumor of the cancer patients, including six different types of tumors.

In conclusion, the knowledge regarding the various roles of platelets has developed massively over the years, and studies showed that platelets have functions far beyond their traditional role in hemostasis, thrombosis, and wound healing. More research about potential roles, abilities, and capabilities of platelets may provide more insight to help develop novel medical treatments and diagnostic methods such as the promising blood-based cancer classification method. For current medical treatments such as the prophylactic treatment of rFVIIa, the research presented in this thesis will already provide a better insight in previously unrecognized roles of platelets.

Relevance for clinical practice

It is almost thirty years ago that rFVIIa became available on the market. Looking back over that period of time shows us that we gained a huge amount of knowledge about for example treatment options, safety, mode of action, and therapeutic response regarding rFVIIa. It has given us more insight how to treat a patient, and how to improve their

lives. Early treatment is an important example, but also the development in given rFVIIa prophylactically to prevent bleeding episodes. The results described in the first part of this thesis extends the knowledge on the mode of action of rFVIIa given prophylactically to inhibitor-complicated patients. Our results will not directly be beneficial for clinical practice at this point in time, but it might be in the future. Further experiments are required to confirm our hypothesis whether lower concentrations of rFVIIa can indeed be used for the prevention of bleeding episodes. Lower concentrations of rFVIIa being effective for a much longer time frame would be beneficial for clinical practice and would result in cost reductions.

Next paragraphs will discuss the role of rFVIIa in a broader perspective. All research done over the last thirty years, including the research described in this thesis, was to optimize the treatment and improve the quality of life of inhibitor-complicated hemophilia patients. What did the hemostatic drug and development in treatment options mean for inhibitor-complicated hemophilia patients? Were we able to improve the lives of these patients? The following paragraphs will discuss these topics from the patient's perspective, and indicates the contribution of the results described in this thesis. Finally, we will give insight in the costs of hemophilia care and speculate what the results of this thesis may mean for this in future.

The treatment of inhibitor-complicated hemophilia patients has been significantly improved by the introduction of rFVIIa. Before 1988, the year in which the first patient was treated with rFVIIa, treatment of inhibitor-complicated hemophilia patients was suboptimal compared with non-inhibitor complicated patients, leading to the development of an alternative treatment [24,25]. At the time recombinant FVIIa became available on the market, it suddenly became possible for inhibitor-complicated patients to treat themselves at home. This home treatment allowed patients to initiate early treatment, which resulted in less pain and morbidity, and thereby improving quality of life (reviewed in [26]). Home treatment has shown to be safe, effective and well tolerated in a multicenter phase III study [27]. The originally marketed rFVIIa required storage at 4°C. In 2008, rFVIIa became available in a room temperature stable formulation and was shown to be bioequivalent to the originally developed rFVIIa [28,29]. This new room temperature stable product is much more user-friendly regarding its transport and storage and much more convenient in the daily practice of patient treatment. The importance of early treatment was demonstrated by analyzing the HemoRec registry, which included hemophilia data regarding initiation of treatment within 2 hours (early treatment) or after 2 hours (late treatment) counted from the onset of bleeding [30]. It was shown that rFVIIa treatment started within 2 hours counted from the onset of a bleeding episode

reduces the incidence of re-bleeding by more than half, compared to rFVIIa treatment started more than 2 hours after bleeding [30]. The effect of various doses of rFVIIa on the incidence of re-bleeding did not differ in the early treatment group (≤2 h), whereas higher doses of rFVIIa were required to control re-bleeding in the late treatment group [30].

Quality of life

The higher the classification of hemophilia (mild, moderate, or severe), the more hemorrhage, spontaneous or upon trauma, the patient might experience despite repeated treatment with factor replacement therapy or bypassing agents in case of inhibitor complicated patients. These (re)-bleedings often occur in joints, muscles or soft tissue, in which hemophilic arthropathy is a long-term consequence of these bleedings in joints. Hemophilia has an appreciable impact on the quality of life of these patients, and this impact has been shown to be more severe in inhibitor-complicated hemophilia patients compared with patients who did not develop inhibitors [31]. It was also shown that the health-related quality of life of inhibitor-complicated hemophilia patients is mainly associated with their orthopaedic status rather than other physical or mental components (e.g., mobility and social functioning) [32]. In order to improve the quality of life of hemophilia patients, it is important to prevent and/or adequately treat the orthopaedic problems in these patients.

Prevention of bleeding episodes would be possible by means of prophylactic treatment. Currently, short-term episodic prophylaxis could be used to optimize treatment outcomes for hemophilia patients with inhibitors [33]. The treatment consists of on-demand treatment with rFVIIa to diminish the bleeding and reducing the pain in the joint. Thereafter, rFVIIa prophylaxis will be given for at least 3 days to stop the hemorrhage completely, to prevent re-bleeding and by means of that to prevent and/or delay the development of target joints and on the long-term arthropathy. Konkle et al. studied the effect of rFVIIa prophylaxis for a longer period of time. In 2007, they published the first and until now only randomized controlled trial of the prophylactic use of 90 or 270 μg/kg body weight rFVIIa [34]. Both doses have been shown to be safe and effective in the reduction of bleeding episodes both during prophylactic treatment and long after the last dose was administered compared to on-demand treatment. Patients included in this randomized trial were also interviewed regarding their health-related quality of life comparing on-demand treatment (pre-prophylaxis period) versus prophylactic treatment (including post-prophylaxis period) [35]. It was shown that prophylaxis with rFVIIa improved the health-related quality of life of inhibitor-complicated patients, with some effects even remaining effective during the post-prophylaxis period. A few

examples of the improvement of the health-related quality of life of inhibitor-complicated hemophilia patients are a reduction in bleeding-related hospitalization (9.5 days (on-demand treatment) vs 1.5 days (prophylaxis)), reduction in the days absent from school or work (18.5 days (on-demand treatment) vs 4.5 days (prophylaxis) and 8.5 days (post-prophylaxis)), reduction in pain (40.9% of patients, end of post-prophylaxis vs on-demand treatment), and an improvement in patient mobility (27.3% of patients, end of post-prophylaxis vs on-demand treatment) [35]. In addition, a systematic review of prospective interventional studies was recently published in which the efficacy of prophylaxis in hemophilia patients and in patients who developed inhibitors was examined [36]. In the majority of trials examined, prophylaxis resulted in the improvement of health-related quality of life for hemophilia patients and inhibitor-complicated patients. In conclusion, rFVIIa prophylaxis results in a significant reduction in bleeding episodes and in important improvements in the quality of life of inhibitor-complicated hemophilia patients. These results underline the importance of prophylactic treatment with rFVIIa for patients. The research presented in this thesis provides more insight in the working mechanisms of rFVIIa prophylaxis.

Cost of hemophilia care

Many studies have assessed the cost-effectiveness of hemophilia treatment, including on-demand versus prophylactic treatment with factor replacement concentrates [37-39] or with bypassing agents (activated prothrombin complex concentrate [APCC] and/or rFVIIa) for inhibitor-complicated patients [40-44]. These cost analyses are based on clinical experience and trials, in which the mean number of (re)-bleeding episodes and the mean number of injections needed to control the bleeding are known. Subsequently the mean of the total dosage given per patient per year can be calculated. Other costs taken into account are the mean amount of hospitalization days and days missing at school and work, and for example surgery costs. An example of a calculation of the cost of care of inhibitor-complicated patients in Italy showed that 47,3% of the monthly health care costs for inhibitor-complicated hemophilia patients consist of rFVIIa treatment, mainly used for surgical intervention such as joint replacements [40]. This indicates the high economic burden of rFVIIa treatment for patients with hemophilia, which means that the cost-effectiveness should be managed and evaluated well. Prophylactic treatment of rFVIIa will not directly lead to cost-reductions, as still significant amounts of rFVIIa need to be administered (90 or 270 μg/kg body weight rFVIIa). However, Konkle et al. showed that prophylactic treatment remains effective for a much longer period of time, even after prophylactic administration of rFVIIa was terminated [34]. For the long term, prophylactic treatment will therefore be much more cost effective as the amount

of bleeding episodes will be reduced, and maybe less surgery will be needed. In addition it improves the quality of life of inhibitor-complicated hemophilia patients which covers indirect health care costs. Furthermore, based on our findings described in chapter 2, we postulate that the low, but hemostatically active levels of rFVIIa found hours after the last bolus administration of rFVIIa would be responsible for the prevention of bleeding episodes. The prophylactic regimen of 90 μg/kg body weight once-daily given, may therefore be reduced substantially, which would lead to even more significant cost reductions.

Role of GPIbα

The GPIb-IX-V complex is the second most abundant receptor complex on the surface of platelets. This receptor is important in the primary hemostatic response to vessel wall injury, as it serves as main receptor for VWF and facilitates platelet adhesion to the damaged vessel wall. The GPIb-IX-V complex is also involved in secondary hemostasis, as several coagulation factors can bind to the N-terminal region of GPIbα which is the most important ligand binding moiety of this complex [45-50]. An example of a known ligand is FVII(a), and it was previously shown that the binding of rFVIIa to the GPIb-IX-V complex contributes to the tissue factor-independent thrombin generation on the surface of activated platelets [46]. Besides the localization of coagulation factors to the surface of platelets, GPIbα appears to modulate coagulation factor function. Therefore the GPIb-IX-V complex is an interesting potential therapeutic target for anti-thrombotic drug development. Current marketed anti-thrombotic drugs target mainly platelets or coagulation factors, and are prescribed for the prevention and/or treatment of arterial and venous thrombosis, respectively. Targeting the GPIb-IX-V complex would be a novel antithrombotic strategy, as it will target both platelets and coagulation at once. The experiments described in chapter 4 are part of an ongoing study in our laboratory to identify which coagulation factors bind GPIbα, to localize the exact binding site, and to identify the role of GPIbα in coagulation reactions.

In the experiments presented in chapter 4 we assessed whether the GPIb-IX-V complex assists in the binding of FIX(a) to the surface of activated platelets, and studied the potential effect of GPIbα on FIXa-mediated propagation of coagulation. We could not provide evidence that the N-terminal region of GPIbα is involved in the binding of FIX(a) to the surface of activated platelets. However, the N-terminal region of GPIbα modulates FIXa-mediated FXa-generation as it seems to reduce the procoagulant activity of FIXa. Several questions remained unanswered, for example whether FIX(a) might bind the GPIb-IX-V complex outside the N-terminal region of GPIbα and/or whether the effect

on the coagulation reaction could be explained by the binding of the other coagulation factors involved (i.e., FVIII and/or FX) rather than FIX(a).

Future experiments will continue with systematic screening of coagulation factors to identify which factors bind GPIbα, the binding site on GPIbα, and to determine the effect of this interaction in coagulation reactions. Eventually, coagulation factors with unique binding sites will be preferred candidates for further development of this novel type of antithrombotic drugs. This drug (e.g., nanobodies) should be designed to specifically inhibit the binding of such coagulation factor with GPIbα. This would result in a specifically targeted platelet-mediated coagulation antithrombotic effect, without affecting other functions of GPIbα. Being more specific, without affecting functions of GPIbα in primary hemostasis (GPIbα-mediated platelet adhesion) and in secondary hemostasis (GPIbα-independent coagulation as well as GPIbα-dependent coagulation in which the binding is not interfered by the nanobody). An example of such targeted antithrombotic drug is anti-VWF aptamer ARC1779, developed and tested in clinical trials in patients with congenital thrombotic thrombocytopenic purpura (TTP). This aptamer binds to the A1 domain of VWF, thereby preventing the interaction of VWF with GPIb. Phase I/II clinical trials showed a dose-dependent inhibition of VWF-dependent platelet function and stabilized or even improved platelet counts in TTP patients who tolerated this drug well [51,52].

Venous thrombosis

Previously, it has been demonstrated that coagulation activation occurs in 17% of a group of healthy individuals after an 8 h flight, whether this was only 3% and 1% in the control situations of an 8 h movie marathon and an 8 h daily life routine, respectively [53]. In addition, these results were mainly found in women using oral contraceptives and/or having the factor V Leiden mutation. It is not known why activation of the coagulation system, measured by increased TAT complexes, occurred in these individuals. Knowledge on the initiating trigger of coagulation activation after air travel may give a clue on the mechanism of air travel-related thrombosis and may point toward the best preventive action to be taken. Currently, anticoagulant drugs (e.g., LMWH), elastic stockings and exercises are proposed for deep venous thrombosis prophylaxis dependent on the risk one individual might encounter. To assess the effect of these interventions, several randomized controlled trials have been conducted. The majority of these trials have been conducted by one research group, and showed that both LMWH and elastic stockings have been proven effective in reducing the incidence of DVT during long-haul flights [54-57]. However, the methodology behind these trials is disputable and the scientific integrity of the named

authors was questioned by the Medical Research Council, which not contribute to the plausibility of these trials [58]. One trial conducted from outside the previous mentioned research group, showed a reduction in symptomless DVT when elastic stockings were worn [59]. However, wearing these elastic stockings caused superficial vein thrombosis in 3% of the patients which not occurred in passengers not wearing elastic stockings. Therefore it has been debated whether such prophylactic measures have an acceptable risk/benefit ratio [60]. A better understanding on the mechanism of air travel-related thrombosis might lead to more insight which preventive action can be taken best to reduce the incidence of thrombosis. Moreover, more insight might lead to the development of novel antithrombotic drugs. With an estimated number of over 2 billion passengers flying each year, air travel might cause over 150.000 extra cases of venous thrombosis [61]. Air travel-related thrombosis is affecting a lot of people, so many studies are eager to identify the exact mechanism behind air travel-related thrombosis. The WRIGHT (WHO Research Into Global Hazards of Travel) project is an initiative which combines several studies analyzing risks, mechanisms and prevention of travel-related thrombosis. The results described in chapter 5 are part of the cohort studies of the WRIGHT project. In chapter 5, we hypothesized that the coagulation activation seen in 17% of the healthy individuals after air travel is initiated via the tissue factor pathway. We measured activity and antigen levels of FVII and FVIIa of all 71 healthy individuals in all three situations (8 h flight, movie, daily life routine), and we used these as marker of extrinsic activation [62]. The FVII and FVIIa activity and antigen levels measured did not provide evidence for air travel-related coagulation activation via the extrinsic pathway. Even in the individuals with an activated clotting system after the flight compared with individuals without an activated clotting system, we did not find differences between activity and antigen levels of FVII and FVIIa. Whether the intrinsic pathway is activated during air travel remains to be elucidated.

In chapter 6, we assessed the role of the tissue factor pathway in the initiation of venous thrombosis [63]. We measured FVII and FVIIa plasma levels in 148 patients diagnosed with acute DVT and in 178 controls. Median levels of FVII in patients with acute DVT and in controls were similar, and individuals with FVII levels above 141.7% had a 1.6-fold increased risk for the presence of a DVT compared to controls. Median levels of FVIIa in patients with acute DVT were substantially lower compared to controls, and individuals with FVIIa levels below 69.9 ng/ml had a 5.5-fold increased risk for the presence of a DVT compared to controls. The decreased levels of FVIIa in patients with acute DVT may indicate ongoing consumption, as in a minority of patients we measured higher FVIIa-AT complex levels compared to controls. However, measuring lower FVIIa levels in patients compared to controls might also be explained by the time blood was

collected from these individuals. We hypothesized to find elevated levels of FVIIa as a result of TF-mediated FVII to FVIIa conversion, as it has been well established that there is active coagulation activation in patients in the acute phase of a DVT. In our study, patients with suspicion of acute DVT were referred to the hospital and upon confirmation of the diagnosis of DVT, patients were included in the study and blood was drawn before the start of anticoagulant treatment. It is possible that higher levels of FVIIa would be measured in patients when blood was drawn earlier. Nevertheless, both situations suggest a contributory role for tissue factor in the initiation of venous thrombosis. Knowing the initiation trigger in the development of venous thrombosis would be specifically of interest to be able to develop novel antithrombotic strategies to prevent venous thrombosis. An example of such novel strategy is using antisense nucleotides towards FXI, which was effective in the prevention of venous thrombosis after a knee replacement surgery [64]. Elevated levels of FXI are an established risk factor for venous thrombosis [65]. It has however not yet been determined whether elevated levels of FXI form a risk because of enhancement of FXIIa- or thrombin-mediated activation of FXI, which is compatible with the intrinsic or the extrinsic pathway as initiating trigger respectively.

References

1. Schut AM, Hyseni A, Adelmeijer J, Meijers JC, de Groot PG, Lisman T. Sustained pro-haemostatic activity of rFVIIa in plasma and platelets in non-bleeding pigs may explain the efficacy of a once-daily prophylaxis in humans. Thromb Haemost 2014; 112: 304-10.
2. Bijsterveld NR, Moons AH, Boekholdt SM, van Aken BE, Fennema H, Peters RJ, Meijers JC, Büller HR, Levi M. Ability of recombinant factor VIIa to reverse the anticoagulant effect of the pentasaccharide fondaparinux in healthy volunteers. Circulation 2002; 106: 2550-4.
3. Friederich PW, Levi M, Bauer KA, Vlasuk GP, Rote WE, Breederveld D, Keller T, Spataro M, Barzegar S, Büller HR. Ability of recombinant factor VIIa to generate thrombin during inhibition of tissue factor in human subjects. Circulation 2001; 103: 2555-9.
4. Villar A, Aronis S, Morfini M, Santagostino E, Auerswald G, Thomsen HF, Erhardtsen E, Giangrande PL. Pharmacokinetics of activated recombinant coagulation factor VII (NovoSeven) in children vs. adults with haemophilia A. Haemophilia 2004; 10: 352-9.
5. Lindley CM, Sawyer WT, Macik BG, Lusher J, Harrison JF, Baird-Cox K, Birch K, Glazer S, Roberts HR. Pharmacokinetics and pharmacodynamics of recombinant factor VIIa. Clin Pharmacol Ther 1994; 55: 638-48.
6. Beeby TL, Chasseaud LF, Taylor T, Thomsen MK. Distribution of the recombinant coagulation factor 125I-rFVIIa in rats. Thromb Haemost 1993; 70: 465-8.
7. Gopalakrishnan R, Hedner U, Clark C, Pendurthi UR, Rao LV. rFVIIa transported from the blood stream into tissues is functionally active. J Thromb Haemost 2010; 8: 2318-21.
8. Gopalakrishnan R, Hedner U, Ghosh S, Nayak RC, Allen TC, Pendurthi UR, Rao LV. Bio-distribution of pharmacologically administered recombinant factor VIIa (rFVIIa). J Thromb Haemost 2010; 8: 301-10.
9. von Hundelshausen P, Weber C. Platelets as immune cells: Bridging inflammation and cardiovascular disease. Circ Res 2007; 100: 27-40.
10. Yeaman MR. The role of platelets in antimicrobial host defense. Clin Infect Dis 1997; 25: 951,68; quiz 969-70.
11. Nocito A, Georgiev P, Dahm F, Jochum W, Bader M, Graf R, Clavien PA. Platelets and platelet-derived serotonin promote tissue repair after normothermic hepatic ischemia in mice. Hepatology 2007; 45: 369-76.
12. Alkozai EM, Nijsten MW, de Jong KP, de Boer MT, Peeters PM, Slooff MJ, Porte RJ, Lisman T. Immediate postoperative low platelet count is associated with delayed liver function recovery after partial liver resection. Ann Surg 2010; 251: 300-6.
13. de Stoppelaar SF, van 't Veer C, van der Poll T. The role of platelets in sepsis. Thromb Haemost 2014; 112: 666-77.
14. Nash GF, Turner LF, Scully MF, Kakkar AK. Platelets and cancer. Lancet Oncol 2002; 3: 425-30.
15. Moore G, Knight G, Blann A. Haematology. Oxford University Press; 2010.

16. Smyth SS, McEver RP, Weyrich AS, Morrell CN, Hoffman MR, Arepally GM, French PA, Dauerman HL, Becker RC, 2009 Platelet Colloquium Participants. Platelet functions beyond hemostasis. J Thromb Haemost 2009; 7: 1759-66.
17. Matsuo R, Ohkohchi N, Murata S, Ikeda O, Nakano Y, Watanabe M, Hisakura K, Myronovych A, Kubota T, Narimatsu H, Ozaki M. Platelets strongly induce hepatocyte proliferation with IGF-1 and HGF *in vitro*. J Surg Res 2008; 145: 279-86.
18. Handagama P, Scarborough RM, Shuman MA, Bainton DF. Endocytosis of fibrinogen into megakaryocyte and platelet alpha-granules is mediated by alpha IIb beta 3 (glycoprotein IIb-IIIa). Blood 1993; 82: 135-8.
19. Cecchetti L, Tolley ND, Michetti N, Bury L, Weyrich AS, Gresele P. Megakaryocytes differentially sort mRNAs for matrix metalloproteinases and their inhibitors into platelets: A mechanism for regulating synthetic events. Blood 2011; 118: 1903-11.
20. Risitano A, Beaulieu LM, Vitseva O, Freedman JE. Platelets and platelet-like particles mediate intercellular RNA transfer. Blood 2012; 119: 6288-95.
21. Kirschbaum M, Karimian G, Adelmeijer J, Giepmans BN, Porte RJ, Lisman T. Horizontal RNA transfer mediates platelet-induced hepatocyte proliferation. Blood 2015; 126: 798-806.
22. Nilsson RJ, Balaj L, Hulleman E, van Rijn S, Pegtel DM, Walraven M, Widmark A, Gerritsen WR, Verheul HM, Vandertop WP, Noske DP, Skog J, Wurdinger T. Blood platelets contain tumor-derived RNA biomarkers. Blood 2011; 118: 3680-3.
23. Best MG, Sol N, Kooi I, Tannous J, Westerman BA, Rustenburg F, Schellen P, Verschueren H, Post E, Koster J, Ylstra B, Ameziane N, Dorsman J, Smit EF, Verheul HM, Noske DP, Reijneveld JC, Nilsson RJ, Tannous BA, Wesseling P, Wurdinger T. RNA-seq of tumor-educated platelets enables blood-based pan-cancer, multiclass, and molecular pathway cancer diagnostics. Cancer Cell 2015; 28: 666-76.
24. Hedner U, Lee CA. First 20 years with recombinant FVIIa (NovoSeven). Haemophilia 2011; 17: e172-82.
25. Hedner U. Activated factor VII: My story. Haemophilia 2012; 18: 147-51.
26. Teitel JM, Barnard D, Israels S, Lillicrap D, Poon MC, Sek J. Home management of haemophilia. Haemophilia 2004; 10: 118-33.
27. Key NS, Aledort LM, Beardsley D, Cooper HA, Davignon G, Ewenstein BM, Gilchrist GS, Gill JC, Glader B, Hoots WK, Kisker CT, Lusher JM, Rosenfield CG, Shapiro AD, Smith H, Taft E. Home treatment of mild to moderate bleeding episodes using recombinant factor VIIa (novoseven) in haemophiliacs with inhibitors. Thromb Haemost 1998; 80: 912-8.
28. Bysted BV, Scharling B, Moller T, Hansen BL. A randomized, double-blind trial demonstrating bioequivalence of the current recombinant activated factor VII formulation and a new robust 25 degrees C stable formulation. Haemophilia 2007; 13: 527-32.
29. Butros L, Boayue K, Mathew P. Current difficulties and recent advances in bypass therapy for the management of hemophilia with inhibitors: A new and practical formulation of recombinant factor VIIa. Drug Des Devel Ther 2011; 5: 275-82.

30. Salaj P, Brabec P, Penka M, Pohlreichova V, Smejkal P, Cetkovsky P, Dusek L, Hedner U. Effect of rFVIIa dose and time to treatment on patients with haemophilia and inhibitors: Analysis of HemoRec registry data from the czech republic. Haemophilia 2009; 15: 752-9.
31. Morfini M, Haya S, Tagariello G, Pollmann H, Quintana M, Siegmund B, Stieltjes N, Dolan G, Tusell J. European study on orthopaedic status of haemophilia patients with inhibitors. Haemophilia 2007; 13: 606-12.
32. Scalone L, Mantovani LG, Mannucci PM, Gringeri A, COCIS Study Investigators. Quality of life is associated to the orthopaedic status in haemophilic patients with inhibitors. Haemophilia 2006; 12: 154-62.
33. Salek SZ, Auerswald G, Benson G, Dolan G, Duffy A, Hermans C, Jimenez-Yuste V, Ljung R, Morfini M, Santagostino E, Lambert T. Beyond stopping the bleed: Short-term episodic prophylaxis with recombinant activated factor FVII in haemophilia patients with inhibitors. Blood Transfus 2015: 1-8.
34. Konkle BA, Ebbesen LS, Erhardtsen E, Bianco RP, Lissitchkov T, Rusen L, Serban MA. Randomized, prospective clinical trial of recombinant factor VIIa for secondary prophylaxis in hemophilia patients with inhibitors. J Thromb Haemost 2007; 5: 1904-13.
35. Hoots WK, Ebbesen LS, Konkle BA, Auerswald GK, Roberts HR, Weatherall J, Ferran JM, Ljung RC, Novoseven (F7HAEM-1505) Investigators. Secondary prophylaxis with recombinant activated factor VII improves health-related quality of life of haemophilia patients with inhibitors. Haemophilia 2008; 14: 466-75.
36. Oladapo AO, Epstein JD, Williams E, Ito D, Gringeri A, Valentino LA. Health-related quality of life assessment in haemophilia patients on prophylaxis therapy: A systematic review of results from prospective clinical trials. Haemophilia 2015; 21: e344-58.
37. Smith PS, Teutsch SM, Shaffer PA, Rolka H, Evatt B. Episodic versus prophylactic infusions for hemophilia A: A cost-effectiveness analysis. J Pediatr 1996; 129: 424-31.
38. Miners AH, Sabin CA, Tolley KH, Lee CA. Assessing the effectiveness and cost-effectiveness of prophylaxis against bleeding in patients with severe haemophilia and severe von willebrand's disease. J Intern Med 1998; 244: 515-22.
39. Fischer K, van der Bom JG, Molho P, Negrier C, Mauser-Bunschoten EP, Roosendaal G, De Kleijn P, Grobbee DE, van den Berg HM. Prophylactic versus on-demand treatment strategies for severe haemophilia: A comparison of costs and long-term outcome. Haemophilia 2002; 8: 745-52.
40. Gringeri A, Mantovani LG, Scalone L, Mannucci PM, COCIS Study Group. Cost of care and quality of life for patients with hemophilia complicated by inhibitors: The COCIS study group. Blood 2003; 102: 2358-63.
41. Lyseng-Williamson KA, Plosker GL. Recombinant factor VIIa (eptacog alfa): A pharmacoeconomic review of its use in haemophilia in patients with inhibitors to clotting factors VIII or IX. Pharmacoeconomics 2007; 25: 1007-29.

42. Jimenez-Yuste V, Nunez R, Romero JA, Montoro B, Espinos B. Cost-effectiveness of recombinant activated factor VII vs. plasma-derived activated prothrombin complex concentrate in the treatment of mild-to-moderate bleeding episodes in patients with severe haemophilia A and inhibitors in spain. Haemophilia 2013; 19: 841-6.
43. Abbonizio F, Giampaolo A, Coppola A, Italian Association of Haemophilia Centres, Arcieri R, Hassan HJ. Therapeutic management and costs of severe haemophilia A patients with inhibitors in italy. Haemophilia 2014; 20: e243-50.
44. Villarrubia R, Oyaguez I, Alvarez-Roman MT, Mingot-Castellano ME, Parra R, Casado MA. Cost analysis of prophylaxis with activated prothrombin complex concentrate vs. on-demand therapy with activated factor VII in severe haemophilia A patients with inhibitors, in spain. Haemophilia 2015; 21: 320-9.
45. De Marco L, Mazzucato M, Masotti A, Ruggeri ZM. Localization and characterization of an alpha-thrombin-binding site on platelet glycoprotein Ib alpha. J Biol Chem 1994; 269: 6478-84.
46. Weeterings C, de Groot PG, Adelmeijer J, Lisman T. The glycoprotein Ib-IX-V complex contributes to tissue factor-independent thrombin generation by recombinant factor VIIa on the activated platelet surface. Blood 2008; 112: 3227-33.
47. Baglia FA, Badellino KO, Li CQ, Lopez JA, Walsh PN. Factor XI binding to the platelet glycoprotein Ib-IX-V complex promotes factor XI activation by thrombin. J Biol Chem 2002; 277: 1662-8.
48. Bradford HN, Pixley RA, Colman RW. Human factor XII binding to the glycoprotein Ib-IX-V complex inhibits thrombin-induced platelet aggregation. J Biol Chem 2000; 275: 22756-63.
49. Joseph K, Nakazawa Y, Bahou WF, Ghebrehiwet B, Kaplan AP. Platelet glycoprotein Ib: A zinc-dependent binding protein for the heavy chain of high-molecular-weight kininogen. Mol Med 1999; 5: 555-63.
50. White TC, Berny MA, Tucker EI, Urbanus RT, de Groot PG, Fernandez JA, Griffin JH, Gruber A, McCarty OJ. Protein C supports platelet binding and activation under flow: Role of glycoprotein Ib and apolipoprotein E receptor 2. J Thromb Haemost 2008; 6: 995-1002.
51. Gilbert JC, DeFeo-Fraulini T, Hutabarat RM, Horvath CJ, Merlino PG, Marsh HN, Healy JM, Boufakhreddine S, Holohan TV, Schaub RG. First-in-human evaluation of anti von willebrand factor therapeutic aptamer ARC1779 in healthy volunteers. Circulation 2007; 116: 2678-86.
52. Jilma-Stohlawetz P, Gilbert JC, Gorczyca ME, Knobl P, Jilma B. A dose ranging phase I/II trial of the von willebrand factor inhibiting aptamer ARC1779 in patients with congenital thrombotic thrombocytopenic purpura. Thromb Haemost 2011; 106: 539-47.
53. Schreijer AJ, Cannegieter SC, Meijers JC, Middeldorp S, Büller HR, Rosendaal FR. Activation of coagulation system during air travel: A crossover study. Lancet 2006; 367: 832-8.
54. Belcaro G, Geroulakos G, Nicolaides AN, Myers KA, Winford M. Venous thromboembolism from air travel: The LONFLIT study. Angiology 2001; 52: 369-74.
55. Cesarone MR, Belcaro G, Nicolaides AN, Incandela L, De S, Geroulakos G, Lennox A, Myers KA, Moia M, Ippolito E, Winford M. Venous thrombosis from air travel: The LONFLIT3 study-prevention with aspirin vs low-molecular-weight heparin (LMWH) in high-risk subjects: A randomized trial. Angiology 2002; 53: 1-6.

56. Belcaro G, Cesarone MR, Shah SS, Nicolaides AN, Geroulakos G, Ippolito E, Winford M, Lennox A, Pellegrini L, Brandolini R, Myers KA, Simeone E, Bavera P, Dugall M, Di Renzo A, Moia M. Prevention of edema, flight microangiopathy and venous thrombosis in long flights with elastic stockings. A randomized trial: The LONFLIT 4 concorde edema-SSL study. Angiology 2002; 53: 635-45.
57. Belcaro G, Cesarone MR, Nicolaides AN, Ricci A, Geroulakos G, Shah SS, Ippolito E, Myers KA, Bavera P, Dugall M, Moia M, Di Renzo A, Errichi BM, Brandolini R, Dugall M, Griffin M, Ruffini I, Ricci A, Acerbi G. Prevention of venous thrombosis with elastic stockings during long-haul flights: The LONFLIT 5 JAP study. Clin Appl Thromb Hemost 2003; 9: 197-201.
58. Kuipers S, Schreijer AJ, Cannegieter SC, Büller HR, Rosendaal FR, Middeldorp S. Travel and venous thrombosis: A systematic review. J Intern Med 2007; 262: 615-34.
59. Scurr JH, Machin SJ, Bailey-King S, Mackie IJ, McDonald S, Smith PD. Frequency and prevention of symptomless deep-vein thrombosis in long-haul flights: A randomised trial. Lancet 2001; 357: 1485-9.
60. Rosendaal FR. Interventions to prevent venous thrombosis after air travel: Are they necessary? no. J Thromb Haemost 2006; 4: 2306-7.
61. Cannegieter SC. Travel-related thrombosis. Best Pract Res Clin Haematol 2012; 25: 345-50.
62. Schut AM, Venemans-Jellema A, Meijers JC, Middeldorp S, de Groot PG, Rosendaal FR, Roest M, Lisman T, Cannegieter SC. Coagulation activation during air travel is not initiated via the extrinsic pathway. Br J Haematol 2015; 169: 903-5.
63. Schut AM, Meijers JC, Lisman-van Leeuwen Y, van Montfoort ML, Roest M, de Groot PG, Urbanus RT, Coppens M, Lisman T. Decreased plasma levels of activated factor VII in patients with deep vein thrombosis. J Thromb Haemost 2015; 13: 1320-4.
64. Büller HR, Bethune C, Bhanot S, Gailani D, Monia BP, Raskob GE, Segers A, Verhamme P, Weitz JI, FXI-ASO TKA Investigators. Factor XI antisense oligonucleotide for prevention of venous thrombosis. N Engl J Med 2015; 372: 232-40.
65. Meijers JC, Tekelenburg WL, Bouma BN, Bertina RM, Rosendaal FR. High levels of coagulation factor XI as a risk factor for venous thrombosis. N Engl J Med 2000; 342: 696-701.