Congenital clotting factor deficiency and cardiovascular disease: protected by nature?

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Congenital clotting factor deficiency and cardiovascular disease: protected by nature?

Congenitale stollingsfactor deficiëntie en cardiovasculaire ziekten: van nature beschermd?
(met een samenvatting in het Nederlands)

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door

Attie Tuinenburg
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Contents

Chapter 1 Introduction and thesis outline

Hemophilia A and B are hereditary X-chromosomal recessive bleeding disorders caused by a deficiency of or a functional defect in clotting factor VIII (FVIII) or IX (FIX), respectively. FVIII and FIX are proteins essential for enhancement of thrombin generation and propagation of fibrin formation. The birth prevalence of hemophilia A is 1 in 5,000 life male births, whereas this is 1 in 20,000 life male births in hemophilia B [1-3]. There are approximately 1,600 patients with hemophilia in the Netherlands. Severity of bleeding symptoms depends on the residual plasma concentration of the deficient clotting factor, expressed as international units (IU) per ml plasma. Severe hemophilia (< 0.01 IU/ml) is characterized by spontaneous bleeds, which are mainly joint and muscle bleeds. Patients with moderate (0.01 – 0.05 IU/ml) or mild (0.06 – 0.40 IU/ml) hemophilia usually experience excessive bleeding after minor trauma, dental procedures or surgery[1-3].

Bleeding episodes are prevented or controlled by intravenous infusion of clotting factor concentrates. Since the introduction of clotting factor concentrates in the 1960s and prophylaxis in the 1970s, life expectancy of patients with hemophilia in developed countries has increased from less than 30 years to over 70 years[4-6]. Consequently, aging hemophilia patients not only experience medical issues associated with the congenital bleeding disorder, the long-term consequences of lack of treatment (e.g. arthropathy), or iatrogenic-induced viral infections (e.g. HIV and HCV). Increasingly, hemophilia patients are confronted with age-related comorbidities, including ischemic cardiovascular disease (CVD)[7,8]. Interestingly, cohort studies reported a reduced mortality due to ischemic heart disease (IHD) in hemophilia patients as compared with the general population[4,6]. In addition, in a recent retrospective study it was shown that nonfatal myocardial infarction occurred less often in patients with severe hemophilia than in patients with moderate or mild hemophilia or in the general male population[9]. Several mechanisms explaining these observations can be considered. Differences in the prevalence of cardiovascular risk factors between hemophilia patients and the general population could account for the lower IHD mortality. A second, very plausible, explanation could be that the hypocoagulable state of hemophilia patients has a protective effect on thrombus formation, which precipitates myocardial infarction. As coagulation plays a role in

atherogenesis, a third possibility could be that congenital clotting factor deficiencies have a protective effect on the development of atherosclerosis.
This thesis focuses on 1) the development of atherosclerosis in patients with hemophilia or von Willebrand disease (vWD), and on 2) the management of IHD in hemophilia patients.

In **chapter 2** an overview of the literature, published until July 2008, on ischemic CVD, cardiovascular risk factors, thrombosis and atherosclerosis in hemophilia patients is given. Studies investigating the possible mechanisms explaining the reduced IHD mortality in hemophilia patients are summarized. In **chapter 3 and 4** the hypothesis that hemophilia patients develop less atherosclerosis than men without hemophilia is investigated using different non-invasive vascular imaging techniques.

Also in patients with vWD, the most common hereditary bleeding disorder and caused by von Willebrand factor (vWF) deficiency or dysfunction, the prevalence of ischemic CVD is lower as compared to the general population[10]. As vWF itself is thought to be involved in atherogenesis and as vWF functions as a carrier protein for FVIII in plasma, it could well be that, in addition to hemophilia patients, patients with vWD develop less atherosclerosis as subjects without a coagulopathy. In **chapter 5**, an overview of the available literature, published until July 2011, on vWF deficiency and atherosclerosis is given.

Although IHD mortality is lower in hemophilia patients as compared to the general male population[4,6], and myocardial infarction occurs less often in patients with severe hemophilia[9], an increase in the incidence and prevalence of IHD is observed in the elderly hemophilia population[7,8]. Treatment of IHD in hemophilia patients is complex, because of the delicate equilibrium between bleeding and thrombosis. As evidence-based guidelines on how to treat IHD in hemophilia patients are lacking, an institutional guideline, based on both experience from hemophilia specialists and existing guidelines on treatment of IHD in non-hemophilic patients, was developed in 2009 and is presented in **chapter 6**. Feasibility and safety of this guideline is assessed in **chapter 7**. Based on the described case series

and developments in new guidelines for non-hemophilic patients with IHD, some adjustments on the 2009 guideline are proposed.

Obesity is an important cardiovascular risk factor and the prevalence of obesity is increasing in hemophilia patients[11]. The study population used in chapter 4 was selected on a body mass index ≥ 30 kg/m^2 or ≤ 25 kg/m^2. In **chapter 8,** a sub-study performed in this study population is described. In the general population, obesity is associated with a prothrombotic state. The effect of obesity on bleeding frequency and clotting factor concentrate usage in hemophilia patients is investigated. In addition, we assess whether hemostatic and fibrinolytic changes observed in obesity differ between men with and without hemophilia.

The process of hemostasis is dependent on the interplay between platelets, coagulation, the vessel wall, and blood rheology. The balance between the determinants of hemostasis results in, what was named by prof. dr. Eikenboom, the hemostasis integral[12]. What happens with the hemostasis integral when patients with FVIII or FIX deficiency are treated with, for example, aspirin and clopidogrel? Current tests use either platelet poor plasma for coagulation assays or platelets devoid of plasmatic components and are mostly performed under static conditions. A field of research which is relatively unexplored considers the investigation of the effects of platelet activation and aggregation on coagulation and vice versa. A global hemostasis test, which integrates the different determinants of hemostasis does not yet exist[12]. In **chapter 9**, we describe the development of a microfluidic device with static mixer, which gives us new possibilities to study the interplay between thrombogenic surfaces, platelets and coagulation under conditions of flow.

Chapter 10 provides a summary and general discussion of the previous chapters.

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Chapter 2 Cardiovascular disease in patients with hemophilia

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Summary

Mortality due to ischemic heart disease in hemophilia patients is lower as compared to the general male population. Differences in the prevalence of cardiovascular risk factors cannot explain this finding. The hypocoagulable state of hemophilia patients might have a protective effect on thrombus formation, which precipitates infarction. It remains unclear whether the deficiency of coagulation factor VIII or IX exerts a protective effect on the development of atherosclerosis. Despite the relative protection against cardiovascular events, the incidence of ischemic cardiovascular disease in hemophilia patients is increasing, because life expectancy of these patients now approaches that of the general population. This review focuses on what is currently known about cardiovascular risk factors, atherosclerosis, arterial thrombosis and ischemic cardiovascular disease in hemophilia patients.

Introduction

Since the introduction of clotting factor concentrates in the 1960s and prophylaxis in the 1970s, life expectancy of patients with hemophilia in developed countries has increased from less than 30 years to over 70 years[1-3]. The Hemophilia in the Netherlands (HiN) surveys demonstrated that between 1972 and 2001, life expectancy of patients with hemophilia did not notably change[3-5]. The life expectancy was 66 years in 1972 and 67 years in 2001. In patients with severe hemophilia, however, life expectancy decreased from 63 to 59 years[3-5]. This was mainly due to the increased mortality caused by the acquired immunodeficiency syndrome (AIDS) and the consequences of hepatitis C virus (HCV) infection. Human immunodeficiency virus (HIV) and HCV were transmitted by infected plasma-derived clotting factor concentrates. In the Netherlands, plasma-derived clotting factor concentrates have been free of HIV since 1985, and free of HCV since 1992[3,6]. When individuals infected with HIV and HCV were excluded, life expectancy of patients with mild and moderate hemophilia approached that of the Dutch male population in general (75 years as compared to 76 years), and life expectancy of patients with severe hemophilia increased to 71 years[3]. Similar results were reported in articles from the UK and Greece[1,7]. Consequently, the aging hemophilia patient not only suffers from medical issues associated with the congenital bleeding disorder, the long-term consequences of lack of treatment (e.g. arthropathy), or iatrogenic-induced viral infections (e.g. HIV and HCV); increasingly, hemophilia patients are confronted with age-related comorbidity. In accordance with this finding, hematologists observe an increase in the number of hemophilia patients with ischemic cardiovascular disease over the last decade. Unfortunately, statistically significant results to support this are lacking[3,8]. Although the incidence and prevalence of ischemic cardiovascular disease appear to be increasing in hemophilia patients, several cohort studies have reported a reduced mortality due to ischemic heart disease in hemophilia patients as compared to the general age-matched male population[1,3-5,7,9].

The delicate equilibrium between bleeding and thrombosis must be taken into account when treating these patients with antithrombotic medication or during cardiac

intervention. It is important that cardiologists and hematologists are informed about the background of this increasing problem, in order to optimize the multidisciplinary approach for these complex patients.

This review focuses on what is currently known about cardiovascular risk factors, atherosclerosis, arterial thrombosis and ischemic cardiovascular disease in hemophilia patients.

Search strategy

We performed a comprehensive literature search of studies published until July 2008 using the Medline [http://www.ncbi.nlm.nih.gov/sites/entrez/] and EMBASE [http://www.embase.com] databases. The following keywords and synonyms of these keywords were used to identify potentially relevant studies: hemophilia, mortality, life expectancy, cause of death, factor VIII, factor IX, cardiovascular disease, ischemic heart disease, cerebrovascular disease, risk factors, atherosclerosis, and arterial thrombosis. Studies concerning venous thrombosis were excluded. The results were screened on title and abstract. References were checked for related articles that did not appear in the initial search. Relevant studies in English were used for this review.

Ischemic cardiovascular disease in hemophilia patients

Cohort studies

Patients with hemophilia seem to be partially protected from ischemic heart disease as compared to the general age-matched male population (Table 1). In the first HiN survey, Rosendaal et al.[4] documented an 80% reduction in mortality caused by ischemic heart disease in hemophilia patients in the late 1980s. Consecutive HiN surveys, conducted by Triemstra et al.[5] and Plug et al.[3] in largely the same cohort of hemophilia patients, confirmed this finding. Plug et al.[3] compared cause-specific mortality between Dutch

Table 1 Standard mortality ratios of ischemic heart disease in hemophilia patients

Author	Period	Hemophilia patients	Observed deaths*	Observed deaths per 1000 patients[II]	Expected deaths*	SMR (95% CI)
Rosendaal et al.[4], 1989 The Netherlands HiN survey	1973 – 1986	- n = 717 - 43 patients died - No deaths caused by AIDS or liver disease	1	1.4	5	0.2 (0.0 – 1.1)
Koumbarelis et al.[7], 1994 Greece	1972 – 1993	- n = 531 - Including HIV-positive and HCV-positive patients - 78 patients died	1	1.9	4	0.25 (0.0 – 1.4)
Triemstra et al.[5], 1995 The Netherlands HiN survey	1986 – 1992	- n = 919 - Including HIV-positive and HCV-positive patients - 45 patients died	1	1.1	5.2	0.2 (0.0 – 1.1)
Soucie et al.[11], 2000 USA	1993 – 1995	- n = 2950 - Including HIV-positive and HCV-positive patients - 236 patients died	8	2.7	NR	3.0 (1.5 – 5.8)
Plug et al.[3], 2006 The Netherlands HiN survey	1992 – 2001	- n = 967 - Including HIV-positive and HCV-positive patients - 94 patients died	6	6.2	12	0.5 (0.2 – 1.1)
Darby et al.[1], 2007 UK	1977 – 2000	- n = 6018 - HIV-negative patients - 862 patients died	104	17.3	166.53	0.62 (0.51 – 0.76)

HiN, Haemophilia in the Netherlands; SMR, standard mortality ratio (calculated by dividing the number of observed deaths by the number of expected deaths); CI, confidence interval; n, number of hemophilia patients in the study cohort; AIDS, acquired immunodeficiency syndrome; HIV, human immunodeficiency virus; HCV, hepatitis C virus; NR, not reported.

*Number of deaths due to ischemic heart disease. [II]Number of deaths due to ischemic heart disease per 1000 hemophilia patients; calculated using the number of patients in the cohort and the number of observed deaths.

hemophilia patients and the general age-matched male population during the period 1992 – 2001. The authors reported a standard mortality ratio (SMR), calculated by dividing the number of observed deaths by the number of expected deaths, of 0.5 for ischemic heart disease. In the UK, Darby et al.[1] also documented a lower SMR for ischemic heart disease in a population of more than 6000 hemophilia patients without HIV in 1977 – 2001. Moreover, even carriers of hemophilia were reported to be relatively protected against fatal ischemic heart disease, with a 36% reduction[10].

In contrast to the low SMRs reported in the previously mentioned studies, Soucie et al.[11] calculated an SMR for acute myocardial infarction of 3.0, indicating an increased mortality of hemophilia patients caused by myocardial infarction as compared to the general male population. The authors did not provide an explanation for this conflicting result. However, when calculating the observed number of deaths caused by ischemic heart disease per 1000 hemophilia patients for all cohort studies, the results were comparable (Table 1).

Although most cohort studies have demonstrated a reduced mortality caused by ischemic heart disease in hemophilia patients as compared to the general male population, the number of events in these studies was very low (Table 1). This increased the uncertainty about whether the association between hemophilia and a decreased risk of ischemic heart disease is true. In addition, no information about the prevalence of cardiovascular risk factors in the general male populations used to calculate the SMR is known. As most studies investigated cause-specific mortality in a hemophilia population including HIV- en HCV positive patients[3,5,7], competing risks may have occurred. Patients may have died of AIDS or end-stage liver disease before having had the time to develop ischemic heart disease. On the other hand, an increased incidence of ischemic cardiovascular disease in HIV-positive subjects receiving highly active antiretroviral therapy (HAART) has been demonstrated[12,13]. Table 2 summarizes all the disadvantages of the cohort studies.

Kulkarni et al.[14] assessed the age-specific prevalence of ischemic heart disease in 3422 American hemophilia patients, using hospital discharge rates between 1993 and 1998. The prevalence was 0.05% in patients under 30 years of age and 15.2% in those 60 years or older. The rate of ischemic heart disease discharges among 45 – 64 year-old hemophilia

patients was one-half that of non-hemophilic males; among hemophilia patients 65 years or older, these discharge rates were nearly 30% lower than that of non-hemophilic males[14].

Table 2 Disadvantages of the cohort studies, case reports and studies investigating intima-media thickness (IMT)

Studies	Disadvantages
Cohort studies[1,3-5,7,11]	- Small cohorts, number of events per study is low - Lack of information about the prevalence of cardiovascular risk factors in the general male populations - Competing risks
Case reports[15,16]	- Publication bias
Studies investigating IMT[39-43]	- Small sample sizes - Several studies include both hemophilia patients and patients with Von Willebrand disease - Differences in risk factors for atherosclerosis - Differences in treatment of the clotting factor deficiency - Comparing subjects with a normal IMT with subjects expected to have even thinner walls is difficult

Little is known about the number of deaths due to ischemic cerebrovascular accidents (CVAs) in patients with hemophilia. Darby et al.[1] reported a lower number of deaths due to ischemic CVA than expected, with an SMR of 0.63. This would be consistent with hemophilia having a protective effect against ischemic CVA as well as ischemic heart disease. However, this was based on only four deaths in a group of 6018 hemophilia patients, and there was no significant difference (95% confidence interval (CI) 0.17 to 1.62). Furthermore, in several studies addressing CVA in hemophilia patients, the authors did not differentiate between mortality due to ischemic or hemorrhagic CVA[4,5,9,10], and in other studies, only mortality from hemorrhagic CVA was investigated[3,7,11]. More studies comparing mortality caused by ischemic CVA between the hemophilia population and the general male population are needed.

Case reports

Girolami et al.[15,16] published two reviews evaluating all reported cases of thrombotic cardiovascular events until 2005 in patients with hemophilia A and B. In these reviews, there seems to be an association between the occurrence of myocardial infarction and recent administration of clotting factor products. In patients with hemophilia A, 22 of 36 myocardial infarctions occurred following infusion of factor VIII concentrate and, more frequently, after administration of activated prothrombin complex concentrate or recombinant factor VIIa concentrate[16]. In 3 cases, myocardial infarction occurred immediately after desmopressin administration[16]. In patients with hemophilia B, 8 of 13 events occurred after infusion of prothrombin complex concentrate, plasma, cryoprecipitate, or activated prothrombin complex concentrate[15]. The results in these reviews should be interpreted with caution, because of substantial publication bias (Table 2). Most case reports consider patients with severe hemophilia, whereas myocardial infarction is expected to occur more often in patients with moderate or mild hemophilia. Additionally, an underestimation of spontaneous ischemic events without previous treatment with clotting factor products is to be expected. These case reports do, however, reflect the need for attentiveness in infusing clotting factor concentrates in hemophilia patients, especially in patients with known cardiovascular risk factors, atherosclerosis, or myocardial ischemia.

The group of Girolami also evaluated case reports regarding ischemic CVA in patients with hemophilia A and B[15,16]. Six ischemic CVAs were described in patients with hemophilia A and one in a patient with hemophilia B. An association with infusion of clotting factor concentrates was found in four of six patients with hemophilia A. The ischemic CVA in the patient with hemophilia B developed after orthopedic surgery, where clotting factor concentrates were given. Again, publication bias will have had an influence on these results.

There is no literature available on the occurrence of peripheral vascular disease in patients with hemophilia.

Cardiovascular risk factors in hemophilia patients

Not much is known about the prevalence of cardiovascular risk factors, for example hypertension, hypercholesterolemia, obesity, and diabetes mellitus, in the hemophilic population as compared to the general population. In Dutch hemophilia patients, mean systolic blood pressure was non-significantly higher (difference 3.7 mmHg, 95% CI -0.3 – 7.7 mmHg) than in the general male population, whereas mean diastolic blood pressure was significantly higher (difference 5.8 mmHg, 95% CI 3.6 – 8.0 mmHg)[17]. After age adjustment, hemophilia patients were twice as often treated with antihypertensive medication as the comparison population. No association was found between the severity of hemophilia and blood pressure[17]. In a cohort of Canadian patients with mild hemophilia, 29% were hypertensive as compared to 18% of the control subjects[18]. Rosendaal et al.[17] suggested that hypertension is more likely to be detected because of the intensive medical care received by hemophilia patients. This appeared not to be the explanation, because even though antihypertensive treatment was twice as common as in the control population, the overall mean blood pressure was still higher than expected[17].

Another possible explanation for the high prevalence of hypertension in hemophilia patients as compared to the general population is discussed by Rosendaal et al.[17] and Kulkarni et al.[19]. The incidence of renal disease, as well as the mortality due to renal insufficiency, was higher among hemophilia patients than in the general male population[4,20]. Besides renal bleeding, HIV infection and acute renal obstruction following treatment of a bleed with tranexamic acid can cause renal disease in hemophilia patients[20-23]. Rosendaal et al.[17] reported that creatinine levels, as a determinant of renal function, were not associated with the occurrence of hypertension. Kulkarni et al.[19] showed that acute and chronic renal disease discharge rates were higher in the hemophilia population than in the general population. There was a clear relationship between hypertension and acute and chronic renal disease. Unfortunately, the etiologic role of hypertension in the development of renal disease was not investigated. The incidence of hypertension in hemophilia patients admitted for kidney bleeds was 10.1% as compared to 4.5% among those admitted for other reasons[19].

The mean cholesterol concentration in Dutch hemophilia patients was significantly lower than in the general population (4.8 versus 5.6 mmol/l). Although not statistically significant, cholesterol levels were lowest in patients with severe hemophilia, which could suggest an association between low cholesterol concentration and the clotting factor deficiency or its treatment[17]. One hypothesis is that viral infections influence both the immune system and liver function, and therefore have an effect on cholesterol concentration[17]. This is supported by the observation that chronic hepatitis C is associated with lower cholesterol concentrations[24]. Hepatitis C status of the patients was not mentioned in the study by Rosendaal et al[17]. From other studies, it is known that at that time 80% of Dutch patients treated with clotting factor concentrates developed chronic hepatitis C[6]. A US study showed a prevalence of hyperlipidemia of 0.4% in patients with hemophilia[14]. Sixty-four per cent of the patients were younger than 30 years. This result was not compared to the prevalence of hyperlipidemia in the general male population.

Rosendaal et al.[17] demonstrated no difference in either body mass index (BMI) or the proportion of smokers between Dutch hemophilia patients and the general male population. Between 1992 and 2001, the prevalence of overweight and of obesity in Dutch adult hemophilia patients increased from 27% to 35% and from 4% to 8%, respectively. The prevalence of overweight was lower in hemophilia patients than in the general male population. However, the prevalence of obesity, and the increase in the prevalence of overweight and obesity, were comparable between these groups[25]. In a Canadian study, the mean BMI for patients with mild hemophilia was 30.2 kg/m^2, as compared to 27.8 kg/m^2 in the control group (95% CI for the difference 0.4 – 4.4)[18]. BMI was positively associated with functional limitations regardless of hemophilia severity[18,26,27].

Published reports show conflicting results on the prevalence of diabetes mellitus in hemophilia patients. In a cohort of Canadian patients with mild hemophilia A, the prevalence of diabetes mellitus was 24%, in comparison with 6% in the control group (95% CI of the difference 3.4 – 33.4%)[18]. The authors do not provide an explanation for this difference. The prevalence of diabetes mellitus in a US cohort of patients with hemophilia was 1.8%[14]. The reasons for the large difference in the prevalence of diabetes mellitus

between the two studies are not clear, but different definitions of diabetes mellitus might have contributed to the conflicting results.

Special consideration is needed for HIV-positive hemophilia patients, who are mostly treated with HAART. HAART, and especially the use of protease inhibitors, is associated with the occurrence of diabetes mellitus, hyperlipidemia, and elevated total cholesterol[12,13,28]. The DAD study group demonstrated an association between HAART therapy and the risk of myocardial infarction[28]. In 2007, this group calculated a relative rate of myocardial infarction per year of protease inhibitor exposure of 1.16 (95% CI 1.10 – 1.23), after adjustment for exposure to another drug class and established cardiovascular risk factors (excluding lipid levels)[13]. The risk of myocardial infarction in HIV-positive hemophilia patients treated with HAART is not known.

Kulkarni et al.[14] demonstrated that known risk factors for cardiovascular disease (e.g. increasing age, hypertension, diabetes mellitus, and hyperlipidemia) were independently associated with ischemic heart disease in individuals with hemophilia, just as in the general population. In 1990, Rosendaal et al.[17] investigated whether the low mortality from ischemic heart disease in hemophilia patients could be attributed to differences in risk factors for myocardial infarction between these patients and the general male population. For smoking, blood pressure, cholesterol, and body weight, the observed risk factor levels were compared with the expected risk factor levels. After combining these factors into an overall risk ratio, the risk for myocardial infarction was 22% lower in hemophilia patients than in the general male population. The investigators concluded that a difference in risk factors could explain only a fraction of the decreased mortality caused by ischemic heart disease among hemophilia patients[17]. This implies that patients with hemophilia are probably protected from ischemic heart disease by the clotting factor deficiency itself or its sequelae.

Atherosclerosis and thrombosis in hemophilia patients

Reduced mortality from ischemic heart disease in patients with hemophilia has been attributed to the hypocoagulable state of these patients. This might have a protective effect on thrombus formation, which precipitates infarction. The fact that elevated levels of factor VIII (FVIII) are a common risk factor for venous thrombosis[29,30], and are associated with an increased risk for ischemic heart disease[31-34] and stroke[35], strengthens this hypothesis. It remains unclear whether the deficiency of FVIII or factor IX (FIX) also exerts a protective effect against the development of atherosclerosis. Studies evaluating the association between elevated clotting factor levels and atherosclerosis report conflicting results[34,36,37].

Atherosclerosis

In 1957, typical angina pectoris and extensive atherosclerosis were described for the first time in a patient with moderate hemophilia[38]. Srámek et al.[39] used B-mode ultrasound to quantify intima-media thickness (IMT), assessing early atherosclerotic changes in carotid and femoral arteries in 76 patients with bleeding disorders (59 hemophilia patients and 17 patients with von Willebrand disease) and in 142 healthy controls. No differences in IMT of the carotid artery were detected between patients with bleeding disorders and healthy controls. IMT of the femoral artery was minimally, but non-significantly, reduced in patients with bleeding disorders as compared to healthy controls (adjusted difference -0.078 mm, 95% CI -0.17 – 0.018 mm). Subgroup analysis revealed that femoral artery walls were thinnest in individuals with moderate to severe hemophilia (adjusted difference -0.10 mm, 95% CI -0.27 – 0.061 mm). From this study, it appears that the hypocoagulability caused by hemophilia has no, or at most a minor, effect on atherogenesis[39]. An Italian group[40], however, demonstrated a significantly lower mean IMT in 50 hemophilia patients (38 severe, 12 moderate) than in 50 age-matched control subjects. At least one atherosclerotic plaque was found in six (12.0%) hemophilia patients as compared to 15 (30.0%) control subjects[40]. This study confirms previous results of the same group[41,42], showing that patients with hemophilia or von Willebrand disease had a lower number and

grade of atherosclerotic plaques (assessed by B-mode ultrasonography in carotid and leg arteries and the abdominal aorta). However, these articles[40-42] do not contain enough information about the control groups. In a more recent study from the Italian group[43], no differences were seen in IMT between 40 hemophilia patients (24 severe or moderate disease, mean age 39.5 years, 16 mild disease, mean age 49.5 years) and 40 age-matched control subjects. The authors suggest that this result is consistent with the middle-aged status of the patient population. However, the mean ages of the hemophilia patients in the previous studies of this group were 58.2, 48.3 and 41.7 years[40-42]. The results of the studies concerning IMT in hemophilia patients are summarized in Table 3.

Endothelial dysfunction precedes structural atherosclerotic changes in the vascular wall. Sartori et al.[43] evaluated endothelial function by measuring flow-mediated dilatation and tissue-type plasminogen activator (t-PA) release before and after 20 minutes of brachial venous occlusion. Flow-mediated dilatation and the mean t-PA release were significantly lower, and thus impaired, in hemophilia patients as compared to control subjects. There is no clear explanation for this finding. As the Italian group was the first to assess endothelial function in hemophilia patients, more studies are needed to confirm these results.

Previous studies are hampered by small sample sizes, patient groups containing both hemophilia patients and patients with von Willebrand disease, differences in risk factors for atherosclerosis, and differences in treatment of the clotting factor deficiency. It is difficult to compare subjects with a normal IMT with subjects expected to have even thinner walls. A study comparing IMT between hemophilia patients with and without cardiovascular risk factors, and non-hemophilic subjects with and without cardiovascular risk factors, might be more informative about the role of FVIII and FIX in atherogenesis. The disadvantages of the studies investigating IMT are summarized in Table 2.

Nowadays, the majority of patients with severe hemophilia are using prophylactic treatment. This may dilute the effect of FVIII deficiency on atherosclerosis. For research purposes, this problem is avoided by using a mouse model, as was done in the study by Khallou-Laschet et al.[44]. In comparison to apolipoprotein E deficient mice (E°) mice, which are atherosclerosis-prone, apolipoprotein E and FVIII double deficient (E°/FVIII°) mice developed dramatically fewer early-stage atherosclerotic lesions. The early lesions in E°

Table 3 Intima-media thickness in hemophilia patients as compared to age-matched control subjects

Author	Subjects	Mean age patients (years)	IMT
Bilora et al.[41], 1999	- 76 patients with either hemophilia A or vWD (46 men, 30 women) - 77 age-matched control subjects (37 men, 40 women)	58.2	- 13.1% of the patients with hemophilia or vWD had carotid plaques vs. 27.2% of the controles (P < 0.05)
Bilora et al.[42], 2001	- 25 patients with hemophilia - 11 mild - 14 severe - 15 patients with vWD - 40 age-matched control subjects	48.3	- 3 of 40 patients with a coagulopathy had plaques in the abdominal aorta vs. 11 of 40 controls - 5 of 40 patients with a coagulopathy had plaques in the leg arteries vs. 17 of 40 controls
Srámek et al.[39], 2001	- 59 patients with hemophilia - 34 mild - 5 moderate - 20 severe - 17 patients with vWD - 142 age-matched control subjects	49	- Carotid artery: no difference - Femoral artery: lowest IMT in patients with moderate to severe hemophilia, adjusted difference -0.10 mm (95% CI 0.27 to 0.016 mm)
Bilora et al.[40], 2006	- 50 patients with hemophilia - 12 moderate - 28 severe - 50 age-matched control subjects	41.72	- Mean IMT is significantly lower in carotid, brachial and femoral arteries and in the abdominal aorta of hemophilia patients
Sartori et al.[43], 2008	- 40 patients with hemophilia - 16 mild - 24 severe-moderate - 40 age-matched control subjects	 49.5 39.5	- Carotid artery: no differences

IMT, intima-media thickness; vWD, von Willebrand disease; vs., versus.

mice contained abundant fibrinogen and fibrin deposits on which few platelets adhered, in contrast to the lesions in E°/FVIII° mice, which were almost devoid of fibrinogen, fibrin, and platelets. At a later stage, FVIII deficiency delayed plaque progression, but did not lead to a difference in plaque composition[44]. These data suggest that in the early development of atherosclerosis, the FVIII-dependent intrinsic coagulation pathway plays an important role, which declines with time.

Presumably, the lower cardiovascular mortality rates in patients with hemophilia are not solely the result of reduced atherogenesis. Although partial protection against atherosclerosis may exist in the hemophilia population, and might be dependent on the severity of hemophilia and the intensity of lifetime treatment with clotting factor concentrates, atherosclerosis does develop and is associated with standard cardiovascular risk factors.

Thrombosis

A plausible explanation for the decreased mortality from ischemic heart disease in hemophilia patients is a decreased tendency to form occlusive thrombi.

Current coagulation tests use either platelet poor plasma for plasmatic coagulation assays, or platelets devoid of plasmatic components. In these tests, the interactions between primary and secondary hemostasis, high shear stress and the endothelium are missing. This problem has been partially overcome by Mizuno et al.[45]. They described a flow chamber system that enables real-time observation of intrathrombus fibrin accumulation during platelet thrombogenesis under flow conditions. Analysis by confocal laser scanning microscopy during perfusion of whole blood, anticoagulated to various extents, revealed that the size and shape of mural thrombi depend on the intrathrombus fibrin development under physiologic high shear rate conditions. The intrathrombus fibrin deposition in a patient with mild hemophilia A was observed to be significantly reduced as compared to that of control thrombi, albeit with normal thrombus volume. In two patients with severe hemophilia A, both the volume and fibrin accumulation of thrombi were significantly reduced as compared to control thrombi. Addition of recombinant factor VIIa

nearly normalized intrathrombus fibrin network formation, with modest improvement in the final thrombus volume and shape[45].

The in vivo hemostatic response in FIX-deficient (FIX°) mice as compared to wild-type mice was morphologically examined at early and late time points following mechanical injury of the endothelium of the carotid artery. In addition, thrombogenesis was studied after chemical injury of a mesenteric arteriole using intravital microscopy. The development of a hemostatic plug and fibrin mass was significantly reduced and delayed in FIX° mice. Injury of the mesenteric arteriole in FIX° mice resulted in few small platelet aggregates, which dissociated easily, in contrast to the stable occlusive platelet plugs formed in wild-type mice[46]. Wang et al.[47] also reported a defect in occlusive thrombus formation in the carotid artery in FIX° mice after ferric chloride-induced endothelial injury measured with a Doppler flow probe. The effect of FIX inhibitors was studied in several animal arterial thrombosis models. These studies showed reduced vascular thrombosis in animals treated with an FIX inhibitor as compared to animals not treated with an FIX inhibitor[48-53].

Although several in vitro experiments and animal models suggest that FVIII or FIX deficiency reduces the tendency to form occlusive arterial thrombi[45-47,49], this is contradicted by the study of Nichols et al.[54]. Animal models in dogs with arterial stenosis in which thrombosis was induced revealed that normal arterial occlusion developed in normal dogs as well as in dogs with hemophilia A[54].

Hemophilia patients with cardiovascular disease: clinical practice

There are no evidence-based guidelines for the treatment of ischemic cardiovascular disease in hemophilia patients. Atherosclerosis and ischemic cardiovascular disease require treatment to decrease the risk of thrombus formation. However, anticoagulant and antiplatelet medications increase the bleeding risk in hemophilia patients, and cardiovascular interventions are frequently complicated by bleeding. To minimize these risks, the clotting factor deficiency has to be corrected. However, infusion of clotting factor concentrates can be followed by ischemic cardiovascular disease[15,16]. The

equilibrium between bleeding and thrombosis must be taken into account when treating ischemic cardiovascular disease in hemophilia patients.

Guidelines for the prevention and treatment of ischemic cardiovascular disease in non-hemophilic patients should, in principle, be applicable to hemophilia patients. The hemophilia comprehensive care team has an important function in signalling cardiovascular risk factors in hemophilia patients. Cardiovascular risk factors such as diabetes mellitus and hypertension can be treated and followed by the internal physician or general practitioner. In the majority of hemophilia patients, antiplatelet therapy is manageable. When bleeding frequency increases, aspirin treatment should be stopped. When clotting factor levels are partially corrected, invasive cardiovascular interventions, anticoagulant therapy or dual antiplatelet therapy are often feasible. Close collaboration between the cardiologist and the hemophilia comprehensive care team is necessary to offer these patients an optimal treatment.

Conclusion

The occurrence of age-related comorbidity in hemophilia patients is increasing, as is the occurrence of ischemic cardiovascular disease. Additionally, there is reasonable evidence that hemophilia patients are relatively protected against fatal cardiovascular events as compared to the general population. An international prospective study in a large cohort of hemophilia patients would provide more accurate information on the incidence of ischemic cardiovascular events and death, and possibly on the prevalence and incidence of cardiovascular risk factors. Currently, not much is known about the underlying mechanisms explaining the reduced mortality of hemophilia patients due to ischemic cardiovascular disease. Knowledge about the role of FVIII or FIX and the intrinsic coagulation pathway in atherogenesis and arterial thrombosis precipitating an ischemic event can help us understand these processes in more detail, and may eventually lead to new prevention strategies or medical treatments in patients both with and without hemophilia.

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Chapter 3 Coronary artery calcification in hemophilia A: no evidence for a protective effect of factor VIII deficiency on atherosclerosis

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Summary

Ischemic heart disease mortality is lower in hemophilia patients than in the general male population. As coagulation plays a role in the inflammatory pathways involved in atherogenesis, we investigated whether the clotting factor deficiency protects hemophilia patients from developing atherosclerosis.

Coronary artery calcification, measured with multi detector-row computed tomography, was compared between 42 men, ≥ 59 years, with severe or moderate hemophilia A, and 613 non-hemophilic men from the Rotterdam Study, a prospective population-based study. None of the study subjects were HIV infected or had a history of cardiovascular disease. Coronary artery calcification was quantified by calculating the Agatston score and calcification mass. Data were analyzed using linear regression. Mean difference (β) of the natural log-transformed Agatston score between men with and without hemophilia was 0.141 (95% confidence interval (CI) -0.602 – 0.885; P = 0.709). Results did not change after adjustment for age, body mass index, hypercholesterolemia, hypertension, and use of antidiabetic medication (β = 0.525, 95% CI -0.202 – 1.252; P = 0.157). Comparable results were found for calcification mass.

The extent of coronary artery atherosclerosis is comparable between elderly men with and without hemophilia. Results from this study underline the importance of screening and treating atherosclerosis risk factors in hemophilia patients.

Introduction

Since the introduction of clotting factor concentrates, life expectancy of hemophilia patients has increased and now approaches that of the general male population[1,2]. Consequently, aging hemophilia patients not only experience medical issues associated with their congenital bleeding disorder, but are increasingly confronted with age-related comorbidities, including ischemic cardiovascular disease (CVD)[3,4]. Interestingly, cohort studies reported a reduced mortality due to ischemic heart disease (IHD) in hemophilia patients as compared with the general male population[1,2].

Several mechanisms explaining this observation have been considered[5]. Rosendaal et al.[6] showed that differences in the prevalence of cardiovascular risk factors between men with and without hemophilia could not account for the lower IHD mortality in hemophilia patients. Another, very plausible, explanation could be that the hypocoagulable state of hemophilia patients has a protective effect on thrombus formation, which precipitates myocardial infarction[7,8]. In this study, we focused on the hypothesis that hemophilia patients develop less atherosclerosis than men without hemophilia. Atherosclerosis is an inflammatory disease, which develops in response to injury of the endothelium[9,10]. Current evidence supports considerable cross-talk between thrombosis and the inflammatory pathways involved in the different stages of atherogenesis (e.g. plaque initiation and expansion, and fibrous cap degradation and rupture)[11,12]. Thrombin is the main effector of the coagulation cascade and links coagulation to inflammation by having proinflammatory effects, via protease-activated receptors, on the cells involved in atherogenesis[11,12]. Activation of prothrombin into thrombin is less in hemophilia patients as compared with non-hemophilic subjects. This might reduce the stimulation of proinflammatory pathways supporting atherogenesis. This is in line with the observation that elevated factor VIII (FVIII) levels have been positively associated with atherosclerosis[13,14].

As compared with apolipoprotein E deficient mice, apolipoprotein E and FVIII double-deficient mice developed considerably fewer early-stage atherosclerotic lesions, which were almost devoid of fibrinogen, fibrin and platelets. At a later stage, FVIII deficiency only

delayed plaque progression[15]. Recently, Fabri et al.[16] showed that FVIII deficiency was associated with an antiatherosclerotic phenotype in apolipoprotein E deficient mice. However, FVIII deficiency did not influence the degree of atherosclerosis in mice lacking the low-density lipoprotein receptor, a model more resembling the human situation[16]. Conflicting results have also been reported by studies comparing intima-media thickness (IMT) between hemophilia patients and non-hemophilic subjects[17-21]. This could be due to heterogeneous study populations including patients with other bleeding disorders than hemophilia, low mean ages, not well chosen and/or defined comparison populations, and variability in tests and outcome parameters.

With multi detector-row computed tomography (MDCT), the amount of coronary artery calcification (CAC) can be assessed noninvasively. Because CAC is correlated with the amount of coronary atherosclerotic plaque in histopathologic studies, it can be used as a measure of the extent of coronary artery atherosclerosis[22,23]. In this cross-sectional study, we investigated the influence of FVIII deficiency on atherosclerosis by comparing CAC between elderly hemophilia A patients and the general male population.

Methods

Study design and study population

Male patients, ≥ 59 years, with severe (FVIII activity < 1%) or moderate (FVIII activity 1 – 5%) hemophilia A, HIV-negative, asymptomatic for CVD, and treated at 1 of the participating Dutch hemophilia treatment centers (University Medical Center Utrecht; Erasmus University Medical Center, HagaZiekenhuis; Radboud University Nijmegen Medical Centre; Maxima Medical Center; Academic Medical Center) were eligible for this study. Symptomatic CVD comprised a history of myocardial infarction, percutaneous coronary intervention, coronary artery bypass graft surgery, atrial fibrillation, or stroke. All hemophilia patients were Caucasian and underwent study examinations between November 2009 and February 2011.

Comparison data on CAC and cardiovascular risk factors were obtained from the Rotterdam Study, a prospective population-based study with the aim to assess the occurrence of chronic diseases in an aging population and to clarify their determinants[24]. In 1990, all inhabitants of a suburb of Rotterdam, the Netherlands, aged ≥ 55 years, were invited, and 7983 agreed to participate (78%). In 2000, the cohort was extended with 3011 subjects. From September 2003 until February 2006, all participants who completed a center visit (the fourth visit for the original cohort and the second visit for the extended cohort) were invited to undergo an MDCT scan of the coronary arteries[25]. Data on CAC were available for 2482 persons (age range 59 – 98 years). For the current study, Caucasian males from the Rotterdam Study without known HIV-infection, bleeding disorders, or symptomatic CVD before scanning were selected. The same definition for symptomatic CVD as in hemophilia patients was used. All eligible males from the Rotterdam Study matching the age of 1 of the hemophilia patients were included.

This study was approved by the Medical Ethics Committee of the University Medical Center Utrecht (Utrecht, the Netherlands). The Rotterdam Study was approved by the Medical Ethics Committee and the Radiation Protection Unit of the Erasmus University Medical Center (Rotterdam, the Netherlands). All participants provided written informed consent.

During the study visit of hemophilia patients to the University Medical Center Utrecht, characteristics of hemophilia patients were assessed. In addition, information on medical history, family history on CVD, medication use, and smoking behaviour was obtained. In the Rotterdam Study, this information was also collected. In both hemophilia patients and persons included in the Rotterdam Study, systolic and diastolic blood pressures were measured at the right brachial artery in a sitting position. The mean of 2 consecutive measurements was used. Hypertension was defined as a systolic blood pressure of ≥ 140 mmHg, a diastolic blood pressure ≥ 90 mmHg, or use of antihypertensive medication. Height and weight were measured, and body mass index (BMI) was calculated as weight divided by height squared (kg/m^2). Total cholesterol, high density lipoprotein (HDL) cholesterol, triglycerides, and glucose were measured in blood samples from both hemophilia patients and Rotterdam Study subjects. Low density lipoprotein (LDL)

cholesterol was calculated using Friedewald's equation. Hypercholesterolemia was defined as a total cholesterol level ≥ 6.5 mmol/l or use of lipid-lowering medication.

Ten-year risk of coronary heart disease (CHD) was calculated for all subjects, using the Framingham Point Scores as recommended in the third report of National Cholesterol Education Program[26]. Points were assigned to categorized risk factors and summed. The summary score corresponds to a 10-year predicted risk of CHD (nonfatal myocardial infarction or fatal CHD) and can be classified into low (< 10%), intermediate (10 – 20%), and high (> 20%) risk categories[26].

MDCT scanning

Hemophilia patients were scanned with a 128x2 detector computed tomography scanner (Brilliance iCT, Philips Healthcare, Cleveland, OH, USA) at the University Medical Center Utrecht. The cardiac scan reached from the tracheal bifurcation to the apex of the heart. Within a single breath hold, an overlapping data set of 3 mm thick slices was acquired (scan parameters: 128x0.625 mm collimation, 120 kVp, 30 – 80 mAs (depending on weight), prospective ECG triggering in mid-diastole). Quantification of CAC was performed by a trained scan reader (A.R.), blinded to the clinical data, using software for calcium scoring (Heartbeat-CS, EBW, Philips Medical Systems, Best, the Netherlands). All voxels with an attenuation of ≥ 130 Hounsfield units were highlighted by the software. Atherosclerotic calcifications in the left main, the left anterior descending, the left circumflex, and the right coronary arteries were manually identified by the scan reader. For each calcified lesion, the Agatston score was calculated as the product of the area of a calcified lesion and a factor was assigned according to the maximum attenuation value of the lesion[27]. The total Agatston score was calculated by adding up the scores of each individual lesion[27]. Because of the limited interscan reproducibility of the Agatston score, an algorithm quantifying the calcification mass, with lower interscan variability, was introduced[28-31]. Calcification mass (mg hydroxyapatite) was calculated as the volume of a calcification times the mean computed tomography number multiplied by a calibration factor c (mg/Hounsfield unitsxcm^3)[29].

The scan protocol and analyses of calcification in the Rotterdam Study have been described in detail previously[25,32]. In short, imaging was performed with a 16-slice (n = 152) or 64-slice (n = 461) MDCT scanner (SOMATON Sensation 16 or 64, Siemens, Forcheim, Germany). Within a single breath hold, 3 mm thick slices were acquired (scan parameters: 12x1.5 mm (16-slice scanner) or 32x0.6 mm (64-slice scanner) collimation, 120 kVp, prospective ECG triggering in mid-diastole). Two trained scan readers, blinded to the clinical data, determined CAC with dedicated software (Syngo Calcium Scoring, Siemens, Forcheim, Germany) that uses the same algorithms for the Agatston score and calcification mass as described above. To investigate whether the Agatston score, obtained with the software and by the scan readers of the Rotterdam Study, could be reproduced with the software and by the scan reader at the University Medical Center Utrecht, the same investigator (A.R.) rescored the CAC of 19 Rotterdam Study scans.

Statistical analyses

Spearman's rank correlation coefficient was calculated to assess reproducibility between the software and scan readers of the Rotterdam Study and the software and scan reader at the University Medical Center Utrecht. The distributions of the Agatston score and calcification mass were highly skewed, and therefore, natural log-transformed (ln-transformed) values were used (e.g. ln(Agatston score + 1)) when analyzing these outcome parameters linearly. The crude association between having hemophilia and the ln-transformed Agatston score was calculated, using univariate linear regression analysis. Imbalances of cardiovascular risk factors across exposure groups were assessed. Then, every potential confounder was included in a bivariate model and the percentage change of the crude regression coefficient was assessed. The covariates age, BMI, hypercholesterolemia, hypertension, and use of antidiabetic medication changed the crude regression coefficient the most (> 20%) and were included in a multivariate linear regression model. The association between having hemophilia and the ln-transformed calcification mass was adjusted for the same confounders. Differences between the ln-transformed Agatston score and calcification mass between patients with severe (FVIII activity < 1%) and moderate (FVIII activity 1 – 5%), and between patients using < 50.000

international units (IU) of clotting factor concentrate per year and patients using > 50.000 IU of clotting factor concentrate per year were assessed using the independent t test. As proposed by Rumberger et al.[33], Agatston scores were divided into 4 categories: absent – minimal (0 – 9), mild (10 – 99), moderate (100 – 399), and severe (≥ 400) degrees of calcification. Using chi-square tests, differences in Agatston score categories and 10-year CHD risk categories between men with and without hemophilia were assessed. Analyses were performed using SPSS 17.0 for Windows (SPSS, Inc, Chicago, IL, USA).

Results

Sixty eligible hemophilia patients were invited, and 42 agreed to participate (70%). Half of the hemophilia patients had a FVIII activity of < 1% (Table 1). Most hemophilia patients were treated on demand, and used less than 50.000 IU of clotting factor concentrate per year. Fourteen (33.3%) patients were infected with hepatitis C, while 16 (38.1%) patients were successfully treated or had spontaneously cleared the virus (Table 1). The selection procedure described earlier resulted in a comparison group of 613 non-hemophilic men.
Cardiovascular risk factors are described in Table 2. Mean age of the study population was 66.5 years (standard deviation 4.6). In both hemophilia patients and non-hemophilic men, age ranged from 59 – 77 years. As compared to men without hemophilia, more hemophilia patients smoked. Mean BMI, mean total cholesterol levels, and the proportion of subjects with hypercholesterolemia were higher in the comparison group than in hemophilia patients. Out of 42 hemophilia patients 18 (42.9%) used antihypertensive medication and 32 (76.2%) were hypertensive, whereas only 26.1% of the comparison population used antihypertensive medication and 65.1% was hypertensive. Mean blood pressure was comparable between the 2 study groups. No differences in 10-year CHD risk category distributions between men with and without hemophilia were found (P = 0.554).
Agatston scores obtained in the Rotterdam Study could be reproduced by the software and scan reader at the University Medical Center Utrecht (Spearman's rank correlation coefficient 0.986; P < 0.001). CAC outcomes are summarized in Table 3. No differences in

Agatston score category distributions between hemophilia patients and non-hemophilic males were found (P = 0.792). The proportion of subjects having an Agatston score of 1000 or more was comparable between men with and without hemophilia (11.9% versus 9.8%, respectively).

The mean ln-transformed Agatston score and calcification mass were slightly higher in hemophilia patients as compared to non-hemophilic men. Univariate linear regression analyses showed no association between the ln-transformed Agatston score and hemophilia (Table 4). Mean difference (β) of the ln-transformed Agatston score between men with and without hemophilia was 0.141 (95% confidence interval (CI) -0.602 – 0.885; P = 0.709). Results did not change after multivariate adjustment for age, BMI, hypercholesterolemia, hypertension, and use of antidiabetic medication (β = 0.525, 95% CI -0.202 – 1.252; P = 0.157). Comparable results were found for calcification mass (Table 4). When comparing the ln-transformed Agatston score and calcification mass between patients with severe (FVIII activity < 1%) and moderate (FVIII activity 1 – 5%) hemophilia, no differences were observed (mean difference -0.983, 95% CI -2.404 – 0.439; P = 0.170, and mean difference -0.871, 95% CI -2.009 – 0.267; P = 0.130, respectively). No statistically significant difference between the amount of clotting factor concentrate usage and the extent of CAC was found (Agatston score P = 0.066, calcification mass P = 0.052).

Table 1 Characteristics of hemophilia patients

Variable	**Patients with hemophilia (n = 42)**
Residual factor VIII activity	
- < 1%	21 (50.0)
- 1 – 5%	21 (50.0)
Current treatment	
- Prophylactic	15 (35.7)
- On demand	27 (64.3)
Amount of clotting factor concentrate used over the last year	
- < 50.000 IU	24 (57.1)
- 50.000 – 100.000 IU	0 (0.0)
- 100.000 – 150.000 IU	7 (16.7)
- 150.000 – 200.000 IU	8 (19.0)
- 200.000 – 250.000 IU	3 (7.1)
Presence of inhibitors	
- Current	2 (4.8)
- Past	1 (2.4)
- Never	39 (92.9)
Hepatitis C infection	
- Current	14 (33.3)
- Past	16 (38.1)
- Never	12 (28.6)

Categorical variables are expressed as numbers and percentages.
IU, international units; n, sample size.

Table 2 Cardiovascular risk factors

Variable	Patients with hemophilia (n = 42)	Non-hemophilic males (n = 613)
Age (years)	65.1 ± 5.1	66.6 ± 4.5
Current smokers	13 (31.0)	123 (20.1)
BMI (kg/m^2)	25.8 ± 3.8	27.5 ± 3.2
Hypercholesterolemia	6 (14.3)	192 (31.3)
Use of lipid lowering medication	3 (7.1)	105 (17.1)
Total cholesterol (mmol/l)	5.1 ± 0.9	5.6 ± 0.9
HDL cholesterol (mmol/l)	1.2 ± 0.3	1.3 ± 0.3
Total cholesterol/HDL cholesterol	4.5 ± 1.2	4.5 ± 1.2
LDL cholesterol (mmol/l)	3.3 ± 0.9	3.5 ± 0.9
Triglycerides (mmol/l)	1.5 ± 1.4	1.6 ± 0.8
Hypertension	32 (76.2)	399 (65.1)
Use of antihypertensive medication	18 (42.9)	160 (26.1)
Systolic BP (mmHg)	146 ± 16	145 ± 19
Diastolic BP (mmHg)	82 ± 12	83 ± 10
Use of antidiabetic medication	4 (9.5)	39 (6.4)
Glucose (mmol/l)	5.8 ± 1.3	5.8 ± 1.3
Family history of CVD	11 (26.2)*	172 (28.1)[II]
10-year CHD risk categories[#]		
- < 10%	4 (9.5)	38 (6.2)
- 10 – 20%	28 (66.7)	451 (73.6)
- > 20%	10 (23.8)	124 (20.2)

Values of continuous variables are expressed as mean ± standard deviation. Categorical variables are expressed as numbers and percentages.

BMI, body mass index; HDL, high density lipoprotein; LDL, low density lipoprotein; BP, blood pressure; CVD, cardiovascular disease; CHD, coronary heart disease; n, sample size.

*History of CVD before the age of 60 years in a first degree relative.

[II]History of CVD before the age of 65 years in a first degree relative.

[#]Ten-year CHD risk based on the Framingham Point Scores.

Table 3 Coronary artery calcification outcomes

Coronary artery calcification	**Patients with hemophilia (n = 42)**	**Non-hemophilic males (n = 613)**
Agatston score	129.60 (17.28 – 380.78)	88.50 (9.35 – 382.95)
ln(Agatston score + 1)	4.20 ± 2.31	4.06 ± 2.38
Categories of Agatston score		
- 0 – 9	10 (23.8)	155 (25.3)
- 10 – 99	9 (21.4)	162 (26.4)
- 100 – 399	13 (31.0)	151 (24.6)
- ≥ 400	10 (23.8)	145 (23.7)
Agatston score ≥ 1000	5 (11.9)	60 (9.8)
Calcification mass	25.55 (2.85 – 71.98)	16.96 (2.35 – 70.65)
ln(calcification mass + 1)	2.92 ± 1.86	2.78 ± 1.85

Values of Agatston score and calcification mass are expressed as median and interquartile range because of skewed distributions. The natural log-transformed Agatston score and calcification mass are expressed as mean ± standard deviation. Categories of Agatston score are expressed as numbers and percentages. Results are unadjusted for confounders.
ln, natural logarithm; n, sample size.

Table 4 Regression coefficients describing the association between the natural log-transformed Agatston score and calcification mass and having hemophilia

	β (95% CI)	**P**
ln(Agatston score + 1): univariate model	0.141 (-0.602 – 0.885)	0.709
ln(Agatston score + 1): multivariate model	0.525 (-0.202 – 1.252)	0.157
ln(calcification mass + 1): univariate model	0.139 (-0.439 – 0.717)	0.637
ln(calcification mass + 1): multivariate model	0.428 (-0.138 – 0.994)	0.138

Multivariate model: adjusted for age, BMI, hypercholesterolemia, hypertension, and the use of antidiabetic medication.
ln, natural logarithm; β, regression coefficient or mean difference; CI, confidence interval; P, p-value.

Discussion

In this study, we found no evidence for a protective effect of congenital FVIII deficiency on the development of CAC measured with MDCT.

Previous studies, using B-mode ultrasonography to compare carotid and femoral IMT between hemophilia patients and non-hemophilic subjects, reported conflicting results[17-21]. Srámek et al.[21] and Sartori et al.[20] found no differences in IMT, whereas 3 other studies[17-19] did show a lower IMT, fewer atherosclerotic plaques, or both in hemophilia patients. In these studies, patients were relatively young; mean age ranged from 40 – 58 years. Possibly, atherosclerotic burden of the study population was so low that it complicated the detection of differences between the groups. A major disadvantage in most studies is the heterogeneous study population, combining patients with severe, moderate or mild hemophilia A or B or von Willebrand disease[17,18,20,21]. Clotting factors VIII and IX, and von Willebrand factor play different roles in the development of atherosclerosis[12,34]. Combining these disorders in 1 study might result in masking a possible relationship between 1 of the disorders and atherosclerosis. Unfortunately, sample sizes are too small for valid subgroup analyses. Moreover, comparison groups were not well chosen and, in some, not well described[17-21]. In addition, confounding was not accounted for in the analyses. These factors make interpretation of the results difficult.

In the current study, only patients with a residual FVIII activity ≤ 5% were included. If a protective effect of FVIII deficiency on the development of atherosclerosis would exists, it can be expected to be most pronounced in patients with severe or moderate hemophilia A. The hemophilia study population had a mean age of 65 years, thereby having a high probability of having developed atherosclerosis. We excluded HIV-positive patients, because most of them are treated with highly active antiretroviral therapy, which influences CVD risk[35]. A well-defined, large sample from the Dutch general male population was used for comparison.

Hemophilia patients were more often hypertensive as compared with men without hemophilia. This result is in accordance with other studies[6,36-39]. However, the underlying

mechanism of this observation is unknown. Mean total cholesterol levels and the proportion of subjects with hypercholesterolemia were lower in the hemophilia patients than in the comparison group, which is consistent with the results of Rosendaal et al.[6]. This could be explained by the association between chronic hepatitis C infection and a favourable lipid profile[40]. We calculated the 10-year CHD risk, using the Framingham Point Scores[26], and found no differences in risk category distributions between men with and without hemophilia. This prediction algorithm is based on and developed for the general population[41]. Consequently, true risks for the hemophilia study population will be different from the calculated risks. However, to compare a summary of the prevalence of cardiovascular risk factors between hemophilia patients and the general male population, in a scientific setting, the Framingham Point Scores can be used. The role of the available cardiovascular risk prediction algorithms in hemophilia patients in a clinical setting is less clear.

The lack of evidence for a lower extent of coronary artery atherosclerosis in hemophilia patients makes that alternative hypotheses, explaining the reduced IHD mortality in these patients, should be considered or reconsidered. In 1990, Rosendaal et al.[6] concluded that only a fraction of the reduced IHD mortality in hemophilia patients as compared with the general male population could be explained by differences in risk factor prevalence. Since then, the elderly hemophilia patient population has expanded and competing risks due to HIV and hepatitis C infection are now less important. Therefore, large prospective studies are needed to reassess the current prevalence of risk factors and their influence on CVD risk in hemophilia patients. As mentioned before, a very plausible explanation would be that the hypocoagulable state of hemophilia patients has a protective effect on thrombus formation. Possibly, hemophilia patients more often develop a mural thrombus upon plaque rupture than a fatal occlusive thrombus as compared with non-hemophilic men[5]. As several cohort studies investigated IHD death in hemophilia patients, only 1 study compared IHD prevalence between men with and without hemophilia[42]. The authors found that the rate of IHD hospital discharges among hemophilia patients 65 years and older was nearly 30% lower than that of non-hemophilic males. This might imply that hemophilia patients experience plaque rupture less often or that they have a 'silent'

plaque rupture more often than non-hemophilic males do. When plaque rupture or erosion is silent and thrombosis does not lead to arterial occlusion, platelets and the thrombotic response are important in the process of plaque progression[43]. Degranulating platelets release platelet-derived growth factor and transforming growth factor-β, which cause a fibrotic healing response, leading to increased collagen accumulation, smooth muscle cell proliferation and accelerated progressive luminal narrowing[43]. This might explain the comparable amount of atherosclerosis found in the current study. The risk of plaque rupture mainly depends on plaque composition[44,45]. Vulnerable plaques have thin or eroded fibrous caps that overlay large lipid cores and contain a lot of inflammatory cells[44,45]. Hemophilia patients might develop less vulnerable plaques. Plaque composition can be investigated with magnetic resonance imaging (MRI)[46]. In addition, markers of plaque vulnerability, such as matrix metalloproteinases and fibrin, can be targeted with antibodies and visualized with molecular MRI[46].

Some limitations of this study need to be discussed. Only 42 patients with hemophilia A were included, which may have affected the results. However, no tendency for a difference in the extent of CAC between men with and without hemophilia was observed. Therefore, we think it is unlikely that larger numbers would have changed the results. Hemophilia patients receive prophylactic or on demand therapy with clotting factor concentrates. If a protective effect of FVIII deficiency would exist, it could have been influenced by this treatment. However, patients with severe hemophilia temporarily become mild hemophiliacs after prophylactic treatment and FVIII activity decreases to ≤ 5% before the next prophylactic treatment in many of them. Thus, FVIII activity is not normalized. In addition, during a large part of the lives of elderly hemophilia patients, treatment with clotting factor concentrates was not (regularly) available. On the other hand, the hemophilia patient who is treated for its disease is the patient we see in our clinic every day, and the cohort studies showing a lower IHD morality are based on this population. It is not clear whether hepatitis C infection is positively related to atherosclerosis, as studies investigating this relation by measuring IMT reported conflicting results[47,48]. Therefore, patients with past or current hepatitis C infection were not excluded. Different MDCT scanners were used to assess CAC in men with and without

hemophilia and different scan readers performed CAC quantification. However, a standard calcium-scoring acquisition protocol with 3 mm thick slices and prospective ECG triggering was used in both groups. In addition, the quantification process has been largely automated, algorithms for calculating the Agatston score and calcification mass have been standardized, and quantification was shown to be reproducible between centers.

In conclusion, the amount of coronary artery atherosclerosis is comparable between elderly patients with hemophilia A and men from the general population. Despite the relative protection against IHD mortality, the incidence of ischemic CVD in hemophilia patients is increasing as life expectancy now approaches that of the general population[1,2,4]. Treatment of these patients is complex, because of the delicate equilibrium between bleeding and thrombosis, and evidence-based treatment guidelines are lacking[3,49]. Results from this study underline the importance of screening and treating atherosclerosis risk factors to minimize the risk of cardiovascular events in hemophilia patients.

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Chapter 4 Factor VIII deficiency does not protect against atherosclerosis

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Summary

Hemophilia A patients have a lower cardiovascular mortality rate than the general population. Whether this protection is caused by hypocoagulability or decreased atherogenesis is unclear. We evaluated atherosclerosis and endothelial function in hemophilia A patients and controls with and without obesity.

Fifty-one obese (body mass index (BMI) ≥ 30 kg/m^2) and 47 non-obese (BMI ≤ 25 kg/m^2) hemophilia A patients, and 92 gender-, age- and BMI-matched non-hemophilic controls were included. Carotid and femoral intima-media thickness (IMT) and brachial flow-mediated dilatation (FMD) were measured as markers of atherogenesis and endothelial function.

Mean age of the study population was 50 ± 13 years. Mean carotid IMT was increased in obese subjects (0.77 ± 0.22 mm) as compared with non-obese subjects (0.69 ± 0.16 mm) (mean difference 0.07 mm (95% confidence interval (CI) 0.02 – 0.13; P = 0.008)). No differences in carotid (mean difference 0.02 mm (95% CI -0.07 – 0.11; P = 0.670)) and femoral (mean difference 0.06 mm (95% CI -0.13 – 0.25; P = 0.550)) IMT between obese hemophilia patients and obese controls were found. Thirty-five percent of the obese hemophilia patients and 29% of the obese controls had an atherosclerotic plaque (P = 0.490), irrespective of hemophilia severity. Brachial FMD was comparable between obese hemophilia patients and obese controls (4.84% ± 3.24% and 5.23% ± 2.37%; P = 0.450).

Hemophilia A patients with obesity develop atherosclerosis to a similar extent as the general male population. Detection and treatment of cardiovascular risk factors in hemophilia patients is equally necessary.

Introduction

Studies assessing the role of hemostasis in ischemic cardiovascular disease (CVD) indicate that hypercoagulability increases the risk of CVD, whereas a bleeding tendency seems to be associated with a lower risk. Previous studies have shown that high levels of factor VIII (FVIII) are associated with an increased risk of both venous and arterial thrombosis[1], and numerous other coagulation factors have also been related to an increased thrombotic risk[2-6]. The opposite also holds true, as patients with a hereditary deficiency of FVIII (hemophilia A) experience considerable protection against mortality caused by CVD[7-9]. A recent meta-analysis showed that, as compared with the general population, hemophilia patients have a 50% reduction in mortality caused by ischemic heart disease[10].

Two processes are required for an arterial thrombotic event to occur: atherogenesis, which gradually leads to the development of an atherosclerotic plaque, and atherothrombosis, the acute formation of an occluding thrombus. A role of hemostasis in the formation of an occluding thrombus is evident, but coagulation factors such as FVIII may also be involved in atherogenesis[11,12].

Whether hemophilia A protects against atherogenesis is unclear. Although FVIII deficiency seemed to protect against atherogenesis in some animal and human studies[13-16], other studies found no association[17-20]. However, the relatively low incidence of cardiovascular events in this group of patients requires large population-based studies. A major drawback of previous studies in hemophilia patients is the low prevalence of cardiovascular risk factors. Measurement of the carotid and femoral intima-media thickness (IMT) and endothelial function by means of brachial flow-mediated dilatation (FMD) allows early detection of atherosclerosis or functional vessel wall abnormalities, but comparing subjects with a low prevalence of risk factors may not be the best way to determine differences in subclinical atherosclerosis. Studies assessing the prevalence of atherosclerosis in hemophilia patients with proatherosclerotic risk factors are lacking. Obesity is such an established and major cardiovascular risk factor[21,22]. In addition, obesity is as prevalent in the hemophilia population as in the general population[23].

To test our hypothesis that a lifelong hypocoagulable state (i.e. FVIII deficiency) reduces the formation of atherosclerosis, we investigated the relationship between hemophilia A and the extent of atherosclerosis in a multicenter cross-sectional study. To assess subclinical atherosclerosis and (impaired) vascular function, we measured IMT and brachial FMD in hemophilia A patients with and without obesity, and in gender-, age- and body mass index (BMI)-matched non-hemophilic controls.

Methods

Participating centers

Hemophilia A patients were recruited from various hemophilia treatment centers across the Netherlands and Belgium. Enrollment in the study took place in 3 study centers: the Academic Medical Center in Amsterdam (AMC), the Netherlands; the University Medical Center Utrecht in Utrecht, the Netherlands; and the University Hospital in Leuven, Belgium. The study was approved by the local ethics committees, and inclusion took place after informed consent had been obtained.

Study population

Hemophilia A patients older than 18 years, who had a BMI ≥ 30 kg/m^2 were eligible for inclusion, irrespective of the severity of hemophilia. These patients were matched for age, and severity of disease with non-obese hemophilia A patients (BM ≤ 25 kg/m^2). Hemophilia patients with a history of symptomatic atherosclerotic disease (i.e. ischemic heart disease, stroke, or peripheral vascular disease) or HIV infection were excluded. Controls were matched with hemophilia patients for BMI, age, and sex. These controls were recruited through placement of an advertisement in local newspapers or were healthy volunteers who had participated in other studies on CVD at the Department of Vascular Medicine of the AMC. To improve accrual, an obesity clinic in the Netherlands was also approached to identify suitable control subjects.

Study regimen and assessment of risk factors

All study subjects were invited to one of the centers after an overnight fast. Patients were instructed to refrain from consuming food and drinks, except water, in the 10 h prior to each measurement. Furthermore, to avoid any influence of a vena puncture on the brachial FMD measurements, patients were asked to refrain from prophylactic infusion of FVIII prior to the visit. Patients with a severe form of hemophilia, however, received FVIII prophylaxis after blood collection, to avoid any bleeding complications as a result of the prolonged blood pressure cuff inflation during the brachial FMD measurement. The study visit included measurement of carotid and femoral IMT and brachial FMD by means of ultrasonography; a vena puncture to assess fasting glucose and lipid levels; and a physical examination, including measurement of weight, length, and waist and hip circumference. BMI was estimated prior to enrollment by using data on weight and length from the hemophilia treatment centers, and was calculated after length and weight had been obtained during physical examination. In all subjects, the ultrasound measurements preceded blood collection. Blood pressure was measured 3 times with the patient in a supine position during the IMT assessment, and the last measurement was registered. Additionally, a medical history was obtained. Levels of total cholesterol, LDL cholesterol, and triglycerides were considered to be increased when they exceeded the 95th percentile of the reference values for the relevant age categories. HDL levels were considered to be low when they were below the 5th percentile of the reference values for the relevant age categories. Dyslipidemia was defined as the use of lipid-lowering drugs and/or any of the cholesterol levels exceeding the reference values for the relevant age categories. Diabetes mellitus was defined as the use of antidiabetic medication and/or fasting glucose levels higher than 7.1 mmol/l. Hypertension was defined as the use of antihypertensive medication, a systolic blood pressure of > 140 mmHg, and/or a diastolic blood pressure of > 90 mmHg.

Carotid and femoral IMT

For assessment of carotid and femoral ultrasound IMT measurements, Acuson Sequoia instruments (Siemens Medical Solutions, Erlangen, Germany) equipped with linear-array

ultrasound transducers (L7, 5 – 12 MHz) were used in all 3 study centers. Sonographers were trained and certified. Instrument application and scanning protocols were standardized as described previously[24]. In each subject, 3 arterial wall segments of the right and 3 segments of the left carotid artery were scanned; in each of the femoral arteries, one segment was scanned. In each center, a maximum of 2 sonographers performed the ultrasound procedures. High resolution images of each of the segments were saved with the use of Digital Imaging and Communication in the diastole of the vessel. From all of the centers, scans were transmitted by secure file transfer protocol to the AMC Vascular Imaging Core Laboratory. Image analysis was performed by 1 certified ultrasound analyst (reader). For the carotid and femoral IMT analyses, ETRACK (Vascular Imaging and Department of Physiology, AMC, Amsterdam, the Netherlands) was used. The reader was blinded to demographic and clinical information of subjects.

The primary ultrasound outcome was the per subject mean IMT of the six carotid arterial wall segments (mean carotid IMT). All other IMT outcomes, such as maximum carotid IMT and femoral IMT, were secondary endpoints. To assess intrasonographer reproducibility and for quality control (QC) purposes, repeat scans were assessed in 17 subjects. The observed mean difference in mean carotid IMT was 0.077 mm, which was well within the predefined intrasonographer QC limits of 0.2 mm.

Plaque

As there is no clear consensus on the definition of plaques in the femoral artery, we only analyzed the presence of atherosclerotic plaque in the carotid artery. All segments of the carotid artery were assessed for the presence of atherosclerotic plaques. A plaque was predefined as a maximum IMT ≥ 1.3 mm of any given segment of the carotid artery[25].

Brachial FMD

Instrument application and scanning protocols were standardized as described previously[26,27]. Sonographers were trained and certified. Each study subject underwent measurement of endothelium-dependent vascular responses of the left brachial artery by B-mode ultrasound imaging. Acuson Aspen (Siemens, Mountain View, CA, USA) ultrasound

systems equipped with L7, 5 – 10 MHz linear arrays were used. Prior to start of the brachial FMD scan, subjects rested for at least 10 min in a quiet and temperature-controlled (21 – 23 °C) examination room. Subsequently, the subjects' left arm was placed in a custom-made transducer holder with arm support, and a blood pressure cuff was placed on the left forearm from the medial epicondyle downwards. A straight, non-branching segment of the brachial artery above the antecubital fossa was identified and scanned longitudinally. Following optimization of depth and gain settings, end-diastolic brachial artery diameters were recorded at a beat-to-beat interval for 1 min (baseline measurement). The cuff was then inflated to 250 mmHg on the forearm for 5 min, after which the cuff was deflated and the segment of the brachial artery was recorded continuously for another 3 min. Clips were stored on a magnetic optical disk. Brachial artery diameter was analyzed qualitatively and quantitatively offline by a certified image analyst at the AMC Vascular Imaging Core Laboratory. For image assessments, a validated automatic edge-detection system (Brachial Analyser; Medical Imaging Application LLC, Coralville, IA, USA) was used. Brachial FMD was expressed as the percentage of the difference between the maximum post-cuff release brachial diameter and the average pre-cuff inflation ('baseline') diameter. For brachial FMD QC purposes, 19 repeat scans were available. To ensure reliable measurements, the intrasonographer QC limit was set at a mean difference in brachial FMD of less than 2%. The mean difference of the paired repeat brachial FMD scans was less (0.79%) than the predefined criteria of 2%.

Statistical analyses

Data are presented as mean (± standard deviation) for continuous variables, median and ranges for variables with a skewed distribution, and frequencies or percentages for categorical variables. Differences in mean values were assessed with t-tests, after log transformation in cases of skewed data, and adjusted by Bonferroni correction for multiple testing. Differences in mean IMT and brachial FMD between the various subgroups were also compared by the use of t-test. Categorical variables were compared by use of chi-square tests. Carotid and femoral IMT were stratified for age and risk factors for CVD to assess the influence of these variables on outcome. To assess the influence of

severity of hemophilia on atherogenesis, IMT, brachial FMD and plaque, data of patients with severe and moderate hemophilia were combined and compared with those of controls.

Results

After the initial selection of potentially eligible hemophilia patients meeting the BMI criteria, 2 patients were excluded because of the presence of HIV, 7 patients were excluded because of a history of CVD, and 27 patients did not want to participate, owing either to difficulty in travelling to the study center, to prior participation in recent studies, or to having no interest in participation in studies. A total of 205 subjects were enrolled at the 3 study centers. Of these 205 subjects, 15 (including both hemophilia patients and controls) were excluded because they did not meet the BMI inclusion criteria (BMI ≥ 30 kg/m^2 or BMI ≤ 25 kg/m^2) during the study visit. The remaining study population (n = 190) consisted of 51 (26.8%) obese hemophilia patients, 47 (24.7%) non-obese hemophilia patients, 42 (22.1%) obese controls, and 50 (26.3%) non-obese controls. As all hemophilia patients are male, the study population consisted entirely of males. Severity of hemophilia was equally distributed among the obese and normal-weight patients (Table 1). The use of prophylactic or on demand treatment with FVIII concentrate and the prevalence of hepatitis C infection were also not different between the 2 groups (Table 1). None of the patients had an inhibitor against FVIII.

Cardiovascular risk factors

Table 2 shows the presence of cardiovascular risk factors in hemophilia patients and controls. As expected, the subgroups were well matched for age and BMI. History of smoking in pack-years was similar in all subgroups (P = 1.000). Mean levels of systolic and diastolic blood pressure were significantly different between obese controls and non-obese controls (mean difference in systolic blood pressure 10 mmHg; P = 0.003, and mean difference in diastolic blood pressure 8 mmHg; P = 0.001). However, there was a higher

prevalence of hypertension in hemophilia patients than in controls (43% and 25%; P = 0.010). Fasting glucose levels were higher in obese than in non-obese subjects in both hemophilia patients and in controls (P = 0.014 and P = 0.020, respectively). Mean HDL levels were lower and triglyceride levels were higher in obese subjects than in non-obese subjects; this difference reached statistical significance in controls, but not in hemophilia patients. Levels of total cholesterol and LDL cholesterol did not differ significantly between the groups. Dyslipidemia was also equally prevalent among hemophilia patients and controls (15% and 12%; P = 0.500).

Table 1 Characteristics of hemophilia patients

Variable	**Hemophilia patients with BMI ≥ 30 kg/m^2 (n = 51)**	**Hemophilia patients with BMI ≤ 25 kg/m^2 (n = 47)**
Residual factor VIII activity		
- < 1%	17 (33)	16 (34)
- 1 – 5%	8 (16)	8 (17)
- 6 – 40%	26 (51)	23 (49)
Current treatment		
- Prophylactic	16 (31)	12 (26)
- On demand	35 (69)	35 (74)
Presence of inhibitors	0 (0)	0 (0)
Hepatitis C infection		
- Current	10 (20)	15 (32)
- Past	13 (25)	7 (15)
- Never	28 (55)	25 (53)

Categorical variables are expressed as numbers and percentages.
BMI, body mass index; n, sample size.

Table 2 Cardiovascular risk factors in hemophilia A patients and control subjects

Variable	Hemophilia patients with BMI ≥ 30 kg/m^2 (n = 51)	Hemophilia patients with BMI ≤ 25 kg/m^2 (n = 47)	Controls with BMI ≥ 30 kg/m^2 (n = 42)	Controls with BMI ≤ 25 kg/m^2 (n = 50)
Age (years)	50 ± 14	49 ± 14	51 ± 12	49 ± 14
Weight (kg)*^{II}	107.8 ± 16.1	75.4 ± 7.5	109.1 ± 13.2	77.1 ± 7.7
BMI (kg/m^2)*^{II}	32.5 (30.1 – 50.2)	23.5 (18.7 – 25.0)	32.4 (30.0 – 50.2)	23.2 (18.5 – 25.0)
Waist circumference (cm) *^{II}	115.7 ± 12.2	89.9 ± 6.6	113.7 ± 11.4	87.0 ± 6.9
Smoking (pack-years)	10 (0 – 55)	9 (0 – 45)	6.5 (0 – 47)	6.5 (0 – 50)
Systolic BP (mmHg)^{II}	135.6 ± 15.8	130.9 ± 16.5	138.2 ± 14.8	126.4 ± 9.8
Diastolic BP (mmHg)^{II}	83.0 ± 9.9	79.1 ± 10.0	84.4 ± 9.0	76.2 ± 7.8
Hypertension*$^{II\#}$	28 (55)	14 (30)	17 (41)	6 (12)
Glucose (mmol/l) *^{II}	5.4 (3.9 – 11.4)	5.2 (3.3 – 10.8)	5.3 (4.4 – 15.5)	5.1 (4.2 – 6.2)
Diabetes mellitus*^{II}	7 (14)	1 (2)	4 (10)	0 (0)
Total cholesterol (mmol/l)	5.01 ± 1.01	5.09 ± 1.07	5.29 ± 1.05	5.20 ± 1.06
HDL cholesterol (mmol/l)^{II}	1.22 ± 0.51	1.42 ± 0.42	1.21 ± 0.31	1.61 ± 0.64
LDL cholesterol (mmol/l)	3.17 ± 0.94	3.22 ± 0.96	3.40 ± 0.99	3.16 ± 0.96
Triglycerides (mmol/l)^{II}	1.20 (0.34 – 4.89)	0.85 (0.27 – 2.50)	1.35 (0.26 – 4.49)	0.81 (0.28 – 2.19)
Dyslipidemia	10 (20)	5 (11)	7 (17)	4 (8)
Family history of premature CVD	11 (22)	8 (17)	15 (36)	9 (18)

Values of continuous variables are expressed as mean ± standard deviation or median and interquartile range. Categorical variables are expressed as numbers and percentages.

BMI, body mass index; BP, blood pressure; HDL, high density lipoprotein; LDL, low density lipoprotein; CVD, cardiovascular disease; n, sample size.

*$P < 0.05$ for the comparison between obese hemophilia patients and non-obese hemophilia patients.

$^{II}P < 0.05$ for the comparison between obese controls and non-obese controls.

$^{\#}P < 0.05$ for the comparison between non-obese hemophilia patients and non-obese controls.

Carotid IMT

The mean carotid IMT in all hemophilia patients (0.74 ± 0.21 mm) was not different from that in all controls (0.72 ± 0.18 mm) (mean difference 0.02 mm (95% confidence interval (CI) -0.03 – 0.08); P = 0.450). Interestingly, mean carotid IMT was increased in obese subjects (0.77 ± 0.22 mm) as compared with non-obese subjects (0.69 ± 0.16 mm) (mean difference 0.07 mm (95% CI 0.02 – 0.13); P = 0.008). Comparison of obese hemophilia patients (0.78 ± 0.23 mm) with obese controls (0.76 ± 0.22 mm), no difference in carotid IM was apparent (mean difference 0.02 mm (95% CI -0.07 – 0.11); P = 0.670) (Fig 1A). The mean carotid IMT was not different in patients with severe or moderate hemophilia from that in controls (mean difference -0.03 mm (95% CI -0.09 – 0.04); P = 0.440).

Femoral IMT

The mean femoral IMT in all hemophilia patients (0.87 ± 0.42 mm) was not different from that in all controls (0.85 ± 0.38 mm) (mean difference 0.02 mm (95% CI -0.09 – 0.14); P 0.700). The effect of obesity on femoral IMT is shown in Fig. 1B. The mean femoral IMT tended to be higher in obese subjects (0.90 ± 0.45 mm) than in non-obese subjects (0.82 ± 0.33 mm) (mean difference 0.08 mm (95% CI -0.03 – 0.25); P = 0.160), although this difference was not statistically significant. The overall mean femoral IMT in obese hemophilia patients (0.92 ± 0.50 mm) was not different from that in obese controls (0.87 ± 0.40 mm) (mean difference 0.06 (95% CI -0.13 – 0.25); P = 0.550). The mean femoral IMT in patients with severe and moderate hemophilia seemed to be lower than that in controls, but the difference did not reach statistical significance (mean difference 0.06 mm (95% CI -0.06 – 0.18); P = 0.330).

Adjusted IMT

Fig. 2 shows the mean carotid and femoral IMT stratified for age. Overall, a similar trend was observed in hemophilia patients and controls, with a gradual increase in IMT of both the carotid and femoral arteries with increasing age. Stratification for the presence of cardiovascular risk factors, such as hypertension, also showed no significant differences in mean carotid or femoral IMT.

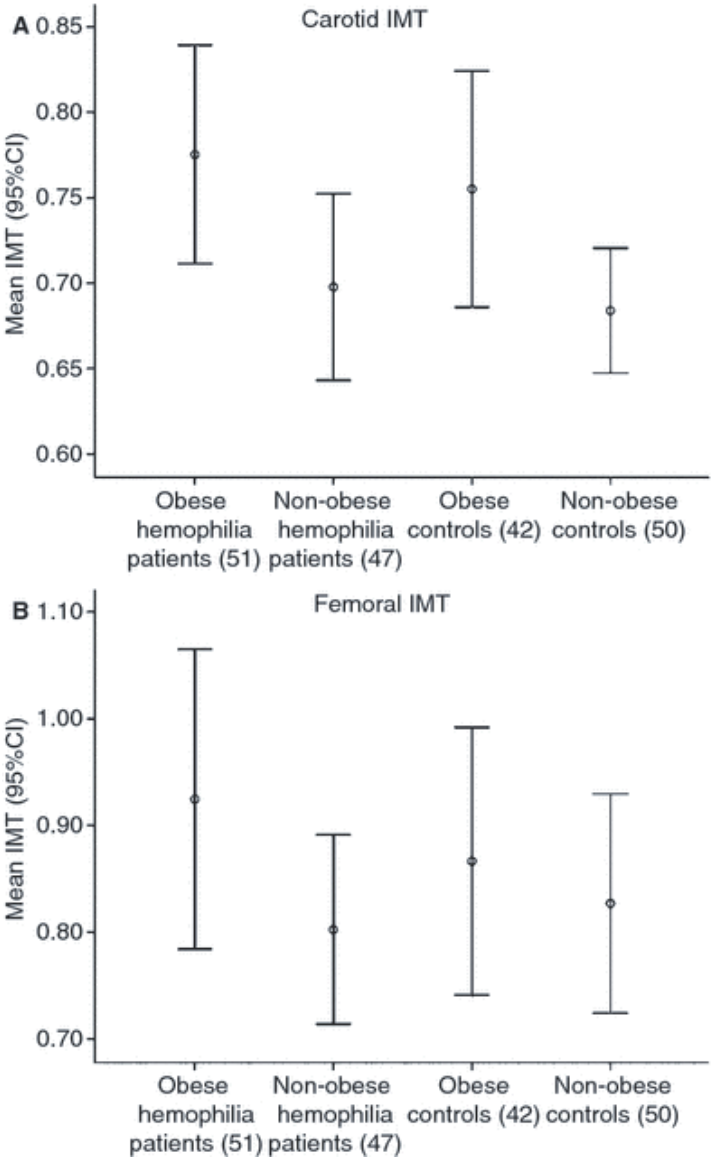

Fig. 1 Intima-media thickness of the carotid (A) and femoral (B) arteries

IMT, intima-media thickness; CI, confidence interval.

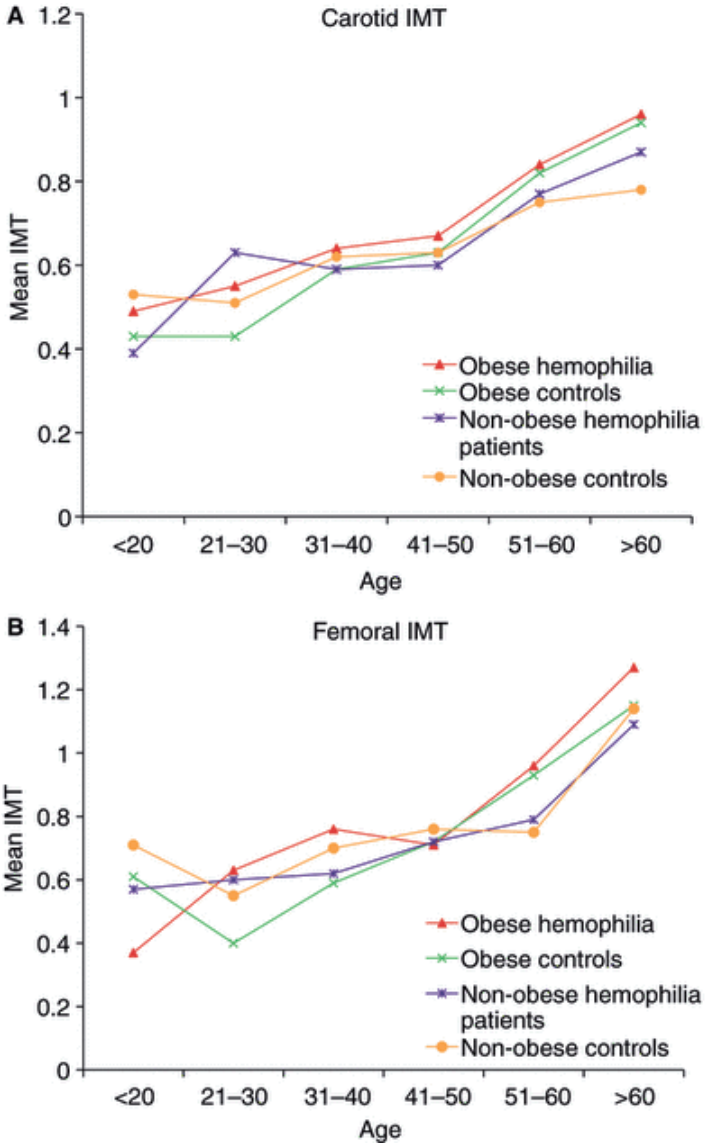

Fig. 2 Intima-media thickness of the carotid (A) and femoral (B) arteries stratified for age

IMT, intima-media thickness.

Plaques in carotid artery

The prevalence of atherosclerotic plaques (carotid IMT ≥ 1.3 mm) was assessed in all 6 segments of the carotid arteries. Of the hemophilia patients, 33% had a plaque in 1 or more of the 6 segments as compared to 25% of controls (P = 0.250). The prevalence of plaques was similar in obese subjects and in non-obese subjects (33% and 25%, respectively; P = 0.230) and in obese hemophilia patients and obese controls (35% and 29%, respectively; P = 0.490). The presence of plaques in patients with severe and moderate hemophilia was also similar to that in controls (27% and 25%, respectively; P = 0.790).

Brachial FMD

Table 3 shows the mean baseline brachial diameter and the mean peak post-occlusion artery diameter, which were similar between hemophilia patients and controls (P = 0.750 and P = 0.830, respectively). The mean brachial FMD in hemophilia patients was also similar to that in controls (4.75 ± 2.84% and 4.93 ± 2.39%; P = 0.660). No effect of obesity on brachial FMD could be detected in the subgroups. The brachial FMD in obese subjects was similar to that in non-obese subjects (5.19 ± 2.79% and 4.51 ± 2.41%; P = 0.090). The brachial FMD in obese hemophilia patients was also similar to that in obese controls (4.84 ± 3.24% and 5.32 ± 2.37%; P = 0.450). On assessment of the influence of severity of hemophilia on brachial FMD, patients with severe or moderate hemophilia had a similar FMD as controls (5.15 ± 3.26% and 4.93 ± 2.39; P = 0.660).

Table 3 Baseline and post-occlusion hemodynamics

	Baseline brachial artery diameter (mm)	Peak brachial artery diameter (mm)	Brachial flow-mediated dilatation (%)
All controls	4.47 ± 0.60	4.68 ± 0.60	4.93 ± 2.40
All hemophilia patients	4.50 ± 0.70	4.70 ± 0.60	4.75 ± 2.80
Obese hemophilia patients	4.67 ± 0.70	4.88 ± 0.60	4.84 ± 3.20
Non-obese hemophilia patients	4.35 ± 0.70	4.55 ± 0.70	4.68 ± 2.50
Obese controls	4.45 ± 0.60	4.69 ± 0.60	5.32 ± 2.40
Non-obese controls	4.48 ± 0.60	4.68 ± 0.60	4.55 ± 2.40
All obese subjects	4.53 ± 0.60	4.76 ± 0.60	5.19 ± 2.80
All non-obese subjects	4.43 ± 0.70	4.63 ± 0.60	4.51 ± 2.40

Values are expressed as mean ± standard deviation.

Discussion

The present study indicates that obesity leads to increased formation of carotid atherosclerosis, but that this process is not affected by a lifelong hypocoagulable state, namely hemophilia A. The overall mean carotid and femoral IMT, the prevalence of atherosclerotic plaques, and endothelial dysfunction, as measured by brachial FMD, did not differ between obese hemophilia patients and obese controls. Moreover, our study shows that atherosclerotic plaques are also prevalent in obese hemophilia patients, predisposing them to future cardiovascular events.

Previously, a protective effect of hemophilia on mortality caused by ischemic heart disease was observed[7-9]. Although the standardized mortality ratio varied (0.20 to 0.62), an overall 50% reduction in the ischemic heart disease mortality rate was observed in hemophilia A patients as compared with the general population[10]. This beneficial effect of hemophilia on fatal atherothrombotic events could be the result of reduced fibrin formation. Thrombin is the key player in both fibrin formation and platelet activation[28]. Thrombin cleaves fibrinogen to form fibrin, but can also trigger platelet activation through protease-activated receptor (PAR)1 and PAR4[29]. This may lead to the formation of thrombi, and ultimately to vascular occlusion. Importantly, thrombin may also influence the process of atherosclerosis. Tissue factor and PARs are expressed at high levels in human atheroma, and are induced in response to injury in animal models[30]. In vitro, PAR

activation induces leukocyte chemotaxis, and smooth muscle cell proliferation and migration, which may lead to arterial remodeling and stenosis[31]. In addition, coagulation factors and PARs are also involved in inflammatory responses and repair after injury[32]. Although patients with hemophilia, who have decreased thrombin formation, may be relatively protected from these atherosclerotic processes, this was not the case in our study.

In previous studies, no clear association between hypocoagulability and IMT was shown[18-20]. In 59 hemophilia A and B patients, mean carotid IMT was not different from that in controls (0.76 mm (95% CI 0.71 – 0.80) and 0.77 mm (95% CI 0.75 – 0.80), respectively)[19], and this was confirmed by Sartori et al.[18]. In patients with severe type III von Willebrand disease, similar results were obtained[20]. Also for femoral IMT, no differences between hemophilia patients and controls were shown[19]. A small protective effect of hemophilia was, however, observed in patients with moderate and severe types of hemophilia, whose mean femoral IMT was somewhat smaller than in controls[19]. Brachial FMD, as a measure of endothelial dysfunction, seemed to be impaired in hemophilia patients as compared with controls (3.8 ± 5.2% and 20.3 ± 13.0%; $P < 0.001$), but brachial FMD in healthy controls was remarkably high[18]. The previous studies had the same drawbacks. Patients were relatively young and had a low prevalence of cardiovascular risk factors. Therefore, a potential protective effect of hypocoagulability on atherosclerosis would have been difficult to detect. In our study, hemophilia patients all had a major risk factor for atherosclerosis, namely obesity. The strengths of this study include careful selection of obese hemophilia patients and controls, as well as the use of validated surrogate markers for atherosclerosis[33,34]. In addition, the ultrasound measurements were of high quality, which was confirmed by the low variation found between the repeated measurements. Furthermore, the number of refusals to participate was very low in this population, as the majority of patients have a good relationship with the hemophilia nurses and physicians who generally recruited these patients. A potential limitation was that patients with severe hemophilia received regular prophylactic treatment, which changes their phenotype to moderate. Nevertheless, if a low FVIII level influences the formation of atherosclerosis, this should have become apparent in our study.

Our study has important clinical implications. We can conclude that hemophilia A patients with cardiovascular risk factors develop atherosclerosis to a similar extent as the general male population. This implies that detection and treatment of these risk factors in hemophilia patients is mandatory. Next, although the cardiovascular mortality rate is lower in hemophilia patients, the increasing life expectancy will lead to more cases of CVD. The anticoagulant treatment of patients with hypocoagulability and consequently a higher bleeding risk is a major challenge[35].

In conclusion, we show that patients with hemophilia and obesity, which is a major risk factor for atherosclerosis, have the same degree of subclinical atherosclerosis as obese control subjects.

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Chapter 5 Von Willebrand factor deficiency and atherosclerosis

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Summary

Von Willebrand factor (vWF) is a large multimeric glycoprotein that plays a major role in hemostasis, illustrated by the bleeding tendency in von Willebrand disease (vWD); the most common hereditary bleeding disorder caused by vWF deficiency or dysfunction. Elevated vWF levels are associated with an increased risk of arterial thrombosis. Whether this relation is causal, or whether increased vWF levels merely reflect disturbances of endothelial function remain to be elucidated. Another possibility is that vWF participates in the process of atherogenesis. The aim of the current review is to determine whether vWF deficiency provides protection against the development of atherosclerosis in humans and animals.

Results from pig studies suggest that vWF deficiency is protective against aortic atherosclerosis at branch point predilection sites. However, no direct support for this was found in pig coronary arteries. Studies in other species, genetically lacking vWF or in which agents were used to prevent vWF binding to GPIb or collagen, indicate that vWF deficiency might be protective against early atherogenesis, most likely based on the disturbed interaction between the vessel wall and platelets. An unequivocal protective effect of vWD on atherosclerosis has not been demonstrated in humans. However, the interpretation of these studies is hampered by several methodological weaknesses. The vWF-platelet or vWF-collagen interaction could prove to be attractive targets in future prevention and treatment of cardiovascular disease, an option that is already being explored in humans.

In conclusion, vWF is probably a significant player in the multifaceted interaction between the hemostatic system and the atherosclerotic process, which deserves further study.

Introduction

Von Willebrand factor (vWF) is a large multimeric glycoprotein (GP), which is secreted by platelet α-granules and endothelial Weibel-Palade bodies. vWF plays a major role in hemostasis. Defects in, or (partial) deficiency of vWF, lead to a bleeding disorder known as von Willebrand disease (vWD). Acting as a carrier protein, vWF protects circulatory factor VIII from inactivation and clearance. At sites of vascular injury, vWF facilitates platelet adhesion by binding to both collagen in the subendothelial matrix and the GPIb-IX-V receptor complex (GPIb) on the platelet surface. Its role in platelet adhesion is particularly important under conditions of high shear stress. After the initial platelet arrest due to vWF-GPIb interaction, further activation of the platelet via intracellular signalling occurs, allowing other receptors to bind to vWF and collagen which reinforces adhesion. In addition, vWF promotes platelet-platelet interaction, thereby supporting platelet aggregation[1].

Elevated vWF levels appear to be associated with an increased risk of arterial thrombosis, including myocardial infarction[2,3] and stroke[4]. Whether the association between vWF and atherothrombotic events is indicative of a causal relationship, or whether increased vWF levels merely reflect disturbances of endothelial function remains to be elucidated[5,6]. Another possibility is that vWF participates in the process of atherogenesis. In vitro, the amount of vWF on the endothelial cell (EC) surface is increased by low-density lipoproteins (LDL), and among the large number of vWF secretion agonists there are numerous factors involved in atherogenesis[6,7]. Furthermore, smooth muscle cells (SMCs) are a major constituent of atherosclerotic plaques and vWF stimulates mouse aortic SMC proliferation in vitro in a direct dose-dependent way[8]. In vivo, cholesterol- and injury-induced plaque formation is associated with a marked increase in vWF expression at plaque predilection sites in rodents[9,10]. In primates, large numbers of Weibel-Palade bodies are found in ECs at sites of early atherosclerotic lesions[11]. In humans, ECs in advanced atherosclerotic plaques contain hyperplastic Weibel-Palade bodies[12]. Finally, atherosclerosis is an inflammatory disease; vWF levels increase during inflammation, and

vWF facilitates neutrophil extravasation in mice at sites of inflammation by destabilisation of the endothelial barrier through platelet recruitment via their GPIb receptor[13,14].

These findings suggest that vWF contributes to the pathogenesis of atherosclerosis. The hypothesis that the extent of atherosclerosis is reduced in patients with vWD as compared to controls with normal vWF levels logically follows. In the current review, studies on atherosclerosis in vWF deficient animals and humans are summarized to determine whether vWF deficiency protects against the development of atherosclerosis. An answer to this question could expand our knowledge about the role of vWF in atherogenesis, and might have future preventive or therapeutic consequences.

Search strategy

A comprehensive literature search was performed using the Medline [http://www.ncbi.nlm.nih.gov/sites/entrez/] database, to identify studies, published until July 2011, on atherosclerosis in patients with all hereditary forms of vWD and in vWF deficient animal models. The following keywords and synonyms of these keywords were used to identify potentially relevant studies: von Willebrand factor, von Willebrand disease, von Willebrand factor deficiency, von Willebrand factor antibody, atherosclerosis, atherogenesis, intima-media thickness, plaque and stenosis. Results were screened on title and abstract. Relevant studies in English were included. Reviews, in vitro studies and studies concerning arterial thrombosis models without induction of atherosclerosis were excluded. References of the selected papers were checked for related articles that did not appear in the initial search. The literature search, review of the data and data extraction was performed by 2 authors independently. A total of 32 relevant studies were included in the current review comprising 27 animal and 5 human studies (Table 1 – 3).

Atherosclerosis in vWF deficient animals

Aortic atherosclerosis in vWF deficient pigs

In the 1970s, Fuster et al.[15] incidentally observed that homozygous vWF deficient ($vWF^{-/-}$) pigs barely suffered from aortic atherosclerosis, whereas control ($vWF^{+/+}$) pigs exhibited significant atherosclerosis. This finding instigated a rush of studies on atherosclerosis in $vWF^{-/-}$ pigs (Table 1). The initial observation could have been confounded, because the control pigs, obtained from a slaughterhouse, carried significantly higher body weights as compared to the $vWF^{-/-}$ pigs[15]. Therefore, subsequent studies implemented diet control. The same authors performed a successive study, in which 3-month-old pigs were fed an atherogenic high cholesterol diet for up to 6 months. Aortic atherosclerosis developed in all control pigs, while less than half of the $vWF^{-/-}$ pigs developed only discrete atherosclerotic plaques[15]. This pronounced difference in the presence and extent of aortic atherosclerosis between $vWF^{-/-}$ and $vWF^{+/+}$ pigs suggests that an absolute deficiency of vWF has a protective effect on the development of atherosclerosis. Spontaneous aortic atherosclerosis, allowed to develop during a 4-year controlled low cholesterol diet, was also less pronounced in $vWF^{-/-}$ pigs than in $vWF^{+/+}$ pigs[16]. In both groups atherosclerotic plaques developed almost exclusively in the distal abdominal aorta. Heterozygous $vWF^{+/-}$ pigs, receiving a high cholesterol diet for 6 months, were not resistant to aortic atherosclerosis. A dose-dependent effect could therefore not be demonstrated[17].

To examine whether the aortas of the $vWF^{-/-}$ pigs were less responsive to atherosclerosis, $vWF^{-/-}$ segments of the distal aorta were cross-transplanted into $vWF^{+/+}$ pigs and vice versa[16]. After transplantation the pigs received an atherogenic diet for 6 months. As a control experiment, $vWF^{+/+}$ pig aortic segments were transplanted into $vWF^{+/+}$ pigs. In these pigs, the extent of atherosclerosis was comparable between the donor and host aortic segments. In $vWF^{-/-}$ pigs, the donor normal aortic segments became resistant to the development of atherosclerosis, whereas $vWF^{-/-}$ aortas transplanted into $vWF^{+/+}$ pigs did develop atherosclerosis. These results suggest that endothelial vWF could be less important than platelet and circulatory vWF in pig aorta atherogenesis[16].

The above mentioned studies were conducted by the same study group of Fuster et al., in pigs of the Mayo Institute Hills farm[15-17]. Another study group (Griggs et al.), using a different pig colony (Chapel Hill), found slightly less extensive aortic atherosclerosis in $vWF^{-/-}$ pigs as compared to $vWF^{+/+}$ pigs, after a 4-month atherogenic diet[18]. This was only significant in the distal part of the aorta, with most prominent involvement of the trifurcation. No differences were found between $vWF^{+/-}$ and $vWF^{+/+}$ or $vWF^{-/-}$ pigs[18]. The effects of carbon monoxide, also considered an atherogenic agent, were investigated by the Griggs study group. The extent of aortic atherosclerosis in this model appeared to be comparable between the 3 phenotypes[19]. In a later publication on this pig stock, the extent of aortic atherosclerosis tended to be lower in vWF-/- pigs than in vWF+/+ pigs. However, the differences were not significant considering the entire aorta or the distal half[20].

In summary, Fuster et al. unequivocally reported protection against aortic atherosclerosis in $vWF^{-/-}$ pigs, while findings of the Griggs study group showed a less clear, location dependent effect. Variations in blood flow dynamics at different areas of the aorta might have an influence on vWF activity and may have caused the observed disparate degree of atherosclerosis.

Coronary atherosclerosis in vWF deficient pigs

Fuster et al.[21] also examined the development of coronary atherosclerosis in their pig colony. Without an atherogenic diet, no spontaneous coronary atherosclerosis or myocardial lesions developed in either the $vWF^{-/-}$ or $vWF^{+/+}$ animals after 4 years of follow-up[21]. Even after a 6-month high cholesterol diet, hardly any coronary atherosclerosis was seen in either phenotype. The authors concluded that, in order to assess the resistance or susceptibility to coronary artery atherosclerosis in the $vWF^{-/-}$ pigs, a longer follow-up would be necessary[21].

Griggs et al.[22] confirmed these results in pigs on a 6-month atherogenic diet. As an alternative, arterial vessel injury can be induced to speed up the process of atherogenesis. The hypothesis that endothelial injury precipitates atherosclerosis is known as the response-to-injury hypothesis[14]. In some of the studies on coronary atherosclerosis that

were performed in the pigs from the Chapel Hill colony (Griggs et al., Table 1) acute arterial injury was incorporated in the model. Ballooning of the coronary arteries to induce injury, was combined with an atherogenic diet for 4 months[18,23], and with exposure to low levels of carbon monoxide[19]. $vWF^{+/+}$, $vWF^{-/-}$ and $vWF^{+/-}$ pigs all developed coronary artery intimal lesions of similar thickness, with comparable extends of calcification and number of coronary vessels displaying gross narrowing[18,19]. Strikingly, none of the $vWF^{-/-}$ pigs had spontaneous myocardial infarction (MI), while 33% of the $vWF^{+/-}$ and $vWF^{+/+}$ pigs in the carbon monoxide group did suffer from MI[19]. The ability to develop occlusive thrombosis of the left anterior descending coronary and carotid arteries after clamping and pinch injury was investigated in pigs on a 6-month atherogenic diet[22]. Although the extent of atherosclerosis was comparable between $vWF^{-/-}$ and $vWF^{+/+}$ pigs, occlusive arterial thrombosis failed to develop in $vWF^{-/-}$ pigs, in contrast to $vWF^{+/+}$ pigs. In both groups, platelets covered the luminal surface of the coronary artery and platelet-fibrin microthrombi were found[22]. Injury-induced coronary vessel wall-platelet interaction was also investigated in 2 other studies[24,25]. Balloon injury resulted in platelet deposition in both $vWF^{-/-}$ and $vWF^{+/+}$ pigs, and when the injury involved the media, platelet-fibrin microthrombi developed. However, in $vWF^{-/-}$ pigs the attached platelets showed less spreading as compared to attached platelets in $vWF^{+/+}$ pigs[24,25]. Although animal numbers were quite small, the inference that vWF might be required to support the progression of microthrombi into occlusive arterial thrombosis can be made[22,24]. In addition, these observations suggest that vWF may play a role in the response of platelets to injury of arteries in which atherosclerosis commonly develops[25].

An important point of discussion regarding the aortic and coronary atherosclerosis findings in $vWF^{-/-}$ and $vWF^{+/+}$ pigs, is the variability in the degree of hypercholesterolemia. Polymorphisms in porcine apolipoprotein B100 genotype significantly influenced the severity of diet-induced hypercholesterolemia and atherosclerotic plaque formation independent of vWF levels[20]. Although no significant difference was found between the mean cholesterol levels between the $vWF^{-/-}$ and $vWF^{+/+}$ groups[15-17,20], apolipoprotein B100 polymorphisms could have influenced the results of studies investigating the relation between vWF deficiency and atherosclerosis.

In summary, no direct support for a role of vWF in coronary atherogenesis was found. However, homozygous vWF deficiency has a protective effect on occlusive coronary artery thrombosis and reduces platelet activation after endothelial injury. It should be noted that models of acute injury differ from the more chronic processes occurring in the development of human atherosclerosis. Additionally, polymorphisms in apolipoprotein B100 genotype of $vWF^{-/-}$ and $vWF^{+/+}$ pigs further complicated the interpretation of results.

Table 1 Animal studies on atherosclerosis and vWF deficiency

Author	Animal	n and vWF phenotype	High chol. diet	Site	Injury induced	Follow-up*	Protection against atherosclerosis in $vWF^{-/-}$ animals
Fuster et al.[15], 1978							
-Original cohort	Pig	$11^{-/-}$; $11^{+/+}$	No	Aorta	No	-	Yes
-Subsequent cohort	Pig	$7^{-/-}$; $11^{+/+}$	Yes	Aorta	No	6 mo	Yes
Fuster et al.[16], 1982	Pig	$5^{-/-}$; $5^{+/+}$	No	Aorta	No	4 yr	Yes
		$4^{-/-}$; $4^{+/+}$	Yes	Aorta	Cross Tx	6 mo	Yes
Fuster et al.[17], 1985	Pig	$5^{-/-}$; $5^{+/-}$; $9^{+/+}$	Yes	Aorta	No	6 mo	Yes
Griggs et al.[18], 1981	Pig	$7^{-/-}$; $8^{+/-}$; $6^{+/+}$	Yes	Aorta	No	4 mo	Yes
				Coronary artery	Balloon injury		No
Griggs et al.[19], 1982	Pig	$7^{-/-}$; $7^{+/-}$; $5^{+/+}$	Yes	Aorta	CO exposure	4 mo	No
				Coronary artery	CO exposure + balloon injury		No
Griggs et al.[20], 1992	Pig	$12^{-/-}$; $9^{+/+}$	Yes	Aorta	No	16 – 26 w	No
		$14^{-/-}$; $13^{+/+II}$		Coronary artery	No		No
Fuster et al.[21], 1985	Pig	$5^{-/-}$; $5^{+/+}$	No	Coronary artery	No	4 yr	No
		$5^{-/-}$; $9^{+/+}$	Yes	Coronary artery	No	6 mo	No
Griggs et al.[22], 1990	Pig	$6^{-/-}$; $8^{+/+}$	Yes	Coronary artery	No	6 mo	No
				Carotid artery	No		No
Griggs et al.[23], 1986	Pig	$9^{-/-}$; $9^{+/+}$	Yes	Coronary artery	Balloon injury	4 mo	No
Griggs et al.[24], 1990	Pig	$8^{-/-}$; $8^{+/+}$	Yes	Coronary artery	No	1 – 16 w	No
Griggs et al.,[25] 1982	Pig	$5^{-/-}$; $5^{+/+}$	No	Coronary artery	Balloon injury	-	Less platelet activation
Griggs et al.[26], 1984	Pig	$10^{-/-}$; $12^{+/+}$	No	Coronary artery	Balloon injury	48 hr	No difference in proliferative response of SMCs
Griggs et al[27]. 1998	Pig	$2^{-/-}$; $3^{+/-}$; $3^{+/+}$	No	Femoral artery	Shear stress	2 w	No difference in neointima formation
				Carotid artery			

Qin et al.[8], 2003	RIIIS/J mouse	$36^{+/-}$; $36^{+/-}$ + DDAVP; $36^{+/+}$	No	Carotid artery	Ligation	2 – 4 w	Reduced intimal hyperplasia
Methia et al.[28], 2001	$vWF^{-/-}LDLR^{-/-}$ mouse	$38^{-/-}$; $34^{+/+}$	Yes	Aorta	No	8 – 37 w	Yes

n, sample size; vWF, von Willebrand factor; chol., cholesterol; $LDLR^{-/-}$, low density lipoprotein receptor deficient; $^{-/-}$, homozygous vWF deficient; $^{+/-}$, heterozygous vWF deficient; $^{+/+}$, normal vWF; DDAVP, 1-deamino-8-D-arginine vasopressin or desmopressin; Tx, transplantation; CO, carbon monoxide; mo, months; yr, years; w, weeks; hr, hours; SMCs, smooth muscle cells. *Follow-up: time from start of the study until sacrifice. [II]Overlap with reference 22, 23, and 24.

Neointima formation in vWF deficient pigs

As was mentioned earlier, SMC proliferation and intimal migration are a hallmark of atherogenesis. In pigs, SMC proliferation was quantified by measuring the incorporation of ^{3}H-thymidine into the DNA of SMCs. No difference was detected in the early proliferative response of SMCs to injury of the coronary artery between $vWF^{-/-}$ and $vWF^{+/+}$ pigs[26] (Table 1). In another study, neointima formation was promoted by inducing shear stress through clamping of the femoral and carotid arteries for 14 days, producing a ≥ 80% stenosis[27]. No differences in neointima lesion size were found between $vWF^{-/-}$, $vWF^{+/-}$ and $vWF^{+/+}$ pigs. Lesions in $vWF^{+/+}$ and $vWF^{+/-}$ pigs contained large amounts of vWF, whereas lesions in $vWF^{-/-}$ pigs had no detectable vWF. This high local concentration of vWF in the neointima might contribute to plaque thrombogenecity, and could thus account for the observed protection against occlusive arterial thrombosis in $vWF^{-/-}$ pigs.

Lessons from vWF deficient mice

In the past decade, 2 relevant studies on atherosclerosis in vWD mice have been published[8,28] (Table 1). Although mouse models provide the advantage of a more defined genetic background, they are less comparable to humans in terms of atherogenesis than pigs[29]. One of the studies used the RIIIS/J mouse strain[8]. Mice of this inbred strain exhibit properties resembling human type 1 vWD. As in humans with type 1 vWD, their prolonged bleeding time can be corrected with desmopressin treatment[30]. In these RIIIS/J mice, mean vWF plasma levels were 21% of normal (± 7% standard error (SE))[8]. In contrast to $vWF^{+/+}$ C57BL/6J control mice, with mean vWF plasma levels of 110% (± 10% SE), the RIIIS/J mice were devoid of any intimal vWF deposition at 2 and 4 weeks after carotid ligation. When treated with 3 µg/kg/d desmopressin during 2 to 4 weeks after carotid ligation, plasma vWF in the RIIIS/J mice rose to 45% (± 8% SE) and some intimal deposition of vWF became apparent. Post-mortem morphometric studies of the carotid arteries revealed significant differences in the intima-media area ratios. Whereas at 2 and 4 weeks graduated and prominent intimal hyperplasia had been induced in the $vWF^{+/+}$ control mice, intimal hyperplasia was only demonstrated in the RIIIS/J mice after the administration of desmopressin[8]. The dose-dependent effect of vWF on intimal

hyperplasia observed in this study is suggestive of a causal relationship between vWF and atherosclerosis.

To reflect the situation in human type 3 vWD, another mouse model has been generated by disruption of the vWF gene in C57BL/6J mice[31]. $vWF^{-/-}$ and $vWF^{+/+}$ C57BL/6J mice were crossed with an atherosclerosis susceptible strain lacking the LDL receptor[28]. The mice were placed on an atherogenic diet when they were 8 weeks old. After 8 weeks on the diet, the fatty streak lesions formed in the aortic sinus of the $vWF^{-/-}LDLR^{-/-}$ mice were 40% smaller than in the $vWF^{+/+}LDLR^{-/-}$ mice. After 22 weeks on the diet, the difference in lesion size persisted. In addition, the $vWF^{-/-}LDLR^{-/-}$ mice had significantly less atherosclerotic plaque calcification compared to $vWF^{+/+}LDLR^{-/-}$ mice. The absence of vWF primarily protected the aorta at branch points of the renal and mesenteric arteries[28], regions of disturbed flow highly prone to atherosclerosis development[32]. After 37 weeks on the diet, the absence of vWF no longer affected lesion size. The diminished extent of plaque calcification, however, persisted at 37 weeks[28]. Despite the stimulatory effect of vWF on SMC proliferation in vitro described in the introduction[8], the proportion of SMCs in this in vivo study remained similar among the 2 genotypes[28]. Nevertheless, the findings in $vWF^{-/-}$ mice suggest that vWF could be involved in the early phases of atherogenesis.

Anti-vWF agents and atherosclerosis in animal models

The role of vWF has not only been investigated in vWF deficient models, but also by the administration of agents that prevent vWF binding to platelet GPIb or collagen (Table 2). AJvW-2, a monoclonal antibody (mAb) against vWF with a specific inhibitory effect on the vWF-GPIb axis, prevented neointima formation in balloon injured guinea pig carotid arteries in a dose-dependent manner[33]. These findings were confirmed in another study, in which an anti-vWF mAb (AJW200) was administered to rabbits with balloon injured iliac arteries, and where a reduction in thrombus formation, neointima cell proliferation, and subsequent neointima growth was shown[34]. The results of these studies suggest that vWF plays a role in promoting neointima formation after thrombus formation, though this finding is not supported by data obtained from $vWF^{-/-}$ pigs discussed earlier[27]. Possibly, the difference between the methods (clamping versus balloon injury) used, influenced the

results[27,33,34]. After balloon injury, organized thrombi were observed in the neointimal lesions. AJvW-2 and AJW200 reduced the deposition of vWF and the extent of organized thrombus formation[33,34]. This suggests that the mAbs prevent neointima formation by inhibition of the initial platelet-mediated thrombus formation rather than by direct inhibition of SMC proliferation. This hypothesis is supported by the observation that proliferation of SMCs was not affected by AJvW-2[33].

In hypercholesterolemic mice it was shown that platelet adhesion preceded the development of manifest atherosclerosis at lesion prone sites. Inhibition of vWF-GPIb binding, by repeatedly injecting a blocking mAb against GPIb, prevented platelet adhesion and dramatically reduced atherosclerotic lesion formation[35]. Another inhibitor of platelet binding to vWF is VCL, a recombinant vWF GPIb binding domain. The administration of this agent also resulted in a reduction of both early platelet adhesion and late intimal thickening in balloon injured femoral arteries in rats[36]. Aurintricarboxylic acid (ATA) is a small molecule which inhibits platelet adhesion by interfering with vWF binding to GPIb. In hamsters, continuous infusion of ATA, fractionated or non-fractionated, resulted in a dose-dependent suppression of neointima formation and inhibited the early proliferation of neointimal SMCs in the injured carotid artery[37,38]. Also in rabbits, after endothelial injury, ATA attenuated aortic intimal thickening[39], and reduced platelet deposition and neointimal hyperplasia in the carotid artery[40]. It should be noted that ATA, besides reducing platelet deposition, blocks procoagulant activity and inhibits GPIIb/IIIa-fibrinogen interaction. In addition, ATA exhibits a diverse range of other effects that have been linked to atherogenesis[39]. Understanding the effects of this agent on neointima formation is complicated; however, interference with the vWF-GPIb interaction could partly explain its anti-atherosclerotic effect. Taken together, results from the above mentioned studies suggest that vWF-GPIb interaction may play a role in early atherogenesis through the promotion of platelet adhesion to the vessel wall.

The vWF-collagen interaction is important for the initial adherence of vWF to the subendothelial matrix that is exposed during vascular injury. Inhibition of this interaction, using a mAb that binds to the vWF A3-domain (82D6A3), did not affect neointima formation in a baboon coronary in-stent stenosis model[41]. In an attempt to resemble

clinical practice in this study, aspirin, clopidogrel and heparin were given to all baboons, which could have masked a potential effect. Another agent interfering with the vWF-collagen interaction is saratin. Topical application of saratin onto exposed subendothelium following carotid endarterectomy decreased platelet adhesion, intimal hyperplasia, luminal stenosis and thrombosis in normal and hyperhomocysteinemic rats[42,43]. This agent also inhibited the development of intimal hyperplasia at a venous anastomosis in a canine dialysis access model[44].

In summary, the observations in animals treated with agents inhibiting the vWF-GPIb or vWF-collagen interaction, point to a protective role for vWF deficiency in early atherogenesis, most likely based on the disturbed interaction between the vessel wall and platelets.

Table 2 Animal studies on atherosclerosis and anti-vWF agents

Author	Animal	Anti-vWF agent / target	n (treated, control)	High chol. diet	Site	Injury induced	Follow-up*	Effect
Kageyama et al.[33], 2000	Guinea pig	AJvW-2 / vWF-GPIb	20, 20	No	Carotid artery	Balloon injury	2 w	Reduced neointima formation
Yamashita et al.[34], 2003	Rabbit	AJW 200 / vWF-GPIb	10, 10	No	Iliac artery	Balloon injury	8 w[II]	Reduced neointima formation
Massberg et al.[35], 2002	ApoE$^{-/-}$ mouse	p0p/B / vWF-GPIb	6, 6	Yes	Carotid artery Aortic sinus Coronary artery	No	18 w	Reduced atherosclerotic lesion formation
Zagher et al.[36], 1995	Rat	VCL / vWF-GPIb	18, 24	No	Femoral artery	Balloon injury	3 – 28 d	Reduced intimal thickening
Matsuno et al.[37], 1997	Hamster	Fractionated ATA / vWF-GPIb	38, 12	No	Carotid artery	Catheter injudy	2 w	Reduced neointima formation
Matsuno et al.[38], 1998	Hamster	ATA / vWF-GPIb	38, 12	No	Carotid artery	Catheter injury	2 w	Reduced neointima formation
Waissbluth et al.[39], 2002	Rabbit	ATA / vWF-GPIb	5, 9	No	Aorta	Balloon injury	2 w	Reduced intimal thickening
Golino et al.[40], 1997	Rabbit	ATA / vWF-GPIb	Unknown	No	Carotid artery	Balloon injury	3 w	Reduced neointimal hyperplasia
de Meyer et al.[41], 2007	Baboon	82D6A3 / vWF-collagen	4, 4	No	Coronary artery	Stent implantation	4 w	No reduction in neointima formation

Cruz et al.[42], 2001	Rat	Saratin / vWF-collagen	15, 10	No	Carotid artery	Endarterectomy	2 w	Reduced intimal hyperplasia
Davis et al.[43], 2004	Hyperhomocysteinemic rat	Saratin / vWF-collagen	6, 12	No	Carotid artery	Endarterectomy	2 w	Reduced intimal hyperplasia
Smith et al.[44], 2003	Dog	Saratin / vWF-collagen	7, 7	No	Femoral artery	Dialysis access graft placement	4 w	Reduced intimal hyperplasia

vWF, von Willebrand factor; n, sample size; chol., cholesterol; ApoE$^{-/-}$, apolipoprotein E deficient; ATA, aurintricarboxylic acid; w, weeks; d, days.
*Time from start atherogenic diet or first injury and start of anti-vWF agent until sacrifice. [||]Four weeks after the first injury, a second injury was performed and a bolus of AJW200 was given. The animals were sacrificed 30 min, 5 days, or 4 weeks after the second injury.

Atherosclerosis in patients with vWD

Congenital vWD is caused by mutations in the vWF gene on chromosome 12, resulting in defects in or reduced levels of vWF. Depending on the mutation, patients can have a partial quantitative (type 1) or complete (type 3) vWF deficiency. Type 2 vWD is characterized by qualitative abnormal variants of vWF[45]. Autopsy studies showed that, even when considering type 3, patients with vWD are not fully protected from developing atherosclerosis[46-48]. In addition, ischemic cardiovascular disease does occur in these patients[49,50]. The prevalence of ischemic cardiovascular disease is, however, significantly lower in patients with vWD as compared to the general population[51]. Several studies, summarized in Table 3, compared the extent of atherosclerosis between patients with vWD and control subjects. A protective effect of coagulopathy on carotid atherosclerotic plaque formation was detected when the degree of carotid atherosclerosis was evaluated by echo Doppler in 76 patients with vWD or hemophilia A[52]. The control group consisted of 77 subjects without coagulopathy and cardiovascular risk factors. Atherosclerotic plaques were defined as lesions thicker than 2 mm. Thirteen percent of the patients with vWD or hemophilia A had carotid plaques versus 27% of the control subjects, a significant difference despite the prevalence of cardiovascular risk factors in the patients with a coagulopathy. Patients with more severe vWD or hemophilia A presented with fewer atherosclerotic plaques and had a milder degree of stenosis as compared to patients with less severe coagulopathy[52]. In a second study, the same group compared the presence of aortic and leg artery atherosclerosis using echo Doppler in 15 vWD and 25 hemophilia A patients and 40 control subjects matched for gender, age and cardiovascular risk factors[53]. Compared to the control subjects, the patients had a significant lower number of atherosclerotic plaques in both arteries. However, no clear protective effect of coagulopathy on intima-media thickness (IMT) of the carotid and femoral arteries could be demonstrated by a Dutch study group that compared 17 patients with mild vWD and 59 patients with hemophilia to 142 healthy controls with a similar prevalence of cardiovascular risk factors[54].

Interpretation of these studies is, however, hampered by several shortcomings. Study populations are heterogeneous, combining patients with vWD or hemophilia with varying severities[52-54]. The effect of vWD on the extent of atherosclerosis was not reported separately from the effect of hemophilia[52,53]. In addition, vWD subtypes were not stated. Furthermore, confounding was not accounted for in the analyses, nor was any information available on treatment with clotting factor concentrate[52-54].

Patients with type 3 vWD completely lack vWF and are therefore the most ideal subjects to study a potential protective effect of vWF deficiency on human atherogenesis. The only study in which the extent of atherosclerosis was assessed in this population included 47 patients with type 3 vWD (mean age 35 years) and 84 control subjects matched for gender, age and region of origin[55]. No differences in carotid and femoral IMT between patients and control subjects were found. Overall, plaques were visible in less than 20% of the study population[55]. This could be explained by the inclusion of fairly young subjects. Possibly, atherosclerotic burden of the study population was so low that it complicated the detection of differences between the groups.

Type 2B vWD is characterized by enhanced vWF binding to the GPIb receptor resulting in a lower concentration of the largest vWF multimers in plasma, and a lower platelet count as a result of microaggregation. The impact of this qualitative vWF defect on the development of atherosclerotic lesions was assessed by echo Doppler of the abdominal aorta, carotid and femoral arteries in a 24 type 2B vWD patients with a mean age of 48 years[56]. The control group consisted of 24 subjects, matched for gender, age, and cardiovascular risk factors. No significant differences in the IMT or in the presence and extent of atherosclerotic lesions, expressed as the percentage of stenosis in the vascular lumen, could be found at any of the sites investigated. At least one atherosclerotic plaque was found in 3 vWD patients versus 5 healthy control subjects. Brachial artery flow-mediated dilatation (FMD) measurement, an accepted indicator of endothelial dysfunction, was also comparable between the groups[56].

In summary, an unequivocal protective effect of vWD on atherosclerosis has not been demonstrated in humans. However, it should be noted that the interpretation of these results is hampered by heterogeneous study populations with relatively low mean ages.

Table 3 Human studies on atherosclerosis in vWD

Author	Subjects	Mean age patients (years)	Findings
Bilora et al.[52], 1999	- 76 patients with either vWD or hemophilia A (46 men, 30 women) - 77 age-matched control subjects (37 men, 40 women)	58.2	- 13.1% of the patients with vWD or hemophilia had carotid plaques vs. 27.2% of the control subjects ($P < 0.05$)
Bilora et al.[53], 2001	- 15 patients with VWD - 25 patients with hemophilia - 40 gender-, age-, and cardiovascular risk factor-matched control subjects	48.3	- 3 of 40 patients with a coagulopathy had plaques in the abdominal aorta vs. 11 of 40 controls - 5 of 40 patients with a coagulopathy had plaques in the leg arteries vs. 17 of 40 controls
Srámek et al.[54], 2001	- 17 patients with VWD - 59 patients with hemophilia - 142 gender and age-matched control subjects	49	- Carotid artery: no difference - Femoral artery: minimally reduced in patients with bleeding tendency; lowest IMT in patients with moderate to severe hemophilia
Srámek et al.[55], 2004	- 47 patients with type 3 vWD - 84 gender-, age-, and region of origin-matched control subjects	35	- IMT carotid artery: no difference - IMT femoral artery: no difference
Bilora et al.[56], 2007	- 24 patients with type 2B vWD - 24 gender-, age-, and cardiovascular risk factor-matched control subjects	48	- No difference in IMT, atherosclerotic lesions or percentage stenosis in the abdominal aorta, carotid arteries or leg arteries

vWD, von Willebrand disease; vs., versus; IMT, intima-media thickness.

Discussion

In the current review we tried to determine whether a deficiency in vWF provides protection against the development of atherosclerosis in animals and humans. Results from pig studies suggest that vWF deficiency is protective against aortic atherosclerosis at branch point predilection sites[15-18]. However, no direct support for this was found in pig coronary arteries[18,19,21-24]. Studies in other species, genetically lacking vWF or in which agents were used to prevent vWF binding to GPIb or collagen, indicate that vWF deficiency might be protective against early atherogenesis. An unequivocal protective effect of vWD on atherosclerosis has not been demonstrated in humans. However, the interpretation of these studies is hampered several methodological weaknesses.

The most plausible explanation for a possible protective effect of vWF deficiency against the development of early atherosclerosis, can be found in the disturbed interaction between the vessel wall and platelets. vWF facilitates platelet adhesion on the inflamed or injured vessel wall. Adherent platelets secrete or express atherogenic mediators, such as cytokines, chemokines, growth factors, adhesion molecules and coagulation factors. This further promotes local recruitment of monocytes, and subsequent monocyte adhesion to the vessel wall, transendothelial migration, and differentiation toward macrophages, which is critical for plaque formation[57,58]. Furthermore, less platelet adhesion and activation could also diminish thrombin generation as activated platelets provide a surface for prothrombin activation. Thrombin is an important player in the inflammatory process of atherosclerosis[57,59,60].

After coronary injury the $vWF^{-/-}$ pigs were, however, protected from MI[19,22,61], which suggests that vWF is required for the progression to occlusive arterial thrombosis. Hereto, impaired platelet activation in $vWF^{-/-}$ pigs could contribute[24,25], as well as less thrombogenecity of plaques since $vWF^{-/-}$ pigs lack neointimal vWF[27]. A protective effect of vWF deficiency on arterial thrombosis in stenosed and injured arteries has been found across animal species[61-63]. In humans, increased vWF levels are clearly associated with the occurrence of ischemic cardiovascular events[2-4,64]. And recently, it has been shown that the prevalence of ischemic cardiovascular disease is significantly lower in patients with

vWD as compared to the general population[51]. Agents inhibiting the vWF-GPIb or vWF-collagen interaction also reduced thrombus size after endothelial injury in $vWF^{+/+}$ animals[33,34,42,43]. In fact, targeting the vWF-GPIb interaction emerges as a promising future therapeutic option that compares favourably to the currently marketed antiplatelet drugs in efficacy and safety, probably because of the selective prevention of platelet adhesion at high shear rates, such as observed in stenosed arteries[65].

In conclusion, vWF deficiency is thought to be protective against the development of atherosclerosis at pig aorta branch point predilection sites, but not in pig coronary arteries. Studies in other species indicate that vWF deficiency might be protective against early atherogenesis, probably via a disturbed interaction between the vessel wall and platelets. The few human studies published are inconclusive, and an unequivocal protective effect of vWD on atherosclerosis has not been demonstrated. However, vWF is probably a significant player in the multifaceted interaction between the hemostatic system and the atherosclerotic process. More experimental and clinical data are needed to further clarify this relationship. If we are able to unravel the complex interplay between vWF, atherosclerosis and arterial thrombosis, we may be able to create new prevention and management possibilities for cardiovascular disease in the near future.

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Chapter 6 Treatment of ischemic heart disease in hemophilia patients: an institutional guideline

Haemophilia. 2009;15:952-958

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Summary

Since the introduction of clotting factor concentrates, life expectancy of hemophilia patients is increasing and now approaches that of the general male population. Increasingly, hemophilia patients are confronted with age-related comorbidity, including ischemic cardiovascular disease. Treatment of stable angina pectoris and the acute coronary syndrome with antithrombotic therapy and percutaneous coronary intervention in hemophilia patients is feasible, but requires a tight co-operation between all specialists involved. As evidence-based guidelines are lacking, we developed a protocol on how we will treat hemophilia patients with ischemic heart disease.

Introduction

Since the introduction of clotting factor concentrates in the 1960s and prophylactic treatment in the 1970s, life expectancy of patients with hemophilia in developed countries as increased form <30 years to over 70 years[1-3]. Consequently, the aging hemophilia patient not only suffers from medical issues associated with his congenital bleeding disorder, the long-term consequences of lack of treatment in the past (e.g. arthropathy), or iatrogenic-induced viral infections (e.g. HIV and hepatitis C). Increasingly, hemophilia patients are confronted with age-related comorbidity. In accordance with this finding, hemophilia specialists observe an increase in hemophilia patients with ischemic cardiovascular disease over the last decade. Kulkarni et al.[4] assessed the age-specific prevalence of ischemic heart disease in 3422 American hemophilia patients, using hospital discharge rates between 1993 and 1998. The prevalence of ischemic heart disease was 0.05% in patients under 30 years of age and 15.2% in those 60 years or older[4]. Although the prevalence and incidence of ischemic cardiovascular disease is increasing in hemophilia patients, mortality due to ischemic heart disease is lower in hemophilia patients as compared with the general age-matched male population[1,3].

Treatment of ischemic cardiovascular disease in hemophilia patients is a major challenge and has never been investigated. Atherosclerosis and ischemic cardiovascular disease require treatment to decrease the risk of thrombus formation. However, antithrombotic therapy and cardiac interventions increase the bleeding risk. To minimize the risk of bleeding, the clotting factor deficiency should be corrected. However, Girolami et al.[5,6] demonstrated that a majority of published cases of thrombotic cardiovascular events in hemophilia patients were related to the infusion of clotting factor concentrates. Although an underestimation of spontaneous ischemic events without previous treatment with clotting factor concentrates is to be expected, these case reports do reflect the need for attentiveness in infusing clotting factor concentrates in hemophilia patients with known cardiovascular risk factors, atherosclerosis or myocardial ischemia.

As evidence-based guidelines are lacking, there is an urgent need for uniform guidelines on how to treat hemophilia patients with ischemic heart disease. We developed a local guideline, both experience and theoretically based, that will be validated prospectively.

General considerations

The spectrum of ischemic heart disease ranges from stable angina pectoris to the acute coronary syndrome (ACS), which represents unstable angina pectoris, non-ST segment elevation myocardial infarction (NSTEMI) and ST segment elevation myocardial infarction (STEMI). NSTEMI can be differentiated from unstable angina pectoris by the presence of elevated cardiac enzymes indicating actual progression to myocardial infarction and necrosis. Both unstable angina pectoris and NSTEMI are differentiated from STEMI in that they are not amenable to either immediate reperfusion therapy with systemic fibrinolytic therapy or immediate percutaneous coronary intervention (PCI)[7].

As in the case of non-hemophilic patients with ischemic heart disease, indications for cardiac intervention should be the same in hemophilia patients with ischemic heart disease. The clotting factor deficiency is not a reason to withhold cardiac intervention from hemophilia patients with ischemic heart disease, because, as with other invasisve procedures, the clotting factor deficiency has to be corrected before the cardiac intervention. Uncomplicated cardiac interventions have been performed in hemophilia patients[5,6,8-11]. As in non-hemophilic patients, primary PCI is indicated for hemophilia patients with STEMI. When angina or ischemic changes on electrocardiography are progressive or relapsing in hemophilia patients with unstable angina pectoris or NSTEMI, primary PCI is also indicated. Although indications for cardiac intervention should be the same in hemophilia patients with ischemic heart disease as in the case of non-hemophilic patients, a specified antithrombotic treatment guideline is needed, but difficult to establish, because of the delicate equilibrium between bleeding and thrombosis in these patients.

Treating hemophilia patients with ischemic heart disease requires a tight co-operation between the hemophilia specialist, cardiologist, nursing staff, chemical laboratory, transfusion facility and pharmacist. Treatment needs to be tailored to the clinical presentation of the patient. In the next paragraphs, we give an overview of specific issues to consider during cardiac intervention and recommendations on antithrombotic therapy and clotting factor correction in hemophilia patients with stable angina pectoris or ACS. A schematic overview of the treatment of stable angina pectoris and the ACS in patients with severe hemophilia is given in Fig. 1.

Technical considerations for cardiac intervention

Choice of arterial access site

Whereas in the general population ischemic complications during PCI have decreased over the last years, bleeding has now emerged as one of the most common complications[12]. When a diagnostic or therapeutic cardiac intervention is indicated, the choice of the access site should be carefully considered. Up to 70% of all major bleedings (access site hematomas and retroperitoneal bleeding) are related to femoral artery access[12]. The safety of radial access has been well established and this should be the default access site in patients with an increased bleeding tendency[12-15]. Therefore, we recommend radial artery access for PCI in hemophilia patients.

Choice of stent

Patients with a drug-eluting stent have a lower 2-year risk of repeat revascularization procedures compared with patients with a bare-metal stent (BMS). They do, however, require longer treatment with antiplatelet therapy. There is no difference in mortality risks after 2 years between patients with drug-eluting stent and patients with a BMS[16]. The risk of restenosis in hemophilia patients is not known. Acute stent thrombosis has been reported in a patient with severe hemophilia B, pretreated with factor IX concentrate and not on antithrombotic therapy[17], stressing the importance of antiplatelet therapy after

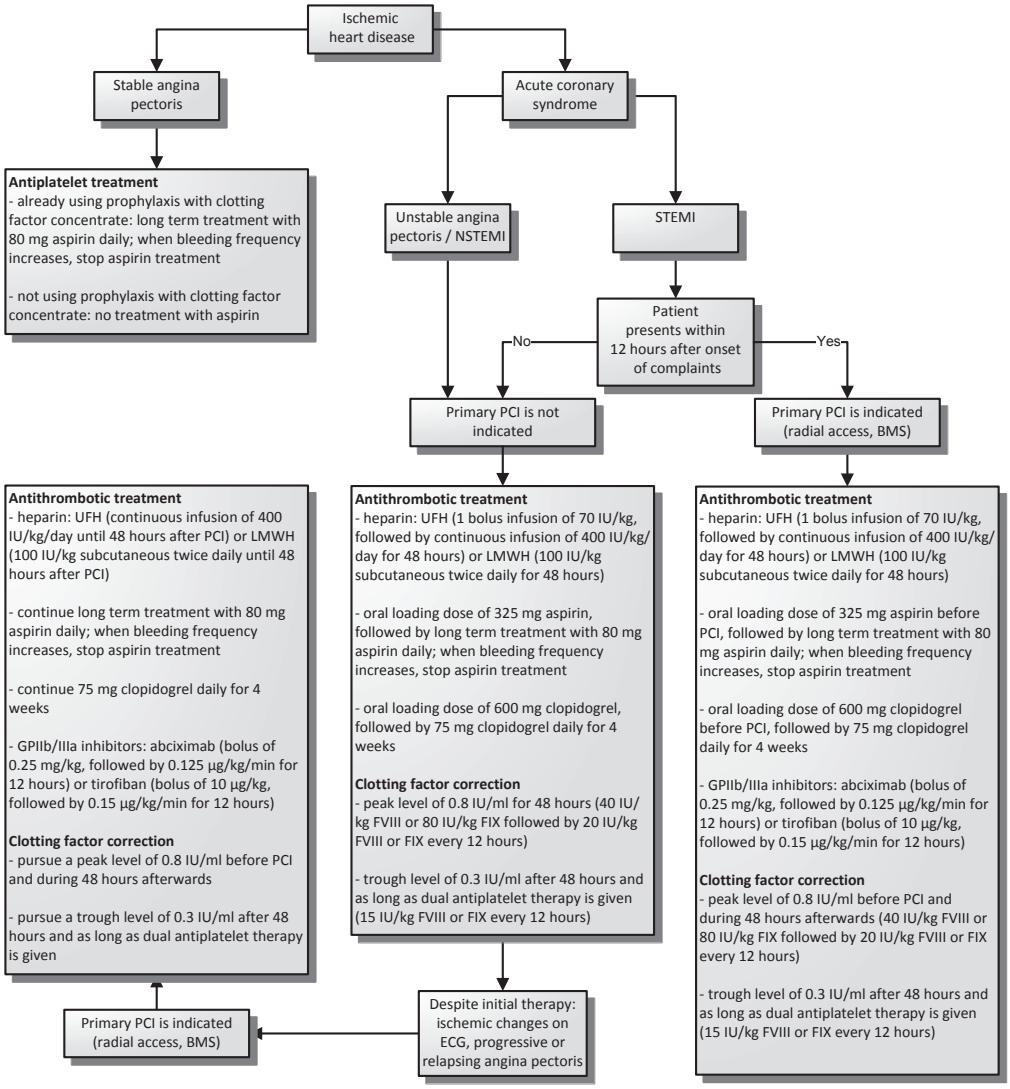

Fig. 1 Flow chart of the treatment of stable angina pectoris and the acute coronary syndrome in patients with severe hemophilia

NSTEMI, non-ST segment elevation myocardial infarction; STEMI, ST segment elevation myocardial infarction; PCI, percutaneous coronary intervention; BMS, bare-metal stent; UFH, unfractionated heparin; LMWH, low molecular weight heparin; FVIII, factor VIII; FIX, factor IX; ECG, electrocardiogram.

stent implantation, also for hemophilia patients. When stent placement is indicated, in a hemophilia patient, we recommend the use of a BMS, because a shorter period of dual oral antiplatelet therapy is needed.

Considerations for antithrombotic therapy

Aspirin

Long-term treatment with low dose aspirin is recommended for all patients with coronary artery disease. Patients with moderate or mild hemophilia often tolerate a low dose of aspirin (80 – 100 mg daily) very well, even without prophylaxis with clotting factor concentrates. Careful follow-up is required during which patients are monitored closely. In case of an increased bleeding frequency, aspirin should be stopped. In patients with severe hemophilia, the use of low-dose aspirin is disputable. In some patients it is manageable, but patients often use prophylaxis in this setting. The decision is mainly based on the underlying condition. In patients with severe hemophilia and ACS, there is rationale for using long-term aspirin treatment, as these patients apparently develop thrombotic vascular occlusion despite their lack of clotting factor. However, in patients with severe hemophilia and stable angina pectoris, the case for using aspirin is less strong. If patients are using prophylaxis with clotting factor concentrates, 80 – 100 mg of aspirin daily can be tried. In case of an increased bleeding frequency, aspirin should be stopped. If no clotting factor prophylaxis is given, we do not routinely prescribe aspirin.

Guidelines recommend the use of an oral loading dose of 325 mg aspirin in non-hemophilic patients with ACS and before PCI, unless on chronic therapy. After PCI, therapy is continued with 325 mg aspirin daily for at least 1 month after BMS, 3 months after sirolimus stents and 6 months after paclitaxel stents[18,19]. As complete correction of the clotting factor deficiency is mandatory during cardiac intervention, the loading dose of aspirin can be given in hemophilia patients before PCI. We do not, however, recommend prolonged treatment with these high dosages after the full clotting factor correction has been tapered down. Instead, following the aspirin loading dose, we recommend a long-

term treatment with 80 – 100 mg aspirin daily under close clinical monitoring. Aspirin treatment in hemophilia patients with unstable angina pectoris or NSTEMI is identical to aspirin treatment in hemophilia patients with STEMI undergoing PCI. Again, aspirin should be stopped when bleeding frequency increases.

Clopidogrel

In patients without hemophilia, clopidogrel is given in combination with aspirin as an oral loading dose of 600 mg, followed by 75 mg daily for at least 4 weeks after PCI with a BMS (if possible up to 6 months), and at least 12 months after a drug-eluting stent[18,20]. As with aspirin, the anti-aggregation effect of clopidogrel is as long as the lifespan of the platelet, i.e. 7 days. The time for endothelialization of a BMS is thought to be 4 weeks, indicating that 4 weeks of clopidogrel is the minimum duration. In recent guidelines, however, the minimum duration of clopidogrel was defined as 2 weeks for patients at high risk for bleeding[18,20]. When dual antiplatelet therapy is prescribed, correction with clotting factor concentrates is mandatory.

We recommend an oral loading dose of 600 mg clopidogrel in hemophilia patients before PCI, followed by 75 mg clopidogrel daily for a minimum of 2 weeks after BMS, in addition to 80 – 100 mg aspirin. If possible, a treatment duration of 4 weeks should be pursued. Clopidogrel treatment in hemophilia patients with unstable angina pectoris or NSTEMI is identical to clopidogrel treatment in hemophilia patients with STEMI undergoing PCI.

GPIIb/IIIa inhibitors

In general, the use of GPIIb/IIIa inhibitors, such as abciximab or tirofiban, is recommended for patients with ACS undergoing PCI[20]. There are little data on safety of abciximab in hemophilia patients, but it has been successfully used[17]. Given the short half-life of several hours and the required correction of the clotting factor deficiency during PCI, the use of abciximab in hemophilia patients is feasible.

In hemophilia patients, we recommend the use of GPIIb/IIIa inhibitors when performing PCI. If abciximab is used, a bolus of 0.25 mg/kg should be given, followed by 0.125 µg/kg/min for 12 h with a maximum of 10 µg/min. If tirofiban is used, a bolus of 10 µg/kg

should be given followed by 0.15 μg/kg/min for 12 h. These dosages are comparable with those used in non-hemophilic patients.

Heparin

During complete clotting factor correction, heparin can be administered for 48 h to hemophilia patients with ACS, according to standard treatment protocols[19]. Heparin has successfully been used in hemophilia patients during coronary stenting[11]. It should not be given before complete clotting factor correction is achieved. Unfractionated heparin (UFH) has the advantage over low molecular weight heparin (LMWH) that its half-life is very short. Furthermore, the SYNERGY trial showed fewer major bleedings in patients using UFH compared to LMWH (9.1% versus 7.6%)[21]. Careful monitoring of the activated partial thromboplastin (aPTT) and clinical signs of bleeding is mandatory. The measurement of the aPTT should not pose a problem when clotting factor is fully corrected. On the other hand, measuring the level of factor VIII (FVIII) or IX (FIX) is hampered when using normal aPTT reagent, due to the effects of heparin. In our institute, we perform routine factor determination in a 1:20 dilution. In this setting, heparin has little inhibitory effects. If necessary, the use of a heparin neutralizer (Hepasorb®, Organon Teknika, Durham, NC, USA) before conducting the factor level measurements can be tried. On the other hand, the problems with aPTT monitoring and interference with FVIII and FIX measurements can be overcome using LMWH.

We recommend UFH or LMWH in ACS. When UFH is used, a bolus infusion of 70 IU/kg followed by a continuous infusion of 400 IU/kg/day for 48 h is needed. Monitoring is done by aPTT measurements, aiming at a 2- to 3-fold increase in baseline value. Further continuation of UFH after an uncomplicated PCI is not indicated. For elective PCI, a single bolus infusion of 70 IU/kg of UFH is given. When LMWH is used, dosages are 100 IU/kg subcutaneous twice daily. These dosages are comparable with those used in non-hemophilic patients.

Bivalirudin

In some centers, heparin has been replaced by bivalirudin, a selective factor II antagonist. It inhibits factor II reversibly and has a short half-life of 1.5 h. Several trials have shown a lower incidence of major bleeding in patients treated with bivalirudin compared with patients treated with heparin and glycoprotein inhibitors[12,22]. Greatest reduction was seen in groin and retroperitoneal bleedings. As the preferred access site in hemophilia patients is the radial artery, it is not clear whether bivalirudin will also exhibit the same benefits in hemophilia patients. Bivalirudin has successfully been used in patients with hemophilia[9,10,23]. Currently, no specific antidote for bivalirudin exists.

The use of bivalirudin in hemophilia seems feasible. The decision to use it over heparin should be made together with the cardiologist. If indicated, a bolus of 0.75 mg/kg should be given, followed by a continuous infusion of 1.75 mg/kg/h (adjusted for creatinin clearance). These dosages are comparable with those used in non-hemophilic patients.

Pentasaccharides

The selective Xa-antagonists, like fondaparinux and idraparinux, are increasingly used in the treatment of unstable coronary heart disease and venous thrombosis. In PCI, they do not have a clear role. There are no data about the usage of Xa-antagonists in hemophilia patients, a specific antidote for pentasaccharides is lacking an therefore heparin is preferred.

Thrombolysis

Thrombolysis is used in STEMI[24,25]. As PCI has a better outcome in non-hemophilic patients than medical intervention and, taking into account the bleeding risk of thrombolysis, we do not recommend thrombolysis in hemophilia patients.

Clotting factor correction during and after cardiac intervention or ACS

Complications during antithrombotic treatment and cardiac intervention are mainly determined by the bleeding risk. To minimize this risk, there is a clear indication to correct the clotting factor deficiency, despite the risk of inducing more thrombosis[5,6]. We do not recommend the use of desmopressin in patients with ischemic heart disease, because desmopressin increases heart rate with 10 – 20%, causes a higher diastolic blood pressure, has a potent antidiuretic effect and increases the risk of arterial thrombosis[26]. Instead, we recommend the use of clotting factor concentrates. Clotting factor concentrate dosages in the following sections are those recommended for patients with severe hemophilia.

Hemophilia patients with STEMI undergoing PCI

If a patient presents within 12 h after onset of complaints, a primary PCI will be performed. If a patients presents after 12 h of onset of complaints, he is treated according to the NSTEMI protocol.

Correction of the clotting factor deficiency before performing a PCI is mandatory. We recommend aiming at a peak level of 0.8 IU/ml before PCI, and until 48 h after PCI. Higher levels should be avoided to prevent occlusive thrombi. This can be achieved by a slow bolus infusion of 40 IU/kg FVIII or 80 IU/kg FIX in 30 min, with a FVIII or FIX recovery assay 15 min after bolus infusion. A peak level of 0.8 IU/ml is pursued for 48 h using slow bolus infusions of 20 IU/kg FVIII or FIX every 12 h.

The duration of clotting factor correction is dependent on the choice of stent and the need for dual antiplatelet therapy with clopidogrel and aspirin. After the initial clotting factor infusion, as described above, we recommend clotting factor substitution aiming at trough levels of 0.3 IU/ml, using 15 IU/kg FVIII or FIX every 12 h, for as long as dual antiplatelet therapy is given. Regular clotting factor level measurements are required to optimize dosing. Afterwards, the regular factor replacement schedule of the patient can be continued.

NSTEMI and unstable angina pectoris

In the acute setting, patients with NSTEMI or unstable angina pectoris are treated with aspirin, clopidogrel and heparin as described above. Primary PCI is not indicated. Clotting factor correction is given, aiming at a peak level of 0.8 IU/ml as long as heparin is given, followed by trough levels of 0.3 IU/ml during treatment with dual antiplatelet therapy. Based on the clinical condition (progressive or relapsing angina pectoris, or ischemic changes on electrocardiography), a PCI can be performed after the initial onset of complaints. Then, the treatment schedule for PCI can be followed.

Coronary angiography and elective PCI

Elective PCI is performed in non-emergency settings and coronary angiography (CAG) is often part of the diagnostic work-up of patients with ischemic heart disease. We recommend clotting factor correction with a peak level of 0.8 IU/ml before elective PCI or CAG, until 24 h afterwards, because only a single bolus infusion of 70 IU/kg of UFH is needed. In case of an elective PCI, the duration of substitution with FVIII or FIX is dependent on the choice of stents and the need for dual antiplatelet therapy. In case of CAG without stent placing, the patient's regular factor replacement treatment schedule can be continued after 24 h.

Conclusion

Hemophilia patients with ischemic heart disease are a challenge for both hemophilia specialists and cardiologists. Although hemophilia specialists observe an increase in hemophilia patients with ischemic heart disease, these patients are not common. This makes it unlikely that well-designed clinical trials can be performed to find the optimal therapy for these complex patients. For the initial management of ACS, we should make a strong effort to follow guidelines that were developed for non-hemophilic patients. For a long-term treatment, however, (dual) antiplatelet therapy should be based on the

individual patient profile, in which hemophilia severity, bleeding phenotype and extensiveness of coronary artery disease play a role.

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Chapter 7 Cardiac catheterization and intervention in hemophilia patients: evaluation of the 2009 institutional guideline

Submitted

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Summary

Aging hemophilia patients are increasingly confronted with ischemic cardiovascular disease. Treatment is complex, because of the delicate equilibrium between bleeding and thrombosis. In 2009, we developed an institutional guideline on how to treat ischemic heart disease in this patient population. Feasibility and safety of the 2009 guideline were evaluated in hemophilia patients who underwent cardiac catheterization or intervention between January 2009 and April 2012.

Nine diagnostic or therapeutic cardiac catheterizations were performed in 6 hemophilia patients. One patient with moderate hemophilia B was included, while the other 5 patients were diagnosed with mild hemophilia A. Age ranged from 49 – 76 years. In 6 out of 9 procedures, access to the circulation was gained via the radial artery. Only bare-metal stents were implanted, after which dual antiplatelet treatment was given for at least 4 weeks. During cardiac catheterization/intervention and dual antiplatelet treatment clotting factor levels were corrected. No thrombotic or bleeding complications occurred. In 1 patient a low titer inhibitor recurred 10 months after catheterization. In-stent restenosis was diagnosed in 1 patient.

The case series described in this article indicates that treatment according to the guideline is feasible and safe. Furthermore, based on the case series and developments in new guidelines for non-hemophilic patients with IHD, some adjustments on the 2009 guideline are proposed.

Introduction

Since the introduction of clotting factor concentrates, life expectancy of hemophilia patients has increased and now approaches that of the general male population[1,2]. Consequently, aging hemophilia patients not only experience medical issues associated with their congenital bleeding disorder, but are increasingly confronted with age-related comorbidities[3]. Although ischemic heart disease (IHD) mortality is lower in hemophilia patients as compared to the general male population[1,2], and myocardial infarction occurs less frequently in patients with severe hemophilia[4], an increase in the incidence and prevalence of IHD is observed in the elderly hemophilia population[5,6].

Treatment of IHD in hemophilia patients is a major challenge for both cardiologists and hematologists. Atherosclerosis and IHD require pharmacological intervention to decrease the risk of thrombus formation. To minimize the risk of bleeding, associated with antithrombotic therapy and cardiac catheterization and intervention, the clotting factor deficiency needs to be corrected. Evidence-based guidelines on how to treat IHD in hemophilia patients are lacking. However, over the last 3 years, several experts have shared their opinions on treatment[5-7]. In 2009, we developed an institutional guideline, based on both experience from hemophilia specialists and existing guidelines on treatment of IHD in non-hemophilic patients[6]. In this guideline, we state that indications for diagnostic or therapeutic cardiac catheterization in hemophilia patients should be the same as in non-hemophilic patients, and that radial artery access is preferred over femoral artery access to minimize the risk for access site bleeding. When stent placement is indicated, we recommend the use of a bare-metal stent (BMS), because a shorter period of dual antiplatelet therapy is needed as compared with the use of drug-eluting stents (DES). We pursue a dual antiplatelet treatment duration of 4 weeks. The clotting factor deficiency should be corrected before start of antithrombotic therapy and cardiac procedure, aiming at a peak level of 0.80 IU/ml, and continued until treatment with heparin ends. We recommend clotting factor substitution aiming at trough levels of 0.30 IU/ml for as long as dual antiplatelet therapy is given. Long-term treatment with low dose aspirin is often well tolerated in patients with mild or moderate hemophilia. If no clotting

factor prophylaxis is given, we do not prescribe aspirin for patients with severe hemophilia. In the present article, we prospectively assessed the feasibility and safety of cardiac catheterizations and interventions in hemophilia patients treated according to the 2009 guideline. Furthermore, based on the case series and developments in new guidelines for non-hemophilic patients with IHD, some adjustments on the 2009 guideline are proposed.

Methods

All patients with hemophilia A or B who presented with IHD at our center since January 2009, were treated according to the 2009 guideline[6]. Hemophilia patients who underwent coronary angiography (CAG) or percutaneous coronary intervention (PCI) between January 2009 and June 2012 were included in the current case series. For every patient, information on hemophilia characteristics, cardiovascular risk factors, cardiovascular diagnosis, cardiac procedure, medical treatment, outcome, and complications was systematically documented at the time of the index procedure. Body mass index (BMI) was calculated as weight divided by height squared (kg/m^2). Hypertension was defined as a systolic blood pressure of ≥ 140 mmHg, a diastolic blood pressure of ≥ 90 mmHg, or use of antihypertensive medication. Hypercholesterolemia was defined as a total cholesterol level of ≥ 6.5 mmol/l, or use of lipid lowering medication. Information on changes in treatment, new cardiovascular events or cardiac catheterizations/interventions, and complications was collected until September 2012. The Medical Ethics Committee of the University Medical Center Utrecht (Utrecht, the Netherlands) approved data collection.

Results

Nine diagnostic or therapeutic cardiac catheterizations were performed in 6 hemophilia patients between January 2009 and June 2012. Hemophilia characteristics and

cardiovascular risk factors of each patient at the time of the index procedure are described in Table 1. Age ranged from 49 – 76 years. One patient with moderate hemophilia B was included, while the other 5 patients were diagnosed with mild hemophilia A. Cardiovascular diagnosis, cardiac procedures, medical treatment, outcomes and complications per patient are described in Table 2.

Patient 1

Patient 1 (moderate hemophilia B) underwent CAG via the radial artery, which showed multiple stenoses. Before catheterization, the clotting factor deficiency was corrected by bolus infusion of 6000 IU factor IX (FIX), which resulted in a peak FIX level of 1.30 IU/ml. In addition, 5000 IU unfractionated heparin (UFH) were given. A second catheterization with fractional flow reserve (FFR) measurements was planned. Because of abnormal FFR results, a BMS was implanted during the same procedure. Again, the radial artery was used to access the circulation. Before PCI, 5000 IU FIX and 7000 IU UFH were infused. During 48 hours after PCI 1000-2000 IU FIX were infused twice daily. The patient was not treated with heparin after the intervention. After a loading dose of aspirin and ticagrelor during PCI, the patient was treated with aspirin and clopidogrel for 4 weeks, and clotting factor levels were corrected with 2000 IU FIX daily. Trough levels of 0.33 IU FIX/ml and 0.29 IU FIX/ml were obtained 7 and 21 days after the intervention, respectively. After dual antiplatelet therapy, aspirin (100 mg) was continued and FIX prophylaxis was stopped.

No thrombotic or bleeding complications occurred during CAG and PCI, or in the days following the procedures. As this patient was treated recently, no long-term follow-up data are available yet. However, no bleeding complications were reported during dual antiplatelet therapy, or during treatment with low dose aspirin only. Inhibitors against FIX did not develop.

Patient 2

Patient 2 (mild hemophilia A) had a cardiac arrest. After successful resuscitation at the Amphia Hospital (Breda, the Netherlands), the cardiologist in charge asked our opinion on how to treat a hemophilia patient with an acute coronary syndrome (ACS). Because of the

Table 1 Patient characteristics at the time of the index procedure

Patient	Age (years)	Type of hemophilia	Residual FVIII / FIX level (IU/ml)	Presence of inhibitors	HCV infection	HIV infection	BMI (kg/m^2)	Cardiovascular risk factors
1	65	B	0.05	No	In the past	No	22.0	Hypertension
2	72	A	0.15	No	No	No	25.9	-
3	76	A	0.28	No	No	No	26.2	Smoking, hypertension, dyslipidemia
4	61	A	0.20	No	No	No	27.2	Hypertension, dyslipidemia
5	57	A	0.20	In the past	No	No	25.7	Dyslipidemia
6	49	A	0.25	No	No	No	25.4	Smoking, hypertension, dyslipidemia

FVIII, factor VIII; FIX, factor IX; HCV, hepatitis C virus; HIV, human immunodeficiency virus; BMI, body mass index.

electrocardiographic findings (signs of inferior myocardial ischemia and ventricular fibrillation) a primary PCI was performed. The femoral artery was used to access the circulation and a BMS was implanted. A bolus of 3000 IU factor VIII (FVIII) was infused to achieve a clotting factor level of 0.80 IU/ml during the intervention. In addition, loading doses of aspirin and clopidogrel were given. A bolus of 70 IU/kg UFH was administered, followed by continuous infusion of 400 IU/kg/day for 48 hours. During heparin treatment, clotting factor levels were corrected by the infusion of 1500 IU FVIII twice daily aiming at peak levels of 0.80 IU/ml. Dual antiplatelet treatment was given for 4 weeks, during which 1000 IU FVIII were infused daily. On day 23 post-intervention a trough level of 0.37 IU/ml was measured. After 4 weeks, low dose aspirin (80 mg daily) was continued and FVIII prophylaxis was stopped. No complications occurred during the procedure and inhibitors against FVIII did not develop. After 10 weeks follow-up the patient experienced hematuria, which was successfully treated with a single bolus of 2000 IU FVIII and aspirin treatment was continued. A year later we were informed that the patient was again admitted at the Amphia hospital and a new IHD episode was suspected. However, cardiologic evaluation showed no signs of this, and an expectative policy followed.

Patient 3

The third patient (mild hemophilia A) presented with symptoms of stable angina pectoris. CAG, performed in a referral hospital, showed 3-vessel disease. Subsequently, an elective PCI was performed at our center via the femoral artery. The culprit lesion appeared to be located in the right coronary artery, which was stented with a BMS. Before PCI a bolus of 2500 IU FVIII was infused, which resulted in a clotting factor level of 1.26 IU/ml during the intervention. In addition, a loading dose of aspirin and clopidogrel, and 2500 IU UFH were given before the intervention. During 48 hours after PCI 1000 IU FVIII were infused twice daily. The patient was not treated with heparin after the intervention. Because of the patients' own residual clotting factor level of 0.28 IU/ml, no clotting factor substitution was given during dual antiplatelet therapy. In addition, dual antiplatelet therapy was extended from 4 weeks to 6 months. During follow-up the patient reported the occurrence of small hematomas on his hands. However, there was no indication to cease

Table 2 Cardiovascular diagnosis, cardiac procedures, medical treatment, outcomes and complications per patient

Patient	Cardiovascular diagnosis / cardiac procedure	Follow-up (months)	Access site / stent	FVIII / FIX level during procedure (IU/ml)	Antithrombotic treatment	Clotting factor correction	Long-term treatment	Outcome / complications
1	Stable AP (NYHA 2) / CAG	7	Radial artery / -	1.30	- Before procedure 5000 IU UFH	- Before procedure 6000 IU FIX	-	Multiple stenoses
	Stable AP (NYHA 2) / FFR + PCI	6	Radial artery / BMS	1.30	- Before procedure 7000 IU UFH - Aspirin loading dose 325 mg - Ticagrelor loading dose 180 mg - DAPT for 4 weeks: aspirin 100 mg daily + clopidogrel 75 mg daily	- Before procedure 5000 IU FIX - 1000 – 2000 IU FIX twice daily for 48 hours - 2000 IU FIX daily for 4 weeks	- Aspirin 100 mg daily	Abnormal FFR
2	STEMI with VF / PCI	42	Femoral artery / BMS	0.80	- Before procedure 70 IU/kg UFH + continuous infusion 400 IU/kg/day for 48 hours - Aspirin loading dose 325 mg - Clopidogrel loading dose 600 mg - DAPT for 4 weeks: aspirin 80 mg daily + clopidogrel 75 mg daily	- Before procedure 3000 IU FVIII - 1500 IU FVIII twice daily for 48 hours - 1000 IU FVIII daily for 4 weeks	- Aspirin 80 mg daily	Hematuria
3	Stable AP (NYHA 3) / PCI	34	Femoral artery / BMS	1.26	- Before procedure 2500 IU UFH - Aspirin loading dose 325 mg - Clopidogrel loading dose 600 mg - DAPT for 6 months: aspirin 100 mg daily + clopidogrel 75 mg daily	- Before procedure 2500 IU FVIII - 1000 IU FVIII twice daily for 48 hours	- Aspirin 100 mg daily	Small hematomas hands
	Stable AP (NYHA 2) / CAG + FFR	10	Radial artery / -	0.90	- Before procedure 7500 IU UFH	- Before procedure 2000 IU FVIII - Afterwards 1000 IU FVIII	- Aspirin 100 mg daily	No significant stenosis

4	Stable AP (NYHA 2) / PCI	18	Radial artery / BMS	0.80	- Before procedure 8000 IU UFH + 2500 IU UFH during procedure - Aspirin loading dose 325 mg - Clopidogrel loading dose 600 mg - DAPT therapy for 4 weeks: aspirin 100 mg daily + clopidogrel 75 mg daily	- Before procedure 3000 IU FVIII - 1500 IU FVIII twice daily for 48 hours - 2000 IU FVIII daily for 4 weeks	- Aspirin 100 mg daily (after 4 months substituted by clopidogrel 75 mg daily)	Stomach complaints
	Stable AP (NYHA 2) / CAG	6	Radial artery / -	0.70	- Before procedure 9000 IU UFH - Ticagrelor loading dose 180 mg	- Before procedure 3000 IU FVIII - 1500 IU FVIII twice daily for 24 hours	- Aspirin 100 mg daily	In stent restenosis, no wire passage possible
5	Retrosternal complaints / CAG	42	Radial artery / -	1.00	- Before procedure 5000 IU UFH	- Continuous infusion of FVIII	- None	No stenoses
6	NSTEMI / PCI	3	Radial artery / BMS	1.25	- Before procedure 7000 IU UFH - Aspirin loading dose 325 mg - Ticagrelor loading dose 180 mg - DAPT for 12 months: aspirin 100 mg daily + clopidogrel 75 mg daily	- Before procedure 2250 IU FVIII - Afterwards 750 IU FVIII	- Aspirin 100 mg daily	-

FVIII, factor VIII; FIX, factor IX; AP, angina pectoris; NYHA, New York Heart Association Classification; STEMI, ST segment elevation myocardial infarction; VF, ventricular fibrillation; NSTEMI, non-ST segment elevation myocardial infarction; CAG, coronary angiography; FFR, fractional flow reserve; PCI, percutaneous coronary intervention; BMS, bare-metal stent; UFH, unfractionated heparin; DAPT, dual antiplatelet therapy.

the dual antiplatelet regime prematurely. While on long-term low dose aspirin treatment, new complaints of angina pectoris occurred 2 years after the first intervention. A CAG with FFR measurement was performed via the radial artery, which showed no significant stenosis. The clotting factor level was corrected by infusion of 2000 IU FVIII before the procedure and 1000 UI FVIII afterwards. In addition, 7500 IU UFH were given before the procedure. No complications occurred and inhibitors against FVIII did not develop.

Patient 4

Because of dyspnoea and complaints of angina pectoris, the fourth patient (mild hemophilia A) was scheduled for a CAG with an option to perform PCI. During the procedure, using the radial artery to access the circulation, the left anterior descending artery was stented. However, after dissection of a large diagonal vessel originating out of the stent, additional stenting of the diagonal vessel was performed. Therefore, in total, 2 stents (BMS) were implanted. Before the intervention 3000 IU FVIII were administered to achieve a clotting factor level of 0.80 IU/ml. 8000 IU UFH were administered before the procedure and during PCI a second bolus of 2500 IU UFH was given, because of the duration of the intervention. During 48 hours after PCI 1500 IU FVIII were infused twice daily. The patient was not treated with heparin after the intervention. Dual antiplatelet therapy was indicated for 4 weeks, which was started after a loading dose of aspirin and clopidogrel. Bleeding risks were minimized by the infusion of 2000 IU FVIII daily, obtaining a trough level of 0.27 IU FVIII/ml on day 15 after PCI. After stopping clopidogrel and FVIII prophylaxis, low dose aspirin treatment was continued. Because of stomach complaints aspirin was substituted by clopidogrel after 4 months. Recently, the patient started to experience new complaints of dyspnoea and chest pain during exercise. A new CAG with an option for PCI was planned. Before the procedure 3000 IU FVIII were administered and a clotting factor level of 0.70 IU/ml was reached. 9000 IU UFH and a loading dose of ticagrelor (180 mg) were given. The patient received 1500 IU FVIII twice daily for the following 24 hours. In-stent restenosis of the side-branch stent had developed. No wire passage was possible through the restenosed stent coming out of the main branch stent.

Therefore, no intervention was done. Afterwards, clopidogrel was switched back to aspirin, and pantozol was added. No inhibitors against FVIII developed.

Patient 5

The fifth patient (mild hemophilia) underwent CAG because of retrosternal complaints and possible indications for minor inferior-lateral ischemia on a perfusion scintigraphy. Nine days before the CAG the patient was admitted at the hospital for a transurethral resection of a bladder tumour. Although no additional retrosternal complaints developed, the CAG was performed during admission because the patient had a history of a low titer inhibitor. Because of the recent surgery the partial FVIII deficiency was corrected by continuous infusion of clotting factor concentrate. 5000 IU UFH were administered before the procedure. CAG showed no abnormalities, and, therefore, no antiplatelet treatment was started. No complication occurred during follow-up. After 10 months and several additional bladder procedures, the patient again developed a low titer inhibitor.

Patient 6

The last patient (mild hemophilia A) was admitted at a referral hospital with a non-ST segment elevation myocardial infarction (NSTEMI). Treatment with aspirin and ticagrelor was started and the clotting factor deficiency was partially corrected. Because of relapsing unstable angina pectoris and the patients' request, he was transferred to our center for CAG with an option to perform PCI. A BMS was implanted in the right coronary artery using the radial artery to access the circulation. Before the procedure 7000 IU UFH and 2250 IU FVIII were administered, obtaining a clotting factor level of 1.25 IU/ml during the intervention. Twelve hours after PCI another bolus of FVIII concentrate (750 IU) were given. Ticagrelor was substituted by clopidogrel and dual antiplatelet treatment was continues. Because of the patients' own residual clotting factor level of 0.25 IU/ml, no clotting factor substitution was given during dual antiplatelet treatment, which will be extended to 12 months. During 3 months of follow-up no complications occurred and no inhibitors against FVIII developed.

Discussion

Since the introduction of our 2009 institutional guideline on how to treat IHD in hemophilia patients, 8 elective diagnostic or therapeutic cardiac catheterizations and 1 primary PCI were performed in 6 patients. To our knowledge this is the first prospectively collected case series of hemophilia patients with IHD who were treated according to a guideline. The case series described in this article indicates that treatment according to this guideline is feasible and safe.

As recommended, in most procedures, access to the circulation was gained via the radial artery. A meta-analysis of randomized clinical trials showed that radial artery access reduced major bleeding by 73% as compared to femoral artery access[8]. A femoral approach was chosen in 2 patients who underwent PCI in 2009. One patient underwent primary PCI, in which sometimes the femoral approach is preferred because of the emergency situation. The second procedure was performed by an operator who was not yet experienced enough to perform a radial procedure. No thrombotic or bleeding complications were observed peri-procedural or during follow-up. During dual antiplatelet or long-term aspirin treatment no significant bleeding occurred. No other stents than BMS were implanted. In-stent restenosis was diagnosed in 1 patient after 1 year follow-up. Three patients reported recurrent chest pain during follow-up, and 2 of them underwent a second catheterization which showed no new stenoses. In 1 patient a low titer inhibitor recurred 10 months after CAG.

Girolami et al.[9,10] published 2 reviews evaluating all reported cases of thrombotic cardiovascular events until 2005 in patients with hemophilia A and B. There appeared to be an association between the occurrence of myocardial infarction and the recent administration of clotting factor concentrate or DDAVP[9,10]. The ACS in patient 2 and 6 were not provoked by infusion of clotting factor concentrate nor DDAVP. Some publication bias in the reviews[9,10] might have occurred. Most case reports considered patients with severe hemophilia, whereas, in a recent publication, myocardial infarction was shown to occur more often in patients with moderate or mild hemophilia[4]. In the current case series, no patients with severe hemophilia and IHD presented. Additionally,

an underestimation of spontaneous ischemic events without previous treatment with clotting factor concentrate is to be expected. These case reports do, however, reflect the need for attentiveness in infusing clotting factor concentrate in hemophilia patients with cardiovascular risk factors or known atherosclerotic cardiovascular disease.

Based on the case series and developments in new guidelines for non-hemophilic patients with IHD, we propose some adjustments on the 2009 institutional guideline. In the original guideline, we recommended aiming at clotting factor trough levels of 0.30 IU/ml by infusing clotting factor concentrate every 12 hours during dual antiplatelet therapy[6]. However, in practice the burden of twice daily infusions appeared unacceptable for patients with mild or moderate hemophilia. Most of these patients do not routinely perform vena punctures themselves. Moreover, the higher residual clotting factor levels in patient with mild or moderate hemophilia made once daily infusions feasible. During dual antiplatelet treatment, homecare services infused clotting factor concentrate daily, with which trough levels of 0.30 IU/ml could be reached. Probably, twice daily infusions are only necessary in patients with severe hemophilia to obtain trough levels of 0.30 IU/ml, and without reaching peak levels which are too high.

Patient 3 and 6 had a residual FVIII level of 0.28 IU/ml and 0.25 IU/ml, respectively. As these levels were close to 0.30 IU/ml, and the burden of increasing these levels to 0.30 IU/ml was thought not to outweigh the benefits, no clotting factor correction was given during dual antiplatelet therapy. After placement of a BMS, endothelialization of the stent is thought to be 4 weeks, indicating that this is the minimal duration for dual antiplatelet therapy[11]. According to treatment protocols in patients from the general population, on which the 2009 guideline was based, dual antiplatelet therapy for 6 – 12 months is preferred[11,12]. In combination with daily correction of clotting factor levels, dual antiplatelet therapy beyond 4 weeks is burdensome and expensive. However, in patients with a residual clotting factor level of 0.25 IU/ml or higher, an extension of the dual antiplatelet regime is worth trying without additional clotting factor infusions. If bleeding frequency increases, clopidogrel should be stopped.

In 2009 we recommended not to use DES, because a longer minimal period of dual antiplatelet therapy was needed as compared with BMS. One of the latest generation DES

(Xience®) has recently received the CE Mark in Europe for a dual antiplatelet treatment length of only 3 months as was mentioned in a press release from Abbott. In general, there is not much evidence for a longer duration of dual antiplatelet treatment than 6 months after DES implantation[13,14]. Therefore, from now on, we will opt for a DES in hemophilia patients with a residual clotting factor level of 0.25 IU/ml or higher and with an indication for a DES.

In the 2009 guideline, we recommended bolus infusion of UFH (70 IU/kg) before PCI, followed by continuous infusion of 400 IU UFH/kg/day for 48 hours. For elective PCI or CAG, a single bolus infusion of UFH (70 IU/kg) was indicated[6]. Nowadays, guidelines for non-hemophilic patients no longer recommend routine use of intravenous UFH after PCI. Also, in most of the described cases, no post-intervention treatment with heparin was performed. Therefore, in future hemophilia patients, we propose the following policy. Before PCI a single bolus infusion of UFH (70-100 IU/kg) should be administered. No additional heparin treatment after intervention is indicated. Before CAG a single bolus infusion of 5000 IU UFH should be administered when choosing radial access to prevent radial artery thrombosis, and 2500 IU UFH when choosing femoral access. However, we still recommend radial artery access. A clotting factor peak level of 0.80 IU/ml during PCI or CAG should be obtained. Trough levels of 0.50 IU/ml should be pursued until 24 hours after the procedure, by giving a single bolus infusion 12 hours after the procedure.

In non-hemophilic patients with an ACS clopidogrel is, nowadays, often replaced by a ticagrelor or prasugrel. Ticagrelor is a new oral, reversible, direct-acting inhibitor of the adenosine diphosphate (ADP) receptor $P2Y_{12}$ and provides faster, greater and more consistent $P2Y_{12}$ inhibition than clopidogrel[15,16]. In patients with an ACS, treatment with ticagrelor as compared with clopidogrel significantly reduced the rate of death from vascular causes, myocardial infarction or stroke at 12 months[17]. This difference was already apparent within the first 30 days of therapy. However, with ticagrelor there were, after 12 months follow-up, more episodes of intracranial bleeding, including fatal intracranial bleeding[17]. Like clopidogrel, prasugrel is a member of the thienopyridine class of ADP receptor inhibitors. Prasugrel inhibits platelet aggregation more rapidly, more consistently, and to a greater extent as compared with clopidogrel[15]. After 15 months

follow-up, prasugrel reduced the combined rate of death from cardiovascular causes, nonfatal myocardial infarction, or nonfatal stroke as compared with clopidogrel in patients with ACS. However, similar to the study with ticagrelor, an increased rate of serious bleedings and fatal bleedings was observed[18]. For this reason, we do not recommend the use of ticagrelor or prasugrel in a dual antiplatelet regime in hemophilia patients with ACS. However, no contraindications seem to exist for the use of a loading dose of ticagrelor or prasugrel after correction of the clotting factor deficiency in this specific patient population.

In conclusion, cardiac catheterizations and interventions in hemophilia patients, who are treated according to the 2009 guideline, are feasible and safe. We proposed some adjustments on the 2009 guideline. In summary, FVIII/FIX trough levels of 0.25 – 0.30 IU/ml during dual antiplatelet treatment can be obtained with once daily infusions of clotting factor concentrate in patients with moderate or mild hemophilia. In patients with a residual clotting factor level of 0.25 IU/ml or higher, administration of clotting factor concentrate is not needed during dual antiplatelet therapy, which can be extended beyond 4 weeks. When there is a risk for restenosis in this subgroup of mild hemophilia patients, a new generation DES may be used. We no longer recommend heparin treatment after PCI and FVIII/FIX trough levels of 0.50 IU/ml should be pursued until 24 hours after cardiac catheterization or intervention. Instead of clopidogrel, a loading dose of ticagrelor or prasugrel can be used in hemophilia patients with an ACS after correction of the clotting factor deficiency. Because of an increased bleeding risk, we do not recommend ticagrelor or prasugrel in a dual antiplatelet regime.

Documentation and evaluation of future hemophilia patients with IHD is needed to increase our knowledge and to improve treatment.

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Chapter 8 Obesity in hemophilia patients: effect on bleeding frequency, clotting factor concentrate usage, and hemostatic and fibrinolytic parameters

Submitted

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Summary

The prevalence of obesity in patients with hemophilia is increasing. In the general population, obesity is associated with a prothrombotic state. We investigated the effect of obesity on bleeding frequency and clotting factor concentrate (CFC) usage in hemophilia patients. In addition, we assessed whether hemostatic and fibrinolytic changes observed in obesity differ between men with and without hemophilia. The number of bleeds and CFC usage were compared between obese (n = 51) and non-obese (n = 46) hemophilia A patients. Markers of hemostasis and fibrinolysis were compared between hemophilia patients, and gender-, age- and BMI-matched non-hemophilic control subjects (n = 91).

Median number of bleeds/patient-month was comparable between obese and non-obese patients with severe hemophilia (P = 0.791). Obese patients with severe hemophilia used 1.4 times more CFC/patient-month than non-obese patients (P = 0.036). When adjusting for weight this difference disappeared (P = 0.451).

vWF:ag levels, FVIII activity and ETP were higher in obese than in non-obese control subjects. Obesity did not influence these markers in hemophilia patients. PAI-1 levels were higher in obese versus non-obese hemophilia patients (P < 0.001), whereas levels were comparable between hemophilia patients and control subjects (P = 0.912). PAP levels appeared to be lower in obese versus non-obese subjects, both within controls (P = 0.011) and hemophilia patients (P = 0.008). However, in hemophilia patients, PAP levels were higher than in controls (P < 0.001).

Obesity is associated with an increase in net CFC usage in hemophilia patients, but has no effect on bleeding frequency. In addition, we found that obesity attenuates hyperfibrinolysis in hemophilia patients. Future research investigating whether obese hemophilia patients need CFC treatment dosed on weight or whether a lower dosage would suffice to prevent and treat bleedings is needed.

Introduction

The prevalence of overweight (body mass index (BMI) 25 – 30 kg/m^2) and obesity (BMI ≥ 30 kg/m^2) in Western countries has increased over the last decades[1]. In 1980, 15% of the adult population in the USA had a BMI ≥ 30 kg/m^2, increasing to 34% in 2008[2]. Obesity is also an increasing health problem in hemophilia patients. The prevalence of overweight and obesity in adult hemophilia patients living in Mississippi, USA, appeared to be 32 and 36%, respectively[3]. Between 1992 and 2001, the prevalence of overweight and obesity in Dutch adult hemophilia patients increased from 27 – 35% and from 4 – 8%, respectively[4]. The prevalence of overweight was lower in hemophilia patients than in the general Dutch male population. However, the prevalence of obesity and the increase in the prevalence of overweight and obesity were comparable between these groups[4].

Obesity is an important risk factor for the development of diabetes mellitus type II, hypertension, cardiovascular disease, malignancies and chronic arthropathy[5]. In hemophilia patients, obesity has been associated with limitations in joint range of motion and with joint mobility loss in lower limbs over time[6,7]. It is not known whether obesity has an influence on bleeding or clotting factor concentrate (CFC) usage in hemophilia patients. Obesity might induce bleeding by increased mechanical stress on the joints. On the other hand, obesity-related hemostatic and fibrinolytic changes, resulting in a prothrombotic state in non-hemophilic subjects[8,9], might also occur in obese hemophilia patients. Consequently, obesity could have a protective effect on bleeding and reduce weight adjusted CFC usage in hemophilia patients.

In this study we evaluated whether obesity has an influence on bleeding frequency and CFC usage in hemophilia patients. In addition, we assessed whether hemostatic and fibrinolytic changes observed in obesity differ between men with and without hemophilia.

Methods

Study design and study population

The current study was embedded in a cross-sectional, multicenter study with the aim to compare the extent of atherosclerosis between men with and without hemophilia. Rationale, study design and selection of the study population have been described in detail previously[10]. Briefly, 51 male patients with severe (FVIII activity < 1%), moderate (FVIII activity 1 – 5%), or mild (FVIII activity 6 – 40%) hemophilia A, and a BMI ≥ 30 kg/m^2 were included. Non-obese (BMI ≤ 25 kg/m^2) hemophilia A patients (n = 47) were matched for age and FVIII activity with obese hemophilia patients. Non-hemophilic control subjects were matched with hemophilia patients for gender, age, and BMI. Forty-two obese and 50 non-obese control subjects were included. Both hemophilia patients and non-hemophilic control subjects were 18 years or older, and excluded when being HIV-positive or having a history of symptomatic cardiovascular disease (i.e. ischemic heart disease, stroke or peripheral arterial disease). Study measurements in hemophilia patients were obtained in 3 study centers: University Hospital, Leuven, Belgium; Academic Medical Center, Amsterdam, the Netherlands; and Van Creveldkliniek, University Medical Center Utrecht, Utrecht, the Netherlands. Control subjects were examined at the Academic Medical Center, Amsterdam, the Netherlands. Subjects were invited to one of the study centers after an overnight fast. Furthermore, hemophilia patients were asked to refrain from clotting factor concentrate infusion 72 hours prior to the visit. All subjects underwent assessment of intima media thickness (IMT) and flow mediated dilatation (FMD), using B-mode ultrasonography. In addition, information on medical history, medication use, and cardiovascular risk factors was collected. In hemophilia patients, information on severity of hemophilia (i.e. residual FVIII activity), type of treatment (prophylactic or on demand), presence of inhibitors, and hepatitis C infection was collected. This study was approved by the Medical Ethics Committees of the 3 study centers. All participating hemophilia patients and control subjects provided written informed consent.

Bleeding frequency and clotting factor concentrate usage

The participating hemophilia treatment centers document bleeding frequency and CFC usage of their patients regularly during control visits. Per patient, the number of registered bleeds and CFC administered within multiple consecutive recent periods were added, as well as the number of months of each included period. The median length of the assembled periods was 24 months, ranging from 7 to 75 months. Then, for each patient, the number of bleeds and CFC usage was calculated per patient-month. To account for the fact that dosage of CFC is based on weight, CFC usage was adjusted for weight (kg). Units of CFC administered because of medical interventions were excluded from the analysis.

Measurement of hemostatic and fibrinolytic parameters

Vena puncture Antecubital vena puncture was performed, after ultrasound measurements, using 23G needles. Blood was collected into Vacutainer® tubes, containing sodium-citrate (3.2%), for analyses of hemostatic and fibrinolytic parameters. Blood was centrifuged 2x 10 min (2000 g, room temperature) to obtain platelet poor plasma (PPP) for soluble platelet activation markers, von Willebrand factor antigen (vWF:ag) and von Willebrand factor (vWF) propeptide measurements. For all other parameters, PPP was obtained by a single centrifugation step (10 min, 2000 g, room temperature). Plasma samples were stored at -80 °C until use.

Assays Platelets release their granule contents as soluble proteins into the circulation upon activation. The most abundant chemokines in the α-granules are β-thromboglobulin (β-TG) and platelet factor 4 (PF4)[11]. A raise in vWF plasma concentration (vWF:ag) accompanied by a raise in vWF propeptide reflects acute endothelial cell activation[12]. At the University Medical Center Utrecht (Utrecht, the Netherlands), plasma levels of soluble platelet activation markers β-TG and PF4, and of vWF propeptide were measured by semi-automated enzyme-linked immunosorbent assay (ELISA) on a TECAN Freedom Evo robot (Tecan, Mannedorf, Switzerland) as described previously with some minor modifications[13,14]. The other parameters were measured at the Academic Medical Center, Amsterdam, the Netherlands. vWF:ag was measured using a home-made ELISA with

antibodies from DAKO (Glostrup, Denmark). FVIII activity was measured using a one-stage clotting assay with FVIII-deficient plasma (Siemens Healthcare Diagnostics, Marburg, Germany). Prothrombin fragment 1 and 2 (F1+2), a marker of thrombogenesis in vivo, was measured using a specific commercially available ELISA according to the instructions of the manufacturer (Enzygnost F1+2, Siemens Healthcare Diagnostics, Marburg, Germany). The endogenous thrombin potential (ETP), indicating the potential to generate thrombin under standardized conditions, was assessed as a measure of overall in vitro thrombin generation, and was determined with a Calibrated Automated Thrombogram (CAT). The CAT assays the generation of thrombin in clotting plasma using a microtiter plate reading fluorometer (Fluoroskan Ascent, ThermoLab Systems, Helsinki, Finland) in combination with Thrombinoscope software (Thrombinoscope BV, Maastricht, the Netherlands). The assay was carried out as described previously[15]. Levels of plasminogen activator inhibitor type 1 (PAI-1), plasmin-α2-antiplasmin (PAP) complexes, and D-dimer were assessed to explore fibrinolytic activity. PAI-1 and PAP levels were measured using specific commercially available ELISAs according to the instructions of the manufacturer (PAI-1, Hyphen, BioMed, Andrésy, France; PAP complexes, DRG Diagnostics GmbH, Marburg, Germany). D-dimer levels were determined with a particle-enhanced immunoturbidimetric assay (Innovance D-dimer, Siemens Healthcare Diagnostics, Marburg, Germany).

Statistical analyses

One-way ANOVA was used to compare age between 4 groups (obese hemophilia patients; non-obese hemophilia patients; obese control subjects; non-obese control subjects). BMI was compared between obese hemophilia patients and obese control subjects, and between non-obese hemophilia patients and non-obese control subjects, using independent t-tests. Hemophilia characteristics (categorical variables) were compared between obese and non-obese hemophilia patients using chi-square tests. Results were tested against an α of 0.05.

Median number of bleeds per patient-month and median CFC usage (prophylaxis, on demand and total) per patient-month (unadjusted and adjusted for weight (kg)) were

compared between obese and non-obese hemophilia patients stratified for hemophilia severity. Results from Mann-Whitney U tests were tested against an α of 0.05.

Data distribution of continuous values of hemostatic and fibrinolytic markers, within the 4 groups described above was assessed by use of histograms and the Kolmogorov-Smirnov test. When data were normally distributed and met the homogeneity of variances assumption (Levene statistic), specific pairwise post-hoc comparisons using the independent t-test were performed. Pairwise post-hoc comparisons included: obese hemophilia patients versus (vs.) non-obese hemophilia patients; obese control subjects vs. non-obese control subjects; obese hemophilia patients vs. obese control subjects; and non-obese hemophilia patients vs. non-obese control subjects. If data were skewed and/or did not meet the homogeneity of variances assumption, the same pairwise post-hoc comparisons, using the Mann-Whitney U test, were applied as described above. When combining obese and non-obese hemophilia patients, and obese and non-obese control subjects, differences in levels of variables between hemophilia patients and control subjects were analyzed, using the independent t-test or Mann-Whitney U test when appropriate. This also accounts for the comparison of all obese vs. all non-obese study participants. The Bonferroni correction was used to adjust for multiple hypotheses testing by dividing an α of 0.05 by the 6 comparisons performed. Thus, results from the 4 post-hoc comparisons and from the comparison between all hemophilia patients and all controls, and between all obese subjects and all non-obese subjects, were tested against an α of 0.008. This is quite a conservative method. However, because of the explorative nature of this study, we used the obtained α of 0.008 more as a guideline than as a strict cut-off for significance.

All analyses were performed using SPSS 16.0 for Windows (SPSS, Inc, Chicago, IL, USA).

Results

Blood could not be collected from 1 non-obese hemophilia patient, because he infused CFC a few hours before the study center visit. In 1 obese control subject, vena puncture failed. These persons were, therefore, left out of the current analyses. This resulted in the following group sizes: 51 obese hemophilia patients, 46 non-obese hemophilia patients, 41 obese control subjects, and 50 non-obese control subjects.

Characteristics of the study population are described in Table 1. Mean age of the study population was 50 years (standard deviation 13). Age ranged from 20 – 76 years in hemophilia patients, and from 20 – 73 years in control subjects. Obese men with and without hemophilia, and non-obese men with and without hemophilia were well matched for BMI. Thirty-two (33.0%) hemophilia patients had a clotting factor activity of < 1%. The distribution of hemophilia severity (P = 0.975) and of patients receiving prophylactic treatment (P = 0.413) was comparable between obese and non-obese groups. FVIII inhibitors were not present in any of the patients. Twenty-four (24.7%) hemophilia patients were currently infected with hepatitis C, whereas 1 (1.1%) non-hemophilic control subject was.

Table 1 Characteristics of the study population

Variable	Obese PWH (n = 51)	Non-obese PWH (n = 46)	Obese controls (n = 41)	Non-obese controls (n = 50)
Age (years)	50 ± 14	49 ± 14	51 ± 12	49 ± 14
BMI (kg/m^2)	33.4 ± 3.8	23.2 ± 1.5	33.2 ± 3.3	23.2 ± 1.4
Residual factor VIII activity				
- < 1%	17 (33.3)	15 (32.6)	NA	NA
- 1 – 5%	8 (15.7)	8 (17.4)	NA	NA
- 6 – 40%	26 (51.0)	23 (50.0)	NA	NA
Prophylactic treatment	16 (31.4)	11 (23.9)	NA	NA
Presence of inhibitors	0	0	NA	NA
Hepatitis C infection				
- Current	10 (19.6)	14 (30.4)	1 (2.4)	0
- In the past	13 (25.5)	7 (15.2)	0	0
- Never	28 (54.9)	25 (54.3)	40 (97.6)	50 (100.0)

Values of continuous variables are expressed as mean ± standard deviation. Categorical variables are expressed as numbers and percentages.
BMI, body mass index; PWH, patients with hemophilia; n, sample size; NA, not applicable.

Bleeding frequency and clotting factor concentrate usage

Median number of bleeds per patient-month (Table 2) was comparable between obese and non-obese patients with severe hemophilia (0.62 (interquartile range (IQR) 0.12 – 0.78) vs. 0.50 (IQR 0.06 – 1.17); P = 0.791). No differences in bleeding frequency per patient-month were found between obese and non-obese patients with moderate or mild hemophilia either.

Obese patients with severe hemophilia used 1.4 times more CFC/patient-month than non-obese patients with severe hemophilia (19167 international units (IU) (IQR 10486 – 28535) vs. 13338 IU (IQR 4982 – 16458), respectively; P = 0.036). When adjusting for weight this difference disappeared (176 IU/kg (IQR 100 – 289) vs. 173 IU/kg (65 – 213); P = 0.451) (Table 2). In addition, no differences between obese and non-obese patients with moderate or mild hemophilia in CFC usage per patient-month adjusted for weight were found. The same results were observed when stratifying for prophylactic and on demand use of CFC.

Table 2 Bleeding frequency and clotting factor concentrate usage

Variable	Obese PWH (n = 51)	Non-obese PWH (n = 46)
Number of bleeds per patient-month		
- Severe PWH	0.62 (0.12 – 0.78)	0.50 (0.06 – 1.17)
- Moderate PWH	0.04 (0.01 – 0.06)	0.06 (0.00 – 0.42)
- Mild PWH	0.00 (0.00 – 0.01)	0.00 (0.00 – 0.04)
CFC used/PM (unadjusted) (IU)		
- Severe PWH	19167 (10486 – 28535)	13338 (4982 – 16458)
- Moderate PWH	211 (0 – 411)	333 (0 – 2135)
- Mild PWH	0 (0 – 300)	0 (0 – 160)
CFC used/PM (weight adjusted) (IU/kg)		
- Severe PWH	176 (100 – 289)	173 (65 – 213)
- Moderate PWH	2 (0 – 4)	5 (0 – 27)
- Mild PWH	0 (0 – 3)	0 (0 – 2)

Values are expressed as median and interquartile range.
PWH, patients with hemophilia; CFC, clotting factor concentrate; PM, patient-month; IU, international units; n, sample size.

Hemostatic and fibrinolytic parameters

Primary hemostasis There was a trend towards higher median vWF:ag levels in obese control subjects as compared to non-obese control subjects (124% (IQR 94 – 151) vs. 97% (IQR 79 – 127); P = 0.009) (Table 3 and Fig. 1A). In obese and non-obese hemophilia patients vWF:ag levels were comparable (125% (IQR 98 – 184) vs. 118% (IQR 98 – 150); P = 0.452). In addition, non-obese hemophilia patients seemed to have higher vWF:ag levels as compared to non-obese control subjects (P = 0.010). This difference was not found between obese hemophilia patients and obese control subjects (P = 0.383). Median vWF propeptide levels were not influenced by obesity (P = 0.230) (Table 4). vWF propeptide levels were comparable between obese hemophilia patients and obese control subjects (7.40 nM (IQR 6.20 – 9.40) vs. 6.95 nM (IQR 5.83 – 8.10); P = 0.133), and between non-obese hemophilia patients and non-obese control subjects (7.30 nM (IQR 6.40 – 8.20) vs. 6.55 nM (IQR 5.80 – 7.65); P = 0.066) (Table 3). vWF:ag levels and vWF propeptide levels were highly correlated (Pearson correlation coefficient 0.751; P < 0.001). No differences in platelet count, β-TG and PF4 levels were found between any of the groups (Table 3+4).

Plasmatic coagulation In controls, mean ETP was increased in obese subjects as compared to non-obese subjects (mean difference 238 nM.min (95% CI 117 – 359); P < 0.001). Median FVIII activity seemed higher in obese than in non-obese control subjects (P = 0.009). Obesity did not change median F1+2 levels in control subjects (Table 3 and Fig. 1B+C+D). In hemophilia patients, obesity did not influence levels of FVIII activity, ETP, or F1+2. ETP (mean difference -353 nM.min (95% CI -441 – -265); P < 0.001) and F1+2 levels (120 pmol/L (IQR 87 – 161) vs. 153 pmol/L (IQR 127 – 196); (P < 0.001) were lower in hemophilia patients than in control subjects (Table 4).

Fibrinolysis Higher median PAI-1 levels were observed in obese control subjects than in non-obese control subjects (65 ng/mL (IQR 45 – 94) vs. 34 ng/mL (IQR 25 – 54); P < 0.001) (Table 3 and Fig. 1E). Also in hemophilia patients, obesity increased PAI-1 levels (58 ng/mL (IQR 40 – 90) vs. 36 ng/mL (IQR 22 – 60); P < 0.001). PAI-1 levels were comparable between hemophilia patients and control subjects (P = 0.912) (Table 4). A trend towards

lower PAP levels in obese than in non-obese control subjects was found (248 µg/L (IQR 193 – 315) vs. 283 µg/l (IQR 218 – 443); P = 0.011). PAP levels also appeared to be lower in obese vs. non-obese hemophilia patients (324 µg/L (IQR 260 – 388) vs. 382 µg/l (IQR 311 – 463); P = 0.008) (Table 3 and Fig. 1F). When compared to controls, PAP levels were higher in hemophilia patients (P < 0.001) (Table 4). This was mainly caused by a difference between obese hemophilia patients and obese control subjects (P = 0.001) (Table 3 and Fig. 1F). Median D-dimer levels did not differ between obese and non-obese subjects (P = 0.336), nor between hemophilia patients and control subjects (P = 0.059) (Table 4).

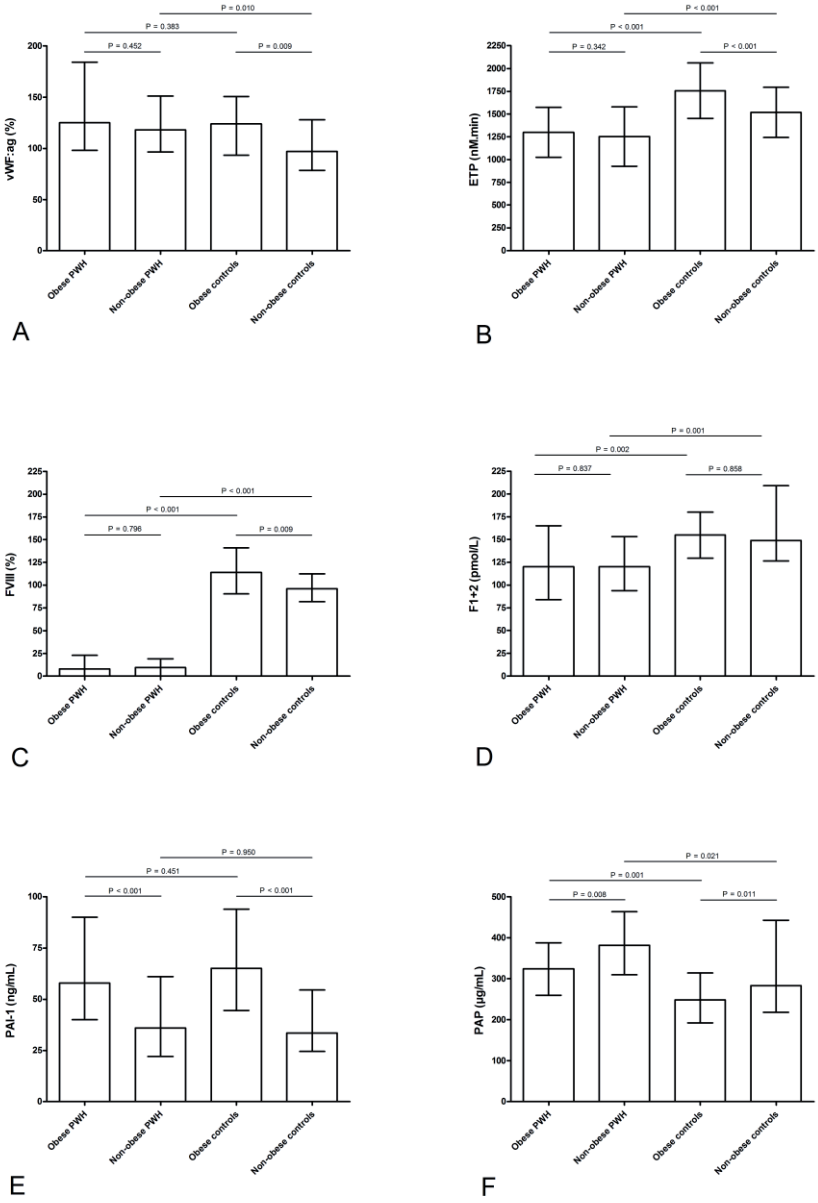

Fig. 1 Hemostatic and fibrinolytic parameters in obese and non-obese hemophilia patients, and in obese and non-obese control subjects

Values are expressed as mean ± standard deviation (B) or median and interquartile range (A, C, D, E, F). vWF:ag, von Willebrand factor antigen; ETP, endogenous thrombin potential; FVIII, factor VIII; F1+2, prothrombin fragment 1 and 2; PAI-1, plasminogen activator inhibitor 1; PAP, plasmin-α2-antiplasmin complex.

Table 3 Hemostatic and fibrinolytic parameters in obese and non-obese hemophilia patients, and in obese and non-obese control subjects

Variable	Obese PWH (n = 51)	Non-obese PWH (n = 46)	Obese controls (n = 41)	Non-obese controls (n = 50)
Primary hemostasis				
- Platelet count (10^9/L)	226 ± 64	235 ± 56	217 ± 52	219 ± 41
- vWF:ag (%)	125 (98 – 184)	118 (98 – 150)	124 (94 – 151)	97 (79 – 127)
- vWF propeptide (nM)	7.40 (6.20 – 9.40)	7.30 (6.40 – 8.20)	6.95 (5.83 – 8.10)	6.55 (5.80 – 7.65)
- β-TG (ng/mL)	17.70 (6.90 – 41.60)	11.30 (7.70 – 20.10)	16.30 (9.90 – 26.88)	13.80 (7.00 – 26.20)
- PF4 (ng/mL)	9.40 (5.20 – 21.20)	6.95 (4.70 – 9.08)	8.70 (5.85 – 14.95)	8.20 (5.50 – 14.00)
Coagulation				
- FVIII activity (%)	8 (0 – 23)	10 (0 – 19)	114 (91 – 141)	96 (82 – 113)
- ETP (nM.min)	1300 ± 276	1241 ± 327	1756 ± 304	1518 ± 276
- F1+2 (pmol/L)	120 (84 – 165)	120 (94 – 152)	155 (129 – 180)	150 (127 – 213)
Fibrinolysis				
- PAI-1 (ng/mL)	58 (40 – 90)	36 (22 – 60)	65 (45 – 94)	34 (25 – 54)
- PAP (μg/L)	324 (260 – 388)	382 (311 – 463)	248 (193 – 315)	283 (218 – 443)
- D-dimer (mg/L)	0.20 (0.17 – 0.28)	0.21 (0.17 – 0.42)	0.27 (0.22 – 0.33)	0.22 (0.17 – 0.30)

Values are expressed as mean ± standard deviation or median and interquartile range.

vWF:ag, von Willebrand factor antigen; vWF, von Willebrand factor; β-TG, beta-thromboglobulin; PF4, platelet factor 4; FVIII, factor VIII; ETP, endogenous thrombin potential; F1+2, fragment 1 and 2; PAI-1, plasminogen activator inhibitor 1; PAP, plasmin-α2-antiplasmin complex; PWH, patients with hemophilia; n, sample size.

Table 4 Hemostatic and fibrinolytic parameters in hemophilia patients and control subjects, and in obese and non-obese subjects

Variable	PWH (n = 97)	Controls (n = 91)	Obese subjects (n = 92)	Non-obese subjects (n = 96)
Primary hemostasis				
- Platelet count (10^9/L)	230 ± 60	218 ± 46	222 ± 59	227 ± 49
- vWF:ag (%)	123 (99 – 158)	108 (83 – 139)	125 (98 – 160)	111 (83 – 141)
- vWF propeptide (nM)	7.40 (6.25 – 8.90)	6.70 (5.80 – 8.03)	7.20 (6.00 – 8.90)	6.90 (6.00 – 8.00)
- β-TG (ng/mL)	11.90 (7.65 – 28.70)	15.30 (7.65 – 26.75)	15.90 (8.50 – 35.60)	12.15 (7.60 – 22.93)
- PF4 (ng/mL)	7.30 (5.05 – 15.30)	8.25 (5.60 – 14.08)	9.25 (5.25 – 18.98)	7.30 (5.20 – 11.85)
Coagulation				
- FVIII activity (%)	9 (0 – 20)	102 (87 – 124)	34 (5 – 104)	55 (10 – 97)
- ETP (nM.min)	1272 ± 301	1625 ± 311	1503 ± 367	1387 ± 330
- F1+2 (pmol/L)	120 (87 – 161)	153 (127 – 196)	145 (106 – 172)	136 (108 – 177)
Fibrinolysis				
- PAI-1 (ng/mL)	49 (29 – 77)	45 (30 – 75)	59 (42 – 93)	35 (23 – 57)
- PAP (μg/L)	343 (281 – 425)	271 (205 – 370)	287 (210 – 348)	354 (268 – 457)
- D-dimer (mg/L)	0.21 (0.17 – 0.30)	0.24 (0.19 – 0.31)	0.23 (0.18 – 0.30)	0.22 (0.17 – 0.32)

Values are expressed as mean ± standard deviation or median and interquartile range.

vWF:ag, von Willebrand factor antigen; vWF, von Willebrand factor; β-TG, beta-thromboglobulin; PF4, platelet factor 4; FVIII, factor VIII; ETP, endogenous thrombin potential; F1+2, fragment 1 and 2; PAI-1, plasminogen activator inhibitor 1; PAP, plasmin-α2-antiplasmin complex; PWH, patients with hemophilia; n, sample size.

Discussion

Obese patients with severe hemophilia used almost 6000 IU of CFC per patient-month more than non-obese patients, whereas bleeding frequency and weight adjusted CFC usage were comparable. In the general population obesity is associated with a prothrombotic state caused by hemostatic and fibrinolytic changes[8,9]. Results from this explorative study indicate that obesity reduces fibrinolysis in both hemophilia patients and control subjects. Several markers of primary hemostasis and plasmatic coagulation were increased in obese control subjects, in contrast to obese hemophilia patients.

We observed a trend towards higher vWF:ag levels in obese control subjects as compared to non-obese control subjects. This is in line with observations from previous studies[16-20]. No obesity related increase in vWF:ag levels was found in hemophilia patients. When comparing non-obese hemophilia patients and control subjects, hemophilia patients seemed to have higher levels of vWF:ag, which is in accordance with a recent paper from Van Bladel et al.[14]. It was hypothesized that a raise in vWF plasma concentration could be a mechanism of the primary hemostatic system to compensate the deficiency in coagulation. The lack of a vWF:ag increase in obese hemophilia patients might be explained by the fact that non-obese hemophilia patients already have elevated levels. vWF propeptide levels were comparable between the different groups. This suggests that the raise in vWF:ag in obese control subjects and the higher levels of vWF:ag in hemophilia patients are not caused by acute endothelial activation.

In men without hemophilia, obesity increased FVIII activity and the potential to generate thrombin under standardized conditions, measured by ETP. This result was expected, since obesity is known to induce a procoagulant state, which involves the increase of tissue factor, fibrinogen, FVII and FVIII[8,9,18-24]. The release of adipokines and cytokines by adipose tissue influences hepatic metabolism and the production of coagulation factors in the liver[8,9]. ETP was not increased in obese hemophilia patients as compared with non-obese hemophilia patients, probably because of an absent or very limited ability to increase FVIII

activity in these patients. In addition, no differences were observed when stratifying for hemophilia severity (results not shown).

PAI-1 levels were increased in obese subjects. Between hemophilia patients and control subjects, no differences in PAI-1 levels were found and the obesity-related increase in PAI-1 levels was comparable. PAI-1 inactivates tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA). As a consequence, less plasminogen is converted into plasmin, thereby reducing fibrinolysis in obese subjects. Although the liver is the major contributor to plasma PAI-1 levels, PAI-1 is also produced by adipocytes[25,26]. In accordance with increased PAI-1 levels, PAP levels were lower in obese subjects as compared to non-obese subjects, both within control subjects and within hemophilia patients. Since, in obesity, the activation of plasminogen into plasmin is reduced by the higher levels of PAI-1, less plasmin is available to bind to antiplasmin.

When comparing PAP levels between hemophilia patients and control subjects, levels were higher in hemophilia patients. Coagulation and fibrinolysis are closely regulated via thrombin activatable fibrinolysis inhibitor (TAFI). After activation, TAFI inhibits fibrinolysis by removing carboxy-terminal lysine residues from fibrin, which function as binding sites for tPA and plasminogen. Activation of TAFI is mediated by relatively high concentrations of thrombin formed via the intrinsic pathway[27-29]. This secondary burst of thrombin formation does not occur in hemophilia patients. Previous studies have shown that premature clot lysis in FVIII deficient plasma was caused by reduced TAFI activation[30,31]. This implies that bleeding in hemophilia patients is not only due to decreased coagulation but also by enhanced fibrinolysis. This might explain the higher PAP levels in hemophilia patients in the current study.

Obesity has been shown to have an adverse influence on the course of pre-existing hemophilic arthropathy[6,7]. Soucie et al.[6] showed a strong association between BMI and limitations in joint range of motion in a cross-sectional study including 4343 hemophilia patients (age 2-19 years). In a consecutive prospective study, Soucie et al[7] showed that

over a 10-year period, joint mobility loss in the lower limbs was faster in obese than in non-obese hemophilia patients. Although obesity attenuates hyperfibrinolysis in hemophilia patients, no effects on bleeding frequency and weight adjusted CFC usage were observed in the current study. This could imply that the adverse influence of obesity on arthropathy is not the result of an increased bleeding frequency. None of the previous mentioned studies investigated CFC usage in obese hemophilia patients[6,7]. Recently, Henrard et al. measured a higher median factor VIII (FVIII) recovery in non-actively bleeding hemophilia A patients with an increased body weight, BMI or fat mass index[32]. It could be that obese hemophilia patients are over-treated with CFC. As the overweight and obese hemophilia population is increasing[4], it would be interesting to investigate whether obese hemophilia patients need CFC treatment dosed on weight or whether a lower dosage, for example adjusted for plasma volume, would suffice to prevent and treat bleedings.

Besides negatively influencing hemophilic arthropathy[6,7], obesity is also an important risk factor of atherosclerosis and cardiovascular disease[5]. The incidence of ischemic cardiovascular disease in hemophilia patients is increasing, as life expectancy now approaches that of the general population[33-35]. Treatment of these patients is complex, due to the delicate equilibrium between bleeding and thrombosis, and evidence-based treatment guidelines are lacking[36]. Taking into account the increasing prevalence and the possible consequences of overweight and obesity in hemophilia patients, (preventive) lifestyle adjustments (e.g. regular exercise and dietary changes) should be recommended in these patients. Results from this study show that the clinical impact of potential beneficial effects of obesity on hyperfibrinolysis are too limited and therefore accessory to the beneficial effects of lifestyle adjustments.

This study was embedded in a study originally designed with the aim to compare the extent of atherosclerosis between men with and without hemophilia[10], which might have had a negative influence on the power of the analyses. Retrospective data on bleeding frequency and CFC usage was collected. CFC usage by hemophilia patients can be

influenced by many factors, such as FVIII half-life, lifestyle, employment and presence of a target joint. The number of bleeds per period was self-reported by the patient. For some patients it can be difficult to distinguish pain caused by arthrosis from pain caused by a bleed. As the patient usually decides if on demand treatment with CFC is necessary, this may lead to unnecessary CFC administration by the patient. In the current study, we did not discriminate between bleeding in weight-bearing or non-weight-bearing joints, muscle bleeds, mucosa bleeds, and other bleeds. In addition, no distinction between traumatic and spontaneous bleeds was made. However, units of CFC administered because of medical interventions were excluded from the analysis.

In conclusion, obesity is associated with an increase in net CFC usage in PWH, but has no effect on bleeding frequency and weight adjusted CFC usage. In addition, we found that obesity attenuates hyperfibrinolysis in hemophilia patients. Future research investigating whether obese PWH need CFC treatment dosed on weight or whether a lower dosage would suffice to prevent and treat bleedings is needed.

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Chapter 9 Microfluidic device with static mixer: new possibilities to study the interplay between platelets and coagulation under conditions of flow

Project in progress

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Summary

The process of hemostasis is dependent on the interplay between platelets, coagulation, the endothelium / subendothelial matrix, and blood rheology. To study hemostasis, the effect of antithrombotic drugs, or thrombotic or bleeding disorders *in vitro*, perfusion chambers have been developed. These devices enable experiments with whole blood, under well controlled conditions of exposure time, thrombogenic surface, and reproducible flow, thereby approaching physiological conditions. Usually, anticoagulated blood is used in these experiments, and, consequently, the influence of thrombin formation on hemostasis is missing. In this chapter, we describe the development of a microfluidic device with static mixer. With this device we can incorporate coagulation in the experimental model, which gives us new possibilities to study the interplay between platelets and coagulation under conditions of flow.

Introduction

The process of hemostasis is dependent on the interplay between platelets, coagulation, the endothelium and subendothelial matrix, and blood rheology. To study hemostasis, the effect of antithrombotic drugs, or thrombotic or bleeding disorders *in vitro* under flow conditions, annular and parallel-plate perfusion chambers have been developed in the late 1960s and early 1980s, respectively[1]. These devices enable experiments with whole blood, under well-controlled conditions of exposure time, thrombogenic surface and reproducible flow, thereby approaching physiological conditions[2]. The set-up of a parallel-plate perfusion experiment is, in general, as follows. A pump aspirates or infuses blood through a channel in the perfusion chamber, which is mounted on a microscope. Platelets adhere to proteins or peptides coated on a coverslip which is attached to the perfusion chamber. Platelet adhesion, spreading and aggregation can be visualized real-time.

The Biorheology Subcommittee of the International Society of Thrombosis and Haemostasis (ISTH) recommends the use of non-anticoagulated blood, preferably with physiological cation concentrations, in the development of new flow assays[3,4]. When blood is drawn from a donor, it is collected in a tube with an anticoagulant buffer, e.g. sodium-citrate. Consequently, the influence of thrombin formation on hemostasis is missing in the model described. Thrombin is a key protein in the coagulation cascade and converts fibrinogen into fibrin, thereby creating a fibrin network which stabilizes platelet aggregates. In addition, thrombin is a potent platelet activator.

Several solutions to incorporate coagulation in the model have been applied. In '*ex vivo* experiments', non-anticoagulated blood from the antecubital vein of a donor was directly perfused through the chamber[5]. However, the experiments were not reproducible, laborious, donor unfriendly, and skilled technicians were needed. Sodium-citrate chelates calcium ions in the blood, which are needed to bind factor II, VII, IX and X, via the Gla-domain, to phospholipids[6]. In principle, by re-adding calcium to the citrated blood, just before the blood flows over the coated coverslip, these clotting factors can be activated and the effects of thrombin formation can be studied. Different methods of restoring calcium concentrations in blood have been described. Adding a calcium solution to a vial

with citrated blood, and gently mixing the fluids by inversion, often caused clotting through contact activation before the blood entered the perfusion chamber[7]. Others described the use of a Y-connection to co-perfuse blood and a calcium solution[8,9]. Some diffusion of the fluids will occur at the interface created, but this is not enough to reach an even distribution of the calcium ions over the blood. Turbulence, which might cause platelet, pre-activation, is created when a calcium solution is infused in the counter direction of the blood flow.

To incorporate coagulation in the experimental model, we developed a disposable microfluidic device with a static mixer coupled to a parallel-plate perfusion channel. Static mixers are widely used to mix fluids and do not have moving parts. In this chapter, we report the development and characteristics of this microfluidic device. This *in vitro* model creates new possibilities to study the interplay between platelets and coagulation under conditions of flow.

Development and characteristics of the microfluidic device with static mixer

Static mixer

The static mixer incorporated in the microfluidic device is a splitting serpentine channel with the DentIncx geometry, developed by P.E. Neerincx and R.P.J. Denteneer at the Eindhoven University of Technology, the Netherlands (Fig. 1)[10]. Chaotic advection is used, applying the baker's transformation, to enhance mixing in laminar flows, which is efficient in the near zero Reynolds regime. With every cycle, the DentIncx mixer splits, rotates and recombines the flow, thereby increasing the number of layers in the fluids exponentially[10,11]. The cross-sectional dimensions of the static mixer are 1 mm x 1 mm and the static mixer consists of 18 cycles, resulting in 2^{18+1} layers with an individual striation thickness of 0.2 nm. Between the layers diffusion occurs. Performance of the static mixer has been evaluated previously by visualizing the flow inside the channels[12]. Water was infused into the mixer by 2 syringe pumps. Water in 1 of the syringes was mixed with a water soluble fluorescent dye and along the length of the mixer the cross channel

intensity was measured. After 2 cycles of splitting, rotating and recombining an intensity plot with 4 peaks and 4 valleys was found, while the distribution was uniform after 10 cycles[12].

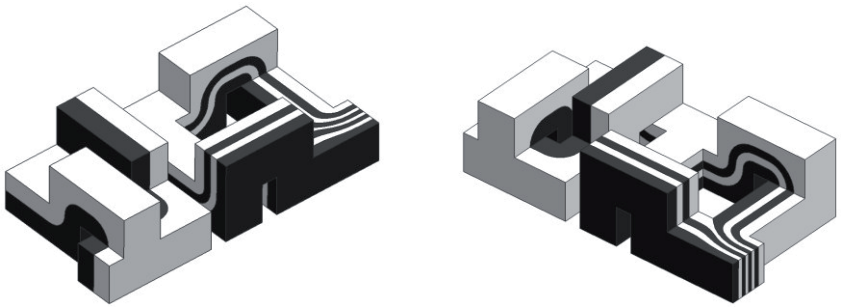


Fig. 1 DentIncx static mixer geometry which is incorporated in the microfluidic device showing fluid flow from left to right. Permission to use this figure was obtained from P.E. Neerincx.

Injection moulding

The microfluidic device is fabricated using 2 component injection moulding technology, which makes combining hard and soft material possible. Polymers are fed into a heated barrel, mixed and forced into a mould where the polymers cool and harden into the configuration of the mould cavity. After development of a mould, this technology allows for cheap mass production of a disposable microfluidic device. The device consists of 2 transparent parts. In both parts, hard polycarbonate (PC; Calibre 303 EP) is used for the casing and soft thermoplastic polyurethane (TPU; Desmopan 9385) is used to ensure optimal connection. A PC bottom plate (Fig. 2a) is connected thermally, during the 2 component injection moulding process, with a sealing TPU plate which contains half of the DentIncx mixer, the outlet and the vacuum channel (Fig. 2b). Next a PC top plate, with the upper half of the static mixer (Fig. 2c), is placed upside-down on the lower part (Fig 2d). The PC top plate is thermally connected with a TPU plate in which the parallel-plate

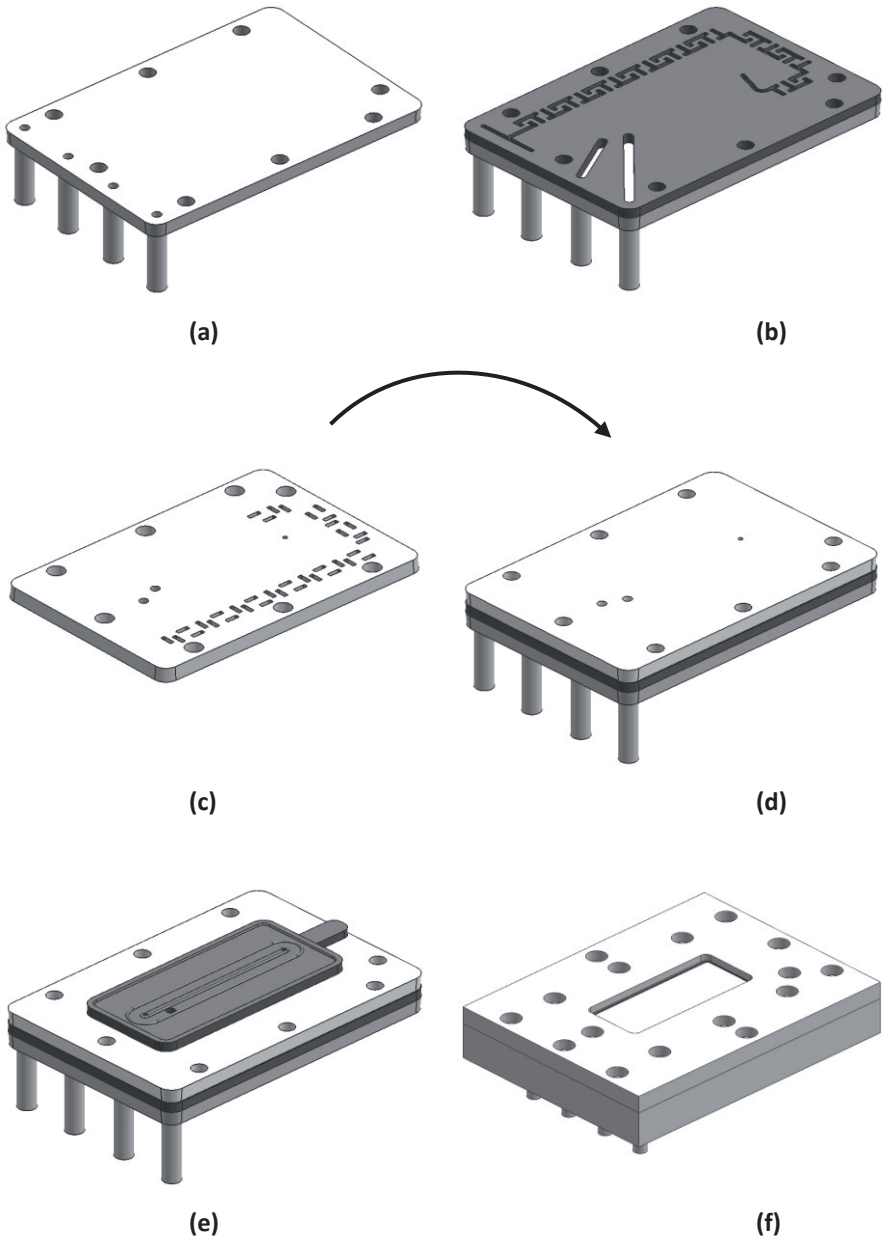


Fig. 2 Parts used to assemble the microfluidic device with static mixer. A PC bottom plate (a) is connected with a sealing TPU plate which contains half of the static mixer, the outlet and the vacuum channel (b). A PC top plate, with the upper half of the static mixer (c), is place upside-down on the lower part (d). The PC top plate is connected with a TPU plate in

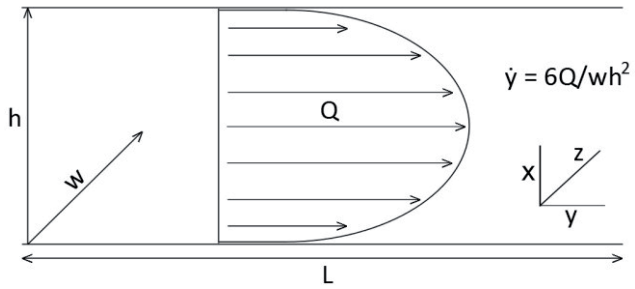
which the parallel-plate perfusion channel and the surrounding vacuum channel are embedded (e). The lower and upper parts are pressed together, and the static mixer and parallel-plate perfusion channel are sealed by an aluminum frame (f). Permission to use this figure was obtained from P.E. Neerincx.

perfusion channel and surrounding vacuum channel are embedded, and on which a coverslip can be placed. The coverslip is sealed to the device using a vacuum force (Fig 2e). The lower and upper parts are pressed together, and the static mixer and parallel-plate perfusion channel are sealed by an aluminium frame containing a window (Fig. 2f). The choice of materials was influenced by the need for good mutual adhesion, transparency and minimization of the possibility of contact activation. PC and TPU were chosen for their inert characteristics, blood compatibility and their frequent use in medical applications[13].

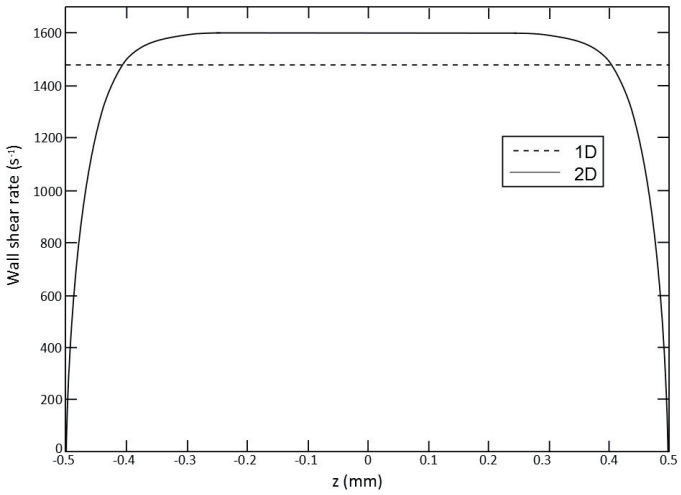
Rheology parallel-plate perfusion channel

In a parallel-plate perfusion channel flow is assumed to be laminar and to have a parabolic velocity profile with the highest velocity at the centerline (Fig. 3a)[14,15]. However, the flow profile is influenced by the dimensions of the parallel-plate perfusion channel. Between 2 fixed, infinitely wide parallel plates separated by a distance (h), the flow profile, in case of a fully developed flow, varies only in 1 dimension (1-dimensional flow profile). The wall shear rate is constant along the width of both surfaces. In this situation the aspect ratio (height:width) is <<1. However, this is not the case for the parallel-plate perfusion channel in the microfluidic device with static mixer, in which the height is 0.125 mm and the width 1 mm, resulting in an aspect ratio of 1:8. Due to the additional boundaries (i.e. the vertical walls) the flow profile is no longer parabolic (2-dimensional flow profile). Besides the horizontal walls, the zero velocity condition at the vertical walls slightly increases the velocity at the center of the perfusion channel. Hence, if the width is much larger than the height, the wall shear rate ($\dot{y}$; expressed as s^{-1}) equals $6Q/wh^2$ in channels with a rectangular cross-section. In this equation Q represents the volumetric flow rate (ml/s), w the width (cm) of the channel, and h the height (cm) of the channel[14,15]. With an aspect ratio of 1:8 the error in wall shear rate is 8.5% (Fig. 3b)[16]. When calculating the volumetric flow rate needed to obtain the desired wall shear rate we accounted for this error. With a

low aspect ratio, the impact of the vertical walls on the overall velocity profile is small. Consequently, the shear rate at the horizontal walls is constant across a large part of the channel width where the flow can be assumed 1-dimensional (Fig. 3b). This, in combination with the fact that platelets near the vertical walls are subjected to shear forces in 2 directions, should be taken into account when choosing a field of view. The wall shear stress (τ) is obtained by multiplying the wall shear rate by the viscosity (η) of the fluid. We should keep in mind that this is a simplified model, as these principles apply to Newtonian fluids. Blood is a non-Newtonian fluid; viscosity of blood varies with local shear rates. In addition, erythrocytes tend to move slightly move away from the walls, resulting in a layer of plasma and platelets near the wall (cell free layer). This leads to blunting of the flow profile.



(a)



(b)

Fig. 3 Parabolic velocity profile (ẏ = wall shear rate (s^{-1}); Q = flow rate (ml/s); w = width (cm); h = height (cm); L = length) (a). Shear rate at horizontal walls of parallel-plate perfusion channel, assuming a fully developed flow (b).

Experimental set-up

A schematic overview of the experimental set-up is depicted in Fig. 4. The microfluidic device with static mixer is mounted on an inverted microscope with the coverslip facing downwards and the inlets and outlet facing upwards. Silicon tubes (Dow Corning, Midland, MI, USA) are attached to the inlets and outlet via luer lock connectors (Qosina, Edgewood, NY, USA). The silicon tubes from the inlets are connected to 2 3-way stopcocks (Qosina, Edgewood, NY, USA). A syringe with citrated blood is connected to the first 3-way stopcock (inner inlet), and a syringe with a calcium solution (10 mM HEPES (VWR International, Leuven, Belgium) buffer pH 7.4 containing 16.7% bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO, USA) and 16 mM $CaCl_2$ (Merck, Darmstadt, Germany)) is connected to the second 3-way stopcock (outer inlet). Via silicon tubes the third outlet of both 3-way stopcocks is connected to a Y-connector (Qosina, Edgewood, NY, USA). In addition, an empty syringe is connected to the Y-connector. The vacuum system (-20 kPa) is attached to the vacuum inlet. A silicone tube connected to the outlet of the perfusion channel is lead into a vial with 10 mM HEPES rinsing buffer containing 16.7% BSA. To generate the required volumetric flow rate, a syringe pump (Harvard Apparatus, Holliston, MA, USA) is used. First, flow through the inner inlet and through the syringe with the calcium solution is blocked. The empty syringe is placed in the pump and by pulling the rinsing buffer through the parallel-plate perfusion channel and static mixer (volumetric flow rate 230 µl/min; wall shear rate parallel-plate perfusion channel 1600 s^{-1}), air bubbles are removed and the system is filled with buffer. Then, the 2 other syringes are placed in the pump and flow from those syringes via the inlets is enabled. Citrated blood and the calcium solution are pushed through the static mixer and parallel-plate perfusion channel. At a flow rate of 115 µl/min per syringe, it takes about 1.30 min for a unit of fluid to flow from inlet to outlet. After the experiment, the aluminum frame is removed and the microfluidic device is disposed.

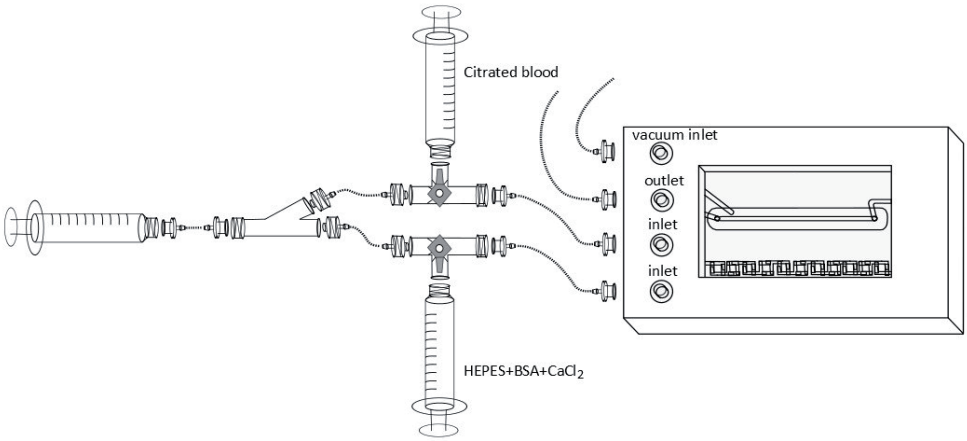


Fig. 4 Schematic overview of the experimental set-up. Permission to use this figure was obtained from N. Vervoort.

Platelet aggregation and fibrin formation under flow

Glass coverslips (0.16 mm x 25 mm x 50 mm; Menzel-Gläser, Braunschweig, Germany) were coated with equine fibrillar type I Horm collagen (60 µl of 100 µg/ml; Nycomed, Linz, Austria) for 90 min at room temperature and subsequently blocked overnight with 10 mM HEPES buffer containing 1% BSA at 4 °C. Blood was collected from healthy donors from the antecubital vein through 20-Gauge needles into vacuum tubes containing 0.105 M trisodium citrate (1/10 vol; BD Diagnostics). As the citrated blood and calcium solution (10 mM HEPES buffer pH 7.4 containing 16.7% BSA and 16 mM $CaCl_2$) were infused at equal volumetric flow rates, one volume of citrated blood is mixed with 1 volume of calcium solution, resulting in a mixed sample with a free Ca^{2+} concentration of approximately 4.5 mM. Wall shear rates of 1600 s^{-1} or 500 s^{-1} were obtained during 15 min. Afterwards, rinsing buffer was perfused through the microfluidic device. Alexa Fluor 488-labeled fibrinogen (6.2 µg/ml; Molecular Probes, Eugene, OR, USA) was added to the citrated blood before perfusion in several experiments. Platelet aggregation and fibrin formation were continuously monitored using differential interference contrast (DIC) or fluorescent microscopy with a Carl Zeiss AxioObserver Z1. A Carl Zeiss EC Plan-NEOFLUAR 40x/0.75

objective was used. Images (400x magnification; 1 frame/5 sec) were recorded with an AxioCam MRm camera using Carl Zeiss AxioVision imaging software (Carl Zeiss MicroImaging GmbH, Gottingen, Germany).

Results

Fig. 5a shows platelet aggregates after 15 min perfusion of citrated blood at a shear rate of 1600 s^{-1}. The appearance of platelet aggregates changes when citrated blood and a calcium solution are perfused through the microfluidic device with static mixer at a high shear rate (Fig. 5b). The aggregates are more dense and homogenous, and a fibrin network can be distinguished with entrapment of erythrocytes. Fibrin deposition is known to increase with decreasing shear rates, as can be observed in Fig. 5c (wall shear rate 500 s^{-1}). After addition of fluorescently labeled fibrinogen, an extensive fibrin network is visualized (Fig. 5d). The development of the fibrin network starts at the platelet aggregates between 5 and 7.30 min after start of the perfusion experiment (Fig. 6a-f). During the 15 min lasting experiments, flow was not obstructed by extensive fibrin clot formation anywhere in the system.

In the process of testing the microfluidic device with static mixer we observed poor mixing when using citrated blood and 10 mM HEPES buffer containing 16 mM $CaCl_2$, showing a separation of the 2 fluids at the inlet of the parallel-plate perfusion channel, which persisted throughout the length of the perfusion channel (Fig. 7a). The origin of this problem was detected at the first mixing element (Fig. 7b-e), where an unusual flow was seen with no vertical interface between the citrated blood and the calcium solution. In Fig. 7b the microfluidic device is oriented with the inlets and outlet facing upwards. The citrated blood chooses to flow via the channel which makes a turn downwards instead of the straight channel, even when the citrated blood is connected to the other inlet (Fig. 7c). When turning the microfluidic device upside down, the straight channel is preferred by the blood flow instead of the other channel which now makes a turn upwards (Fig. 7d). A

detailed schematic overview of the static mixer and the preference route of blood are shown in Fig. 7e. Density differences between the citrated blood and the calcium solution appeared to be the cause of this effect. The density of citrated blood was 1049 kg/m^3, and after adjusting the density of the calcium solution using BSA, proper mixing was obtained as is shown in Fig. 8a, where blood flows into both splitting channels. Fig. 8 b shows the layer development in the first 10 cycles of splitting, rotating and recombining, which is comparable with the intended layer development in the previous described static mixer performance experiment[12]. At the inlet of the static mixer a clear interface can be seen, and after 10 cycles the layers are no longer visible. In addition, a homogeneous flow in the parallel-plate perfusion channel was observed.

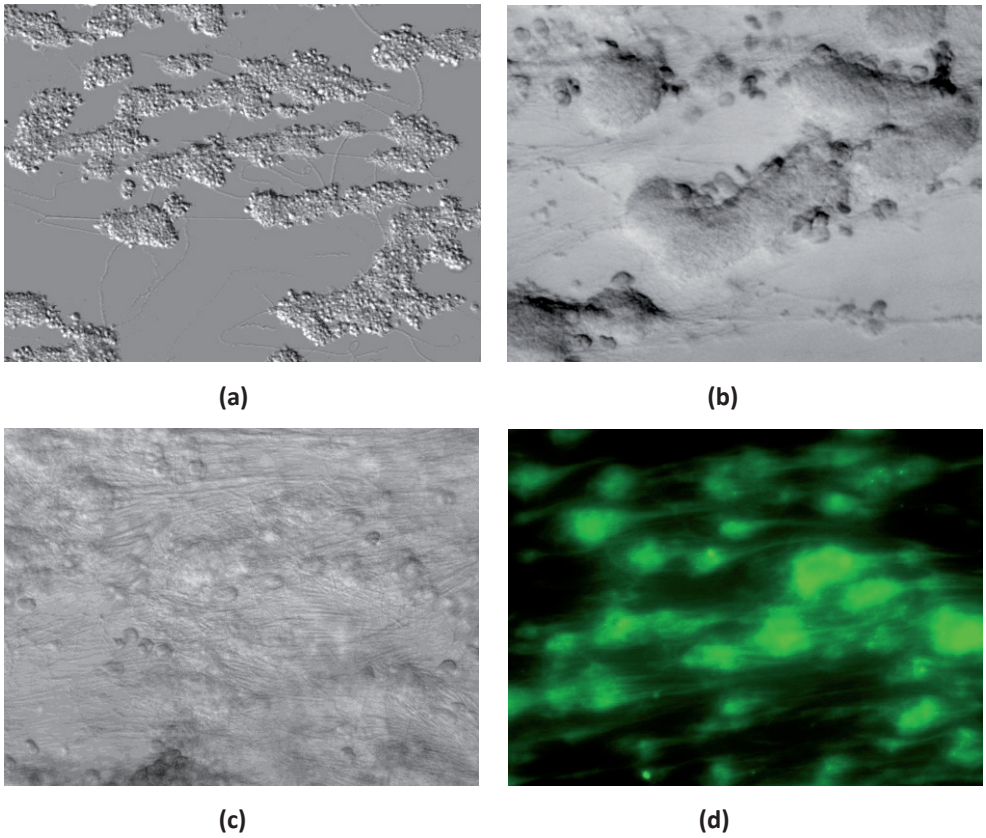


Fig. 5 Platelet aggregates after 15 min perfusion of citrated blood at a shear rate of 1600 s^{-1} (a). Platelet aggregates and fibrin network after 15 min perfusion of citrated blood mixed with a calcium solution at a shear rate of 1600 s^{-1} (b); at a shear rate of 500 s^{-1} (c); at a shear rate of 500 s^{-1} in the presence of Alexa Fluor 488-labeled fibrinogen (d).

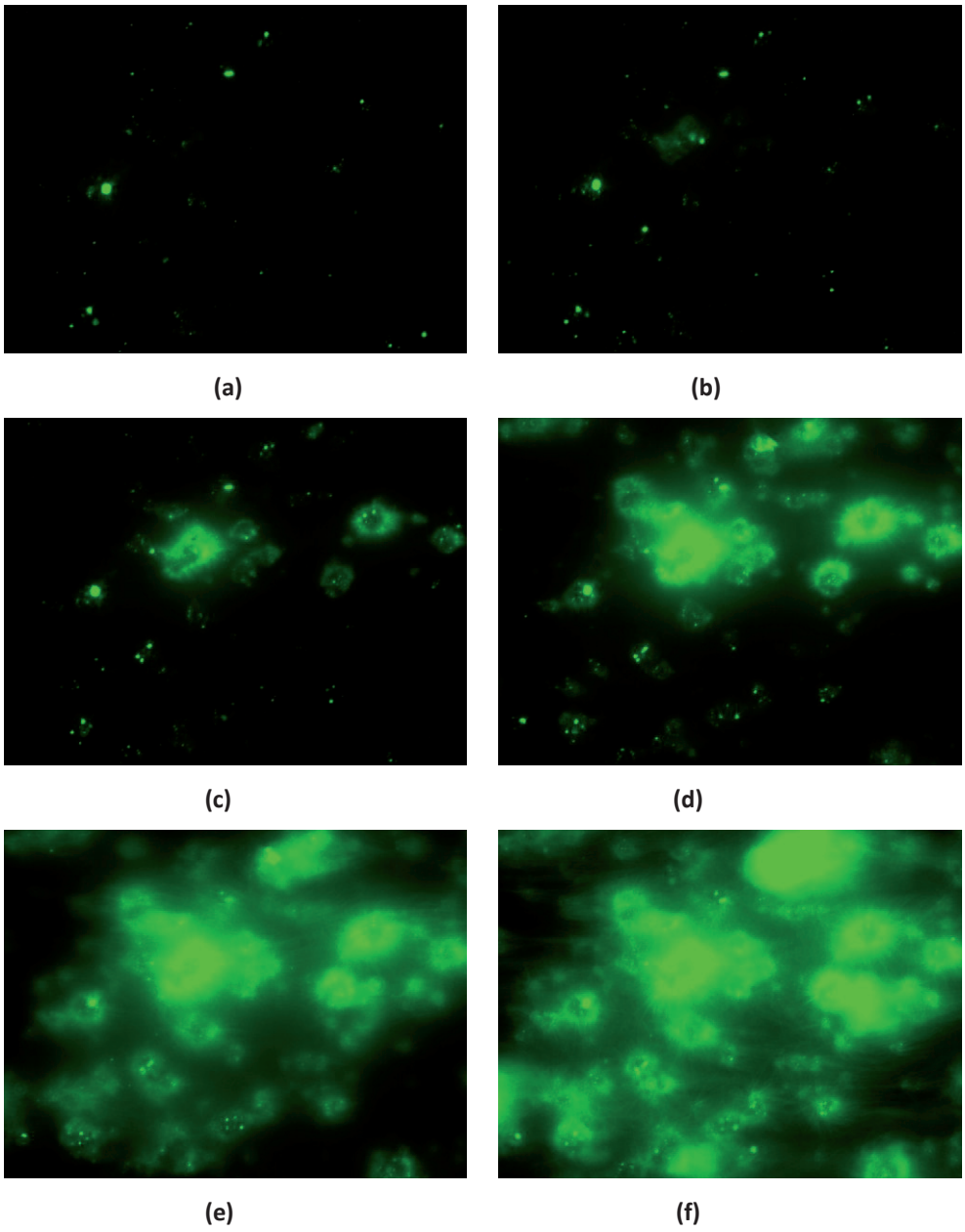
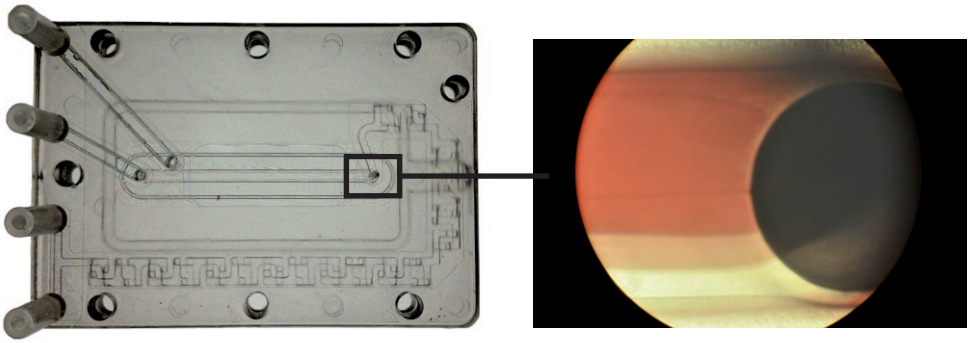


Fig. 6 Development of fibrin network during perfusion of citrated blood mixed with a calcium solution at a shear rate of 500 s^{-1} in the presence of Alexa Fluor 488-labeled fibrinogen. Fibrin network after 2.30 min (a); after 5 min (b); after 7.30 min (c); after 10 min (d); after 12.30 min (e); and after 15 min (f).



(a)

(b)

(c)

(d)

(e)

Fig. 7 Poor performance in mixing. Separation of citrated blood and HEPES 10 mM, pH 7.4 + 16 mM $CaCl_2$ in the perfusion channel (a). Citrated blood appears to have a preference route as is shown in the first mixing element; microfluidic device oriented with inlets and outlet facing upward, citrated blood connected to outer inlet (b) and to inner inlet (c); microfluidic device turned upside down (d). A schematic representation of the preference route of citrated blood (e). Permission to use figure 5e was obtained from A.D. Barendrecht.

(a)

(b)

Fig. 8 Improved mixing by adding 16.7% BSA to the calcium solution to match citrated blood density. Flow in first mixing element over time; blood connected to inner inlet (a). Layer development throughout the first 10 cylces of splitting, rotating and recombining of the static mixer (b).

Discussion

Using a static mixer we recalcified citrated blood, which was then perfused over a collagen coated surface. Subsequently, the development of platelet aggregates and a fibrin network could be visualized. We demonstrated that, in principle, the microfluidic device with static mixer does what it is designed for; reproducible and thorough mixing is obtained and coagulation is incorporated in the perfusion model. However, before using the microfluidic device with static mixer in biomedical research, the model has to be further characterized and validated.

First, to assess the extent of contact activation caused by the materials used in the microfluidic device with static mixer, we will co-perfuse blood, anti-coagulated with trisodium citrate, and a $CaCl_2$ solution over an albumin coated coverslip. Markers of coagulant activity, such as thrombin-anti-thrombin (TAT) complexes, fragment 1 + 2 (F1+2) and fibrinopeptide A (FPA), will be measured in blood before and after flowing through the device. If coagulant activity increases after perfusion, the experiment will be repeated in the presence of corn trypsin inhibitor (CTI) or active-site inhibited factor VIIa (FVIIai) to differentiate between activation via the intrinsic or extrinsic pathway. If necessary, we could investigate the use of anti-biofouling coatings in the microfluidic device with static mixer, to reduce interactions between the material and plasma proteins. Second, the extent of platelet activation caused by the perfusion of blood through the microfluidic device with static mixer, will be studied by assessing platelet P-selectin expression using flow cytometry. In addition, free hemoglobin, a marker of hemolysis, will be measured using spectrophotometry. Then, platelet aggregation and fibrin formation need to be quantified to be able to compare between experiments. After perfusion, samples will be fixed and end point measurements will be performed by confocal microscopy. Finally, reproducibility of experiments needs to be assessed. Blood from healthy donors will be used in multiple experiments performed on different days and results will be compared within donors and between donors. To investigate whether the experimental model can differentiate between normal and defective coagulation, fibrin formation in blood from healthy donors and patients with hemophilia of different severities will be compared. Citrate, as a chelator of divalent cations, lowers both Ca^{2+} and Mg^{2+} concentrations[17]. Mg^{2+} plays a role in factor IX activation[18] and platelet integrin, especially α2β1, function[19]. So, in future experiments we will substitute Mg^{2+} as well. Currently, we mix equal volumes of citrated blood and a calcium solution, thereby changing the flow characteristics of blood and diluting plasma components. We will investigate whether we can increase the citrated blood volume in comparison with calcium solution volume, without losing mixing capabilities.

In vivo, the major trigger of coagulation is tissue factor (TF), which is exposed to the flowing blood upon endothelial injury. In the experiments described in this chapter, no tissue factor (TF) was added to the citrated blood nor was TF immobilized on the coverslips. There are several hypotheses on how coagulation could have been activated in the recalcified citrated blood flowing over collagen coated coverslips. Coagulation might be stimulated by the accumulation of leukocyte derived TF-bearing microparticles into the developing aggregates via an interaction between microparticle P-selectin glycoprotein ligand 1 (PSGL-1) and platelet P-selectin[20]. Incorporation of monocytes expressing TF into the developing thrombus might be an additional source of TF[21]. Another possibility could be that factor XII is activated, initiating fibrin formation, via the plasma contact system upon contact with polyphosphates released from dense granules of platelets[22,23]. Other studies showed that, under conditions where TF is limited, collagen triggers the intrinsic coagulation pathway via activation of factor XII, and thus subsequent factor XI activation, in addition to its role in platelet recruitment and activation[9,24,25]. To distinguish between TF and factor XII initiated fibrin formation, we will perform perfusion experiments over collagen in the presence of FVIIai or CTI. Blocking antibodies will be used to investigate whether the P-selectin-PSGL-1 interaction is necessary for fibrin formation. Phosphatase, which hydrolyzes polyphosphates, will be used to study polyphosphate depend FXII activation.

Increasingly, investigators have turned to animal models of thrombosis and hemostasis, especially in mice[26]. *In vivo*, all the components of the hemostatic process are present. As the microfluidic device with static mixer selectively combines these components *in vitro*, the amount of animals needed in thrombosis and hemostasis research could be reduced. Other advantages are that, human blood can be used, and components of the hemostatic process can be varied relatively easy. A disadvantage of the microfluidic device with static mixer is that relatively large amounts of blood (± 2 ml for an experiment lasting 15 min with a wall shear rate of 1600 s^{-1} in the parallel-plate perfusion channel) are needed, making it impossible to use blood from smaller animals. Hopefully, we can reduce the size of the device in the future.

In future biomedical research, the microfluidic device with static mixer can be used to further investigate the interplay between platelets and coagulation. Current tests use either platelet poor plasma for coagulation assays, or platelets devoid of plasmatic components. The relatively unexplored field of research in which the effects of platelet activation and aggregation on coagulation and vice versa is investigated, can be further studied using, next to animal models, the microfluidic device with static mixer. In addition, current tests often do not show abnormalities in patients with a bleeding tendency, and prediction of severity of a bleeding tendency is difficult. Combining platelet aggregation and coagulation, with flow and thrombogenic surfaces, selected depending on the diagnostic or research question might help us unravel more of the underlying problems in these patients. Another interesting field of research, in which the microfluidic device with static mixer might prove its value, is that of drugs influencing hemostasis. For example, the effects of new antithrombotic medication, such as direct thrombin inhibitors and factor Xa inhibitors, on platelet activation / aggregation and fibrin formation can be investigated.

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Chapter 10 Summary and general discussion

Summary

Since the introduction of clotting factor concentrates, life expectancy of hemophilia patients has increased and now approaches that of the general male population[1,2]. Consequently, aging hemophilia patients are increasingly confronted with age-related comorbidities, including ischemic cardiovascular disease (CVD)[3,4]. Interestingly, cohort studies reported a reduced mortality due to ischemic heart disease (IHD) in hemophilia patients as compared with the general population[1,2]. Differences in cardiovascular risk factor prevalence between men with and without hemophilia, a protective effect of factor VIII (FVIII) or IX (FIX) deficiency on thrombus formation or on the development of atherosclerosis could be possible explanations for this observation.

Chapter 2 provides an overview of the literature, published until July 2008, on ischemic CVD, cardiovascular risk factors, thrombosis and atherosclerosis in hemophilia patients. Cohort studies investigating IHD mortality reported a standard mortality ratio (observed deaths in patients divided by expected deaths in general male population) of 0.20 – 0.62[1,2,5-7]. The prevalence of hypertension appeared to be higher and cholesterol levels were lower in hemophilia patients than in the general population[8,9]. However, differences in the prevalence of cardiovascular risk factors could not account for the lower IHD mortality in hemophilia patients[8]. *In vitro* studies and animal models supported the explanation that the hypocoagulable state of hemophilia patients could have a protective effect on thrombus formation, which precipitates myocardial infarction[10,11]. Apolipoprotein E and FVIII deficient mice developed fewer early-stage atherosclerotic lesions as compared to apolipoprotein E deficient mice with normal FVIII levels[12]. However, studies comparing intima-media thickness (IMT) between hemophilia patients and non-hemophilic controls reported conflicting results[13-17]. Knowledge about the role of FVIII or FIX (deficiency) in arterial thrombosis and atherogenesis could help us understand these processes in more detail, and may lead to future prevention or treatment strategies of CVD in patients with hemophilia.

In **chapter 3**, we focus on the hypothesis that hemophilia patients develop less atherosclerosis than men without hemophilia. The amount of coronary artery calcification (CAC), measured using multi detector-row computed tomography, was compared between 42 men, ≥ 59 years, with moderate or severe hemophilia A, and 613 non-hemophilic age-matched males from the Rotterdam study, a prospective population-based study. The amount of CAC, expressed as the Agatston score and calcification mass, is known to be correlated to the extent of underlying atherosclerotic plaque burden[18,19]. Univariate linear regression showed no association between the natural-log transformed Agatston score or calcification mass and hemophila. Results did not change after adjustment for age, body mass index (BMI), hypercholesterolemia, hypertension, and use of antidiabetic medication. In conclusion, no evidence for a protective effect of congenital FVIII deficiency on the development of coronary artery atherosclerosis was found. Results from this study underline the importance of screening and treating atherosclerosis risk factors in hemophilia patients.

The conclusion obtained in chapter 3 is confirmed in **chapter 4**, in which the extent of early atherosclerosis and endothelial dysfunction were compared between 51 obese (BMI ≥ 30 kg/m^2) and 47 non-obese (BMI ≤ 25 kg/m^2) hemophilia A patients, and 92 gender-, age-, and BMI-matched non-hemophilic control subjects. Mean carotid and femoral IMT was comparable between obese men with and without hemophilia, and between non-obese men with and without hemophilia. In addition, mean brachial flow-mediated dilatation and the prevalence of atherosclerotic plaques in the carotid arteries was comparable between obese hemophilia patients and obese control subjects.

Also in patients with von Willebrand disease (vWD), the prevalence of ischemic CVD has been shown to be lower as compared to the general population[20]. In **chapter 5**, the literature, published until July 2011, on vWF deficiency and atherosclerosis is summarized. Results from pig studies suggested that vWF deficiency is protective against the development of atherosclerosis at pig aorta branch point predilection sites, but not in pig coronary arteries. Studies in other species indicated that vWF deficiency might be

protective against early atherogenesis, probably via a disturbed interaction between the vessel wall and platelets. The few human studies published are inconclusive, and an unequivocal protective effect of vWD on atherosclerosis has not been demonstrated. In conclusion, the potential role of vWF as a significant player in the multifaceted interaction between the hemostatic system and the atherosclerotic process deserves further study.

Although IHD mortality is lower in hemophilia patients as compared to the general male population[1,2], an increase in the incidence and prevalence of IHD is observed in the elderly hemophilia population[3,4]. Treatment of IHD in hemophilia patients is complex, because of the delicate equilibrium between bleeding and thrombosis. As evidence-based guidelines on how to treat IHD in hemophilia patients are lacking, an institutional guideline, based on both experience from hemophilia specialists and existing guidelines on treatment of IHD in non-hemophilic patients, was developed in 2009 and is presented in **chapter 6**. Briefly, indications for cardiac catheterization or intervention in hemophilia patients should be the same as in non-hemophilic patients, and radial artery access is preferred over femoral artery access. Before catheterization and start of antithrombotic treatment the clotting factor deficiency should be corrected. When stent placement is indicated, we recommend a bare-metal stent and dual antiplatelet treatment for 4 weeks, during which clotting factor trough levels of 0.30 IU/ml should be pursued. Long-term low dose aspirin treatment is given to patients with mild or moderate hemophilia, and to patients with severe hemophilia on clotting factor prophylaxis. The case series of hemophilia patients who underwent cardiac catheterization or intervention, which is described in **chapter 7**, indicates that treatment according to the 2009 guideline is feasible and safe. Based on this case series and developments in new guidelines for non-hemophilic patients with IHD, some adjustments on the 2009 guideline were proposed.

Using the same study population as described in chapter 4, the effect of obesity on bleeding frequency and clotting factor concentrate (CFC) usage in hemophilia patients is assessed in **chapter 8**. Obese patients with severe hemophilia used 1.4 times more

CFC/patient-month than non-obese patients, whereas bleeding frequency and weight adjusted CFC usage were comparable.

In addition, we investigated whether hemostatic and fibrinolytic changes observed in obesity differ between non-hemophilic control subjects and hemophilia patients. vWF antigen levels, FVIII activity and endogenous thrombin potential were higher in obese than in non-obese control subjects. Obesity did not influence these markers in hemophilia patients. Plasminogen activator inhibitor type 1 (PAI-1) levels were higher in obese versus non-obese hemophilia patients, whereas levels were comparable between hemophilia patients and control subjects. Plasmin-α2-antiplasmin (PAP) levels appeared to be lower in obese versus non-obese subjects, both within controls and hemophilia patients. However, in hemophilia patients PAP levels were higher than in control subjects.

The prevalence of obesity in patients with hemophilia is increasing[21], and obesity has been shown to have an adverse influence on the course of pre-existing hemophilic arthropathy[22,23]. Although obesity attenuates hyperfibrinolysis in hemophilia patients, no effects on bleeding frequency and weight adjusted CFC usage were observed in the current study. It could be that obese hemophilia patients are over-treated with CFC. As the overweight and obese hemophilia population is increasing, it would be interesting to investigate whether a lower dosage, for example adjusted for plasma volume, would suffice to prevent and treat bleedings.

The effects of antithrombotic therapy in hemophilia patients with IHD on the hemostasis integral[24], described in the introduction of this thesis, are not known. A test of hemostasis in whole blood, which integrates the effects of platelets, coagulation, the vessel wall and blood rheology, does not yet exist. In **chapter 9**, the development and characteristics of a global hemostasis test, which integrates the different determinants of hemostasis, is described. In this disposable microfluidic device with static mixer, sodium-citrate anticoagulated blood and a calcium solution enter the static mixer and pass a certain repeating geometry by flowing through it. Then, the recalcified blood is lead to a parallel-plate perfusion channel and flows over a thrombogenic surface. We were able to visualize platelet aggregation and fibrin formation. This device creates new possibilities to study the

interplay between thrombogenic surfaces, platelets and coagulation under conditions of flow.

General discussion

Link between thrombosis, inflammation and atherosclerosis

Atherosclerosis is an inflammatory disease, which develops in response to injury of the endothelium[25,26]. Besides the important role of platelets and coagulation in atherothrombosis, current evidence supports considerable cross-talk between thrombosis and the inflammatory pathways involved other stages of atherogenesis, e.g. plaque initiation and expansion, and fibrous cap degradation and rupture[27-29].

Platelets play a prominent role in atherogenesis[30,31]. vWF and P-selectin, secreted by the pro-atherogenic endothelium, recruit platelets via the interaction with glycoprotein Ibα. Firm adhesion of platelets is mediated by glycoptotein IIb-IIIa[31-33]. Activated platelets secrete cytokines, chemokines, growth factors, adhesion molecules and coagulation factors that participate in the atherogenic process. For example, P-selectin expression after platelet activation, binds to P-selectin glycoprotein ligand 1 on leukocytes, promoting the adhesion of leukocytes to vascular cell adhesion molecule 1 (VCAM-1) and other adhesion molecules on activated endothelial cells. Secretion of the chemokine RANTES (Regulated upon Activation, Normal T-cell Expressed, and Secreted) triggers recruitment of monocytes and platelet factor 4 promotes differentiation of monocytes into macrophages. Endothelial cells, vascular smooth muscle cells and macrophages within the atherosclerotic plaque express CD40, the receptor for CD40 ligand which is secreted by activated platelets. Endothelial cell activation induced by CD40 ligand induces expression of pro-inflammatory cytokines including interleukin (IL) 1, IL6 and IL8, and also stimulates endothelial expression of intracellular adhesion molecule 1 (ICAM-1), VCAM-1 and E-selectin. In addition, CD40 ligand increases metalloproteinase (MMP) and tissue factor (TF) production[27,30,31].

The main effector of the coagulation cascade is thrombin, a serine protease, which is generated when disruption of the vascular integrity allows plasma coagulation factor VII (FVII) to contact extravascular TF. Thrombin links coagulation to inflammation by having pro-inflammatory effects, via protease-activated receptors (PARs), on the cells involved in atherogenesis[28,29,34]. Activated factor II (FIIa) is a potent platelet activator, stimulating the above mentioned processes[28]. Endothelial cell activation increases inflammatory gene transcription and stimulates expression of leukocyte adhesion molecules. In addition, thrombin promotes recruitment of monocytes and T-cells into the vessel wall by inducing monocyte chemotactic protein 1 (MCP-1) production in endothelial cells and vascular smooth muscle cells[34-36]. Induction of cell retraction and reorganization of cadherins at endothelial junctions increases endothelial permeability and promotes platelet deposition and leukocyte extravasation[28,29]. Production of extracellular matrix proteins and TF by vascular smooth muscle cells, and proliferation and migration of these cells, is also stimulated by thrombin[28,29]. Systemic inflammation and a prothrombotic response is promoted via the secretion IL6 by thrombin-activated endothelial cells, vascular smooth muscle cells and platelets[28]. Macrophages are central cellular effectors in atherogenesis. The in vivo importance of thrombin-induced macrophage activation is not clear. However, macrophages do respond to the inflammatory mediators produced by endothelial cells, vascular smooth muscle cells and platelets following thrombin activation[28,36]. Besides thrombin, TF-FVIIa and FXa also contribute to activation of PARs[37,38].

Early fatty streak lesions progress gradually over time, by proliferative and inflammatory responses, into more advanced fibroatheromata, which eventually lead to clinical manifestations[26]. Besides continuous atheroma growth, lesions suddenly expand after physical disruption of plaques and subsequent thrombus formation[25,39,40]. In these processes, the hemostatic system also plays an important role. After fibrous cap erosion or rupture collagen and TF are exposed to the flowing blood. Platelets adhere, via vWF, to collagen and are activated, and TF initiates thrombin formation. Most episodes of plaque erosion or rupture do not cause clinical symptoms[39,40]. When the fibrinolytic mechanisms outweigh the pro-coagulant pathways, a limited mural thrombus forms, instead of an

occlusive thrombus. Thrombin stimulates smooth muscle cell migration and proliferation and triggers platelet release of growth factors from their alpha granules. Platelet-derived growth factor (PDGF) further promotes smooth muscle cell migration and proliferation, and transforming growth factor β (TGF-β) stimulates interstitial collagen synthesis by smooth muscle cells. Together with resorption of the mural thrombus these processes lead to fibrous tissue formation and expansion of the atherosclerotic plaque[25]. Disruption of microvessels, formed in atherosclerotic plaques, also promote sudden plaque progression[41]. As a result of neo-angiogenesis, plaques develop microvascular channels which are fragile and prone to hemorrhage[42]. Repeating intraplaque hemorrhages (IPH) contribute to lipid-core expansion through the accumulation of free cholesterol from erythrocyte membranes[43]. The subsequent thrombotic responses are similar to those after fibrous cap erosion or rupture[25]. Besides sudden plaque growth, fibrous cap erosions or ruptures and IPH also promote plaque destabilization and vulnerability[39,41,44,45].

In conclusion, the hemostatic system promotes atherogenesis in multiple ways and influences composition and phenotype of the atherosclerotic plaque.

Hemophilia and atherosclerosis

Activation of prothrombin into thrombin is less in hemophilia patients as compared with non-hemophilic subjects. We hypothesized that this might reduce the stimulation of proinflammatory pathways supporting atherogenesis. This is in line with the observation that elevated FVIII levels have been positively associated with atherosclerosis[46,47].

In chapter 3 and 4 of this thesis, we clearly show a lack of evidence for a protective role of FVIII deficiency on the development of atherosclerosis in hemophilia patients, which makes that alternative hypotheses, explaining the reduced IHD mortality in these patients, should be considered or reconsidered. In 1990, Rosendaal et al.[8] concluded that only a fraction of the reduced IHD mortality in hemophilia patients as compared with the general male population could be explained by differences in cardiovascular risk factor prevalence. Since then, the elderly hemophilia population has expanded and competing risks due to HIV and hepatitis C infection are now less important. Fransen van de Putte et al.[48] assessed the prevalence of cardiovascular risk factors, between January 2009 and July

2011, in 709 Dutch and English hemophilia patients. As compared to the general male population, a higher prevalence of hypertension, and lower prevalences of obesity and hypercholesterolemia were found in hemophilia patients. In addition, hemophilia patients had an overall more unfavorable CVD risk profile. Currently, this study population is prospectively being followed to determine the incidence of CVD and to assess the influence of cardiovascular risk factor prevalence on CVD occurrence[48].

As was discussed in chapter 2, a plausible explanation for the lower IHD mortality would be that the hypocoagulable state of hemophilia patients has a protective effect on thrombus formation. Possibly, hemophilia patients more often develop a mural thrombus upon plaque rupture or erosion than a fatal occlusive thrombus as compared with non-hemophilic men. Although several cohort studies investigated IHD death in hemophilia patients[1,2,5-7], only 2 studies compared IHD prevalence between men with and without hemophilia[49,50]. Kulkarni et al.[50] found that the rate of IHD hospital discharges among hemophilia patients aged 65 years and older was nearly 30% lower than that of non-hemophilic males. In addition, in a recent retrospective study it was shown that nonfatal myocardial infarction occurred less often in patients with severe hemophilia than in patients with moderate or mild hemophilia or in the general male population[49]. This might imply that when the occurrence of plaque erosions or ruptures is comparable between men with and without hemophilia, patients with hemophilia more often develop an asymptomatic thrombus. Another possibility could be that plaque ruptures occur less often in hemophilia patients. The risk of plaque rupture mainly depends on plaque composition and is influenced by inflammation[25,39,45]. Vulnerable plaques have thin or eroded fibrous caps that overlay large lipid cores and contain a lot of inflammatory cells[25,39,45]. As we hypothesized that lower levels of thrombin in hemophilia patients could reduce the stimulation of proinflammatory pathways supporting atherogenesis, this might also result in the development of less vulnerable plaques. On the other hand, IPH is an important determinant of plaque vulnerability[41]. Coumarin-type anticoagulation use was shown to be independently related with carotid IPH[51]. Possibly, patients with hemophilia are more prone to IPH resulting in more plaque destabilization as compared to non-hemophilic men. However, this contradicts the reduced fatal and not-fatal IHD in

hemophilia patients. There might be a balance between protective effects on atherosclerosis and atherothrombosis and effects promoting plaque vulnerability, resulting in a net protection against the occurrence of fatal and non-fatal IHD.

Also in patients with von Willebrand disease (vWD), the prevalence of ischemic CVD has been shown to be lower as compared to the general population[20]. Studies comparing IMT between subjects with and without vWD do not demonstrate an unequivocal protective effect of vWD on atherosclerosis[13,14,17,52,53]. As vWF is necessary to facilitate adhesion of platelets to collagen after atherosclerotic plaque rupture and functions as a carrier protein for FVIII, the same hypotheses explaining reduced fatal and non-fatal IHD in hemophilia patients, also apply for patients with vWD.

In the near future, our group, together with other hemophilia treatment centers, will further explore mechanisms explaining the reduced occurrence of IHD in patients with hemophilia or vWD. Plaque morphology can be assessed with magnetic resonance imaging (MRI)[54,55]. Carotid plaque composition (lipid core, fibrous cap, wall thickness, luminal thrombus) and the presence of IPH will be investigated. In addition, markers of plaque vulnerability, such as MMPs and fibrin, can be targeted with antibodies and visualized with molecular MRI[56]. This will be done in low density lipoprotein receptor (LDLR) and FVIII double deficient mice.

Future studies investigating atherosclerotic plaque phenotype in patients with hemophilia or vWD are important in further understanding the role of the hemostatic system in atherogenesis, and might have implications for prevention and targeted treatment of atherosclerotic cardiovascular disease in both patients with a coagulopathy or patients from the general population.

In conclusion, the protective effect of congenital clotting factor deficiencies on fatal and non-fatal IHD is most likely mediated by effects on both atherothrombosis and atherosclerotic plaque phenotype.

Treatment of atherosclerotic disease in hemophilia patients

Although hemophilia specialists observe an increase in hemophilia patients with IHD, these patients are not common. This makes it unlikely that well-designed clinical trials can

be performed to find the optimal therapy for these complex patients. Therefore, we developed an institutional guideline, presented in chapter 6 of this thesis, which is based on both experience from hemophilia specialists and existing guidelines on treatment of IHD in non-hemophilic patients. Documentation and evaluation of hemophilia patients with IHD, as we did in chapter 7, is needed to keep improving treatment. We propose to establish a collaboration on this subject between Dutch comprehensive hemophilia treatment centers. By combining data obtained in multiple centers, we could gather more knowledge on feasibility, outcomes, and safety of our current guideline, and improve treatment faster. In addition, in such a collaboration, consensus on treatment of IHD in patients with hemophilia or vWD can and should be obtained.

The balance between the determinants of hemostasis results in the hemostasis integral[24]. As no clinical trials exist, we cannot depend on epidemiological data and knowledge to assess the effects of antithrombotic drugs in combination with (partial) clotting factor substitution on the hemostasis integral in hemophilia or vWD patients with IHD. Future research, using the microfluidic device with static mixer, could help us understand hemostatic processes in these patients better and might result in optimization of dosages of both antithrombotic drugs and clotting factor concentrate. Before we can use the microfluidic device to investigate the hemostasis integral and especially the interplay of platelets and coagulation, we have to further characterize and validate the model. In addition, we need to invest in the development of read-off parameters and quantification.

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Nederlandse samenvatting

Congenitale stollingsfactor deficiëntie en cardiovasculaire ziekten: van nature beschermd?

Dit proefschrift behandelt 1) de vorming van slagaderverkalking bij patiënten met hemofilie of de ziekte van von Willebrand, en 2) de behandeling van hemofiliepatiënten met hart- en vaatziekten.

Hemostase

Bij beschadiging van een bloedvat zorgt de bloedstolling, ofwel hemostase, ervoor dat er minimaal bloedverlies optreedt. Falen van de bloedstolling leidt tot een verhoogde bloedingsneiging, terwijl bij een te hoge activiteit van het bloedstollingsmechanisme ongewenste bloedstolsels (trombose), welke bloedvaten verstoppen, kunnen ontstaan. Hemostase is een zeer complex proces waarbij bloedplaatjes en een groot aantal eiwitten in het bloed (stollingsfactoren) samenwerken. Onder normale omstandigheden klonteren bloedplaatjes niet samen. Bij een beschadiging van de binnenbekleding van de vaatwand (endotheel) komen de bloedplaatjes in contact met onderliggende structuren, bijvoorbeeld collageen. De hechting van bloedplaatjes aan collageen vindt plaats onder invloed van de von Willebrand factor (vWF). vWF is een groot eiwit wat bindt aan zowel collageen als aan een receptor (glycoproteïne Ib) op de bloedplaatjes. vWF vangt de bloedplaatjes uit het stromende bloed. Hoe harder het bloed stroomt, des te belangrijker is vWF voor de binding van bloedplaatjes aan collageen. De bloedplaatjes worden geactiveerd, waardoor een andere receptor (glycoproteïne IIb/IIIa) op de bloedplaatjes beschikbaar wordt. vWF en het bloedstollingseiwit fibrinogeen kunnen aan deze receptor binden en zorgen ervoor dat de bloedplaatjes ook onderling samenklonteren. De prop van bloedplaatjes kan uiteen vallen zodat de bloeding weer opnieuw begint. Om de prop te stabiliseren, moeten de bloedstollingseiwitten ook geactiveerd worden. Bij beschadiging van het bloedvat komt het eiwit weefselfactor vrij, welke bindt aan stollingsfactor VII in het bloed. Het complex wat hierbij vormt, is de start van een reeks enzymatische reacties waarbij stollingsfactoren geactiveerd worden en elkaar versterken. Uiteindelijk leidt dit tot de omzetting van fibrinogeen in fibrine door trombine. Een stevig netwerk van fibrinedraden versterkt de prop van bloedplaatjes. Er zijn ook eiwitten die de stolling weer

afremmen, zodat de bloedstolsels niet te groot worden. Het stolsel wordt uiteindelijk weer opgeruimd door een proces genaamd de fibrinolyse.

Hemofilie

Hemofilie is een erfelijke stollingsstoornis die voornamelijk bij mannen voorkomt. Vrouwen kunnen draagster zijn, wat inhoudt dat zij de aandoening aan hun zoons kunnen doorgeven. Er zijn ongeveer 1600 hemofiliepatiënten in Nederland. Vijfentachtig procent van de patiënten heeft hemofilie A. Bij hen is er een tekort aan stollingsfactor VIII. Het overige deel heeft hemofilie B, wat veroorzaakt wordt door een tekort aan stollingsfactor IX. Deze stollingsfactoren zijn nodig voor de vorming van trombine. Het fibrine netwerk wat hierdoor gevormd kan worden, stabiliseert de bloedplaatjes prop (zie Hemostase). De ernst van de hemofilie wordt bepaald door de hoeveelheid stollingsfactor VIII of IX die door het lichaam zelf nog wordt aangemaakt. Het tekort aan stollingsfactor resulteert, vooral bij de ernstige vormen van hemofilie, in bloedingen in gewrichten en spieren, maar ook in organen. De steeds terugkerende gewrichtsbloedingen kunnen, bij niet adequate behandeling, kraakbeenschade veroorzaken. Elke hemofiliepatiënt heeft een verhoogd risico op bloedingen na medische ingrepen, operaties en trauma's.
Bloedingen worden voorkomen of behandeld door (profylactische) toediening van het ontbrekende eiwit via een ader. Voor de introductie van deze stollingsfactorconcentraten in de jaren 60, overleden de meeste patiënten met ernstige hemofilie voor het 30ste levensjaar. In de jaren 70 en 80 zijn veel hemofiliepatiënten besmet met HIV (human immunodeficiency virus) en HCV (hepatitis C virus) als gevolg van toediening van besmette stollingsproducten gewonnen uit humaan bloed. In Nederland zijn stollingsfactorconcentraten sinds 1985 vrij van HIV en sinds 1992 van HCV. De levensverwachting van hemofiliepatiënten is de laatste jaren gestegen en is tegenwoordig vrijwel gelijk aan die van Nederlandse mannen zonder hemofilie.

Hemofilie en hart- en vaatziekten

Door de gestegen levensverwachting worden hemofiliepatiënten steeds vaker geconfronteerd met leeftijdsgerelateerde aandoeningen, zoals hart- en vaatziekten. Hart- en vaatziekten, zoals een hartinfarct of beroerte, zijn het gevolg van slagaderverkalking en vormen een belangrijk gezondheidsprobleem in de oudere bevolking. Hoewel we steeds vaker hart- en vaatziekten bij de ouder wordende hemofiliepatiënt zien, blijken met name hartinfarcten minder vaak voor te komen bij hemofiliepatiënten dan bij mannen uit de algemene bevolking. Het lijkt erop dat hemofiliepatiënten op de een of andere manier beschermd worden tegen de ontwikkeling van hart- en vaatziekten. In **hoofdstuk 2** van dit proefschrift bespreken we 3 mogelijke verklaringen voor deze observatie aan de hand van de beschikbare literatuur.

De eerste verklaring betreft risicofactoren. Een aantal factoren verhoogt het risico op het ontwikkelen van slagaderverkalking en hart- en vaatziekten, zoals hoge bloeddruk, suikerziekte, hoog cholesterol gehalte, roken, overgewicht, weinig lichaamsbeweging, en het voorkomen van hart- en vaatziekten bij familieleden. Uit verschillende onderzoeken blijkt dat hemofiliepatiënten minder vaak een hoog cholesterolgehalte hebben, maar vaker een hoge bloeddruk. Berekeningen laten zien dat verschillen in het voorkomen van risicofactoren tussen hemofiliepatiënten en mannen zonder hemofilie, het minder vaak optreden van hartinfarcten bij hemofiliepatiënten niet kunnen verklaren. Een tweede verklaring zou kunnen zijn dat de verminderde stollingsneiging van hemofiliepatiënten een beschermend effect heeft op de vorming van het bloedstolsel wat voorafgaat aan een hartinfarct. Proefdier en laboratorium onderzoek ondersteunen deze verklaring. De laatste verklaring betreft de vorming van slagaderverkalking en wordt besproken in de volgende paragraaf.

Hemofilie en atherosclerose

Het onderliggende probleem van hart- en vaatziekten is slagaderverkalking, ofwel atherosclerose. Iedereen ontwikkelt atherosclerose. Dit proces neemt tientallen jaren in beslag en begint vaak al op vrij jonge leeftijd. Kleine beschadigingen van de binnenbekleding van de vaatwand worden veroorzaakt door hoge bloeddruk, verhoogd suiker- en/of cholesterolgehalte, en nicotinedeeltjes. Door de beschadigingen ontstaan ontstekingsprocessen, die aan de basis van atherosclerose liggen. Cholesterol dringt de wand van de slagader binnen. Het afweersysteem reageert op deze situatie. Ook witte bloedcellen dringen de vaatwand binnen en nemen grote hoeveelheden cholesterol in zich op. Er ontstaat een ophoping van met cholesterol gevulde witte bloedcellen en deze zogenaamde 'foam cellen' verergeren de ontstekingsprocessen. Als reactie hierop groeit de vaatwand langzaam naar binnen en ontstaat een zogenaamde plaque welke het bloedvat vernauwt. Uit wetenschappelijk onderzoek blijkt dat bloedplaatjes en de stollingseiwitten een belangrijke rol spelen in de ontstekingsprocessen die aan de vorming van atherosclerose ten grondslag liggen. Bloedplaatjes die hechten aan de beschadigde vaatwand en geactiveerd worden, helpen de witte bloedcellen de vaatwand binnendringen. Trombine, gevormd door de activatie van de stollingsfactoren, zet niet alleen fibrinogeen om in fibrine, maar activeert ook de bloedplaatjes en de spiercellen in de vaatwand die de groei van de plaque bevorderen. Dit zijn slechts enkele voorbeelden van de invloeden van stolling op ontsteking en atherosclerose. Door de afwezigheid van stollingsfactor VIII of IX kunnen hemofiliepatiënten minder trombine vormen. Dit zou kunnen betekenen dat de onstekingsprocessen nodig voor de vorming van atherosclerose minder gestimuleerd worden in hemofiliepatiënten, waardoor ze minder atherosclerose vormen.

In **hoofdstuk 3** wordt dit onderzocht door de mate van atherosclerose in de kransslagaderen te vergelijken tussen patiënten met hemofilie A en mannen zonder hemofilie. Verkalking van de slagaders rond het hart is gemeten met een beeldvormende techniek, genaamd multi detector-row computed tomography (MDCT). De hoeveelheid

gemeten verkalkingen, de zogenaamde kalkscore, is een maat voor atherosclerose. De kalkscore bleek vergelijkbaar tussen mannen met en zonder hemofilie. We kunnen concluderen dat er geen bewijs is voor een beschermend effect van de afwezigheid van stollingsfactor VIII op de ontwikkeling van atherosclerose.

In **hoofdstuk 4** wordt deze conclusie bevestigd. In dit hoofdstuk is echografie gebruikt om een vroeger stadium van atherosclerose op te sporen in de halsslagaders van hemofiliepatiënten zonder en met overgewicht, een belangrijke risicofactor voor hart- en vaatziekten. Hemofiliepatiënten met overgewicht hebben meer atherosclerose dan patiënten zonder overgewicht. Er blijken geen verschillen te zijn tussen hemofiliepatiënten en mannen zonder hemofilie (met en zonder overgewicht).

De hoeveelheid atherosclerose blijkt uit onze onderzoeken niet te verschillen tussen mannen met en zonder hemofilie. Daarom moeten we andere mogelijke verklaringen of hypothesen overwegen. Een plaque wordt zelden zo groot dat hij een slagader helemaal afsluit. Wel bestaat het gevaar dat er een scheurtje in de plaque ontstaat waardoor de inhoud van de plaque in aanraking komt met het bloed. Het bloed stolt (zie Hemostase) en er een ontstaat een bloedstolsel op de plaque, welke in korte tijd de slagader geheel of gedeeltelijk kan afsluiten. Hierdoor kan er geen bloed en zuurstof naar het achterliggende weefsel gebracht worden. Dit geeft klachten en als het weefsel afsterft, ontstaat er een infarct. Het risico op het ontstaan van deze scheurtjes wordt bepaald door de samenstelling van de plaque en wordt beïnvloedt door ontsteking. Misschien treden deze scheurtjes wel minder vaak op bij hemofiliepatiënten. Aan de andere kant worden plaques meer gevoelig voor scheurtjes door bloedingen die in de plaque kunnen optreden. Deze zogenaamde intraplaque bloedingen zouden juist weer vaker kunnen optreden bij hemofiliepatiënten. In de nabije toekomst zal dit onderzocht worden met behulp van MRI (magnetic resonance imaging), waarmee de samenstelling en de aanwezigheid van intraplaque bloedingen zichtbaar gemaakt kan worden. Waarschijnlijk bestaat er in hemofiliepatiënten een balans tussen beschermende effecten op atherosclerose en trombose op plaques, en effecten die de gevoeligheid van plaques op scheurtjes

verhogen. Uiteindelijk resulteert deze balans in een bescherming tegen het optreden van hart- en vaatziekten.

Ziekte van von Willebrand

De ziekte van von Willebrand is ook een erfelijke stollingsstoornis, en komt mogelijk bij 1% van de Nederlandse bevolking voor, zowel bij mannen als bij vrouwen. Er bestaan verschillende typen van deze aandoening en de mate van ernst kan sterk variëren. Er zijn mensen die nauwelijks last hebben van de ziekte van von Willebrand. Bij anderen komt de aandoening tot uiting via slijmvliesbloedingen (neus- en tandvleesbloedingen). Vrouwen kunnen zeer hevige bloedingen tijdens de menstruatie of na een bevalling ondervinden. Bij ernstiger vormen van de ziekte van von Willebrand komen ook spier- en gewrichtsbloedingen voor, vergelijkbaar met hemofilie. Bij de ziekte van von Willebrand is er sprake van een tekort aan of van een gestoorde functie van de von Willebrand factor (vWF). Het eiwit is nodig voor de hechting van bloedplaatjes aan de beschadigde vaatwand en aan elkaar (zie Hemostase). Daarnaast zorgt de von Willebrand factor voor het transport van stollingsfactor VIII.
Ook bij patiënten met de ziekte van von Willebrand blijken hart- en vaatziekten minder vaak voor te komen dan bij de algemene populatie. In **hoofdstuk 5** beschrijven we de literatuur die gepubliceerd is over de relatie tussen de afwezigheid van vWF en het voorkomen van atherosclerose. Er kan geen eenduidige conclusie getrokken worden, maar hoogstwaarschijnlijk speelt ook vWF een belangrijke rol in de ontwikkeling van atherosclerose. De bovenstaande hypothesen zijn hier ook van toepassing en in de nabije toekomst zal ook verder onderzoek naar de samenstelling van plaques bij patiënten met de ziekte van von Willebrand gedaan worden.

Behandeling van hemofiliepatiënten met hart- en vaatziekten

De behandeling van hemofiliepatiënten met hart- en vaatziekten is ingewikkeld. De hemofilie wordt behandeld door de afwezige stollingsfactor toe te dienen, terwijl hart- en vaatziekten met antistolling medicatie worden behandeld. Omdat hemofilie een zeldzame aandoening is, kunnen er geen grote wetenschappelijke studies opgezet worden om de optimale behandeling te achterhalen en te onderzoeken. We hebben een behandelingsrichtlijn geschreven voor hemofiliepatiënten met hart- en vaatziekten (**hoofdstuk 6**). Deze is gebaseerd op ervaring van hemofilieartsen / hematologen en op richtlijnen over de behandeling van hart- en vaatziekten bij de algemene populatie. In **hoofdstuk 7** laten we, aan de hand van de beschrijving van 9 casus, zien dat onze richtlijn haalbaar en veilig is. Documentatie en evaluatie van de behandelde hemofiliepatiënten met hart- en vaatziekten is nodig om de behandeling te blijven verbeteren. Eveneens is samenwerking met andere hemofiliebehandelcentra van groot belang.

Zoals besproken in de eerste paragraaf van deze Nederlandse samenvatting beïnvloeden bloedplaatjes, stollingsfactoren, de vaatwand en de bloedstroming elkaar en de hemostase. We weten niet precies wat er met de bloedstolling van hemofiliepatiënten gebeurd als we hen volgens onze richtlijn behandelen voor hart- en vaatziekten. Bij het laboratorium onderzoek van de bloedstolling wordt altijd naar een deel van de hemostase gekeken. De functie van de bloedplaatjes wordt, bijvoorbeeld, onderzocht in afwezigheid stollingsfactoren en de bloedstroming, terwijl de interacties tussen deze onderdelen zo belangrijk zijn. We hebben, samen met de Technische Universiteit Eindhoven, een experimenteel model ontwikkelt waarin we de invloeden van bloedplaatjes, stollingsfactoren, onderdelen van de vaatwand en stroming op elkaar en op bloedstolling kunnen onderzoeken. Deze ‘perfusie kamer met statische mixer’ is beschreven in **hoofdstuk 9**. Voordat we de perfusie kamer met statische mixer in ons onderzoek kunnen gebruiken, zullen we het model eerst moeten valideren.

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Curriculum Vitae

Attie Tuinenburg was born on February 26, 1982 in Gorinchem, the Netherlands. After completing high school at the Merewade College in Gorinchem, she studied Medicine at the University of Utrecht, Utrecht, the Netherlands. A major internship on apoptosis of endothelial progenitor cells cultured in hyperglycemic conditions at the Laboratory of Vascular Medicine at the University Medical Center Utrecht (UMCU) under supervision of prof. dr. M.C. Verhaar initiated her interest in science. Attie received her MD degree in 2007.

Following graduation she began working as a PhD student at the Van Creveldkliniek / Department of Hematology at the UMCU under supervision of dr. R.E.G. Schutgens, prof. dr. M.C. Verhaar and prof. dr. D.H. Biesma. In 2009 she started at the Department of Clinical Chemistry and Hematology under supervision of dr. M. Roest and prof. dr. Ph.G. de Groot. The results of her research performed at these departments are described in this thesis. In 2010 Attie was awarded the CSL Behring prof. Heimburger Award (chapter 3) and in 2011 she obtained a Master's degree in the Postgraduate Clinical Epidemiology program of Biomedical Sciences.