Von Willebrand Factor and ADAMTS13 in Cardiovascular Disease

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Von Willebrand Factor en ADAMTS13 in hart- en vaatziekten

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Hartstichting

A ship in harbor is safe, but that is not what ships are built for

John A. Shedd

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1

GENERAL INTRODUCTION AND OUTLINE OF THE THESIS

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Hemostasis

Hemostasis is a complex balance between procoagulant and anticoagulant factors to prevent individuals from bleeding. Multiple factors are involved in the hemostatic system, which consists of three different parts: primary hemostasis by which the clot is formed,mainly by platelets, secondary hemostasis which strengthens the clot by fibrin formation, and the fibrinolytic system by which the clot is dissolved after wound healing. An important player in primary hemostasis is Von Willebrand Factor (VWF). This multimeric glycoprotein mediates platelet adhesion and aggregation which leads to the formation of a platelet plug (1).

Von Willebrand Factor

Von Willebrand Factor (VWF) is mainly produced by endothelial cells and megakaryocytes (1, 2), and is synthesized as a precursor propeptide and forms dimers via disulfide bonds in the endoplasmic reticulum. These VWF dimers are transported to the Golgi apparatus where VWF multimers are formed. The VWF multimers differ in size and some can consists of up to 500 monomers. In the trans-Golgi network the propeptide is cleaved from the subunits and mature VWF proteins remains. VWF is secreted into the circulation constitutively or tubulized and packaged into Weibel Palade Bodies (WPBs) within the endothelial cells (2-4). A small portion is stored in the alpha-granules of platelets (5). Upon stimulation endothelial cells release ultra-large high molecular weight (ULHMW) VWF multimers, which are the most procoagulant forms and can lead to thrombus formation. ULHMW VWF multimers are cleaved by the metalloprotease ADAMTS13 (A Disintegrin And Metalloprotease with ThromboSpondin motif repeats 13) into smaller, less procoagulant forms (6, 7).

The VWF gene is located on the short arm of chromosome 12 and encompasses 52 exons. VWF plasma levels in normal individuals differ between around 0.60 and 1.40 IU/mL (8). Different factors, genetic and non-genetic, influence the plasma levels including age, inflammation, and ABO blood group(9). Individuals with blood group O have 20-25% lower VWF plasma levels than individuals with blood group non-O (10, 11). This is due to the fact that individuals with blood group O have a different glycosylation profile of VWF which influences its clearance (11, 12). In addition, several single nucleotide polymorphisms (SNPs) in and outside the VWF gene are known to influence VWF plasma levels (13, 14) (Figure 1). VWF functions also as a carrier protein for coagulation factor VIII (FVIII), thereby preventing FVIII from proteolytic degradation (15, 16). FVIII is an important factor for secondary hemostasis and changes in VWF levels in circulation will also results in variability of FVIII levels.

The VWF protein consists of four types of homologous domains. Each of the domains has a specific function and binding properties. ADAMTS13 cleaves VWF in the A2 domain (figure 2) between Tyrosine 1605 and Methionine 1606 (6, 7). This scissile bond becomes exposed

when VWF undergoes a conformational change at moments of high shear stress or when it is bind to subendothelial structures. Other domains of VWF are involved in binding with GPIb on platelets, collagen, heparin, ristocetin, GPIIb/IIIa, or FVIII (2, 17).

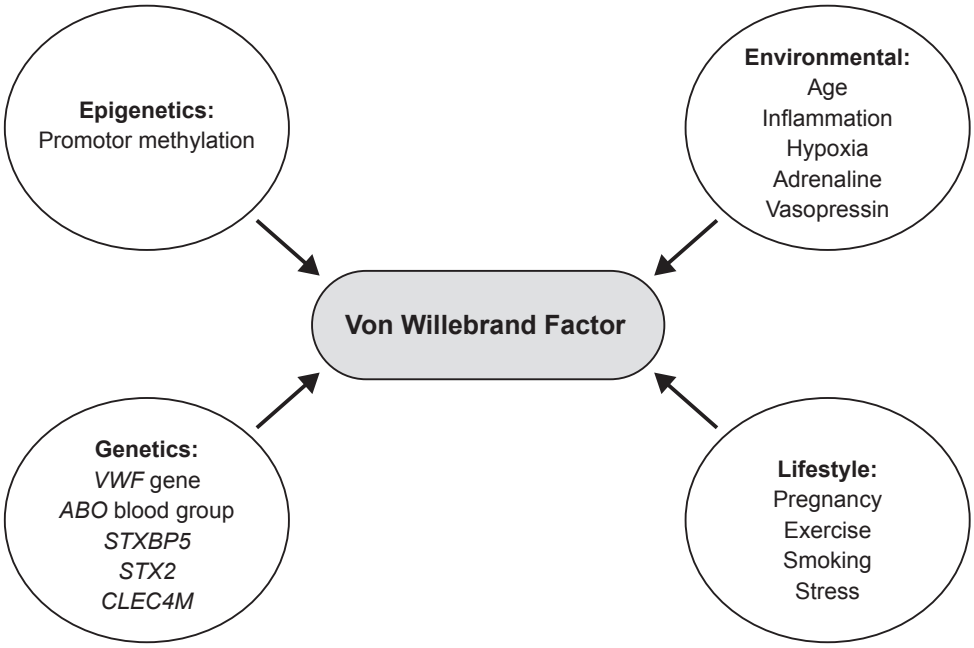

Figure 1. Determinants of VWF plasma levels

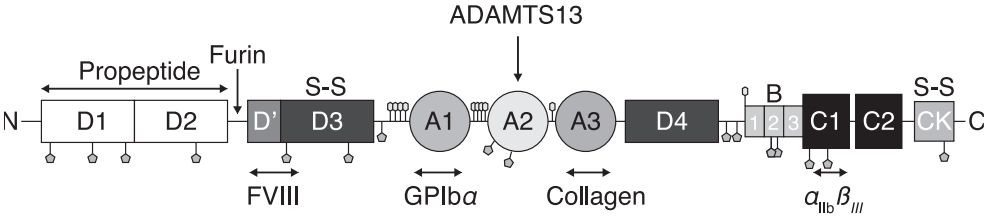

Figure 2. Domain organization of VWF, ADAMTS13 cleaves VWF at the A2 domain. Adopted from J.T.B. Crawley et al. Blood 2011.

ADAMTS13

The ADAMTS13 gene is located on chromosome 9q34 and compises 29 exons (18). ADAMTS13 is part of the ADAMTS family which shares the homology of domain structures, a signal peptide and a propeptide (7, 19, 20). ADAMTS13 consists of a catalytic domain, a disintegrin domain, TSP1-motif, a cysteine-rich domain, a spacer domain, seven other TSP-1 domains (2-8) and two CUB-domains.

ADAMTS13 is synthesized in hepatic stellate cells in the liver and also vascular endothelial cells are found to synthesize and secrete ADAMTS13 (21, 22). ADAMTS13 is also released from platelets upon activation (23).

After initiation of the hemostasis, platelets will adhere to ULHMW VWF which is anchored to the activated endothelial cell or the exposed subendothelium. A conformational change of VWF is induced when shear stress forces are present and when VWF is bound to the glycoprotein Ib receptor on platelets via the A1 domain, thereby exposing the A2 domain for ADAMTS13 binding. ADAMTS13 cleaves VWF at the Tyr1605-Met1606 peptide bond, predominantly at or close to the anchoring sites of the ULHMW VWF multimers. After cleavage the remaining strings of VWF are less biologically active and less susceptible for cleavage (24). So far this is the only function known for ADAMTS13

The importance of ADAMTS13 is exemplified in patients with Thrombotic Thrombocytopenic Purpura (TTP). In these patients there is a complete deficiency or strongly reduced ADAMTS13 level which results in the inability to cleave ULHMW VWF into smaller , less procoagulant forms. Subsequently this can result into microthrombi formation and microangiopathy. TTP is characterized by a classical pentad of signs and symptoms including hemolytic anemia, neurological symptoms, renal dysfunction, fever, and profound thrombocytopenia (25). Most individuals presenting with TTP are acquired, however TTP may also be congenital, caused by mutations in the ADAMTS13 gene. In acquired TTP the cause is the formation of auto-antibodies against ADAMTS13 (26-28).

ADAMTS13 deficiency has also been described in other diseases, including liver cirrhosis and systemic lupus erythematosus (SLE) (29-31). Patients with pre-eclampsia and the anti-phospholipid syndrome also have reduced levels of ADAMTS13 and are prone for thrombotic complications (32-34).

It has been suggested that ADAMTS13 is involved in inflammation. Recent studies have shown an association between ADAMTS13 levels and severe sepsis, septic shock, severe Plasmodium Falciparum malaria and Dengue virus infection (35-38). Therefore, ADAMTS13 may be a link between inflammation and thrombosis.

Cardiovascular disease, atherosclerosis and atherothrombosis

Cardiovascular disease, including myocardial infarction and stroke, are a major cause of morbidity and mortality in the world. In the Netherlands, approximately 40,000 individuals die each year due to cardiovascular disease (39). The pathogenesis of cardiovascular disease

is complex. Many risk factors, environmental and genetic, are known to influence the risk of cardiovascular disease, including smoking, hypertension, diabetes, family history and hypercholesterolemia. One of the most important risk factor for cardiovascular disease is atherosclerosis. Atherosclerosis is a chronic disease characterized by lipid accumulation and inflammation (40). Atherosclerosis starts already in the childhood with a focal thickening of the intima called fatty streaks, and lipid accumulation (41). The lesions may progress by migration of smooth muscle cells from the media to the intima of the vessel wall where the cells proliferate and synthesize several matrix components. Smooth muscle cells within the deep layer of the fatty streaks are susceptible to apoptosis and form a necrotic core (42). The layer of this core is covered with a fibrous cap which stabilizes the plaque. Multiple factors contribute to the development of atherosclerosis, including inflammation and endothelial dysfunction. Endothelial dysfunction occurs early in the process of atherosclerosis and has been shown to contribute to the formation, progression, and complications of an atherosclerotic plaque (43). Additionally, many inflammatory cells play a role in the formation of an atherosclerotic plaque (44).

Different components of an atherosclerotic plaque can be distinguished and may be used as a marker of atherosclerosis. One of the components are calcifications which can be present in advanced atherosclerotic lesions which consist of a necrotic lipid rich core (45). However, calcifications may also be present at earlier stages of atherosclerosis (46). Previous studies have shown an association between calcified lesions and the total plaque area or the presence of cardiovascular disease (47-50). In addition, intraplaque hemorrhages are commonly observed in atherosclerotic plaques and it has been shown to be associated with plaque progression and risk of ischemic stroke (51, 52).

Rupture of an unstable atherosclerotic plaque triggers coagulation and may lead to platelet aggregation (53) and finally to thrombus formation and clinical symptoms such as myocardial infarction and stroke. Plaque rupture may also be silent leading to progression of atherosclerosis, stenosis and arterial remodeling (54, 55). Studies have shown that different plaque characteristics, including thin-cap fibroatheromas and a minimal luminal area, predict plaque rupture and major adverse cardiovascular events (56, 57).

Although many risk factors for cardiovascular disease are known, there are still many patients suffering from cardiovascular disease in whom no risk factor can be found. Therefore, there is still a need to identify new risk factors.

Additionally, there is a lack of good therapeutic options in especially ischemic stroke patients. Currently antithrombotic agents, such as platelet inhibitors, thrombolysis, and thrombectomy are used as treatment options (58, 59). However, thrombolysis and thrombectomy have only shown to improve outcome when given within 4-6 hours after onset of symptoms. Therefore, there is also a need to identify new therapeutic goals.

Von Willebrand Factor, ADAMTS13 and arterial thrombosis

Variations in coagulation factor levels have shown to be associated with a higher risk of arterial thrombosis (60, 61). Previous studies have shown an association between high VWF levels and the risk of cardiovascular disease, including myocardial infarction and stroke, suggesting that VWF is a risk factor for cardiovascular disease (62-65).

Because high levels of VWF are associated with arterial thrombosis risk, it has been hypothesized that ADAMTS13, its main cleavage enzyme, may also modify the risk of thrombosis. Theoretically low levels of ADAMTS13 may reduce cleavage of VWF, resulting in more active VWF, increased platelet aggregation and therefore to a higher thrombosis risk. So far the association between ADAMTS13 and cardiovascular disease has not yet been established because of controversial results of a limited number of studies (66-68). However, so far this has only been investigated in case-control studies, which are inconclusive. Therefore large prospective studies on the association of ADAMTS13 levels and arterial thrombosis, including ischemic stroke and myocardial infarction are urgently needed.

The exact pathogenic role of VWF and ADAMTS13 in arterial thrombosis is yet unknown. Due to the fact that endothelial dysfunction is related with atherosclerosis and VWF is released from WPBs in case of endothelial dysfunction, there might be an association between VWF and the extent of atherosclerosis. Two previous studies have focused on the association between VWF levels and atherosclerosis, measured by the intima-media thickness (IMT) and ankle brachial index, and both found a significant association, suggesting that there is an association between VWF and atherosclerosis (69, 70). Previous animal studies have shown less formation of atherosclerosis in VWF deficient mice and pigs (71-73). These studies suggested that VWF is causally involved in the formation of atherosclerosis. Controversially, studies in humans with Von Willebrand disease (VWD) type 3, characterized by a total deficiency of VWF, have shown a similar extent of atherosclerotic lesions compared with healthy individuals (74-76). This suggests that in humans there is no causal role for VWF in the development of atherosclerosis. However, VWF increase due to endothelial dysfunction accompanying atherosclerosis may lead to increased thrombus formation and an increased risk of cardiovascular events. However, further studies are needed to establish this association using more accurate, objective measurements of atherosclerosis.

Aim and outline of this thesis

The aim of this thesis is to obtain more insight in the role of VWF and ADAMTS13 in the pathogenesis of cardiovascular disease, including ischemic stroke and myocardial infarction. In the first part of this thesis we will study the association between VWF and atherosclerosis and cardiovascular disease. The second part of the thesis will focus on the association between ADAMTS13 and cardiovascular disease.

First, we will review the current literature on the association between VWF, ADAMTS13 and cardiovascular disease by performing a systematic review and meta-analysis (chapter 2).

In the first part of this thesis the association between atherosclerosis and VWF levels is studied. First, we will study the association in the Erasmus Stroke Study, which includes consecutive patients suffering from ischemic stroke and Transient Ischemic Attack (TIA) referred to the Erasmus University Medical Center (chapter 3). In these patients we will measure the calcification volume as a marker of atherosclerosis. Additionally, we investigate the association between VWF levels and outcome of stroke in these patients. In chapter 4 we investigate the association between coronary atherosclerosis and VWF levels. The association between atherosclerosis and VWF will be studied in patients with either an acute coronary syndrome or a stable angina pectoris. Atherosclerosis will be measured by plaque burden using intravascular ultrasound (IVUS) at the moment of coronary catheterization. We will also investigate the association between VWF levels and outcome after 1 year. Lastly, different markers of atherosclerosis will be measured in the ParisK study. This study investigate the association between carotid atherosclerosis and VWF levels and ADAMTS13 activity to obtain more insight in this association. The Parisk study is a multicenter prospective study in which all ischemic stroke and TIA patients with a mild to moderate (30-69%) carotid artery stenosis were included. These patients have a high risk of recurrence and do not have beneficial effect of carotid endarterectomy, with respect to patients with a carotid artery stenosis of 70% and more. Atherosclerosis is measured by different markers including calcification volume, the presence of ulcerations, and intraplaque hemorrhage. We studied whether these markers were associated with VWF levels and ADAMTS13 activity (chapter 5).

Next, we investigate in a large population based cohort study, the Rotterdam Study, the association between ADAMTS13 activity and both stroke (chapter 6) and coronary heart disease (chapter 7). With these studies the association between ADAMTS13 and cardiovascular disease will be assessed. The hypothesis is that low ADAMTS13 is associated with an increased risk of stroke and myocardial infarction and may be used to predict the risk of these diseases in healthy elderly individuals. In the Rotterdam Study, we will also investigate the association between VWF levels, ADAMTS13 activity and the risk of all-cause and cardiovascular mortality (chapter 8).

Subsequently, we investigate determinants of the secretion mechanism of VWF to determine factors which can influence VWF levels. We will study the effect of important mediators, including lifestyle factors, environmental factors and common genetic variations in *STXBP5, STX* and VWF on the release of VWF by endothelial cell activation, known to be associated with the increase of VWF levels during heavy exercise in healthy individuals (chapter 9).

In the last chapter (chapter 10), we will summarize and discuss the most important findings of this thesis. Also, the possible clinical implications and future perspectives are discussed.

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2

VON WILLEBRAND FACTOR AND ADAMTS13 IN ARTERIAL THROMBOSIS: A SYSTEMATIC REVIEW AND META-ANALYSIS

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ABSTRACT

Von Willebrand Factor (VWF) plays an important role in hemostasis by mediating platelet adhesion and aggregation. Ultralarge VWF multimers are cleaved by ADAMTS13 in smaller, less procoagulant forms. An association between high VWF levels and cardiovascular disease has frequently been reported, and more recently also an association has been observed between low ADAMTS13 levels and arterial thrombosis. We reviewed the current literature and performed meta-analyses on the relationship between both VWF and ADAMTS13 with arterial thrombosis. Most studies showed an association between high VWF levels and arterial thrombosis. It remains unclear whether ADAMTS13 is a causal independent risk factor because the association between low ADAMTS13 and arterial thrombosis is so far only shown in case-control studies. Prospective studies are awaited. A causal role for ADAMTS13 is supported by mice studies of cerebral infarction where the infusion of recombinant human ADAMTS13 reduced the infarct size.

1. INTRODUCTION

Arterial thrombosis, specifically coronary heart disease and ischemic stroke, is associated with a high mortality and morbidity in the Western World. Multiple risk factors for arterial thrombosis are known, including the classical risk factors hypertension, diabetes and obesity. Many studies have shown that the variation in coagulation factors may also confer a higher risk of arterial thrombosis (1-4). Previous studies have suggested a role for Von Willebrand Factor (VWF) in the pathogenesis of arterial thrombosis. VWF, a large multimeric glycoprotein, has an important function in primary hemostasis. It facilitates platelet adhesion and aggregation by interacting with the GPIb receptor on platelets and it is a carrier protein of factor VIII, thereby protecting factor VIII from proteolytic degradation (5). ADAMTS13, *A Disintegrin and Metalloprotease with TrompoSpondin motif repeats 13,* cleaves ultralarge very active VWF multimers into smaller, less procoagulant forms (6, 7). Hypothetically, lower levels of ADAMTS13 are associated with increased VWF activity and can thereby contribute to arterial thrombosis. Many studies have focused on the role of VWF in the pathogenesis of arterial thrombosis, both on the occurrence of the first event and on recurrence. A limited number have been published on the role of ADAMTS13 in arterial thrombosis yet. In this review we summarize the current literature on VWF and ADAMTS13 in arterial thrombosis. We additionally present meta-analyses of the published studies on this research topic.

2. METHODS

2.1 Search strategy and selection criteria

Medline and Embase were used to search literature till October 1st 2013. The following search was used:

('von Willebrand Factor'/de OR 'Von Willebrand Factor cleaving proteinase'/de OR ('Willebrand Factor' OR ADAMTS13 OR 'ADAMTS 13'):ab,ti) AND ('Brain ischemia'/exp OR (stroke* OR CVA OR ((brain OR cerebr* OR neural) NEAR/4 (ischem* OR ischaem* OR accident* OR circulat* OR flow*))):ab,ti OR 'Ischemic heart disease'/exp OR (((myocardial OR heart OR cardia* OR cardio*) NEAR/4 (ischem* OR ischaem* OR infarct*)) OR (coronary NEAR/4 (syndrome* OR disease* OR obstruct* OR occlusi* OR stenos* OR thromb* OR atherosclero*)) OR angina):ab,ti).

The search strategy was restricted to published data and the English language. In total, 2656 citations were found of which titles and abstracts were screened. Potentially suitable studies were read in full text. Studies were selected when the following criteria were met: 1) prospective cohort studies or case-control studies; 2) association between VWF or ADAMTS13 and myocardial infarction, coronary heart disease, (unstable) angina pectoris or ischemic stroke.

2.2 Statistical analysis

Odds ratios were presented in the forest plots using random effects models. The overall effects were determined using the Z-test. P-values <0.05 were considered statistically significant and 95% confidence intervals were given. Statistical heterogeneity between studies was determined by a Tau^2 test, a Chi^2 test and I^2 statistics. Log odds ratios and standard errors were calculated of all studies based on the odds ratio of the highest VWF/lowest ADAMTS13 group compared with the lowest VWF / highest ADAMTS13 group. Some studies had slightly differences in odds ratios after calculating using the log odds ratio and SE. Only studies which gave an odds ratio were included in the meta-analyses. All analyses were performed using Review Manager version 5.2 (The Nordic Cochrane Center, Copenhagen, Denmark).

3. Von Willebrand Factor (VWF)

Von Willebrand Factor (VWF) is a multimeric glycoprotein that plays a crucial role in primary hemostasis. At moments of vascular injury or high shear conditions, VWF acts as a bridging molecule for platelets, resulting in platelet adhesion and aggregation, the initial steps in thrombus formation (5). VWF also acts as a carrier protein for coagulation factor VIII, thereby preventing factor VIII from proteolytic degradation (8, 9). The importance of VWF is highlighted in von Willebrand's disease, which is the most common inherited bleeding disorder, characterized by a deficiency of VWF or qualitative abnormal VWF, resulting in various, mainly mucocutaneous, bleeding symptoms (10).

The VWF gene is located on chromosome 12 and is composed of 178 kilobases (kb) and 52 exons (11-14). The VWF plasma concentration varies widely and is determined by multiple factors, including age, pregnancy and stress. Sixty percent of the VWF level variability is accounted by genetic variation, of which ABO blood group has the largest effect and explains around 25%. Individuals with blood group O have an increased clearance of VWF, resulting in lower VWF levels than individuals with non-O blood groups (15), because the sugar groups that VWF shares with the red blood cells in blood group A and B individuals, protects VWF from clearance (16).

VWF is synthesized mainly in endothelial cells and also to some extent in alpha-granules of megakaryocytes and stored in Weibel-Palade bodies in the endothelial cells. VWF undergoes a process of dimerization and multimerization (5, 17) and the biological activity of VWF depends largely on its multimer size; the larger the multimer size, the more active the VWF molecule. VWF multimers, released from the Weibel-Palade bodies by agonists such as thrombin, epinephrine and collagen, are larger than those are normally present in plasma. Circulating VWF is almost completely of endothelial origin and in case of endothelial damage, plasma levels of VWF increase. Therefore VWF is considered as a marker of endothelial damage or dysfunction (18). VWF mulitmers are cleaved in circulation by ADAMTS13 (A Disintegrin and Metalloprotease with ThromboSpondin Motif repeats 13), in the A2 domain between residues Y1605 and M1606, which converts ultralarge VWF multimers to smaller, less procoagulant forms (6, 7).

3.1 VWF and arterial thrombosis

3.1.1 VWF and coronary heart disease

As VWF plays an important role in thrombus formation, several studies have been performed to assess the relationship between VWF and myocardial infarction and coronary heart disease. Two meta-analyses have been published previously on the association between VWF and coronary heart disease, including more than 15 prospective cohort studies consisting of 899 and 6556 cases, respectively (19, 20). Both analyses showed a significant association (OR 1.5 95% CI 1.1 – 2.0 and OR 1.16 95% CI 1.10 – 1.22) (19, 20). In Table 1 we summarized all prospective cohort studies, of which most were included in these meta-analyses. A number

of these studies found a positive association between VWF and coronary heart disease (1, 19-30), including the large prospective ARIC study that included 1802 coronary heart disease cases aged 45 – 64 years (30). This was also observed in many case-control studies, listed in table 2 (3, 31-49), suggesting a pathogenic role for VWF in coronary heart disease. However, there are doubts whether VWF is causal or mainly a marker of endothelial damage. Some other prospective cohort and case-control studies did not find this association (Table 1, 2) (4, 50-69). This might be due to differences in adjustment for a variety of possible confounders. In some studies the observed associations between VWF and coronary heart disease disappeared after adjustment for C-reactive protein (CRP) or factor VIII (1, 21, 23, 29, 30, 56, 57, 69). This may suggest that the association between VWF and arterial thrombosis may be driven by other conditions known to increase VWF levels, such as inflammation. Because VWF levels are increased during the acute phase of an event due to endothelial dysfunction or acute phase response (70, 71), the moment of blood sampling will influence the results and this needs to be controlled. Especially in the case-control studies, the time between the event and the blood sampling should be at least several months. One study measured VWF levels both in the acute phase and after reconvalescence and only observed a significant difference in VWF levels between MI patients and controls in the acute phase of the event (42). Furthermore, most studies have measured VWF:antigen (VWF:Ag) levels as representative of the VWF plasma levels. However, it might be possible that not the concentration of VWF is associated with coronary heart disease, but rather the functional activity of VWF, however this has hardly been studied so far.

Although studies on the association between VWF and coronary heart disease have been different in design and population and therefore difficult to compare, most studies found a positive association. Therefore, the data allow the conclusion that high VWF levels are associated with an increased risk of coronary heart disease.

Several studies have studied the association between VWF levels and cardiovascular outcome, which in most studies was defined as cardiovascular mortality. High VWF levels were associated with cardiovascular outcome (4, 23, 24, 34, 47, 50, 72-75), although others could not confirm these findings (59, 60, 63, 76). Whincup et al. have shown in a meta-analysis that in patients with a pre-existent vascular disease that high VWF levels are associated with recurrent coronary heart disease and cardiovascular mortality (OR 1.6 95% CI 1.0 - 2.5) (19).

3.1.2 VWF and ischemic stroke

Coronary heart disease and ischemic stroke have several risk factors in common and because VWF is associated with CHD, it has been hypothesized that VWF may also be associated with the risk of ischemic stroke. In table 3, we summarized the prospective cohort studies and in table 4 the case-control studies. An association between high VWF levels and ischemic stroke has been found in most studies (2, 25, 30, 33, 77-92). However, two recent studies did not observe an association between VWF levels and subclinical cerebral infarction on MRI

(93, 94). The fact that not all studies observed an association between VWF and ischemic stroke (4, 48, 51, 53, 60, 95-97) might be explained by a lack of power of some studies due to small numbers of patients. We performed a meta-analysis of all prospective cohort studies, consisting of 1567 cases, on the association between VWF and ischemic stroke and found an odds ratio of 1.17 [95% CI 1.08 – 1.26] (Figure 1). This clearly indicates a positive association between high VWF levels and ischemic stroke. In addition, if we included the case-control studies, of in total 2532 patients, in the meta-analysis we found an OR of 1.55 (95% CI 1.31 – 1.83) (data not shown).

The origin of ischemic stroke is very diverse and can be classified into different subtypes based on the etiology using the widely used TOAST or ASCO classification (98, 99). Large artery atherosclerosis subtype of ischemic stroke is classified as ≥ 70% atherosclerosis in the carotid arteries and has been associated with higher levels of VWF in several studies (83, 84, 88, 100, 101). However, ischemic stroke due to cardio-embolism and lacunar stroke, which are not associated with atherosclerosis, were also associated with VWF levels (84, 86, 100). Most studies even found an association between high VWF levels and ischemic stroke mortality (72, 78, 80, 89, 92, 102). In the study by Lip et al., VWF levels were found to be significantly higher in ischemic stroke patients who died, when compared with patients who were still alive at 12 months follow-up (81).

In conclusion, the studies performed so far, indicate that VWF levels are associated with ischemic stroke and with subtypes of ischemic stroke, mainly with large artery atherosclerosis. In addition, VWF levels are also associated with outcome of ischemic stroke.

3.2 VWF deficiency and atherosclerosis in animals and humans

An important role of VWF in the development of atherosclerosis has been shown in animal studies using VWF-deficient pigs and mice. VWF knock-out animals showed less atherosclerosis compared with wild type animals (103-106). These studies suggest a causal role for VWF in the formation of atherosclerosis. However, other animal studies showed contradictory results, which has been attributed to the influence of a polymorphism in Apolipoprotein B100, which has a major role in determining the severity of diet-induced hypercholesterolemia and thereby results in a higher degree of atherosclerosis (106, 107). Despite the causal role for VWF suggested in animal models, human studies on the pathogenesis of VWF in atherosclerosis are limited. Several studies in individuals with severe von Willebrand disease (VWD), characterized by a deficiency of VWF in the circulation, did not observe reduced atherosclerosis (108-110). In patients with VWD type 3, characterized by a total deficiency of VWF, the extent of atherosclerosis was similar to that in controls (111). This suggests that VWF does not have an important causal role in the formation of atherosclerosis in humans. Recently, a lower prevalence of myocardial infarction and ischemic stroke was observed in a large group of moderate and severe adult VWD patients (N=635) compared with control populations (112), which stresses the importance of VWF in atherothrombosis, which may be caused by less thrombus formation.

4. ADAMTS13

Von Willebrand factor (VWF) cleavage protease was discovered simultaneously by Furlan et al and Tsai in 1996 (113, 114). A few years later, the gene was cloned and the protease was discovered as part of the family of *A Disintegrin and Metalloprotease with TromboSpondin motif* and was designated as ADAMTS13 (7, 115, 116). The ADAMTS13 gene is located on chromosome 9q34 and is composed of 37 kb, spans 29 exons and encodes a protein with 1427 amino acids (117). So far, the only known physiological function of ADAMTS13 is the cleavage of ultralarge VWF multimers into smaller, less procoagulant multimers (6). The cleavage occurs at a single site of the VWF molecule, the Tyr1605-Met1606 bond within the A2 domain (118). ADAMTS13 is mainly produced by the liver (119), although it can also be synthesized by vascular endothelial cells (120). The clinical importance of ADAMTS13 is exemplified by the potentially fatal thrombotic disorder Thrombotic Thrombocytopenic Purpura (TTP). TTP is characterized by a severe deficiency of ADAMTS13, which leads to the characteristic hallmarks of the disease, including micro-angiopathic hemolytic anemia, profound thrombocytopenia, renal dysfunction and neurologic deficits by micro-vascular thrombotic complications (121). In addition, TTP patients regularly develop ischemic stroke and acute myocardial infarction. More recent studies have indicated that ADAMTS13 may also be involved in the pathogenesis of inflammation and determine outcome of other disease states such as severe sepsis (122-126).

4.1 ADAMTS13 assays

Several assays to measure ADAMTS13 activity or antigen levels in plasma have been developed. The first activity assays were based on the loss of collagen binding when VWF is cleaved by ADAMTS13 (127). A disadvantage of this test is the high analytical coefficient of variation. Furthermore, the original test was very elaborate, time-consuming and measures ADAMTS13 under non-physiological conditions. Several ADAMTS13 assays have been developed since then. Antigen assays (ELISAs) have become available using different antibodies (128-130). In 2005, a test based on the FRETS (Fluorescence Resonance Energy Transfer Substrate) principle was developed, which uses a VWF peptide containing the ADAMTS13 cleavage site (131). An advantage of this test is the short duration of less than 4 hours. The disadvantage may be that it uses a peptide as ADAMTS13 substrate and not the native VWF protein. However, the activity tests have been extensively compared and give similar test results in healthy controls and TTP patients (132, 133). The FRETs assay is the most widely used nowadays.

4.2 ADAMTS13 in congenital and acquired TTP

Congenital ADAMTS13 deficiency was first demonstrated in 1998 during episodes of TTP, mostly preceded by infectious periods or other stressful situations (134). In several of these individuals, mutations in the ADAMTS13 gene have been described in the last years

(117, 135-137). Treatment consists of regular infusions of fresh frozen plasma to maintain ADAMTS13 levels to prevent TTP exacerbations. In the future it may be treated by infusion of recombinant ADAMTS13, as it has been shown to be effective in mice models to treat TTP and (sporadically) in humans (138, 139).

Acquired TTP was first reported in 1952 by Moschcowitz (140). Acquired ADAMTS13 deficiency is due to the formation of auto-antibodies against ADAMTS13 (141). Acquired TTP is characterized by a classical pentad of thrombocytopenia, micro-angiopathic hemolytic anemia, fever, neurological symptoms and renal insufficiency (121). This is caused by the presence of ultra large (UL)VWF multimers, due to the absence of ADAMTS13-induced VWF cleavage, that are highly active and spontaneously form platelet aggregates and microthrombi.

4.3 ADAMTS13 and arterial thrombosis

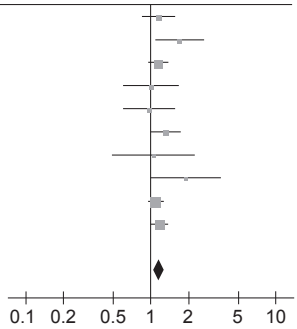
4.3.1. ADAMTS13 and coronary heart disease

Because strongly reduced levels or absence of ADAMTS13 is associated with the occurrence of microangiopathies in TTP and because high VWF levels are associated with arterial thrombosis, it has been hypothesized that a reduction of ADAMTS13 levels increases the risk of arterial thrombosis. So far only eight case-control studies have been performed of which four showed a significant association between low levels of ADAMTS13 and risk of myocardial infarction (MI) or coronary heart disease (Table 5) (33, 41, 46, 142). Remarkably, one study in elderly men found exactly the opposite, as high levels of ADAMTS13 were associated with a significant increased risk of MI (31). Others did not find a significant difference in ADAMTS13 levels between MI patients and controls (32, 42, 143). We performed a meta-analysis of all studies which included cases with a first MI or coronary heart disease (N = 1578) and provided an odds ratio (31-33, 41, 46). We did not find a significant association between ADAMTS13 levels and myocardial infarction or coronary heart disease (OR 1.45, 95% CI 0.71-2.98) (figure 2). We found a high odds ratio but a wide confidence interval, suggesting a lack of power to find statistical significance. This suggests that there is no association between ADAMTS13 and coronary heart disease. Three other studies have shown that ADAMTS13 was associated with the recurrence of cardiovascular events in patients with a prevalent coronary heart disease (40, 45, 61). The role of ADAMTS13 in coronary heart disease was found to be independent of VWF. Individuals with high VWF levels and low ADAMTS13 levels confer the highest risk of arterial thrombosis (33), which suggests that ADAMTS13 and VWF are independent risk factors.

In conclusion, several studies suggested an association between low ADAMTS13 levels and AMI or CHD that is independent of VWF levels, although the studies performed did not include sufficient numbers of patients. Another problem is that all published studies have a case-control design, therefore prospective studies are needed to definitely assess the role for ADAMTS13 levels in AMI or coronary heart disease.

Figure 1. Forest plot of the association between VWF and iscemic stroke

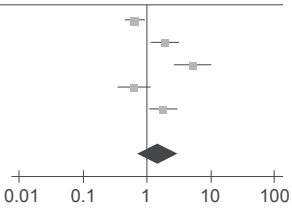
Study or Subgroup	Cases Total	Controls Total	Odds Ratio IV, Random, 95%	Odds Ratio IV, Random, 95% CI
Smits 1997	45	1174	1.15 [0.85, 1.55]	
Folsom 1999	191	14522	1.71 [1.09, 2.68]	
Knuiman 2001	159	1234	1.15 [0.96, 1.37]	
Johansson 2002	87	216	1.01 [0.61, 1.68]	
Smith 2005	133	2090	0.97 [0.61, 1.55]	
Tzoulaki 2007	168	177	1.33 [1.02, 1.73]	
Carcaillon 2009	111	1055	1.06 [0.49, 2.28]	
Gottesman 2009	196	214	1.90 [1.00, 3.61]	
Wieberdink 2010	290	5960	1.10 [0.96, 1.26]	
Wannamethee 2012	187	3171	1.18 [1.02, 1.36]	
Total 95% CI)	**1567**	**29813**	**1.17 [1.08, 1.26]**	



Heterogeneity: $Tau^2 = 0.00$; $Chi^2 = 7.65$, df = 9 (P = 0.57); $I^2 = 0\%$
Test for overall effect: Z = 4.03 (P < 0.0001)

Figure 2. Forest plot of the association between ADAMTS13 and coronary heart disease

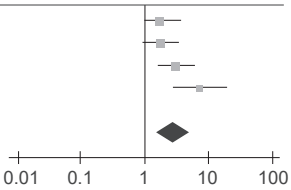
Study or Subgroup	Cases Total	Controls Total	Odds Ratio IV, Random, 95%	Odds Ratio IV, Random, 95% CI
Chion 2007	551	635	0.64 [0.45, 0.91]	
Crewley 2007	488	484	1.89 [1.14, 3.12]	
Bongers 2009	218	332	5.20 [2.67, 10.13]	
Peyvandi 2010	138	199	0.63 [0.35, 1.12]	
Andersson 2011	205	638	1.80 [1.10, 2.94]	
Total 95% CI)	**1578**	**2288**	**1.45 [0.71, 2.98]**	



Heterogeneity; $Tau^2 = 0.60$; $Chi^2 = 41.31$, df = 4 (P < 0.00001); $I^2 = 90\%$
Test for overall effect: Z = 1.01 (P = 0.31)

Figure 3. Forest plot of the association between ADAMTS13 and ischemic stroke

Study or Subgroup	Cases Total	Controls Total	Odds Ratio IV, Random, 95% CI	Odds Ratio IV, Random, 95% CI
Bongers 2006	124	125	1.70 [0.74, 3.90]	
Bongers 2009	109	332	1.76 [0.90, 3.43]	
Andersson 2011	175	638	3.10 [1.60, 6.00]	
Lambers 2013	20 8	12 5	7.30 [2.74, 19.45]	
Total (95% CI)	**616**	**1220**	**2.72 [1.52, 4.86]**	



Heterogeneity: $Tau^2 = 0.20$; $Chi^2 = 6.86$, df = 3 (P < 0.08); $I^2 = 56\%$
Test for overall effect: Z = 3.36 (P = 0.0008)

4.3.2. ADAMTS13 and Ischemic Stroke

Only a few studies have been performed on the association between ADAMTS13 levels and ischemic stroke (Table 6) (33, 46, 85, 144). Of those studies, two found a significant increased risk of ischemic stroke in individuals with low ADAMTS13 levels (33, 144) and the other two studies had similar risk estimates, but did not reach statistical significance (46, 85), probably because of a lack of power due to small number of patients (46, 85). We performed a meta-analysis of these studies, consisting of 616 cases, and showed a clear association between ADAMTS13 and ischemic stroke (OR 2.72, 95% CI 1.52 – 4.86) (Figure 3), indicating that low ADAMTS13 levels are associated with an increased risk of ischemic stroke. An association between stroke subtype and ADAMTS13 levels could not be observed in a pediatric cohort study (144), but also in this study only a small number of patients were included. Taken together, all studies on the association between ADAMTS13 and ischemic stroke have limited numbers of patients and a case-control design and therefore it is difficult to draw conclusions from these studies, although our meta-analysis showed a significant association. Prospective cohort studies are needed to definitely confirm the role of ADAMTS13 in the pathogenesis of ischemic stroke.

4.3.3. ADAMTS13 and Atrial Fibrillation (AF)

Atrial fibrillation (AF) is the most common cardiac arrhythmia and is associated with an increased risk of stroke (145). There have been a limited number of studies performed investigating the role of ADAMTS13 in atrial fibrillation and the risk of cardiovascular events such as ischemic stroke. A study by Uemura et. al showed significant lower ADAMTS13 levels in AF patients compared with controls (146), which could not be confirmed by another study (147). Furthermore, low ADAMTS13 levels were found to be a predictor of major cardiovascular events in patients with AF, although these levels were not associated with all-cause mortality (76).

4.5 ADAMTS13 and atherosclerosis in mouse models

Studies in ADAMTS13 deficient mice showed more extensive atherosclerosis and plaque formation in the major vessels compared with wild type mice (148, 149). Others have shown that inducing cerebral or myocardial ischemia in ADAMTS13 deficient mice results in larger infarctions compared with wild type mice (150-155). Infusion of recombinant human ADAMTS13 (rhADAMTS13) after induced focal cerebral or myocardial ischemia, reduced the infarct size and improved the functional outcome without producing more bleeding symptoms (150, 152, 155). Additionally, ADAMTS13 deficient mice have more leukocytes and macrophages infiltration compared with wild type mice suggesting a role for ADAMTS13 in inflammation (148, 149, 151-154, 156). Although human studies showed that VWF and ADAMTS13 are independent risk factors, ADAMTS13 deficient and ADAMTS13/VWF deficient mice showed similar reduction of infarct size and inflammatory parameters,

suggesting that the role of ADAMTS13 on ischemia and inflammation is mediated via VWF (150, 153, 154, 156). Infusion of recombinant ADAMTS13 after occlusion may result in smaller VWF multimers and less platelet aggregation and thrombosis. In conclusion, previous mice studies showed a causal role for ADAMTS13 in myocardial and cerebral ischemia and atherosclerosis and infusion of rhADAMTS13 reduces infarct size.

5. Possible mechanism for the role of VWF and ADAMTS13 in arterial thrombosis

The mechanism by which VWF and ADAMTS13 lead to myocardial infarction and ischemic stroke is not yet known. It has been hypothesized that there might be a role for both these factors in atherosclerosis, as previous mouse models have shown more atherosclerosis in ADAMTS13 and less atherosclerosis in VWF deficient mice (148, 149). We and others have shown that the degree of measured atherosclerosis in humans is strongly associated with VWF levels. Our recent study has shown a positive association between well-defined atherosclerosis, measured by the calcification volume in the carotid arteries and aortic arch, and VWF in ischemic stroke patients possibly related to endothelial damage (101). However, another study found no association between VWF levels and the intima media thickness (IMT), in healthy individuals (30). As VWF seems to play a role in both ischemic stroke and myocardial infarction and was found to be associated with large artery atherosclerosis, it may be suggested that VWF is a marker of atherosclerosis and/or inflammation and not a risk factor in itself. A causal role of VWF in cardiovascular disease is challenged by the fact that, despite the strong effect of ABO blood group on VWF levels, the role of ABO blood group in arterial thrombosis is weak and not consistent (157, 158). On the other hand, we and others have seen that genetic variations in the VWF and ADAMTS13 gene and other related genes, that are associated with VWF and ADAMTS13 levels, are sometimes related with arterial thrombosis (159), but not always, as we have recently reviewed (54, 159-161). This suggests that there is no causal role for VWF in the development of atherosclerosis, but once atherosclerosis has developed this is associated with increased VWF levels and these levels may contribute to thrombus formation and arterial thrombosis.

6. Conclusion

In the last few years many studies have investigated the association between both VWF levels and ADAMTS13 levels and the risk of arterial thrombosis. Most previous prospective cohort studies and meta-analyses of more than 6500 cases have shown that high VWF levels are associated with an increased risk of myocardial infarction. We also confirmed this in a meta-analysis for ischemic stroke. An association between ADAMTS13 and arterial thrombosis has been shown in a few small cross-sectional and case-control studies, therefore it remains unclear whether ADAMTS13 is an independent risk factor. Our current meta-analysis of the association between ADAMTS13 and ischemic stroke showed a highly significant OR of 2.72 (95% CI 1.52 – 4.86) for low levels of ADAMTS13. This result is in accordance with

mice studies that show that ADAMTS13 deficient mice have a higher risk of myocardial or cerebral ischemia and have shown a promising role for rhADAMTS13 as therapeutic option (148-155).

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Table 1. Prospective cohort studies on the association between Von Willebrand Factor and coronary heart disease (CHD) or myocardial infarction (MI)

Study	N	Mean follow-up time	VWF level (IU/ml) in cases	**VWF level (IU/ml) in controls**	**P-value**	**Disease endpoint OR [95% CI]**
ARIC study (30)	14904	n.a.	White men: 1.22 ± 2 Black men: 1.34 ± 4 White women: 1.15 ± 2 Black women 1.39 ± 3	White men: 1.11 ± 1 Black men: 1.30 ± 2 White women: 1.11 ± 1 Black women 1.34 ± 1	White men: P<0.05 White women: P<0.05	Men: 1.12 P<0.05 Women: 1.02, NS
NPHS (50)	1393	16.1 years	0.77 (0.71 - 0.84)	0.75 (0.73 - 0.77)	NS	Fatal IHD: 1.34 [1.00 - 1.79] Non fatal IHD: 0.89 [0.59 - 1.33] Total IHD: 1.16 [0.92 - 1.47]
ARIC study (1)	14477	4-7 years	n.a.	n.a.	n.a.	RR men 1.05 [0.91-1.20] RR women 1.02 [0.84-1.25]
Edinburgh artery study (4)	1592	5 years	1.15 (0.82 - 1.56)	1.05 (0.78 - 1.38)	P<0.05	MI: 0.95 [0.81 - 1.12] AP: 1.00 [0.80 - 1.25]
VIP and MONICA study (57)	234	n.a.	Men: 1.42 Women 1.62	Men: 1.31 Women: 1.34	NS P=0.044	2.58 [0.87 - 7.63]
Caerphilly heart study (21)	1997	61 months	1.28 ± 0.44	1.19 ± 0.42	P=0.048	1.20 [1.01 - 1.43]
British regional heart study (19)	1891	n.a.	1.21 ± 0.43	1.13 ± 0.44	P=0.0003	1.53 [1.10 - 2.12]
Reykjavik study (29)	6428	17.5 years	n.a.	n.a.	n.a.	1.11 [0.97 - 1.27]
Caerphilly study (51)	2223	13.4 years	n.a.	n.a.	n.a.	HR 1.09 [0.83 - 1.45]
PRIME study (22)	296	n.a.	n.a.	n.a.	n.a.	HR 1.34 [1.07 - 1.67]
BWHHS (52)	3582	4.7 years	1.44 (1.16 - 1.78)	1.39 (1.12 - 1.75)	P=0.21	HR 0.97 [0.69 - 1.37]

Edinburgh artery study (25)	1592	17 years	CVD: 1.15 (0.87-1.48) MI: 1.16 (0.87-1.55)	CVD: 1.05 (0.78-1.37) MI: 1.05 (0.78-1.37)	P<0.001 P<0.001	1.33 [1.02 – 1.74]
Fletcher challenge study (56)	720	n.a.	1.40 ± 0.61	1.27 ± 0.52	n.a.	1.24 [0.56 - 2.73]
PRIME study (28)	AP: 321 ACS: 486	5 years	AP: 1.11 (0.85-1.47) ACS: 1.17 (0.91 - 1.50)	AP:1.16 (0.88 - 1.41) ACS: 1.14 (0.84 - 1.40)	P=0.71 P=0.005	ACS: 2.43 [1.31 - 4.51] AP: 0.87 [0.43 - 1.79]
British regional heart study (23)	3217	7 years	MI: 1.46 AP: 1.38	MI: 1.37 AP: 1.37	P=0.005 P=0.87	MI: 1.24 [0.87 - 1.76] AP: 1.06 [0.78 - 1.45] HR 1.19 [1.04 - 1.37]
Three City study (3C) (53)	1254	4 years	1.44 (1.19 - 1.67)	1.30 (1.10 - 1.58)	P=0.046	CHD: 1.58 [0.29 - 8.57] MI: 1.52 [0.75 - 3.10]
ARIC study (24)	14009	12 years	incidence rate: SCD: 19.8 (16.2 - 23.4) NSCD: 14.9 (11.8 - 18) MI: 51.1 (45.5 - 56.8)	n.a.	n.a.	SCD: 3.34 [2.26 - 4.93] NSCD: 2.11 [1.40 - 3.19] MI: 1.40 [1.17 - 1.67]
WHI-HT (27)	1064	7.1 and 5.6 years	0.99 (0.73 - 1.39)	0.90 (0.66 - 1.19)	P<0.0001	1.21 [1.02 - 1.43]
Rotterdam Study (54)	5801	6.4 years	1.42 ± 0.8	1.30 ± 0.6	n.a.	HR 1.39 [0.98 - 1.76]
Rotterdam Study (55)	5933	n.a.	n.a.	n.a.	n.a.	HR 1.2 [0.9 - 1.6]
VIP and MONICA study (26)	1364	n.a.	1.60 (1.27 - 2.03)	1.41 (1.13 - 1.79)	P<0.001	2.52 [1.72 - 3.67]
Reykjavik study (20)	5541	19.4 years	n.a.	n.a.	n.a.	1.08 [1.02 - 1.15]

Table 2. Case-control studies on the association between Von Willebrand Factor and coronary heart disease (CHD) or myocardial infarction (MI)

Participants	N total (% cases)	Blood sampling after event	Mean follow-up time	VWF level (IU/ml) in cases	VWF level (IU/ml) in controls	P-value	Disease endpoint OR [95% CI]
First ever MI (31)	1186 (46)	6 months		1.38 ± 0.51	1.35 ± 0.66	NS	1.44 [1.00 - 2.07]
First ever MI (69)	106 (47)	Acute and 8.4 years	8.4 years	Acute: 1.36 (1.15-1.91) Chronic: 1.89 (1.59-2.37)	Acute: 1.36 (0.97-1.67), NS Chronic: 1.72 (1.36-2.14)	significant	Acute: 0.99 [0.4 - 2.5] Chronic: 1.04 [0.4 - 2.6]
First ever MI (32)	138 (41)	0-3 days		38 nmol (23.5 - 53.6)	30.7 nmol (20.7 - 42.5)	P=0.002	2.0 [1.1 - 3.8]
First ever MI (33)	843 (24)	23-146		10.8 microg/ml	8.6 microg/ml	n.a.	4.2 [2.2 - 8.0]
MI (58)	281 (53)	3-6 months		Men: 1.14 ± 0.55 Women 1.62 ± 0.69	Men: 1.04 ± 0.41 Women 1.00 ± 0.35	NS P<0.01	n.a.
MI (34)	123 (37)	3 months after discharge	4.9 years	1.76 (1.56 - 1.96)	1.43 (1.31 - 1.55)	P<0.001	R 0.15 P<0.001
MI or SAP (3)	MI: 335 (5.7) SAP: 123 (9.8)	At inclusion and 12 months	2 years	MI: 1.73 (0.76 - 1.52) SAP: 1.54 (0.60-1.56)	MI: 1.24 (0.58 - 1.16) SAP: 1.27 (0.48-1.29)	P=0.004 P=0.026	MI: 1.68 [1.18 – 2.40] SAP: 1.78 [1.07-2.95]
MI or AP (35)	AMI: 46 (30) AP: 42 (24)	Within 8 hours		AMI: 1.80 ± 0.114 AP: 1.68 ± 0.099	1.02 ± 0.088	P<0.001	n.a.
MI (36)	111 (35)	6 weeks	46 months	1.36 ± 0.39	1.22 ± 0.36	P=0.001	n.a.
MI (63)	1045 (7.8)	2 months	26 months	1.58 ± 0.74	1.48 ± 0.68	NS	n.a.
MI (68)	194 (19)	n.a.	2 years	1.16 (0.58 - 2.24)	1.13 (0.47 - 3.30)	P=0.70	RR 1.4 [0.36 - 5.5]
MI (37)	347 (25)	3 months	2-3 years	Men: 1.69 ± 0.77 Women: 1.66 ± 0.43	Men: 1.34 ± 0.54 Women: 1.36 ± 0.48	n.a.	2.3 [1.3 - 4.0]
MI (61)	71 (42)	Acute		AMI: 2.15 ± 0.97	SEA: 1.45 ± 0.93 CPS: 1.43 ± 0.76	P<0.0001 P<0.0001	2.57 [0.88 - 7.51]
MI (38)	826 (24)	5 year		1.34	1.07	n.a.	4.7 [2.3 - 9.7]
MI or UAP (39)	AMI: 80 (71) UAP: 159 (86)	Acute		AMI: 1.39 ± 0.095 UAP: 1.15 ± 0.0456	0.95 ±0.095	P<0.05	n.a.
MI (40)	132 (70)	Admission, 1,3,7,14 days		n.a.	n.a.	P<0.0001	day 0: 1.11 [0.63 – 1.96] day 3: 1.46 [1.06 – 2.01]

MI (41)	950 (49)	3-9 months		1.86 ± 0.68	1.64 ± 0.60	P<0.0001	n.a.
MI (42)	56 (46)	At PCI and chronic phase		1.51 ± 0.58	2.05 ± 0.90	P<0.05	n.a.
MI (66)	237 (60)	3 months		1.13 (0.82 - 1.44)	1.04 (0.70 - 1.37)	P=0.032	1.004 [0.99 - 1.01]
MI (67)	142 (29)	3 months		1.20 ± 0.30	1.12 ± 0.32	P=0.242	0.79 [0.25 - 2.47]
MI or UAP (59)	840 (42)	1-3 months	4.2 years	1.19 ± 0.7	1.04 ± 0.4	n.a.	1.33 [0.21 - 8.23]
ACS or SAP (49)	ACS: 100 (54) SAP: 154 (70)	Acute		ACS: 1.77 ± 0.40 SAP: 1.43 ± 0.20	1.42 ± 0.80	P<0.05	n.a.
ACS (75)	156 (31)	Acute and 48 hours	30 days and 1 year	ACS: 2.15 ± 0.59 CAD: 1.47 ± 0.32	vs. 1.21 ± 0.20	n.a.	1.02 [1.00- 1.03] HR 14.4 P<0.001
ACS (62)	155 (74)	Acute		2.85 ± 1.27	1.05 ± 0.26	P<0.01	Beta 0.91 [0.81 - 1.03]
CHD (43)	791 (39)	n.a.		1.46 (1.20 - 1.68)	1.31 (1.10 - 1.46)	P=0.0002	1.6 [1.1 - 2.4]
CHD (44)	386 (50)	16 days and 6 months		1.59 (1.27 - 1.94)	1.28 (1.00 - 1.60)	P<0.001	P<0.001
CAD (64)	259 (61)	Within 12 hours		0.506 (0.501 - 0.512)	0.49 (0.483 - 0.497)	P<0.001	NS
CAD (65)	141 (73)	Before PTCA	13.2 months	0.679 (0.45-1.14)	0.689 (0.37 - 0.86)	P=0.58	2.5 [0.5 - 13.5]
CAD (45)	325 (69)	At angiography and 2-14 days	22.3 months	1.86 (1.43 - 2.39)	1.63 (1.33 - 2.15)	P=0.041	HR 3.15[1.93 - 5.14]
CVD (46)	550 (66)	1-3 months		1.20 (0.9 - 1.6)	1.04 (0.8 – 1.4)	P<0.001	2.1[1.3 – 3.3]
AF (47)	2806 (3.8)	n.a.	2 years	1.38 ± 0.49	1.25 ± 0.49	P=0.05	1.24 [1.00-1.53]
Nonvalvular AF (48)	994 (6.8)	At inclusion		n.a.	n.a.	n.a.	RR 2.5 [1.2 – 5.0]
Intermittent claudication (60)	363 (44)	n.a.	6-7 years	Total IHD: 1.43 (0.99-1.90) Fatal IHD: 1.51 (0.96 - 1.88)	1.13 (1.06 - 1.65)	P<0.05	1.04 [0.90 – 1.19]

Table 3. Prospective cohort studies on the association between Von Willebrand Factor and ischemic stroke

Study	N	Mean follow-up time	VWF level (IU/ml) in cases	VWf level (IU/ml) in controls	P-value	Disease endpoint OR (95% CI)
ARIC study (30)	14904	n.a.	White men: 1.22 ± 2 Black men: 1.34 ± 4 White women: 1.15 ± 2 Black women: 1.39 ± 3	White men: 1.11 ± 1 Black men: 1.30 ± 2 White women: 1.11 ± 1 Black women: 1.34 ± 1	White men: P<0.05 White women: P<0.05	Men: NS Women: 1.3, P<0.05
Edinburgh artery study (4)	1592	5 years	1.26 (0.93 - 1.52)	1.05 (0.78 - 1.38)	NS	RR 1.15 [0.85 - 1.57]
ARIC study (2)	14713	6-9 years	1.36	1.17	P<0.0001	1.71 [1.1 - 2.7]
ARIC study (94)	1393	n.a.	n.a.	n.a.	n.a.	1.15 [0.97 - 1.37]
VIP and MONICA study (96)	87	n.a.	1.49 ± 1.31	1.30 ± 0.87	n.a.	1.01 [0.61 - 1.67]
Caerphilly study (51)	2223	13.4 years	n.a.	n.a.	n.a.	HR 0.97 [0.61 - 1.56]
Edinburgh artery study (25)	1592	17 years	1.17 (0.87 - 1.57)	1.05 (0.78 - 1.37)	P<0.01	HR 1.33 [1.02 - 1.74]
Three City study (3C) (53)	1254	4 years	1.34 (1.09 - 1.58)	1.31 (1.09 - 1.58)	P=0.469	HR 1.06 [0.49 - 2.28]
ARIC study (93)	464	n.a.	1.48 (1.30 - 1.69)	1.33 (1.07 - 1.47)	P=0.01	1.9 [1.0 - 3.4]
Rotterdam Study (95)	6250	4 years	n.a.	n.a.	n.a.	HR 1.10 [0.95 - 1.26]
British regional heart study (92)	3358	9 years	1.50 ± 0.45	1.37 ± 0.51	P<0.0001	1.18 [1.02 - 1.38]

Table 4. Case-control studies on the association between Von Willebrand Factor and ischemic stroke

Participants	N total (% cases)	Blood sampling after event	Mean follow-up time
First ever ischemic stroke (88)	405 (49)	Within 7 days and 3-6 months	
First ever ischemic stroke (85)	249 (50)	7-14 days	
First ever ischemic stroke or TIA (82)	197 (48)	7-14 days	
First ever ischemic stroke or TIA (77)	549 (50)	7-14 days and 3 months	
First ever ischemic stroke (33)	813 (22)	23-146 months	
Ischemic stroke (79)	40 (48)	72 hours	
Ischemic stroke (80)	392 (53)	Within 10 days and 3-4 months	6 months or till death
Ischemic stroke (78)	196 (83)	n.a.	
Ischemic stroke (91)	114 (65)	3-10 days and 1-3 months	
Ischemic stroke (83)	138 (60)	Within 48 hours and after 1 months	
Ischemic stroke (81)	121 (71)	At admission, 48hr, 1 week, 2 weeks, 3 and 6 months	12 months
Ischemic stroke (90)	296 (28)	Acute	
Ischemic stroke (86)	243 (49)	n.a.	
Ischemic stroke (84)	1200 (50)	Within 10 days and 3 months	
TIA or cerebral infarct or cerebral embolism (87)	CVA: 68 (50) TIA: 22 (50) CE: 18 (50)	n.a.	
TIA or minor ischemic stroke (97)	331 (29)	At least 4 weeks	
Non-valvular AF (48)	994 (3.9)	At inclusion	
Non-valvular AF with ischemic stroke (102)	91 (67)	Within 48 hours and on day 7, 21 and 90	8.8 months
Non-valvular AF (89)	373 (13.7)	72 hours	3 years
Intermittent claudication (60)	282 (28)	n.a.	6-7 years

VWF level (IU/ml) in cases	VWF level (IU/ml) in controls	P-value	Disease endpoint OR (95% CI)
Baseline: 1.84 Chronic: 1.78	Baseline: 1.64 Chronic: 1.64,	P=0.17 P=0.21	2.8 [1.5 – 5.2]
1.47 ± 0.66	1.23 ± 0.5	P=0.002	3.2 [1.4 - 7.5]
VWF:Ag: 1.32 (0.98 - 1.85) VWF:Act: 1.2 (0.9 - 1.8)	VWF:Ag: 1.22 (0.84 - 1.56) VWF:Act: 1.3 (0.9 - 1.7)	P=0.04 NS	n.a.
1.47 ± 0.68 1.25 ± 0.56	1.25 ± 0.5	P=0.03 P<0.001	1.9 [1.0 - 3.3] 1.9 [1.1 – 3.1]
1.0	1.1	n.a.	6.7 [3.2 - 13.8]
1.85 ± 0.67	1.34 ± 0.50	P<0.05	n.a.
Acute: 1.86 (1.75 - 1.97) Chronic: 1.51 (1.42 - 1.60)	Acute: 1.26 (1.19 - 1.33) Chronic: 1.26 (1.19 - 1.33)	P=0.0001 P=0.0001	n.a.
1.44 ± 0.21	1.14 ± 0.16	P=0.0002	n.a.
Acute: 2.31 ± 0.90 Chronic: 1.98 ± 1.03	Acute: 1.41 ± 0.41 Chronic: 1.41 ± 0.41	P<0.001 P<0.01	n.a.
Acute: 1.78 (1.26 - 2.05) Chronic: 1.80 (1.55 - 2.15)	Acute: 1.33 (1.02 - 1.66) Chronic: 1.33 (1.02 - 1.66)	P<0.001 P<0.001	n.a.
1.51 ± 0.39	1.00 ± 0.28	P<0.0001	n.a.
n.a.	n.a.	n.a.	20.14 [6.61 - 85.39]
1.00 (0.66 - 1.00)	0.40 (0.30 - 0.80)	P=0.0001	n.a.
n.a.	n.a.	P<0.0001	Acute: 1.87 [1.54 - 2.27] Chronic: 1.36 [1.15 - 1.62]
CVA: 2.08 ± 0.59 TIA: 1.78 ± 0.95 CE: 1.03 ± 0.40	CVA: 0.998 ± 0.26 TIA: 0.99 ± 0.28 CE: 0.98 ± 0.26	P<0.001 P<0.02 NS	n.a.
1.37 ± 0.65	1.15 ± 0.57	P=0.004	1.42 [0.57 - 3.52]
n.a.	n.a.	n.a.	RR 2.3 [1.0-5.6]
0.97 ± 0.16	0.92 ± 0.14	P=0.202	n.a.
n.a.	n.a.	n.a.	3.69 [1.96 - 4.5]
1.26 (0.99 - 1.84)	1.31 (1.06 - 1.65)	NS	RR: 0.97 [0.78 - 1.21]

Table 5. Case-control studies on the association between ADAMTS13 and myocardial infarction

Participants	N total (% cases)	Blood sampling after event	ADAMTS13 assay	ADAMTS13 in cases	ADAMTS13 in controls	P-value	Disease endpoint OR [95% CI]
First ever MI (31)	1186 (46)	6 months	ELISA	101 (40 - 350)	100 (41 - 432)	P=0.99	1.56 [1.10 - 2.22]
First ever MI or UAP (46)	550 (40)	1-3 months	ELISA activity and antigen	74.5 (50.6 – 98.2)	97.4 (81.7 – 111)	P<0.001	5.20 [2.67 – 10.13]
First ever MI (32)	337 (41)	0-3 days	ELISA antigen	118 (97 - 142)	116 (96 - 140)	P=0.48	0.63 [0.35 – 1.12]
First ever MI (33)	843 (24)	23-146 months	ELISA antigen	100	110	n.a.	1.8 [1.1 - 3.0]
MI (61)	71 (58)	Acute	ELISA antigen, FRETS activity	76.8 ± 27	89.3 ± 27 93.6 ± 29	P=0.0014 P<0.0001	0.006 [5.9 x 10^{-4} - 0.591]
MI (41)	950 (49)	3-9 months	ELISA	111 ± 35.9	112.6 ± 32.6	P=0.36	0.53 [0.32 - 0.88]
MI (40)	132 (70)	Admission, 1,3,7,14 days	ELISA antigen	n.a.	n.a.	P<0.0001	day 0: 1.92 [0.16 - 21.9] day 3: 0.06 [0.002 – 2.12]
MI (42)	56 (46)	At PCI and chronic phase	ELISA activity	51 ± 15	54 ± 19	NS	n.a.
CHD (143)	55 (62)	several years	ELISA activity	83 (47 - 113)	91 (42 - 122)	NS	n.a.
CHD (142)	29 (48)	n.a.	ELISA activity	n.a.	n.a.	P<0.01	n.a.
CAD (45)	325 (69)	At angiography and 2-14 days	ELISA antigen	73.5 (62.0 – 88.8)	82.5 (66.5 – 95.5)	P=0.008	HR 0.621 [0.412 - 0.933]

Table 6. Case-control studies on the association between ADAMTS13 and ischemic stroke

Participants	N total (% cases)	Blood sampling after event	ADAMTS13 assay	ADAMTS13 level in cases	ADAMTS13 level in controls	P-value	Disease endpoint OR [95% CI]
First ever ischemic stroke (85)	249 (50)	7-14 days	Collagen binding	96 ± 41	103 ± 44	P=0.23	1.7 [0.7 - 3.9]
First ever ischemic stroke or TIA (46)	441 (25)	1-3 months	ELISA activity and Antigen	96.4 (70.3 – 112.2)	109.5 (93 – 123.8)	P<0.001	1.76 [0.90 – 3.44]
First ever ischemic stroke (33)	813 (22)	23-146 months	ELISA antigen	100	110	n.a.	3.1 [1.6 - 5.8]
Ischemic stroke (144)	333 (62)	6-12 months	ELISA activity	98 (11.1 - 81)	103 (11.2 - 91)	P=0.03	7.30 [2.73 - 19.5]

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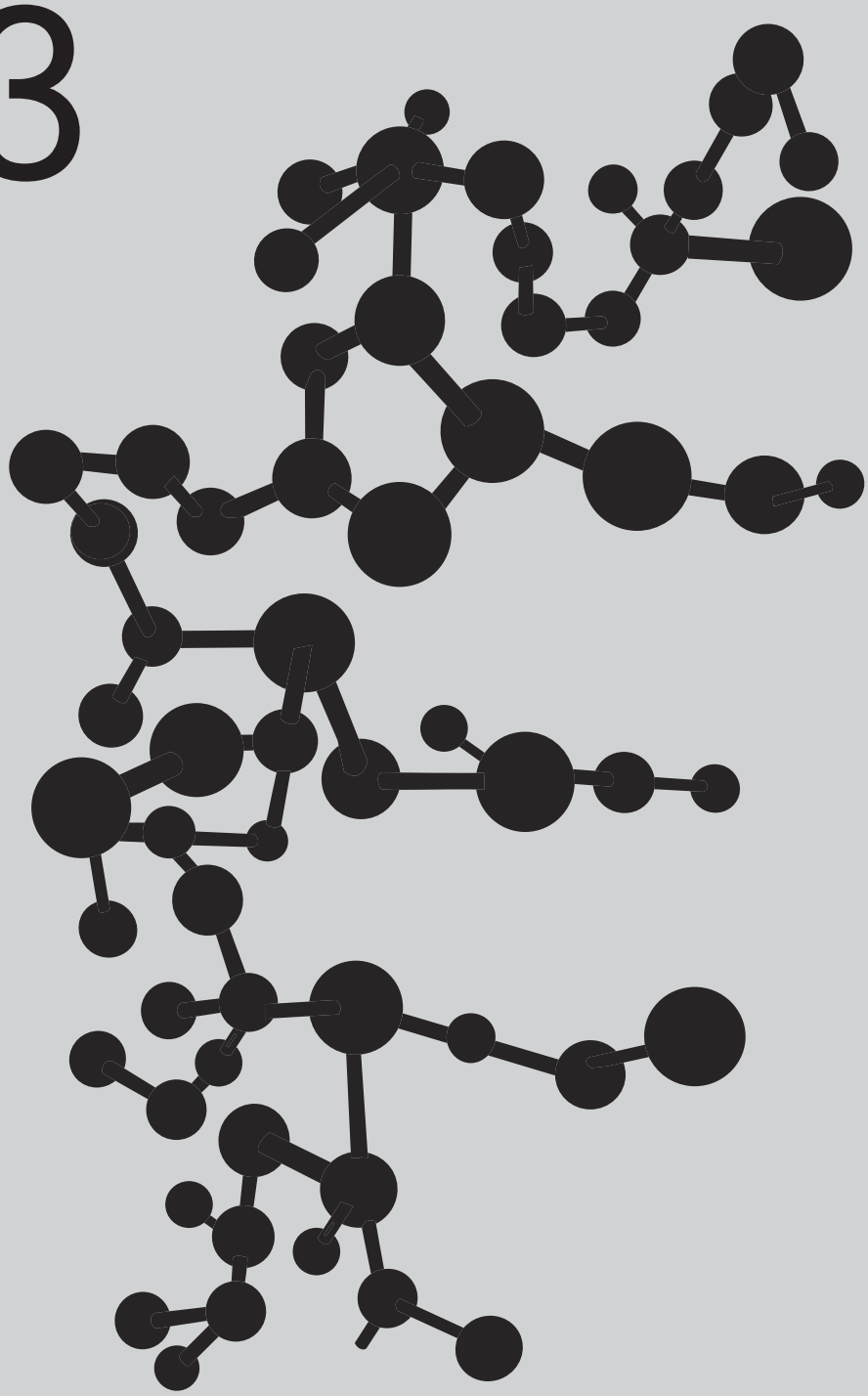

RELATIONSHIP OF VON WILLEBRAND FACTOR WITH CAROTID ARTERY AND AORTIC ARCH CALCIFICATION IN ISCHEMIC STROKE PATIENTS

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ABSTRACT

Background

Large population studies have revealed that increased von Willebrand Factor (VWF) levels are associated with an increased risk of ischemic stroke. In previous studies VWF was associated with atherosclerosis in healthy individuals. However, it is yet unknown what the association is between atherosclerosis and VWF levels in patients with ischemic stroke.
The aim of our study was to determine the association of atherosclerosis, measured with recent developed techniques, and VWF levels in a large, well characterized, cohort of ischemic stroke patients and to determine the prognostic value.

Methods

We included 925 consecutive patients with transient ischemic attack (TIA) or ischemic stroke. Calcification volumes (mm^3) were scored in the aortic arch and both carotid arteries using multidetector computed tomography (CT) angiography. VWF antigen (VWF:Ag) levels were measured using ELISA.

Results

Mean VWF:Ag levels were significantly higher in the presence of calcification in either the aortic arch (1.47 vs. 1.37 IU/ml [P=0.039]) or the carotid arteries (1.49 vs. 1.34 IU/ml [P=0.001]). Patients with a large artery atherosclerosis ischemic stroke had significantly higher VWF:Ag levels then the other TOAST subtypes (P<0.0001). High VWF:Ag levels were associated with an unfavorable outcome (modified Rankin Scale >2 vs. ≤2; 1.64 vs. 1.41 IU/ml, [P<0.0001]).

Conclusion

Our study showed a strong association between the extent of atherosclerosis in both the aortic arch and the carotid arteries and VWF levels in patients with TIA or ischemic stroke. Higher VWF levels are found in large artery atherosclerosis and are associated with a poor outcome.

INTRODUCTION

Von Willebrand Factor (VWF) plays a crucial role in platelet adhesion and aggregation, the initial steps in thrombus formation. VWF is a multimeric plasma protein that is produced by endothelial cells and megakaryocytes (1). Since VWF plasma levels increase as the result of endothelial damage, VWF levels can be used as a marker of endothelial dysfunction (2).
Previous studies have shown a positive association between levels of VWF and risk of coronary heart disease and stroke (3-6). In ischemic stroke, a particular association of VWF levels with etiologic subtypes, such as large artery atherosclerosis and cardioembolic stroke, was found(7). Despite the fact that prospective studies have identified VWF levels as a predictor of ischemic stroke (8-10), the mechanism by which increased VWF levels are related to stroke is still unclear.
We have previously shown that genetic variation strongly determines VWF levels, however these genetic variations are not or minimally associated with ischemic stroke risk (11, 12). Therefore it has been suggested that the VWF levels may predominantly be determined by endothelial dysfunction and atherosclerosis (13).
Endothelial dysfunction is the first phase in the development of atherosclerotic plaques. Because endothelial activation is related to atherosclerosis and an association of VWF with ischemic stroke has been found, atherosclerosis may be a determinant of VWF levels (14). Previous studies have shown a significant association between atherosclerosis, measured by the ankle-brachial index and intima-media thickness, and increased VWF levels in healthy individuals (15, 16).
In recent years, new techniques have been developed to study the extent of atherosclerosis more precisely. Calcification volume is an important indicator of atherosclerosis severity and may have a strong association with VWF levels (17-19).
We hypothesized that a higher degree of calcification volume, measured both in the aortic arch and carotid arteries, is associated with higher levels of VWF in patients with ischemic stroke, which may provide more insight in the relationship between VWF levels and ischemic stroke risk and the prognostic value of VWF.

MATERIALS AND METHODS

Study population

We studied 925 consecutive patients with TIA or ischemic stroke from the Erasmus Stroke Study, an ongoing registry of patients with cerebrovascular diseases treated at our hospital, from December 2005 until December 2010 of whom plasma, DNA and a CT angiography was available (20). TIA was defined as a focal neurological deficit of presumed vascular origin lasting less than 24 hours, with imaging studies showing no abnormalities. Ischemic stroke was defined as a focal neurological deficit of presumed vascular origin lasting ≥24 hours, with brain imaging studies showing no abnormalities or typical signs of infarction. Patients were classified according to the Trial of Org 10172 in Acute Stroke Treatment (TOAST) criteria(21) and additionally to a phenotypic classification which is a variant of the A-S-C-O score (22). This score is characterized by 9 categories: definite lacunar stroke, definite atherothrombotic stroke, probable atherothrombotic stroke, definite cardiac cause, possible cardiac cause, definite hematologic cause, possible hematologic cause, other cause of stroke and unknown cause of stroke. Definitions are described in the supplemental information. Patients who have multiple causes of stroke did not fit into one of the categories and are therefore excluded from the analysis (N=125). Hypertension and hypercholesterolemia were defined as the use of antihypertensive or cholesterol lowering drugs, respectively, before the inclusion event. Diabetes mellitus was defined as the use of oral and/or parenteral antidiabetic drugs before the event. Smoking status (smoking versus non-smoking) was assessed at the time of the event. Patients were considered to have a history of ischemic heart disease when they had a documented myocardial infarction, angina pectoris or cardiac revascularization therapy. Peripheral arterial disease was defined as a history of intermittent claudication or peripheral vascular surgery or amputation due to lower limb ischemia. A history of cardiovascular disease indicates a history of ischemic heart disease and/or peripheral arterial disease and/or atrial fibrillation and/or TIA or ischemic stroke. The National Institutes of Health Stroke Scale (NIHSS)(23) was assessed at admission from the stroke unit or outpatient clinic. The functional outcome was assessed using the modified Rankin scale (mRS)(24) at discharge and was dichotomized as favorable (≤2) or unfavorable (>2) outcome.

All participants provided written informed consent. The study was approved by the Medical Ethics Committee of the Erasmus University Medical Center.

MDCTA angiography

A multidetector CT angiography (MDCTA) was performed routinely according to a standard protocol. MDCTA was performed at a median of 5 days (interquartile range 2-14 days) after onset of symptoms. Image acquisition was performed using a 16, 64 or 128 slice multi-detector CT system (Sensation 16, Sensation 64, Definition, Definition AS+ or Definition flash, Siemens Medical Solutions, Erlangen, Germany) using a standardized optimized contrast-enhanced protocol (120 kVp, 180-200 mAs, collimation 16 x 0.75 mm; 32 x 2 x 0.6 mm; 64 x

2 x 0.6 mm, pitch < 1). The scan range extended from the ascending aorta to the intracranial circulation. All patients received 80 ml of contrast agent (320 mg/mL iodixanol, Visipaque, Amersham Health, Little Chalfont, UK), followed by 45 ml saline bolus chaser, both at an injection rate of 4 or 5 ml/s. Real-time bolus tracking at the level of the ascending aorta was used to synchronize passage of contrast agent and data acquisition. Image reconstructions were made with field of view of 120 mm, matrix size 512 × 512, slice thickness 0.75 or 1.0 mm, increment 0.4 - 0.6 mm and with an intermediate reconstruction algorithm. All MDCTA studies were evaluated by trained readers blinded for clinical data. Dedicated commercially available software (Syngo CalciumScoring, Siemens) was used to quantify calcifications at the aortic arch and the carotid arteries; expressed as calcification volume in mm^3. The aortic arch was defined as the origin of the aortic arch to the first 1 cm of the common carotid arteries, the vertebral arteries and the subclavian arteries beyond the origin of the vertebral arteries. Both carotid arteries were scored within 3 cm proximal and distal of the bifurcation and calcification volume of both carotid arteries was added. A threshold of 600 Hounsfield units (HU) was used to differentiate calcifications from contrast material in the lumen. A detailed description of the measurement is provided elsewhere(17, 25).
Reasons for not performing MDCTA were poor renal function, significant comorbidity with resultant very short life expectancy and very severe stroke with likely fatal outcome. Patients with a time interval between event and MDCTA of more than 180 days were excluded (n=20). Because of poor image quality of both the aortic arch and the carotid arteries caused by artifacts, 17 scans were not gradable. In 7 patients the aortic arch and in 10 patients the carotid arteries could not be analyzed due to dissection of the carotid artery, presence of artifacts or stents.

Blood samples, VWF measurement and blood group assessment
Blood sampling was performed at a median of six days (interquartile range 3-14 days) after onset of symptoms. There was no significant correlation between VWF levels and time from event till blood sampling (ß -0,0001 IU/ml per day; P=0.33).
Citrated blood was centrifuged at 1700g for 15 minutes at room temperature, and stored at -80°C within 2 hours from collection. DNA was isolated from blood using MagNA Pure (Roche Diagnostics) and stored at -80°C. VWF antigen (VWF:Ag) levels were determined with an in-house ELISA, using rabbit anti-human VWF antibodies (DakoCytomation, Glostrop, Denmark) for catching and tagging. Reference standard plasma, calibrated against the international standard (Cryocheck Reference, Kordia, Leiden, the Netherlands), was used as a calibrator. The intra-assay coefficient of variation was 3.2%. Blood groups were assessed with a standard test of blood group antibodies in 572 patients. In the remaining 353 patients, we genotyped rs687289, which can be used to discriminate blood group O from non-O status, using custom TaqMan Genotyping Assays (Applied Biosystems, Foster City, CA, USA)(26). Genotyping was successful in 99% of all patients.

Statistical analysis

Levels of VWF:Ag and the calcification volumes in the aortic arch and carotid arteries were normalized by logarithmic transformation. The data are presented as geometric means and 95% confidence interval (CI). Correlation between two groups was assessed with Spearman rank correlation. The aortic arch and carotid calcification volume were divided into four subgroups. The first group consists of all patients with a calcification volume of 0 mm^3, the remaining patients were divided into tertiles. VWF:Ag levels were divided into two categories: low level below the median (≤1.43 IU/ml) and high level above the median (>1.43 IU/ml). Groups were compared using independent T-tests or ANOVA with Bonferroni correction. Linear regression analysis was used to analyze the relationship between calcification volume with VWF:Ag levels. All analyses were adjusted for potential confounders: age, sex, ABO blood group, smoking, hypertension, hypercholesterolemia, diabetes mellitus and a history of cardiovascular disease. Only confounders that were significantly associated with VWF:Ag levels were used in the multivariate analyses. All analyses were performed using SPSS version 20.0 (IBM, Somers, NY, USA). A P value <0.05 was considered to indicate statistical significance.

RESULTS

Baseline characteristics of the 925 patients in this study are shown in Table 1. Mean age was 62 ± 13.6 years and 47% were females. As expected in our cohort of patients with TIA or ischemic stroke, they frequently suffered from hypertension, hypercholesterolemia and the majority of patients smoked. The geometric mean of the VWF:Ag levels in our patients was 1.43 IU/ml (95% CI 0.63-3.24). Patients with ischemic stroke had significantly higher levels of VWF than patients with TIA (1.50 vs. 1.35 IU/ml; P<0.0001). Patients with blood group O had significantly lower levels of VWF:Ag compared with patients with non-O blood groups (1.24 vs 1.62 IU/ml, p<0.0001). 113 patients had a time interval between event and MDCTA of more than one month. The results of the study did not change, when excluding those patients.

Table 1. Baseline characteristics of the study population

		Patients in whom both calcification volume were determined (N = 908)		
	Total cohort N = 925	**No calcification N = 256**	**Any calcification N = 652**	**P-value**
Age, years	62.0 (13.6)	49.0 (11.5)	67.1 (10.7)	<0.0001
Female sex	443 (46.8)	137 (53.5)	290 (44.5)	0.014
Smoking	283 (30.6)	82 (32.0)	195 (29.9)	0.677
Body mass index (kg/m^2)	27.3 (13.5)	27.9 (5.8)	27.1 (15.6)	0.543
Hypertension	488 (52.8)	76 (29.7)	400 (61.3)	<0.0001
Hypercholesterolemia	311 (33.6)	43 (16.8)	258 (39.6)	<0.0001
Diabetes Mellitus	128 (13.8)	21 (8.2)	104 (16.0)	0.002
History of CVD	367 (39.7)	54 (21.1)	303 (46.5)	<0.0001
Bloodgroup				
O	416 (45)	115 (44.9)	294 (45.1)	0.888
Non-O	504 (54.5)	141 (55.1)	353 (54.1)	0.888
Diagnosis				
TIA	428 (46.3)	130 (50.8)	289 (44.3)	0.079
Stroke	497 (53.7)	126 (49.2)	363 (55.7)	0.079
TOAST classification				
Large artery atherosclerosis	154 (16.6)	16 (6.3)	133 (20.4)	<0.0001
Cardioembolism	113 (12.2)	29 (11.3)	79 (12.1)	0.741
Small vessel occlusion	183 (19.8)	51 (19.9)	129 (19.8)	0.963
Other determined etiology	50 (5.4)	25 (9.8)	25 (3.8)	<0.0001
Undetermined etiology	425 (45.9)	135 (52.7)	286 (43.9)	0.016

Data are presented as N(%), unless for age and body mass index, where mean (SD) are shownCVD indicates cardiovascular disease

Calcification volume of the aortic arch and VWF:Ag levels

The geometric mean calcification volume of the aortic arch of the total group was 17.59 mm^3 (95% CI 0-3374.8 mm^3). Patients with calcifications in the aortic arch (n=593) had significantly higher VWF:Ag levels compared with patients without calcifications in the aortic arch (n=325; 1.47 vs. 1.37 IU/ml, P=0.039). VWF:Ag levels increased linearly with increasing groups of the aortic calcification volume (P for trend 0.003; Figure 1a). After multivariate adjustment, age and blood group were significantly associated with levels of VWF:Ag (P<0.0001; P<0.0001; respectively). Patients with blood group O, with mean VWF:Ag levels of 1.24 IU/ml, had similar aortic calcification volume compared with patients with blood group non-O, who had a mean VWF:Ag level of 1.62 IU/ml (17.2 vs. 18.0 mm^3, P=0.80). The association between the calcification volume and VWF:Ag levels was seen both in blood group O and non-O and this was not statistically significant different (P for indication = 0.49).

Figure 1

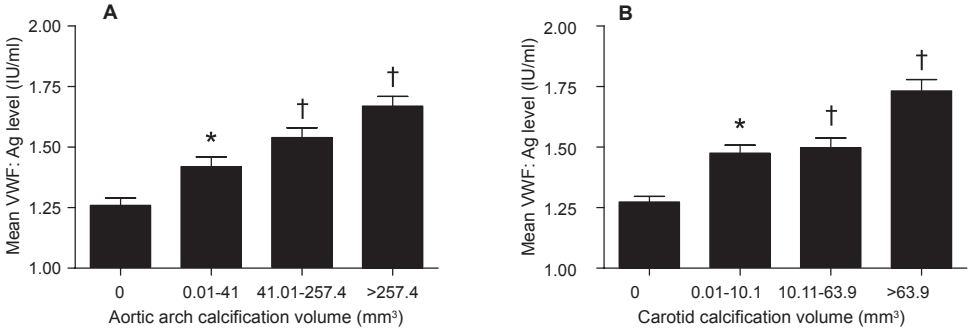

Figure 1. Levels of mean VWF:Ag per group of calcification volume
Mean and standard error (SE) VWF:Ag levels (in IU/ml) in the aortic arch calcification volume groups (* P=0.006 compared with the first group; †P<0.0001 compared with the first group)
Mean and standard error (SE) VWF:Ag levels (in IU/ml) in the carotid calcification volume groups (* P=0.001 compared with the first group; † P<0.0001 compared with the first group)

Calcification volume of the carotid arteries and VWF:Ag levels

The geometric mean of the carotid calcification volume of the total group was 5.7 mm^3 (95% CI 0.0-396.9 mm^3). Patients with calcifications (n=529) had significantly higher levels of VWF:Ag compared with those without calcifications (n=386; 1.49 vs 1.34 IU/ml, P=0.001). VWF:Ag levels increased linearly in increasing groups of the calcification volume (P for trend <0.0001; Figure 1b). Age and blood group were significantly associated with levels of VWF:Ag after multivariate analysis (P<0.0001; P<0.0001, respectively). Patients with blood group O, with a mean VWF:Ag levels of 1.24 IU/ml, had a similar carotid calcification volume compared with patients with blood group non-O, with a mean VWF:Ag level of 1.61 IU/ml (6.4 vs. 5.3 mm^3, P=0.25).

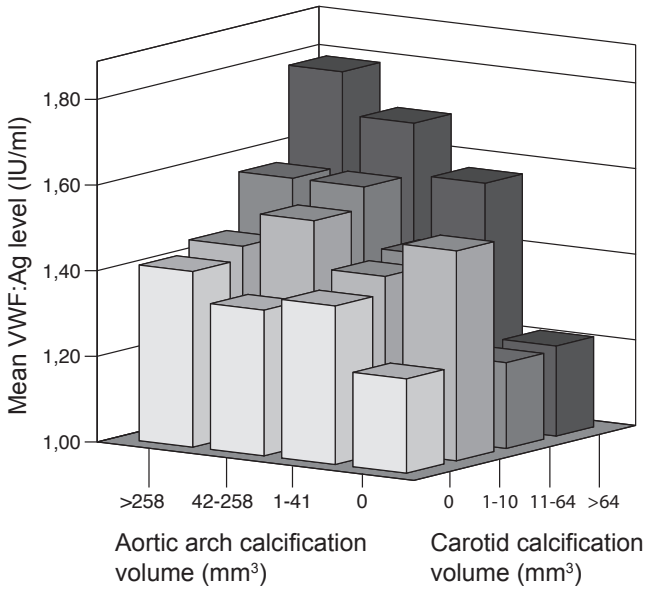

Figure 2. Relationship between aortic arch and carotid calcification volume and mean VWF:Ag levels

Calcification volume of the aortic arch and of the carotid arteries were highly correlated (R=0.69; P<0.0001). Patients with calcifications in both arteries were older (67 vs. 49 years, P<0.0001) and had more cardiovascular risk factors than those without calcifications (Table 1). This was similar in the separate arteries. Assessing the mean VWF:Ag level in patients using both calcification volume, showed the highest levels in patients with both the highest calcification volume (Figure 2). The association between the calcification volume and VWF:Ag levels was seen both in patients with blood group O and non-O and this was not statistically significant different (P for interaction = 0.11).

Etiologic subtypes of TIA or stroke and levels of VWF:Ag

Levels of VWF:Ag differed significantly between etiologic subtypes of TIA and ischemic stroke (P=0.006; Figure 3). Levels of VWF:Ag were significantly increased in patients with a large artery atherosclerosis of TIA or ischemic stroke, compared with the other subtypes (1.59 vs. 1.40 IU/ml, P<0.0001). Patients with a small vessel occlusion etiology had significantly lower levels of VWF:Ag compared with the other patients (1.34 vs. 1.45 IU/ml, P=0.009).

Patients with a definite atherothrombotic stroke using the A-S-C-O score variant had significantly higher VWF:Ag levels (1.58 IU/ml) compared with patients with a probable atherothrombotic stroke (1.40 IU/ml; P=0.001), other cause of stroke (1.24 IU/ml; P=0.009) and unknown cause of stroke (1.32 IU/ml; P<0.0001). Patients with a definite lacunar stroke had significantly higher VWF:Ag levels (1.66 IU/ml) compared with probable atherothrombotic (P=0.04), other cause of stroke (P=0.013) and with unknown cause of stroke (P=0.009).

VWF levels were significantly correlated with the NIHSS score at admission (R=0.183, P<0.0001) and with functional outcome of the patients, as determined by the modified Rankin Scale (mRS) at discharge (R=0.222, P<0.0001). Patients with an unfavorable outcome (mRS >2) had significantly higher VWF:Ag levels compared with patients with a favorable outcome (mRS ≤2) (1.64 IU/ml vs. 1.41 IU/ml, P<0.0001). However, these patients did not have a significant higher calcification volume in both the aortic arch (23.1 vs. 17.0 mm^3, P=0.17) and carotid arteries (6.6 vs. 5.6 mm^3, P=0.45), compared with patients with a favorable outcome. Patients with a high VWF:Ag level (>1.43 IU/ml) had a higher risk of an unfavorable outcome compared with patients with a low VWF:Ag level (≤ 1.43 IU/ml; OR 1.45, P<0.0001) and this difference remained after additionally adjustment for aortic arch and carotid calcification volume. However, there was no significant difference in outcome between patients with blood group O and non-O (OR 0.95, P=0.40).

Figure 3

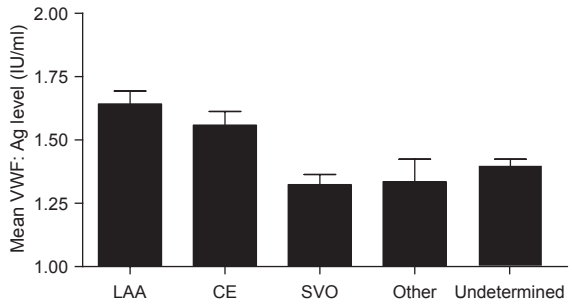

Figure 3. Levels of mean VWF:Ag per etiologic subtypes of TIA and ischemic stroke
Mean and standard error (SE) VWF:Ag levels (IU/ml) in the etiologic subtypes of TIA and ischemic stroke according to the TOAST criteria.
* indicates P<0.0001; † P<0.05
LAA indicates large artery atherosclerosis; CE, cardioembolism; SVO, small vessel occlusion; Other, stroke of other determined etiology; Undetermined, cerebral ischemia of undetermined etiology

Table 2. Association between calcification volume and VWF:Ag levels

Calcification volume (mm^3)	**Mean VWF:Ag (IU/ml) Model 1**	**Mean VWF:Ag (IU/ml) Model 2**
Aortic arch		
0	1.26 ± 0.03	1.36 ± 0.03
0.01-41	1.42 ± 0.04	1.41 ± 0.04
41.01-257.4	1.54 ± 0.04	1.48 ± 0.04
>257.4	1.67 ± 0.04	1.54 ± 0.05
P for trend	<0.0001	0.03
Carotid artery		
0	1.27 ± 0.03	1.34 ± 0.03
0.01-10.1	1.47 ± 0.04	1.43 ± 0.04
10.11-63.9	1.49 ± 0.05	1.46 ± 0.04
>63.9	1.73 ± 0.05	1.61 ± 0.05
P for trend	<0.0001	<0.0001

Mean VWF:Ag levels (mean ± SE) per calcification volume subgroup
Model 1: univariate
Model 2: adjusted for age, sex, blood group and cardiovascular risk factors

DISCUSSION

The main result of our study is a strong positive association between the extent of atherosclerosis, determined by the calcification volume in both the aortic arch and the carotid arteries, and VWF:Ag levels in patients with ischemic stroke. In addition, we observed that levels of VWF were significantly higher in patients with a large artery atherosclerosis type of ischemic stroke compared to other stroke subtypes.

To the best of our knowledge, our study is the first to investigate the extent of atherosclerosis, using newly developed quantitative measurements, and VWF levels in patients with TIA or ischemic stroke. We found similar positive associations in both aortic arch calcification volume and carotid calcification volume with VWF:Ag levels. Furthermore, patients with the highest score for both the aortic arch calcification volume and carotid calcification volume had the highest VWF:Ag levels. VWF:Ag levels were strongly associated with large artery atherosclerosis.

It is still debated whether VWF itself plays a pathogenetic role in atherogenesis. Despite the fact that several animal models indicated that VWF may lead to a reduction of atherosclerosis(27, 28), multiple human studies failed to confirm this observation. In these studies a similar extent of atherosclerosis in individuals with severe von Willebrand disease (VWD), characterized by reduced levels of VWF, was shown compared with controls (29-33). It is well known that genetic variations, both within the VWF gene and outside the VWF gene, strongly determine VWF levels (11, 12, 34-36). One of the main genetic determinants of VWF levels is the ABO blood group, resulting in 25-30% lower levels in individuals with blood group O compared to non-O(37). Also in our study, ischemic stroke patients with blood group O had 30% lower VWF:Ag levels compared to patients with blood group non-O, but did not have lower calcification volumes. This finding suggests that VWF levels do not have a major pathogenetic role in atherosclerosis. In addition, it is still debatable whether blood group is a risk factor for ischemic stroke (38, 39). An earlier study of our group already showed that, despite the fact that several genetic variations were strongly associated with VWF levels, the genetic variations did not influence the risk of ischemic stroke(11). This suggests that VWF levels are merely a marker of atherosclerosis and thereby determine the previously found association between increased VWF:Ag levels and risk of stroke, as atherosclerosis is a well-known important risk factor for TIA and ischemic stroke.

Patients with a small vessel occlusion ischemic stroke had lower levels of VWF:Ag than the other stroke patients. This was comparable with another study, which showed that patients with a small vessel disease type of stroke, had lower VWF levels compared with the large vessel disease and cardio embolism group(7). These lower VWF levels may be explained by the fact that in these patients atherosclerosis does not play a major role in the etiology of stroke. However, the small arterioles of the brain are presumed to be affected by various vascular risk factors(40). This may also lead to endothelial activation, but only to certain subtypes of lacunar infarcts (41). However, lacunar stroke described in the A-S-C-O score

variant was associated with high VWF:Ag levels. This might be explained by the difference in the number of patients (N=22 A-S-C-O score, N=183 TOAST classification) and a difference in definition. In this study, VWF:Ag levels were associated with stroke severity, determined by the NIHSS at admission and with poor outcome, determined by the mRS at discharge. This is in agreement with one previous study in patients with acute ischemic and hemorrhagic stroke in which high VWF levels were associated with a poor modified Rankin score (42). Recently we showed in the prospective population-based Rotterdam study that increased VWF levels are a predictor of stroke (9). In this study we have shown that increased VWF levels and not blood group are a predictor of stroke outcome suggesting that VWF levels could serve as a risk marker of stroke outcome. Additionally, because levels of VWF are increased due to presence of atherosclerosis, VWF could also serve as a useful marker of atherosclerosis and thereby as a risk marker of stroke.

Some methodological issues have to be addressed. Strengths of our study are the large number of ischemic stroke patients of all ages, with availability of well-documented clinical information, extensive scoring (>96%) of MDCT angiography of the aortic arch and the carotid arteries, and the availability of plasma of nearly all consecutive patients.

Furthermore, we determined calcifications of the aortic arch and carotid arteries using dedicated commercially available software in which calcifications of the aortic arch and carotid arteries can be reproducible quantified in a specified range. So far only limited data was available regarding calcifications in the carotid arteries as a marker of atherosclerosis. In this study, we found a significantly higher prevalence of cardiovascular risk factors in patients with calcifications in the aortic arch and carotid arteries than in patients without calcifications. This is in agreement with another study, which showed an independent relationship between several cardiovascular risk factors and carotid calcifications and similar risk factor profiles were found in different vessel beds including the coronary arteries, aortic arch and carotid arteries (17). Calcifications in the aortic arch and carotid arteries were associated with the presence of stroke and luminal stenosis in previous studies (18, 19, 43, 44). This all shows that calcifications in the aortic arch and carotid arteries can be used as a marker of atherosclerosis.

A potential drawback of our study is that calcifications below the threshold of 600 HU or outside the scan range were not detected. Another possible limitation concerns the fact that calcifications are one of the components of an atherosclerotic plaque and a soft atherosclerotic plaque that contains no calcification is therefore not detected (43). However, it has been shown that the majority (82%) of atherosclerotic plaques contains some calcifications (25) and therefore this would only minimally affect our results. In addition, time between stroke and blood sampling was variable. However, there was no significant correlation between VWF levels and time from event till blood sampling. This suggests that the time of blood drawn did not influence the levels of VWF. Furthermore, patients with significant comorbidity, very severe stroke or those who died within 24 hours were not included in this study, resulting in a relatively less severely affected cohort.

CONCLUSION

In conclusion, our study in patients with TIA or ischemic stroke indicates that the extent of atherosclerosis, determined by calcifications in the aortic arch and carotid arteries, is strongly associated with VWF levels. In addition, highest VWF levels are found in large vessel disease, whereas the levels are lower in small vessel disease. Furthermore, our study suggests that VWF may have prognostic value in patients with ischemic stroke.

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SUPPLEMENTAL INFORMATION

Lacunar stroke is defined as a small deep infarct on scan <15 mm in the territory corresponding to symptoms in a patient with a clinical syndrome compatible with small deep infarct. Definite atherothrombotic stroke is defined by (1) an ipsilateral internal carotid stenosis ≥50% or (2) an ipsilateral stenosis ≥50% in another intra/extracranial artery (also in vertebrobasilar system if applicable), or (3) mobile thrombus in the aortic arch. Probable atherothrombotic stroke is defined as patients with ipsilateral internal carotid or other intra/extracranial artery <50% stenosis, contralateral stenosis; patients with ≥2 of the following risk factors for atherothrombotic disease: hypertension (as defined above), diabetes mellitus, smoking at time of event, high cholesterol. Definite cardiac cause is defined as patients with atrial fibrillation, atrial flutter, sick sinus syndrome, prosthetic valve, mitral stenosis, recent myocardial infarction (<6 weeks), left ventricular thrombus, atrial myxoma, infective endocarditis, non-ischemic dilating cardiomyopathy, non-bacterial thrombotic endocarditis. Possible cardiac cause is defined by patients with calcific aorta stenosis, mitral valve prolaps, mitral annulus calcification, patent foramen ovale, atrial septal aneurysm, ventricular aneurysm, ventricular septal defect, other structural cardiac abnormalities not mentioned above. Definite hematologic cause is defined as patients with disseminated intravascular coagulation, myeloproliferative disorders, essential thrombocythemia, polycythemia vera and antiphospholipid syndrome (the full syndrome). Possible hematologic cause is defined as patients with protein C deficiency, protein S deficiency, antithrombin deficiency, factor V Leiden, isolated lupus anticoagulans, single increased antiphospholipid antibodies (not confirmed with second increased measurement), and other hematologic causes. In other causes of stroke carotid or vertebral dissection, vasculitis, AVMs, Moyamoya, Fabry, other vascular causes, hemodynamic stroke, migrainous stroke, neoplasm and miscellaneous causes are included. Unknown cause stroke is defined as patients whom, based on all available data, cannot be categorized into either of the above categories.

4

VON WILLEBRAND FACTOR IN RELATION TO CORONARY PLAQUE CHARACTERISTICS AND CARDIOVASCULAR OUTCOME: RESULTS OF THE ATHEROREMO-IVUS STUDY

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ABSTRACT

Backgrounds

High VWF plasma levels are associated with an increased risk of coronary artery disease. It has been suggested that the increase of VWF levels is partly due to endothelial dysfunction and atherosclerosis. Our aim was to investigate the association between coronary plaque burden, the presence of high-risk coronary lesions as measured by intravascular ultrasound virtual histology (IVUS-VH) and VWF levels. In addition, we studied the association between VWF levels and 1-year cardiovascular outcome.

Methods

Between 2008 and 2011, IVUS-VH imaging of a non-culprit coronary artery was performed in 581 patients undergoing coronary angiography for acute coronary syndrome (ACS) (n= 318) or stable angina pectoris (SAP) (n= 263). Arterial blood was sampled prior to the coronary angiography. VWF antigen (VWF:Ag) levels were measured using ELISA (n= 577).

Results

Patients with ACS had significantly higher VWF:Ag levels than SAP patients (median 1.73 IU/ml [IQR 1.27-2.31] vs. 1.26 IU/ml [0.93-1.63], p<0.001). High coronary plaque burden was associated with higher VWF:Ag levels (ß= 0.12, p=0.027) in SAP patients, but not in ACS patients. In ACS patients, VWF:Ag levels were associated with 1-year MACE (HR 4.14 per SD increase of lnVWF:Ag, 95% CI 1.47-11.6), whereas in SAP patients VWF:Ag levels predicted 1-year all-cause death and hospitalisation for ACS (HR 7.07 95% CI 1.40-35.6).

Conclusions

Coronary plaque burden was associated with VWF:Ag levels in SAP patients undergoing coronary angiography. In ACS and SAP patients, high VWF levels are predictive of adverse cardiovascular outcome and death during 1-year follow-up.

INTRODUCTION

Von Willebrand Factor (VWF) is a multimeric protein that plays a crucial role in primary hemostasis by mediating platelet adhesion and aggregation (1). VWF is produced by endothelial cells and megakaryocytes and stored in Weibel-Palade bodies in the endothelium and alpha-granules of platelets. VWF plasma levels are increased at moments of endothelial damage and are a marker of endothelial dysfunction (2).

It is well known that high VWF levels are associated with an increased risk of coronary heart disease and ischemic stroke in the general population (3-8). However, the underlying mechanisms of this association are still unclear. As high VWF levels are seen in situations with endothelial dysfunction, which is an important early process in atherosclerosis development, it has previously been suggested that VWF has a pathogenic role in atherosclerosis. This hypothesis is supported by results from animal studies (9-11). However, studies in patients with type 3 von Willebrand disease, characterized by a total deficiency of VWF in the circulation, revealed no reduction in atherosclerotic lesions (12-14). The role of VWF in the development of atherosclerosis in humans is therefore still unresolved. In a recent study, we observed a strong association between the extent of atherosclerosis, measured by the calcification volume in the aortic arch and carotid arteries, and VWF levels in ischemic stroke patients (15). Because VWF also plays a pivotal role in platelet aggregation and thrombus formation, these high VWF levels may further increase the risk of coronary events in patients with high risk atherosclerotic lesions.

Intravascular ultrasound (IVUS) can accurately quantify coronary atherosclerosis (16, 17). A previous study in 697 patients with an acute coronary syndrome at inclusion showed that half of the incident recurrent cardiovascular events occurred in patients with non-culprit lesions present at baseline, assessed by IVUS imaging (18). High-risk coronary lesions that are predictive for events include lesions with a plaque burden of at least 70%, a minimal luminal area of 4.0 mm^2 or less, or the presence of IVUS virtual histology (VH)-derived thin-cap fibroatheroma lesions (VH-TCFA) (18).

In order to gain further insight into the relationship between VWF levels and cardiovascular outcome, the aim of the present study was to investigate the associations of coronary plaque burden, and the presence of high-risk coronary lesions as assessed by virtual histology intravascular ultrasound (VH-IVUS) with VWF levels, as well as to investigate the association of VWF with 1-year cardiovascular outcome in patients with coronary artery disease (CAD).

METHODS

Study population

The design of The European Collaborative Project on Inflammation and Vascular Wall Remodeling in Atherosclerosis – Intravascular Ultrasound (ATHEROREMO-IVUS) study has been described in detail elsewhere (19). In brief, 581 patients who underwent diagnostic coronary angiography or percutaneous coronary intervention (PCI) for an acute coronary syndrome (ACS) or stable angina pectoris (SAP) have been included between 2008 and 2011 in the Erasmus MC, Rotterdam, the Netherlands.

The ATHEROREMO-IVUS study was approved by the medical ethics committee of the Erasmus MC. The study was performed in accordance with the criteria described in the declaration of Helsinki. Written informed consent was obtained from all included patients. This study is registered in ClinicalTrials.gov, number NCT01789411.

Von Willebrand Factor measurement

Blood samples were drawn from the arterial sheath prior to the coronary angiography procedure. The blood samples were transported to the clinical laboratory of the Erasmus MC for further processing and storage at temperature of -80^{o}C within 2 hours after blood collection. VWF antigen (VWF:Ag) levels were determined (N=577) using citrate blood with an in-house ELISA using rabbit anti-human VWF antibodies (DakoCytomation, Glostrop, Denmark) for catching and tagging. Reference standard plasma was calibrated against the international standard (Cryocheck Reference, Kordia, Leiden, The Netherlands) and was used as a calibrator. The intra- and inter-assay coefficients of variation were 2.6% and 4.7%..

Intracoronary ultrasound imaging

Following the standard coronary angiography procedure, IVUS imaging of a non-culprit coronary artery was performed. Selection of the non-culprit vessel was predefined in the study protocol. The order of preference for selection of the non-culprit vessel was: 1. left anterior descending (LAD) artery; 2. right coronary artery (RCA); 3. left circumflex (LCX) artery. All IVUS data were acquired with the Volcano s5/s5i Imaging System (Volcano Corp., San Diego, CA, USA) using a Volcano Eagle Eye Gold IVUS catheter (20 MHz). An automatic pullback system was used with a standard pull back speed of 0.5 mm per second. The baseline IVUS images were sent to an independent core laboratory (Cardialysis BV, Rotterdam, the Netherlands) for offline analysis. The core laboratory personnel were blinded for baseline patient characteristics and clinical outcome data. The IVUS virtual histology analyses were performed using pcVH 2.1 and qVH (Volcano Corp., San Diego, CA, USA) software.

The external elastic membrane and luminal borders were contoured for each frame (median interslice distance, 0.40 mm). Extent and phenotype of the atherosclerotic plaque were assessed. Plaque burden was defined as plaque and media cross-sectional area divided by

external elastic membrane cross-sectional area (FIGURE 1). A coronary lesion was defined as a segment with a plaque burden of more than 40% in at least 3 consecutive frames. Three types of high-risk lesions were identified: 1. Virtual histology-derived thin-cap fibroatheroma (VH-TCFA) lesion, defined as a lesion with presence of >10% confluent necrotic core in direct contact with the lumen; 2. lesion with large plaque burden, defined as a lesion with a plaque burden of ≥70%; 3. stenotic lesion, defined as a lesion with a minimal luminal area of ≤4.0 mm^2 (Figure 1) (18, 20-22).

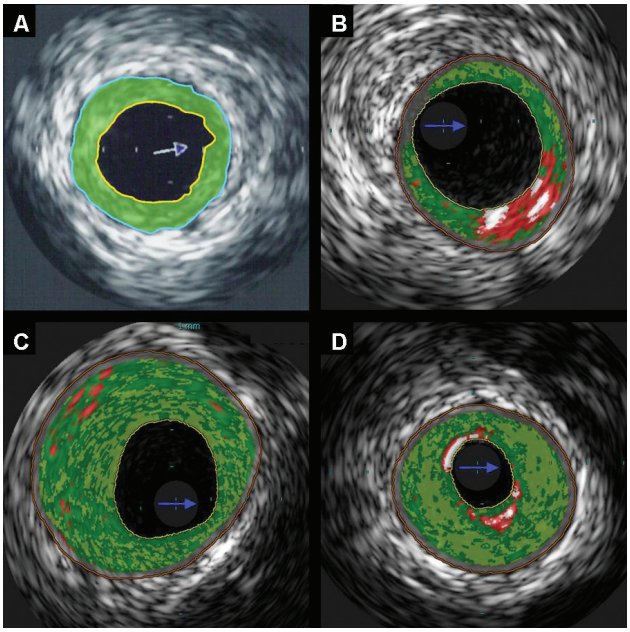

Figure 1. Measurement of plaque burden and identification of high risk lesions with intravascular ultrasound virtual histology
A: Plaque burden is defined as plaque and media cross-sectional area (green) divided by external elastic membrane cross-sectional area (contoured in blue). B: Thin-cap fibroatheroma lesion, defined as a lesion with presence of >10% confluent necrotic core (red) in direct contact with the lumen. White indicates dence calcium, light green indicates fibrofatty tissue, and dark green indicates fibrous tissue. C: Lesion with plaque burden of ≥70%. D: Lesion with a minimal luminal area of ≤4.0 mm^2.

Clinical endpoints

Clinical follow-up started at inclusion and lasted 1 year. Post-discharge survival status was obtained from municipal civil registries. Post-discharge rehospitalizations were prospectively assessed during follow-up. Questionnaires focusing on the occurrence of major adverse cardiac events (MACE) were sent to all living patients. Subsequently, hospital discharge letters were obtained and treating physicians and institutions were contracted for additional information whenever necessary.

The primary endpoint was MACE, defined as all-cause mortality, ACS or unplanned coronary revascularization. ACS was defined as the clinical diagnosis of ST segment elevation myocardial infarction (STEMI), non-STEMI or unstable angina pectoris in accordance with the guidelines of the European Society of Cardiology (23). Unplanned coronary revascularization was defined as unplanned repeat PCI (either culprit or non-culprit coronary artery) or coronary artery bypass grafting (CABG). The secondary endpoint was defined as the composite of all-cause mortality or ACS. The endpoints were adjudicated by a clinical event committee that had no knowledge of the VWF:Ag levels and IVUS data.

Statistical analysis

The distributions of the continuous variables, including VWF levels and the IVUS parameters, were tested for normality by visual examination of the histogram. Normally distributed continuous variables are presented as mean ± standard deviation (SD). Non-normally distributed continuous variables are presented as median and interquartile range (IQR). VWF levels were not normally distributed and were therefore natural logarithmically (ln) transformed (lnVWF:Ag), where after a normal distribution was acquired. Categorical variables are presented as numbers and percentages. We examined associations of plaque burden and presence of high-risk coronary lesions with VWF:Ag levels. VWF:Ag levels and plaque burden were divided into tertiles. To test for linear association, we used linear regression analyses with continuous ln-transformed VWF:Ag level as dependent variable. In multivariable analyses, the covariates age, gender, diabetes mellitus, hypertension, hypercholesterolemia, smoking and history of myocardial infarction were considered as established cardiovascular risk factors and as potential confounders, and were therefore entered into the full model.

Patients lost to follow-up were considered at risk until the date of last contact, at which time-point they were censored. Cumulative event rates were estimated according to the Kaplan-Meier method. Cox proportional hazards regression analyses were performed to evaluate the associations between VWF:Ag levels and study endpoints. Analyses were adjusted for age, gender and plaque burden. The final results are presented as crude and adjusted hazard ratios (HR) with 95% confidence interval (95% CI).

We a priori expected that there might be heterogeneity in effect estimates between patients with ACS and patients with stable angina pectoris, since VWF:Ag levels are known to be elevated in the acute phase of an ACS (24, 25). Therefore, all statistical analyses were performed separately for patients with ACS and patients with stable angina pectoris at inclusion. Data were analyzed with SPSS software (SPSS 20.0, IBM corp., Armonk, NY, USA). All statistical tests were two-tailed and p-values <0.05 were considered statistically significant.

Table 1. Baseline characteristics

Patient characteristics	ACS patients (n=315)	SAP patients (n=262)
Age, years	59.7 ± 11.8	63.6 ± 10.2
Men, n (%)	232 (73.7)	203 (77.5)
Diabetes mellitus, n (%)	40 (12.7)	59 (22.5)
Hypertension, n (%)	138 (43.8)	161 (61.5)
Hypercholesterolemia, n (%)	139 (44.1)	180 (68.7)
Smoking, n (%)	117 (37.1)	50 (19.1)
Positive family history, n (%)	145 (46.0)	155 (59.2)
Previous MI, n (%)	80 (25.4)	104 (39.7)
Previous PCI, n (%)	57 (18.1)	128 (48.9)
Previous CABG, n (%)	7 (2.2)	11 (4.2)
Previous stroke, n (%)	11 (3.5)	15 (5.7)
Peripheral artery disease, n (%)	12 (3.8)	24 (9.2)
History of renal insufficiency, n (%)	13 (4.1)	19 (7.3)
History of heart failure, n (%)	6 (1.9)	13 (5.0)
Von Willebrand Factor, IU/mL	1.73 [1.27-2.31]	1.26 [0.93-1.63]
Procedural characteristics		
Coronary artery disease		
No significant stenosis, n (%)	18 (5.7)	25 (9.5)
1-vessel disease, n (%)	174 (55.2)	133 (50.8)
2-vessel disease, n (%)	88 (27.9)	78 (29.8)
3-vessel disease, n (%)	35 (11.1)	26 (9.9)
PCI performed, n (%)	293 (93.0)	214 (81.7)
IVUS segment characteristics		
Imaged coronary artery		
Left anterior descending, n (%)	120 (38.1)	88 (33.6)
Left circumflex, n (%)	110 (34.9)	84 (32.1)
Right coronary artery, n (%)	85 (27.0)	90 (34.4)
Segment length, mm	44.1 [33.0-54.3]	44.3 [34.3-57.2]

Data are presented as mean ± standard deviation or as median [interquartile range].
ACS = acute coronary syndrome; CABG = coronary artery bypass grafting; MI = myocardial infarction; PCI = percutaneous coronary intervention; SAP = stable angina pectoris.

Table 2. Number of patients with incident major adverse cardiac events

Number of patients	ACS Patients (n=315)	SAP Patients (n=262)
Composite of major adverse cardiac events	26	29
Death from any cause	13	4
Definite cardiac or unexplained sudden death	6	2
Acute coronary syndrome	7	7
Myocardial infarction	4	3
Unplanned coronary revascularization	6	18
Composite of death or acute coronary syndrome	20	11

ACS = acute coronary syndrome; SAP = stable angina pectoris.

RESULTS

In total 577 patients were included, 315 had an ACS and 262 had a SAP. Patients had a mean age of 61.5 years and 75% were men (Table 1). Over half of the patients had single vessel disease. SAP patients had a higher prevalence of cardiovascular risk factors than ACS patients. ACS patients were more likely to smoke. ACS patients had significantly higher VWF:Ag levels than patients with SAP (median 1.73 IU/ml [IQR 1.27-2.31] vs. 1.26 IU/ml [0.93-1.63], p<0.001) (Table 1).

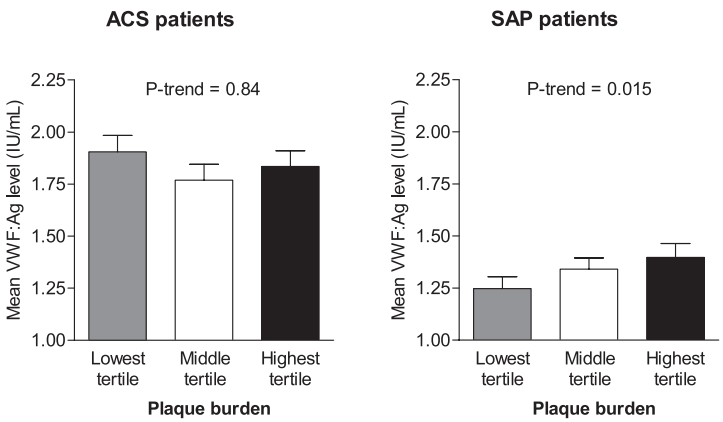

Figure 2. Coronary plaque burden of imaged coronary segment in relation to Von Willebrand Factor levels Mean ± standard error VWF:Ag levels per tertile coronary plaque burden. ACS = acute coronary syndrome; SAP = stable angina pectoris; VWF:Ag = von Willebrand Factor antigen.

Plaque burden was significantly higher in SAP patients than in ACS patients (39.7 ± 11.0 % vs. 36.9 ± 11.8 %, p = 0.005). In SAP patients, higher plaque burden was associated with higher VWF:Ag levels (P for trend 0.015) (Figure 2). Also after adjustment for established cardiovascular risk factors in multivariable analysis, higher plaque burden remained

associated with higher VWF:Ag levels (p = 0.027) in patients admitted with SAP. In ACS patients, the coronary plaque burden was not associated with VWF:Ag levels (P for trend 0.84). VWF:Ag levels were not significantly different between patients with and without high risk coronary lesions in both ACS and SAP patients (Figure 3).

For 575 (99.7%) patients the vital status at 1-year follow-up could be acquired, and the response rate to the questionnaires that were sent to all living patients was 93.4%. After 1 year of follow-up, 55 patients (9.6%) had experienced a MACE (Table 2). The cumulative Kaplan-Meier incidences of the 1-year MACE was 8.3% for patients with ACS, and 11.1% for patients with SAP. The risk of all-cause death and ACS was significantly associated with higher VWF:Ag levels in both ACS patients (HR 7.45, 95% CI 2.15-25.9, P=0.002) and patients with SAP (HR 7.07 95% CI 1.40-35.6, P=0.018). Additional adjustment for plaque burden did not affect the risk estimate for all-cause death and ACS in ACS patients (HR 7.65 95% CI 2.16-27.2), while the risk in SAP patients was slightly lower (HR 4.05 95% CI 0.88-18.7). Higher VWF:Ag levels were also significantly associated with a higher incidence of MACE in ACS patients (HR 4.14, 95% CI 1.47–11.6, P=0.007), but not in patients with SAP (HR 1.31, 95% CI 0.52-3.29, p=0.57) (Table 3, Figure 4). Additional adjustment for plaque burden did not change the results.

Table 3. Associations between von Willebrand Factor level and cardiovascular outcome

	ACS patients		**SAP patients**	
	HR (95%CI)*	P	HR (95%CI)*	P
MACE				
Unadjusted	4.28 (1.61-11.4)	0.004	1.39 (0.56-3.42)	0.48
Adjusted for age and gender	4.14 (1.47-11.6)	0.007	1.31 (0.52-3.29)	0.57
Adjusted for age, gender and plaque burden	4.13 (1.47-11.6)	0.007	1.08 (0.43-2.70)	0.87
Composite of death or ACS				
Unadjusted	7.15 (2.21-23.1)	0.001	7.62 (1.58-36.8)	0.011
Adjusted for age and gender	7.45 (2.15-25.9)	0.002	7.07 (1.40-35.6)	0.018
Adjusted for age, gender and plaque burden	7.65 (2.16-27.2)	0.002	4.05 (0.88-18.7)	0.073

* Hazard ratio per SD increase in ln-transformed Von Willebrand Factor level.
ACS = acute coronary syndrome; MACE = major adverse cardiac event; SAP = stable angina pectoris.

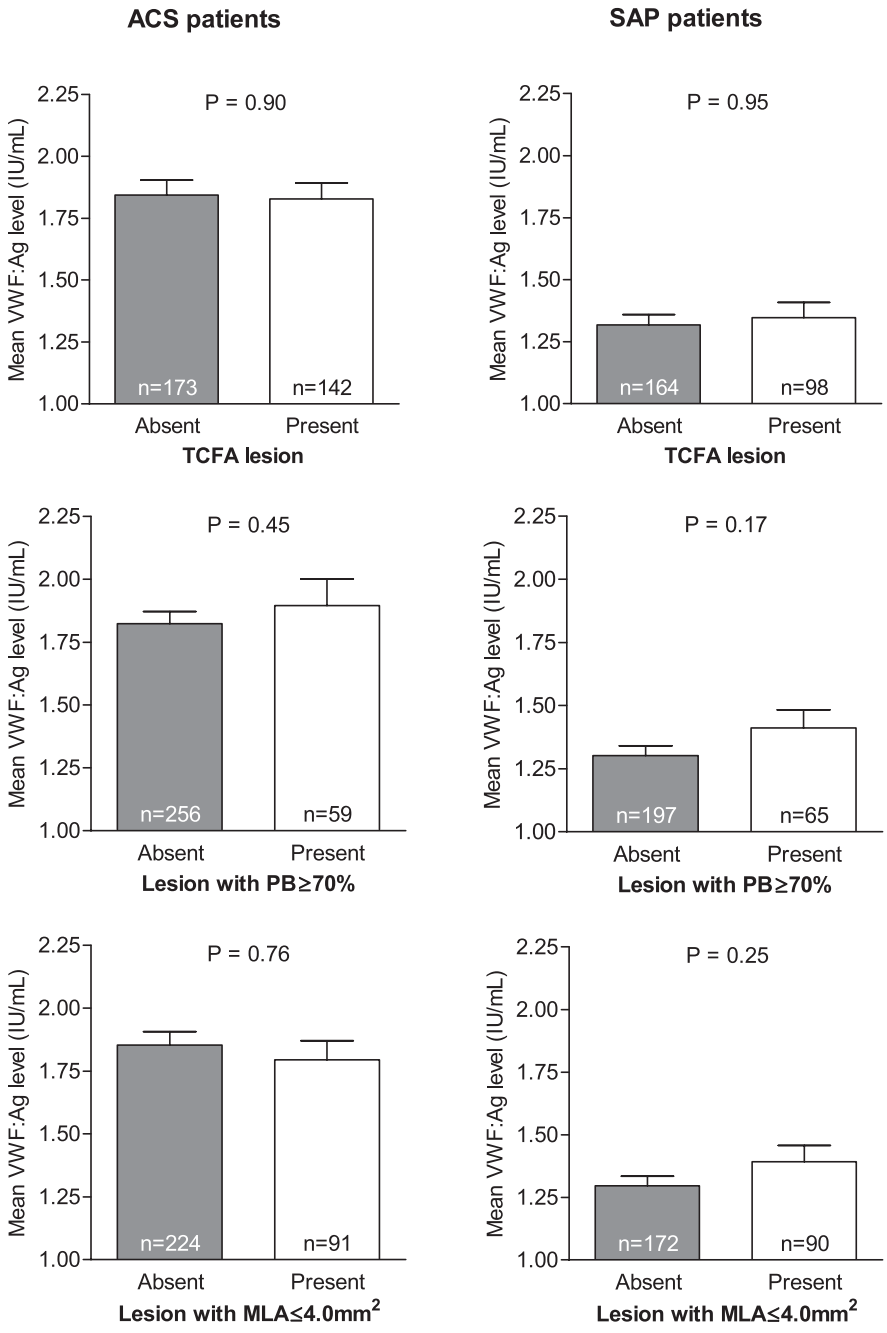

Figure 3. High risk coronary lesions in relation to Von Willebrand Factor levels
Mean ± standard error VWF:Ag levels between high-risk coronary lesions present or absent. ACS = acute coronary syndrome; MLA = minimal luminal area; PB = plaque burden; SAP = stable angina pectoris; TCFA = thin-cap fibroatheroma; VWF:Ag = von Willebrand Factor antigen.

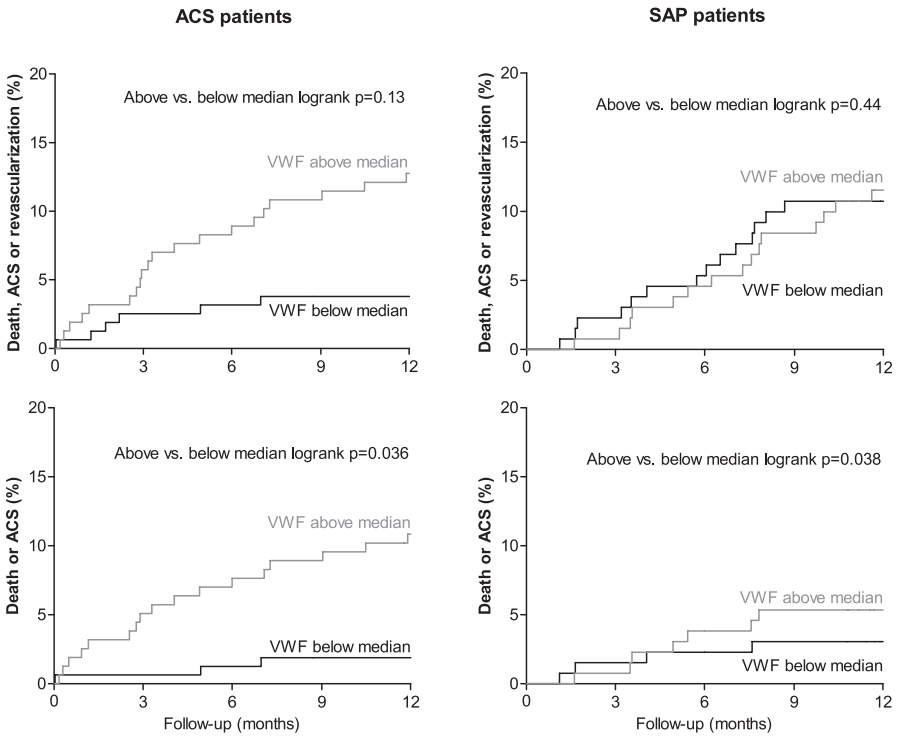

Figure 4. Von Willebrand Factor and cardiovascular outcome
Kaplan-meier curve for the cumulative event-free survival of MACE or death and hospitalization for ACS per VWF:Ag above and below the median (1.45 IU/ml). ACS = acute coronary syndrome; SAP = stable angina pectoris.

DISCUSSION

This is the first study that has investigated the association between invasive measured coronary atherosclerosis by VH-IVUS and VWF:Ag levels. We have shown that patients with an ACS have significantly higher VWF levels than patients with SAP. In patients with SAP, coronary plaque burden was positively associated with VWF:Ag levels. In addition, high VWF:Ag levels were associated with death and ACS at 12 months follow up and this was also observed for all MACE in patients with ACS.

The exact pathophysiologic role of VWF in cardiovascular disease has not been elucidated yet. First, it has been hypothesized that VWF may play a causal role in the development of atherosclerosis, thereby increasing the risk of CAD. This was suggested by animal studies with VWF deficient mice, which showed less development of atherosclerosis (9-11). However, human studies, for instance in patients with type 3 von Willebrand disease who have a complete deficiency of VWF, could not confirm these findings (12, 14). However, these patients may incidentally receive VWF concentrates and some use prophylaxis at regular basis and are therefore not completely VWF deficient. It is now suggested that the association between atherosclerosis and VWF is mainly driven by the fact that VWF is a marker of endothelial damage, which is also observed in atherosclerosis (26, 27).

In this study we found that patients with ACS had significantly higher VWF:Ag levels compared with SAP patients, which is in line with a previous study (24). The finding that plaque burden was associated with VWF:Ag levels in SAP patients confirms our previous findings that VWF is associated with the extent of atherosclerosis. In our previous study in ischemic stroke patients, we observed that a higher calcification volume in the aortic arch and carotid arteries was associated with higher VWF:Ag levels (15). The fact that there was no association between plaque burden and VWF:Ag levels in ACS patients might be explained by the strongly increased VWF:Ag levels in these patients due to an acute phase response, which is well known for VWF (2, 25).

We observed no association between several types of high-risk coronary lesions, including thin-cap fibroatheroma lesions, lesions with plaque burden ≥70% or lesions with a minimal luminal area ≤4.0mm^2 and VWF:Ag levels. High risk lesions are precursors of plaque rupture and may thereby account for the occurrence of coronary thrombi (18, 22, 28). Our results suggest that although VWF is associated with the extent of atherosclerosis, it is not associated with the phenotypic more vulnerable atherosclerotic lesions and might be more involved in stable atherosclerosis. However, a previous mice study showed, by molecular imaging, that activated VWF was found in atherosclerotic disease with high risk features (29). This difference might be explained by the VWF measurement, as only locally activated

VWF was measured in the mice study and in our study we measured circulating VWF:Ag plasma levels. In addition, a difference in the pathophysiologic mechanism of destabilising the plaque between mice and human could also influence the results (30-33).

Our data on the association between VWF:Ag levels and MACE in ACS patients strengthens findings of previous studies suggesting that VWF has a predictive role in cardiovascular outcome (34-39). These results were not affected by additional adjustment for plaque burden, suggesting a role for VWF in cardiovascular outcome. In SAP patients, we found an association between high VWF levels and risk of death or ACS. After additional adjustment for plaque burden the association was not significant anymore in SAP patients, which may be explained by the small sample size, resulting in reduced power. These data suggest that the high VWF levels observed in ACS patients, the most severe CAD patients, at inclusion predict MACE at follow-up. However, in the definition of MACE unplanned revascularisation was included which may be considered as a weaker end-point and could therefore have influenced the adverse outcome risk (40). Overall these data supports the role for VWF in the prognosis of patients with a CAD, independent of plaque burden.

There are some limitations of this study. First, blood was sampled in the acute phase at the moment of the coronary angiography. This may explain the higher VWF:Ag levels in ACS patients compared with SAP patients. Therefore, this could have influenced our results. However, we separated the ACS and SAP patients for all analyses. Secondly, a single non-culprit coronary vessel was imaged in this study. This may have led to an underestimation of the association between the presence of high risk lesions in the overall coronary tree and VWF:Ag levels. However, a previous study have shown that culprit and non-culprit lesions were equally related to MACE (18). In addition, the spatial resolution of IVUS-VH (150 μm) is insufficient to exactly replicate histopathologic definitions of a thin fibrous cap (<65 μm) (41). Therefore, IVUS-VH tends to overestimate the number of thin-cap fibroatheroma lesions. Nevertheless, the presence of VH-TCFA lesions has been shown to carry prognostic information (18, 22). Finally, due to the cross-sectional design our data is not able to distinguish whether VWF is causal or a marker of atherosclerosis.

In conclusion, the extent of coronary atherosclerosis is associated with VWF:Ag levels in SAP patients undergoing coronary angiography, but not in ACS patients which might be explained by the acute phase response. High VWF:Ag levels have a predictive role for adverse cardiovascular outcome, and also for MACE in ACS patients, independent of plaque burden.

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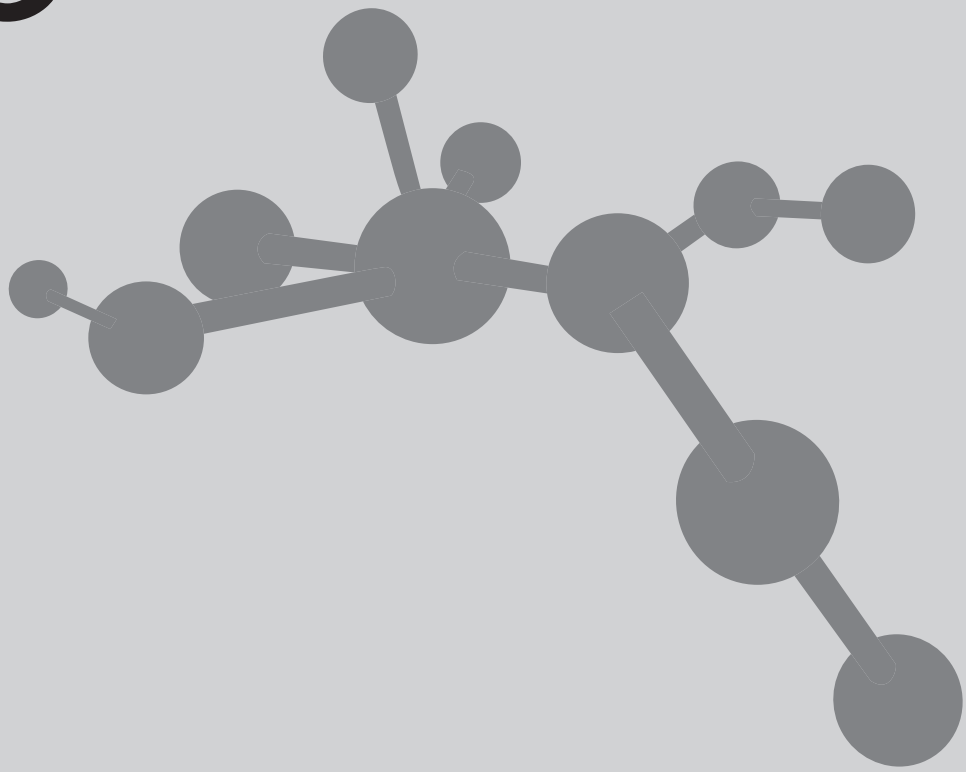

IMAGING BIOMARKERS OF ATHEROSCLEROSIS ARE NOT ASSOCIATED WITH VWF:AG LEVELS OR ADAMTS13 ACTIVITY. THE PLAQUE AT RISK STUDY (PARISK).

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ABSTRACT

Background

High Von Willebrand Factor (VWF) and low ADAMTS13 levels are associated with an increased risk of ischemic stroke and myocardial infarction. One of the candidate mechanisms underlying this association may be the increase of VWF levels by atherosclerosis. Therefore, we assessed the association between novel imaging biomarkers of the advanced atherosclerotic plaque and VWF levels and ADAMTS13 activity.

Methods

In 180 patients of the PARISK-study with a recent TIA or ischemic stroke and a symptomatic mild-to-moderate carotid artery stenosis (Plaque-At-RISK; clinicaltrials.gov NCT01208025), we measured VWF antigen (VWF:Ag) and ADAMTS13 activity. Imaging biomarkers of carotid atherosclerosis were determined by MDCTA (n=158) and MRI (n=169). In this cross-sectional analysis, we used linear regression analysis to assess the association between imaging biomarkers and VWF:Ag levels and ADAMTS13 activity.

Results

Age and blood group were associated with VWF:Ag (β=0.01 IU/ml, p=0.001; β=0.22 IU/ml, p<0.001). Age and time between event and blood withdrawal were inversely associated with ADAMTS13 (β=-0.60%, p=0.002; β=-0.13%, p=0.008). None of the imaging biomarkers were associated with VWF:Ag or ADAMTS13.

Conclusion

In our study, we found no association between imaging biomarkers of advanced atherosclerosis and VWF and ADAMTS13 in patients. It remains unclear whether the blood biomarkers mark widespread atherosclerosis or have a more complex role in atherosclerotic plaque development.

INTRODUCTION

Atherosclerosis of the carotid arteries is an important risk factor for ischemic strokes. With improved imaging techniques like multidetector-row computed tomography (MDCTA) and magnetic resonance imaging (MRI), atherosclerotic burden can be quantified by for example degree of stenosis, maximum vessel wall area or calcification volume; and characteristics of the vulnerable – rupture-prone – plaque can be visualized like plaque ulceration, intraplaque hemorrhage (IPH) and lipid core (1, 2). Vulnerable plaque rupture is crucial in the pathophysiological cascade from atherosclerotic plaque development to thrombus formation and eventually ischemic stroke or TIA.

Von Willebrand Factor (VWF) has an important function in primary hemostasis via its role in platelet adhesion and aggregation, and increases as a result of endothelial damage. *A Disintegrin And Metalloprotease with ThromboSpondin motif repeats 13* (ADAMTS13) cleaves large VWF multimers into smaller and less prothrombotic forms. Levels of VWF are increased in ischemic stroke patients and the highest levels of VWF are found in large vessel disease and cardio-embolic strokes (3, 4). High VWF and low ADAMTS13 levels are associated with an increased risk of ischemic stroke and myocardial infarction (5-7). One of the candidate mechanisms underlying the association between VWF and ischemic stroke may be atherosclerosis. Previous studies suggest that atherosclerosis (and not only thrombus formation) is a determinant of VWF levels (8). On the other hand, *in vitro* and *in vivo* studies suggest that VWF might contribute to the pathogenesis of atherosclerosis (9). In addition, studies on animals with a VWF deficiency suggest that VWF deficiency has a protective effect against atherosclerosis and VWF may be a determinant of atherosclerosis (9-11). When taking all these studies together, the data on this topic are inconclusive (9, 12, 13). We expect that, due to the role of ADAMTS13 in the metabolism of VWF, atherosclerosis might play a role in the association between ADAMTS13 and ischemic events as well.

The aim of our study was to evaluate the association between novel imaging biomarkers of the atherosclerotic plaque and VWF and ADAMTS13 in patients with a recent TIA or ischemic stroke and a symptomatic mild-to-moderate carotid artery stenosis to help clarify the association between atherosclerosis, VWF and ADAMTS13.

METHODS

Study population

Patients were derived from the PARISK-study (Plaque-At-RISK; clinical trials.gov NCT01208025); details of the study design have been previously described (14). The PARISK-study is a prospective multicenter cohort study using non-invasive plaque imaging to identify patients with an ipsilateral mild-to-moderate carotid artery stenosis (30-69%) with an increased risk of recurrent stroke. All included patients had a recent TIA, including amaurosis fugax, or minor stroke in the carotid artery territory prior to inclusion. Institutional Review Board approval was obtained and all patients gave written informed consent.

Between September 2010 and December 2014, 240 patients were included in the PARISK-study; 180 patients had either a MDCTA (n=158) or MRI (n=169) of the carotid arteries, and had an available blood sample.

MDCTA and 3T MRI data acquisition and analysis

Standardized, previously described, contrast-enhanced MDCTA and multi-sequence contrast-enhanced MRI protocols were used (14). All imaging studies were evaluated by trained readers blinded for clinical data and other imaging tests (15).

MDCTA images were reviewed using dedicated 3D analysis software (Leonardo and syngo. via; Siemens, Erlangen, Germany). First, image quality was rated on a 3-point scale; poor (not eligible for analysis), moderate and good (eligible for analysis) (15). The most severe stenosis in the symptomatic carotid bifurcation and internal carotid artery was measured according to the ECST criteria, perpendicular to the central lumen line (16). Additionally, we defined plaque ulceration as an extension of contrast material of >1mm into the atherosclerotic plaque on at least 2 orthogonal planes (17, 18). Finally, a custom-made plug-in for the freely available Image J software (National Institutes of Health, Bethesda, Maryland) was used to quantify calcifications in the symptomatic carotid artery within 3 cm proximal and distal to the bifurcation. We used a threshold of 600 HU to differentiate calcifications from contrast material in the lumen; calcification volume was expressed in cubic millimeters. A detailed description of the measurements is provided elsewhere (19).

MR images were evaluated with dedicated vessel wall analysis software (Vesselmass, Department of Radiology, Leiden University Medical Center, Netherlands). Image quality was rated on a 5-point scale; low SNR (not eligible for analysis) to marginal and high SNR (eligible for analysis) (15, 20). MR images were automatically registered by delineating the lumen and outer vessel wall of the symptomatic carotid artery. Registration was manually corrected if needed. Plaque components of the symptomatic carotid artery (lipid, calcifications, IPH) were manually segmented. Fifteen transverse adjoining slices of 2 mm each covering the entire plaque were annotated. Maximum vessel wall area and plaque component volumes of the symptomatic carotid artery were derived from these annotations.

Blood sampling, VWF levels and ADAMTS13 activity measurements

Citrated blood was centrifuged at 2000 g for 10 minutes; then the plasma was centrifuged at 14000 g for 10 minutes and stored in aliquots at -80ºC. VWF:Antigen (VWF:Ag) levels were measured with an in-house ELISA, using polyclonal rabbit anti-human VWF antibodies (Dakocytomation, Glostrup, Denmark) for catching and tagging. ADAMTS13 activity was measured using the Fluorescence Resonance Energy Transfer Substrate VWF 73 (FRETS-VWF73) (21). The inter- and intra-assay coefficient of variation for VWF:Ag levels and ADAMTS13 activity were 8.7% and 1.9% (VWF:Ag); 13.1% and 2.9% (ADAMTS13), respectively.

Statistical analysis

In this cross-sectional analysis, linear regression models were used to investigate the association between imaging biomarkers of the symptomatic carotid artery and VWF:Ag levels or ADAMTS13 activity, respectively. VWF:Ag levels were not normally distributed and therefore log-transformed. All quantitative imaging biomarkers were divided into tertiles. Adjustments were made for age and gender (model 1), and additionally for cardiovascular risk factors (model 2; age, gender, current smoking, hypertension, hypercholesterolemia, diabetes mellitus, cardiovascular history, BMI). In the analyses with VWF:Ag, we also adjusted for blood group. The analyses were repeated after adding interval event-blood withdrawal to the covariates. Statistical analyses were performed using STATA software (version 13.1, StataCorp, College Station, Texas). $P<0.05$ was considered statistically significant.

RESULTS

Baseline clinical characteristics, VWF:Ag levels, ADAMTS13 activity and imaging characteristics are shown in Table 1. Mean age was 68 years, 74% were male and prevalence of cardiovascular risk factors was high. We found a median VWF:Ag level of 1.45 IU/ml [1.10 - 1.81] and a mean ADAMTS13 activity of 98.6% (± 22.8%). There was no correlation between VWF:Ag levels and ADAMTS13 activity (R=0.06, P=0.42). Mean symptomatic carotid artery stenosis (ECST) was 55 ± 15 %.

Table 1. Clinical characteristics, blood measurements and imaging biomarkers*

Clinical characteristic (n=180)	
Age (years)	68 ± 9
Male	133 (74%)
Current smoking	39 (22%)
BMI	26.7 ± 4.4
Hypertension	127 (71%)
Hypercholesterolemia	139 (77%)
Diabetes Mellitus	44 (24%)
History of CVD and PAD	85 (47%)
Classification event	
TIA	
Stroke	
Amaurosis fugax	
77 (43%)	
80 (44%)	
23 (13%)	
Blood measurements (n=180)	
Interval event-blood withdrawal (days)	47 [32-67]
VWF:Ag (IU/ml)	1.45 [1.10-1.81]
ADAMTS13 activity (%)	98.6 ± 22.8
Blood group non-O	106 (59%) Imaging biomarkers (symptomatic artery)
Degree of stenosis (ECST) (%)†	55 ± 15
MDCTA (n=158)	
Interval event–MDCTA (days)	32 [12-54]
Presence plaque ulceration	43 (27%)
Presence calcifications	142 (90%)
Calcification volume (mm^3)	25.9 [5.1-80.7]
MRI (n=169)	
Interval event–MRI (days)	47 [30-67]
Maximum vessel wall area (mm^2)	72.7 [57.2-88.1]
Presence IPH	66 (39%)
IPH volume (mm^3)	0.0 [0.0-39.7]
Presence lipid	106 (63%)
Lipid volume (mm^3)	26.2 [0-146.0]

* Data are mean ± SD, absolute numbers of patients (%), or median [25th–75th percentile]; † If MDCTA was absent, degree of stenosis was assessed at MRI (n=22); CVD, cardiovascular disease; PAD, peripheral arterial disease; TIA, transient ischemic attack; ECST, European Carotid Surgery Trial; IPH, intraplaque hemorrhage

Increasing age was associated with higher VWF:Ag levels (β= 0.01IU/ml/year, p=0.001) and individuals with blood group non-O had higher VWF:Ag levels compared with individuals with blood group O (β= 0.22 IU/ml, p<0.001). None of the other clinical characteristics were significantly associated with VWF:Ag levels. Age was inversely associated with ADAMTS13 activity (β= -0.60%/year, p=0.002). We also found an inverse association between the time from event to blood withdrawal and ADAMTS13 activity (β= -0.13%/day, p=0.008). None of the other clinical characteristics were associated with ADAMTS13 activity.

None of the qualitative and quantitative measures of atherosclerosis were associated with VWF:Ag levels or ADAMTS13 activity in model 1 and 2 (Table 2). Additional adjustment for interval event–blood withdrawal did not change the results.

Table 2. Imaging characteristics and VWF:Ag levels and ADAMTS13 activity

Characteristic	VWF (model 1)*		ADAMTS13 (model 1)*	
	Beta [95% CI]	P value	Beta [95% CI]	P value
Degree of stenosis (ECST)†	-0.02 [-0.09;0.04]	0.53	-2.29 [-6.30;1.71]	0.26
Maximum vessel wall area†	-0.03 [-0.11;0.05]	0.41	2.50 [-2.11;7.10]	0.29
Calcification volume†	0.00 [-0.07;0.08]	0.91	-1.87 [-6.44;2.71]	0.42
Lipid volume†	-0.05 [-0.12;0.02]	0.14	1.43 [-2.76;5.62]	0.50
IPH volume†	-0.04 [-0.10;0.02]	0.18	0.70 [-3.11;4.51]	0.72
Plaque ulceration	-0.12 [-0.24;0.01]	0.08	0.56 [-7.40;8.52]	0.89

* Adjusted for age and gender; additionally for blood group non-O in case of VWF; † in tertiles; ECST, European Carotid Surgery Trial; IPH, intraplaque hemorrhage

DISCUSSION

This study does not show associations between novel imaging biomarkers of atherosclerosis and VWF:Ag levels or ADAMTS13 activity in patients with a recent TIA or ischemic stroke and a symptomatic mild-to-moderate carotid artery stenosis.

The strength of our study is that we characterized the carotid atherosclerotic plaque using two image modalities (MDCTA and MRI) and assessed atherosclerotic burden as well as vulnerable plaque characteristics. Furthermore, this is the first study investigating the relationship between novel imaging biomarkers with VWF:Ag levels and ADAMTS13 activity.

A limitation of our study was the cross-sectional design, which precludes the unraveling of cause and effect. We had a median delay of 47 days between clinical event and blood sampling/imaging, which might have influenced the association via a change in plaque composition and VWF levels (22). VWF is known to be increased in the acute phase of an event (23). However, imaging and blood sampling were performed at the same moment and we found no significant correlation between the interval event-blood withdrawal and VWF:Ag. We found a slight correlation between the interval event-blood withdrawal and ADAMTS13 activity, however this might be a chance finding. Nonetheless, adjustment for the interval did not influence the association between imaging and blood biomarkers.

In a previous study, we found a strong correlation between calcification volume in the aortic arch and carotid arteries and VWF levels in patients with an ischemic stroke or TIA. In accordance to literature we also found in this previous study significantly higher VWF levels in patients with large artery atherosclerosis compared to other etiological subtypes of TIA or stroke (3, 4, 24). No previous studies are known investigating the association between ADAMTS13 and atherosclerosis. In the current study, all patients had a carotid artery stenosis and we assessed plaque volume as well as vulnerable plaque characteristics; no associations were found between any of the novel imaging biomarkers and VWF and ADAMTS13. It might be that VWF levels are differently associated with plaque volume measurements than with vulnerable plaque characteristics. For instance, in acute coronary syndrome patients, the presence of atherosclerosis measured by IVUS was associated with VWF:Ag levels, but VWF:Ag levels were not associated with high risk, prone-to-rupture atherosclerotic lesions (25). However, due to the clear role of VWF in thrombus formation and the less clear role of VWF and ADAMTS13 in atherosclerotic plaque development, we expected to find the opposite. It seems that neither the local disturbance of blood flow nor the disruptive plaque surface in the carotid bifurcation causes an increase in VWF or a decrease in ADAMTS13. It may be that the systemic alteration of the endothelial layer due to widespread atherosclerotic disease causes the change in VWF. If this is the case, blood coagulation markers are then a marker of a widespread atherosclerotic disease and not a modifiable risk marker for secondary prevention. However, a complex role of VWF and ADAMTS13 in atherosclerotic plaque development cannot be ruled out.

CONCLUSIONS

Imaging biomarkers of atherosclerosis were not associated with VWF:Ag levels or ADAMTS13 activity in patients with a recent TIA or ischemic stroke and a symptomatic mild-to-moderate carotid artery stenosis. Whether the blood biomarkers are simply a marker of widespread atherosclerosis or have a more complex role in atherosclerotic plaque development, remains unclear.

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6

LOW ADAMTS13 ACTIVITY IS ASSOCIATED WITH AN INCREASED RISK OF ISCHEMIC STROKE

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ABSTRACT

Background

ADAMTS13 (A Disintegrin and Metalloprotease with TromboSpondin motif repeats 13) has antithrombotic properties because it cleaves Von Willebrand factor (VWF) in smaller, less active multimers. The aim of our study was to investigate prospectively the association between ADAMTS13 activity and ischemic stroke.

Methods

We included 5941 individuals ≥ 55 years without a history of stroke or Transient Ischemic Attack (TIA) of the Rotterdam Study, a population-based cohort study. ADAMTS13 activity was measured at inclusion with the FRETS-VWF73 assay and VWF antigen (VWF:Ag) levels by ELISA. We assessed the association between ADAMTS13 activity, VWF:Ag levels and ischemic stroke by Cox proportional hazard analysis. The added value of ADAMTS13 activity above the traditional risk factors for ischemic stroke risk prediction was examined by the c-statistic and the net reclassification improvement index (NRI). All individuals were followed for incident stroke or TIA.

Results

Over a median follow-up time of 10.7 years (56403 total person years), 461 participants experienced a stroke, 306 of which ischemic. After adjustment for cardiovascular risk factors, individuals with ADAMTS13 activity in the lowest quartile had a higher risk of ischemic stroke (absolute risk 7.3%) than those in the reference highest quartile (absolute risk 3.8%; HR 1.65, 95% CI 1.16–2.32). Adding ADAMTS13 to the model in prediction of ischemic stroke, increased the c-statistic by 0.013 (P=0.003) and provided 0.058 (95% CI -0.002–0.119) NRI.

Conclusion

Low ADAMTS13 activity is associated with the risk of ischemic stroke and improves the accuracy of risk predictions for ischemic stroke beyond traditional risk factors.

INTRODUCTION

Ischemic stroke is a major cause of mortality and morbidity in the Western World [1]. Although many risk factors have been identified, its pathogenesis remains still largely unclear and treatment options are still limited. The discovery of new risk factors might thus support the development of new preventive measures and treatment strategies.

ADAMTS13, part of the family of *A Disintegrin and Metalloprotease with TromboSpondin motif* [2,3], has antithrombotic properties by cleaving ultralarge Von Willebrand Factor (VWF) multimers into smaller forms[4]. Ultralarge VWF multimers are the most procoagulant forms leading to platelet adhesion and aggregation and finally thrombus formation. Several studies have reported an association between high VWF levels and the risk of ischemic stroke [5-7]. The importance of ADAMTS13 in circulation is exemplified by patients who develop thrombotic thrombocytopenic purpura (TTP) due to a severe deficiency of ADAMTS13, resulting in reduced cleavage of high molecular weight VWF multimers. This leads to an increase of these procoagulant VWF multimers, which in turn can result in microthrombus formation, frequently leading to disturbances of the cerebral circulation or other organs. In TTP patients microthrombi may lead to focal neurological deficit, seizures and even coma [8]. The importance of circulating ADAMTS13 was also shown in animal models. Several animal studies have indicated a pathogenic role of ADAMTS13 in the development or progression of ischemic stroke. Studies in which focal cerebral ischemia was experimentally induced showed larger cerebral infarctions in ADAMTS13-deficient mice than in wild-type mice [9-11].

These data and small case-control studies suggest that ADAMTS13 plays a role in cardiovascular disease, but no prospective studies have yet established the relationship between the VWF cleavage protease ADAMTS13 and the risk of ischemic stroke (IS)[7,12-15].To investigate the role of ADAMTS13 and VWF in the pathogenesis of ischemic stroke, we investigated prospectively the longitudinal association of ADAMTS13 activity, and its interaction with VWF and the risk of ischemic stroke in a large population based cohort of nearly 6000 individuals above the age of 55. We also examined whether addition of ADAMTS13 activity to the traditional risk factors would lead to improvements in ischemic stroke risk prediction.

MATERIAL AND METHODS

Study design and study population

We included participants from the Rotterdam Study (RS), a prospective population-based cohort study among individuals of 55 years and older living in a suburb in the city of Rotterdam, the Netherlands[16,17]. The study started in 1990 (RS-I). In 1999, additional individuals who had turned 55 years or had moved into the study district since the start of the study were added to the cohort (RS-II). All participants visited the research center every 3 to 4 years, where established cardiovascular risk factors are assessed. For this study, we used data obtained from participants at the third examination of the original cohort (RS-I-3, 1997-1999) and the first examination of the extended cohort (RS-II-1, 2000-2001). Protocols for the original and the extended cohort were similar. Previous findings of the Rotterdam Study are summarized in table 6 (online supplement). The Rotterdam Study has been approved by the medical ethics committee according to the Population Study Act Rotterdam Study, executed by the Ministry of Health, Welfare and Sports of the Netherlands. A written informed consent was obtained from all participants.

Assessment of stroke and TIA

History of stroke and TIA was determined during the baseline interview and verified in medical records. After enrollment in the Rotterdam Study, participants were continuously monitored for incident strokes and TIAs through automated linkage of the study database with files from general practitioners, the municipality, and nursing home physicians. Additional information was obtained from hospital records. Potential strokes were reviewed by research physicians, and verified by an experienced stroke neurologist (P.J.K.). Events were structured based on this information and a diagnosis was made based on the criteria independent of the diagnosis of the initial caregivers. Subarachnoid hemorrhages and retinal strokes were excluded. Strokes were classified as ischemic, hemorrhagic or unspecified. Ischemic stroke was diagnosed if a CT or MRI scan carried out within four weeks after the event ruled out other diagnoses. A hemorrhagic stroke was diagnosed if a relevant hemorrhage was shown on CT or MRI scan. If no neuroimaging was performed, the stroke was classified as unspecified. TIA was defined by focal symptoms that started suddenly and improved within seconds with a maximum duration of 24 hours.

Baseline characteristics

At inclusion in the study (RS-I-3 and RS-II-1), a detailed interview was taken from all participants, as well as an extensive set of examinations, including a physical examination and blood sampling. Blood pressure was calculated as the mean of two measurements using a random-zero sphygmomanometer at the right brachial artery while the individual was in a sitting position. Antihypertensive drugs was defined as the use of antihypertensive

medication indicated for the treatment of high blood pressure (≥ grade 1 hypertension according to World Health Organization criteria[18]). Grade 1 hypertension was defined as systolic >140 mmHg or a diastolic blood pressure >90 mmHg. Grade 2 hypertension was defined as systolic >160 mmHg or a diastolic blood pressure >100 mmHg. Antithrombotic medication was defined as the use of vitamin K antagonists, platelet aggregation inhibitors, and direct thrombin inhibitors. Diabetes mellitus was defined as the use of blood glucose-lowering medication and/or a fasting serum glucose level ≥ 7.0 mmol/L. Lipid reducing agents was defined as the use of statins. Total cholesterol and high-density lipoprotein cholesterol were measured using an automated enzymatic procedure. Body mass index was calculated as the weight (in kilograms) divided by the square of the height (in meters). Smoking status was defined as current, former and no smoking. Blood group antigen phenotypes were reconstructed by haplotypes analysis of 4 single nucleotide polymorphisms, rs687289, rs507666, rs8176704, and rs8176749, which collectively serve as tagging SNPs for the O, A1, A2, and B allele.

ADAMTS13 activity measurements

ADAMTS13 activity was measured in a kinetic assay using the Fluorescence Resonance Energy Transfer Substrate VWF 73 (FRETS-VWF73) as previously described[19]. Samples were measured against a reference curve of serial dilutions of normal human plasma defined to have an ADAMTS13 activity of 1 U/ml. Normal ADAMTS13 activity is 100% with a range between 50% and 150%[20,21]. 10% of the samples were retested and all were within 25% variation.

VWF antigen (VWF:Ag) levels were determined with an in-house ELISA, using polyclonal rabbit anti-human VWF antibodies (DakoCytomation, Glostrup, Denmark) for catching and tagging.

Statistical analysis

Data on baseline characteristics are shown as mean and standard deviation for continuous variables and as counts and percentages for categorical variables. The association between ADAMTS13 and baseline characteristics was assessed by univariate and multivariable linear regression and presented as beta-coefficients. Beta-coefficients represent the change in ADAMTS13 activity with an increase of 1 unit of the specific variable. In case of categorical variables, this means that having the characteristic is associated with a change of beta in ADAMTS13 activity compared with participants who did not have the characteristic. To assess proportional hazards assumption, we tested the log minus log plots. The association between ADAMTS13 and stroke, was performed by Cox proportional hazards regression analysis using ADAMTS13 quartiles, derived from the whole cohort. We examined the

association of ADAMTS13 activity with all strokes (hemorrhagic, ischemic and unspecified), ischemic strokes, TIA and all cerebrovascular events (TIA and all strokes). Analyses with stroke were censored for TIA occurring before the stroke and analyses with TIA were censored for stroke. Consequently, participants were followed from inclusion to stroke, TIA, death, last health status update where they were known to be free of stroke or TIA, or January 1, 2012, whichever came first. Follow-up was complete until January 1, 2012, for 95.4% of potential person years[22]. The total number of person years was 56,403. A curve with cumulative incidence of TIA and all strokes during follow-up was constructed for each of the ADAMTS13 activity quartiles. For the interaction between VWF:Ag and ADAMTS13 and the risk of stroke, we performed a Cox proportional hazard regression analysis with addition of an interaction term to the model. For the association between ADAMTS13 and VWF with the risk of stroke, a combination of ADAMTS13 activity ≤ and > the 25th percentile and VWF:Ag levels ≥ and < the 75th percentile were used. We adjusted all analyses for age and sex, and additionally for antithrombotic agents, antihypertensive drugs, diabetes mellitus, lipid reducing agents, BMI, smoking, total cholesterol, HDL cholesterol, systolic blood pressure, and diastolic blood pressure.

To address the additional predictive value of ADAMTS13 in ischemic stroke risk prediction, we formed 2 models. The first model consisted of the variables included in the recent American Heart Association Pooled Cohort Equation; age, sex, systolic blood pressure, treatment for hypertension, total and high density lipoprotein cholesterol, smoking and diabetes [23]. The second model additionally included quartiles of ADAMTS13 activity. We then compared the two models using likelihood ratio chi-square test for global model fit, c-statistic for model discrimination, and net reclassification improvement index (NRI). Discrimination refers to the ability of the model to assign a higher risk to individuals who will develop an event compared to the individuals who will not. NRI specifies the amount of correct reclassification of individuals with event and individuals without event after extension of the model with ADAMTS13. The NRI estimates were based on the 10-year ischemic stroke risk categories of <5%, 5% to 7.5%, and >7.5%[24].

Missing values of these covariates (0-4.7%) were imputed five times using a multiple imputation method including all covariates. Statistical analyses were performed on all five different datasets and pooled into one final result using SPSS software. Data were analyzed with SPSS version 21 (SPSS 21.0. IBM, Somers, NY, USA) and R, version 3.1.2. All statistical tests were two-tailed and p-values <0.05 were considered statistically significant.

RESULTS

The Rotterdam Study started with 7,983 participants (out of 10,215 invitees) and was extended with an additional 3,011 participants (out of 4,472 invitees). We included all participants of whom blood was sampled (n = 6494). All participants with a history of stroke (n = 257) or TIA (n = 296) at the moment of blood sampling (1997 – 2001) were excluded from the analysis. A total of 5941 individuals over 55 years were included in this study. Over a median follow-up time of 10.7 years (IQR 7.9 – 11.6) and 56403 total person years, 461 individuals experienced a stroke, 306 of which (66%) ischemic stroke, 48 (10%) hemorrhagic and 107 (23%) unspecified stroke. A TIA occurred in 315 individuals during follow-up. The mean age of all individuals was 69 years (± 8.1) and 57% was female (Table 1). The mean ADAMTS13 activity in the total cohort was 91.9 ± 17.8%. The mean time between ADAMTS13 activity measurement and the occurrence of stroke or TIA were 5.80 and 5.46 years, respectively.

Baseline characteristics and ADAMTS13 activity

ADAMTS13 activity was associated with age and sex, also after adjustment for cardiovascular risk factors. In this cohort we observed a decrease of 5.68% in ADAMTS13 activity per 10 year increase of age (Beta coefficient 5.68%, P<0.001) and we found 8.6% higher ADAMTS13 activity in women vs. men (P<0.001; table 2). Several cardiovascular risk factors were also associated with ADAMTS13 activity, including diabetes mellitus, cholesterol, and smoking (Table 2).

ADAMTS13 activity and the risk of stroke

Compared with individuals in the highest quartile of ADAMTS13 activity, individuals with an ADAMTS13 activity within the lowest quartile had an increased risk of all strokes after adjustment for age and sex (absolute risk lowest quartile 11.0%, highest quartile 5.3%; HR 1.52 95% CI 1.15–2.02) and there was no statistically significant change after additional adjustment for cardiovascular risk factors (HR 1.49 95% CI 1.12–2.00) (Table 3). There was no graded response effect from quartile 4 to quartile 1. Taking ADAMTS13 activity as a continuous variable (per SD decrease), we found an increased risk for stroke (HR 1.12 95% CI 1.01-1.24) (Table 3). For ischemic stroke (absolute risk 7.3%) the HR was 1.65 (95% CI 1.16–2.32). The risk for ischemic stroke was also increased when taking ADAMTS13 as a continuous variable (HR 1.19 95% CI 1.05–1.34). Individuals in the lowest quartile of ADAMTS13 activity had also an increased risk of TIA (absolute risk 6.8%) and all cerebrovascular events (absolute risk 17.8%) (HR 1.64 95% CI 1.17–2.31; HR 1.56 95% CI 1.25–1.94). These results were similar for all outcomes if we adjusted additionally for prevalent coronary heart disease (Table 7, online supplement) and for blood group. The cumulative incidence of TIA and all strokes per ADAMTS13 quartile over the total follow-up period is shown in figure 1.

Table 1. Baseline characteristics of the total study population

N = 5941	**Total cohort N (%) or mean ± SD**
Age (years)	69.0 ± 8.1
Female sex	3401 (57.2%)
Smoking	
Current	1020 (17.3%)
Former	2907 (49.4%)
BMI (kg/m^2)	27.0 ± 4.1
Use of antithrombotic medication	952 (16.8%)
Use of antihypertensive drugs	1314 (23.2%)
Use of lipid reducing agents	716 (12.6%)
Presence of diabetes Mellitus	599 (10.2%)
Total cholesterol (mg/dL)[1]	224.7 ± 37.7
HDL cholesterol (mg/dL)[1]	53.8 ± 14.9
Hypertension	
Systolic blood pressure (mmHg)	143 ± 21
Diastolic blood pressure (mmHg)	77 ± 11
Grade I hypertension	2309 (39.1%)
Grade II hypertension	879 (14.9%)
Blood group O	2288 (45.3%)
ADAMTS13 activity (%)	91.89 ± 17.8

[1] SI conversion factors. To convert total cholesterol and HDL cholesterol to mmol/L, multiply values by 0.0259.

ADAMTS13 activity and VWF:Ag levels

There was no relevant correlation between VWF:Ag levels and ADAMTS13 activity, although it was significant ($R^2$0.01, P<0.001). Individuals who had both the lowest ADAMTS13 activity (≤25 percentile) and the highest VWF:Ag levels (≥75 percentile) had an increased risk of all strokes (absolute risk 13.5%) and ischemic stroke (absolute risk 9.1%) compared to the remaining individuals (HR 1.49 95%CI 1.11–2.01; HR 1.71 95% CI 1.19-2.45, respectively) (Table 4). These data did not change when we additionally adjusted for prevalent coronary heart disease (Table 8, online supplement). When comparing individuals with a low ADAMTS13 activity and high VWF:Ag to individuals with a high

Table 2. Association between ADAMTS13 and baseline characteristics

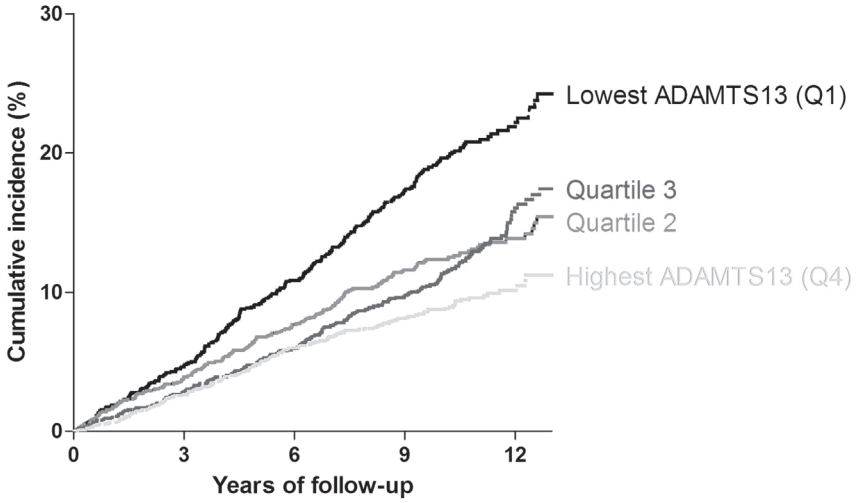
	Univariate Beta-coefficient (95% CI)	P-value	MultivariableBeta-coefficient (95% CI)	P-value
Every 10 year increase of age	-5.23 (-5.77 - -4.68)	<0.001	-5.68 (-6.28 - -5.09)	<0.001
Female sex	7.19 (6.29 – 8.08)	<0.001	8.60 (7.64 – 9.55)	<0.001
Antihypertensive drugs	0.35 (-0.75 – 1.45)	0.53	-0.24 (-1.31 – 0.84)	0.668
Lipid reducing agents	1.62 (0.20 – 3.05)	0.026	2.12 (0.69 – 3.54)	0.004
Antithrombotic medication	-4.52 (-5.76 - -3.28)	<0.001	-1.64 (-2.90 - -0.38)	0.011
Diabetes mellitus	4.10 (2.59 – 5.61)	<0.001	5.17 (3.72 – 6.62)	<0.001
Total cholesterol (mg/dL)	0.05 (0.04 – 0.06)	<0.001	0.03 (0.02 – 0.04)	<0.001
HDL cholesterol (mg/dL)	-0.02 (-0.05 – 0.02)	0.315	-0.12 (-0.15 - -0.08)	<0.001
Every 10 mmHg increase in systolic blood pressure	-0.11 (-0.33 – 0.10)	0.302	0.19 (-0.08 – 0.46)	0.162
Every 10 mmHg increase in diastolic blood pressure	0.57 (0.16 – 0.98)	0.006	-0.11 (-0.61 – 0.40)	0.679
BMI (kg/m^2)	0.30 (0.19 – 0.41)	<0.001	-0.04 (-0.15 – 0.07)	0.472
Current smoking	-3.17 (-4.37 - -1.98)	<0.001	-4.95 (-6.09 - -3.82)	<0.001

Univariate and multivariable linear regression analysis, beta-coefficient represents the change in ADAMTS13 activity with 95% confidence interval per unit increase of the selected variable.

ADAMTS13 activity and low ischemic stroke (absolute risk 9.1%) (HR 2.94 95% CI 1.49–5.78; HR 3.51 95% CI 1.60–7.70, respectively) (Table 9, online supplement). Formal statistical testing did not reveal a significant interaction between VWF:Ag levels and ADAMTS13 activity in the association with all strokes, nor with ischemic stroke (P=0.93 and P=0.78, respectively).

Added predictive value of ADAMTS13 activity in ischemic stroke risk prediction

Table 5 summarizes the added value of ADAMTS13 for the prediction of ischemic stroke above the traditional risk factors. Addition of ADAMTS13 to the model including the traditional risk factors, improved the model fit; i.e. the Likelihood ratio test statistics (X^2) increased from 123.7 to 136.9 (P=0.013). The model including ADAMTS13 also increased the c-statistic by 0.013 (P=0.003) in prediction of ischemic stroke. Addition of ADAMTS13 to the traditional risk factor model provided an NRI of 0.058% (95%CI -0.002 - 0.119) for the total population. Among subjects in the 5%-7.5%, which can be considered an intermediate risk category for ischemic stroke, the NRI was 0.212 (95% CI 0.048 - 0.376), composed of NRI of 13.5% for events and 7.7% for non-events. Adding VWF to the model including traditional risk factors and ADAMTS13 did not provide any improvement in risk prediction.



No. at risk					
Quartile 1	1486	1304	1079	846	253
Quartile 2	1485	1366	1232	1053	292
Quartile 3	1485	1394	1270	1104	309
Quartile 4	1485	1399	1295	1153	279

Figure 1. Number of events per ADAMTS13 quartile

Number of all cerebrovascular events (TIA and all strokes) per quartile of ADAMTS13 activity. Cut-off points (%) of ADAMTS13 activity quartiles were: ≤80.73%, 80.74 – 91.44%, 91.45 – 102.26%, and ≥ 102.27%.

Table 3. Cox proportional hazard regression analysis between ADAMTS13 quartiles and stroke

ADAMTS13 level	Mean ADAMTS13 activity (95% CI)	Number of cases / Total number at risk	Absolute risk (%)	Model 1 HR (95% CI)	Model 2 HR (95% CI)
All strokes (N= 461)					
Quartile 1	70.3 (69.8 – 70.8)	163 / 1486	11.0	1.52 (1.15 – 2.02)	1.49 (1.12 – 2.00)
Quartile 2	86.3 (86.2 – 86.5)	108 / 1485	7.2	1.10 (0.82 – 1.47)	1.08 (0.80 – 1.45)
Quartile 3	96.6 (96.4 – 96.7)	111 / 1485	7.5	1.18 (0.88 – 1.58)	1.17 (0.88 – 1.57)
Quartile 4	114.4 (113.8 – 114.9)	79 / 1485	5.3	1 (ref)	1 (ref)
Per SD decrease				1.13 (1.02 - 1.24)	1.12 (1.01 – 1.24)
Ischemic strokes (N= 306)					
Quartile 1	70.3 (69.8 – 70.8)	109 / 1486	7.3	1.61 (1.15 – 2.26)	1.65 (1.16 – 2.32)
Quartile 2	86.3 (86.2 – 86.5)	70 / 1485	4.7	1.07 (0.75 – 1.53)	1.08 (0.75 – 1.54)
Quartile 3	96.6 (96.4 – 96.7)	71 / 1485	4.8	1.12 (0.79 – 1.60)	1.13 (0.80 – 1.62)
Quartile 4	114.4 (113.8 – 114.9)	56 / 1485	3.8	1 (ref)	1 (ref)
Per SD decrease				1.18 (1.04 – 1.33)	1.19 (1.05 – 1.34)
TIA (N= 315)					
Quartile 1	70.3 (69.8 – 70.8)	101 / 1486	6.8	1.55 (1.11 – 2.18)	1.64 (1.17 – 2.31)
Quartile 2	86.3 (86.2 – 86.5)	78 / 1485	5.3	1.20 (0.85 – 1.69)	1.25 (0.89 – 1.76)
Quartile 3	96.6 (96.4 – 96.7)	77 / 1485	5.2	1.19 (0.84 – 1.67)	1.21 (0.86 – 1.71)
Quartile 4	114.4 (113.8 – 114.9)	59 / 1485	4.0	1 (ref)	1 (ref)
Per SD decrease				1.17 (1.03 – 1.24)	1.19 (1.05 – 1.34)
Any cerebrovascular event (N= 776)					
Quartile 1	70.3 (69.8 – 70.8)	264 / 1486	17.8	1.54 (1.24 – 1.91)	1.56 (1.25 – 1.94)
Quartile 2	86.3 (86.2 – 86.5)	186 / 1485	12.5	1.14 (0.91 – 1.42)	1.15 (0.92 – 1.44)
Quartile 3	96.6 (96.4 – 96.7)	188 / 1485	12.7	1.18 (0.95 – 1.48)	1.19 (0.95 – 1.48)
Quartile 4	114.4 (113.8 – 114.9)	138 / 1485	9.3	1 (ref)	1 (ref)
Per SD decrease				1.14 (1.06 – 1.23)	1.15 (1.06 – 1.24)

Model 1 adjusted for age and sex
Model 2 additionally adjusted for antithrombotic medication, antihypertensive drugs, diabetes mellitus, lipid reducing agents, BMI, smoking, total cholesterol, HDL cholesterol, systolic blood pressure and diastolic blood pressure. All strokes indicates ischemic, hemorrhagic and unspecified strokes. Any cerebrovascular event indicates all strokes and TIA. Cut-off points (%) for quartiles were: ≤80.73%, 80.74 – 91.44%, 91.45 – 102.26%, and ≥ 102.27%

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Table 4. Cox proportional hazard regression analysis between ADAMTS13 and VWF and stroke

ADAMTS13 and VWF:Ag level	Number of cases / Total number at risk	Absolute risk (%)	Model 1 HR (95% CI)	Model 2 HR (95% CI)
All strokes (N= 461)				
VWF < p75 and ADAMTS13 > p25	223 / 3395	6.6	1 (ref)	1 (ref)
VWF ≥ p75 and ADAMTS13 > p25	76 / 1055	7.2	0.91 (0.70 – 1.19)	0.92 (0.71 – 1.20)
VWF < p75 and ADAMTS13 ≤ p25	101 / 1032	9.8	1.24 (0.97 – 1.58)	1.24 (0.97 – 1.60)
VWF ≥ p75 and ADAMTS13 ≤ p25	61 / 451	13.5	1.53 (1.14 – 2.06)	1.49 (1.11 – 2.01)
Ischemic strokes (N= 306)				
VWF < p75 and ADAMTS13 > p25	153 / 3395	4.5	1 (ref)	1 (ref)
VWF ≥ p75 and ADAMTS13 > p25	44 / 1055	4.2	0.83 (0.59 – 1.16)	0.83 (0.59 – 1.17)
VWF < p75 and ADAMTS13 ≤ p25	68 / 1032	6.6	1.31 (0.98 – 1.76)	1.34 (1.00 – 1.81)
VWF ≥ p75 and ADAMTS13 ≤ p25	41 / 451	9.1	1.72 (1.20 – 2.47)	1.71 (1.19 – 2.45)
TIA (N= 315)				
VWF < p75 and ADAMTS13 > p25	156 / 3395	4.6	1 (ref)	1 (ref)
VWF ≥ p75 and ADAMTS13 > p25	58 / 1055	5.5	1.06 (0.78 – 1.44)	1.06 (0.78 – 1.44)
VWF < p75 and ADAMTS13 ≤ p25	72 / 1032	7.0	1.44 (1.08 – 1.92)	1.49 (1.11 – 1.98)
VWF ≥ p75 and ADAMTS13 ≤ p25	29 / 451	6.4	1.27 (0.84 – 1.91)	1.29 (0.85 – 1.95)
Any cerebrovascular event (N= 776)				
VWF < p75 and ADAMTS13 > p25	379 / 3395	11.2	1 (ref)	1 (ref)
VWF ≥ p75 and ADAMTS13 > p25	134 / 1055	12.7	0.97 (0.80 – 1.19)	0.97 (0.80 – 1.19)
VWF < p75 and ADAMTS13 ≤ p25	173 / 1032	16.8	1.32 (1.10 – 1.59)	1.34 (1.11 – 1.61)
VWF ≥ p75 and ADAMTS13 ≤ p25	90 / 451	20.0	1.44 (1.13 – 1.83)	1.43 (1.12 – 1.82)

Model 1 adjusted for age and sex
Model 2 additionally adjusted for antithrombotic medication, antihypertensive drugs, diabetes mellitus, lipid reducing agents, BMI, smoking, total cholesterol, HDL cholesterol, systolic blood pressure and diastolic blood pressure
All strokes indicates ischemic, hemorrhagic and unspecified strokes.
Any cerebrovascular event indicates all strokes and TIA.
ADAMTS13 ≤ 25 percentile represents ≤ 80.72%, VWF ≥ 75 percentile ≥ 1.58 IU/ml.

Table 5. Additional predictive information after extending the traditional risk factor model with ADAMTS13 in prediction of ischemic stroke

	Model fit	Model discrimination
Models	**Likelihood Ratio Test Statistics (X^2)**	**C-Statistic (95% CI)**
Traditional risk factor model	123.7	0.694 (0.665, 0.723)
Traditional risk factor model + ADAMTS13	136.9 [a]	0.707 (0.678, 0.735) [a]

Reclassification after addition of ADAMTS13 to the traditional risk factors, total population (N=5941)

Subjects with event (%) [b]		Subjects without event (%) [b]		NRI (95% CI) [c]
Up	**Down**	Up	Down	
15.0%	**9.4%**	8.8%	9.0%	0.058 (-0.002, 0.119)

Reclassification after addition of ADAMTS13 to the traditional risk factors, among the 5%-7.5% risk category (N=1179)

Subjects with event (%) [b]		Subjects without event (%) [b]		
Up	**Down**	Up	Down	**NRI (95% CI)** [c]
33.3%	**19.8%**	20.3%	28.0%	0.212 (0.048, 0.376)

X^2, Chi-square statistic; CI, confidence interval; NRI, net reclassification improvement.
The traditional risk factor model is composed of the variables included in the American Heart Association Pooled Cohort Equation; age, sex, systolic blood pressure, treatment for hypertension, total and high density lipoprotein cholesterol, smoking, and diabetes.
[a] P-value <0.01 for the increase in model fit and for the increase in the c-statistic after addition of ADAMTS13 to the traditional risk factors.
[b] Percentages of subjects with or without an event who move to a higher or lower risk category after adding ADAMTS13 to the traditional risk factors. The risk categories are based on 10-year ischemic stroke risk <5%, 5%–7.5%, and >7.5%.
[c] NRI is estimated as ([number of events reclassified higher minus number of events reclassified lower]/number of events) +
([number of nonevents reclassified lower minus number of nonevents reclassified higher]/number of nonevents).

DISCUSSION

In this prospective cohort study of nearly 6000 elderly individuals with a median follow-up of 10.7 years (56403 total person years), we observed an association between baseline ADAMTS13 activity and the development of ischemic stroke. Individuals with low ADAMTS13 activity had a higher risk of experiencing an ischemic stroke than individuals with high ADAMTS13 activity.

The association between ADAMTS13 levels and ischemic stroke has previously been investigated in small case-control studies [12-15]. Although these studies also suggested that low ADAMTS13 levels are associated with an increased risk of stroke, they all had a cross-sectional design. Therefore prospectively designed cohort studies, like the Rotterdam Study, are necessary to assess the role of ADAMTS13 as a risk factor for stroke.

The association between low ADAMTS13 activity and ischemic stroke risk is likely to be explained by less cleavage of high molecular weight multimers of VWF, which are the most prothrombotic forms. This results in more large procoagulant VWF multimers and consequently a prothrombotic state and may lead to thrombus formation at sites of endothelial damage and especially at sites with high shear stress, as in the arterial circulation. Nearly all individuals in the lowest ADAMTS13 quartile had ADAMTS13 activity in the normal range (50 – 150%)[20]. Nonetheless, our study clearly suggests that moderately reduced ADAMTS13 activity is associated with an increased risk of thrombotic complications, even though the levels are not as low as characteristic for TTP patients. We did not find a graded response effect from quartile 4 to quartile 1, and also previous case-control studies failed to find a graded pattern with decreasing ADAMTS13 quartiles [14,15,25]. This suggests that there is more a low level-threshold rather than a clear dose association for ADAMTS13 and ischemic stroke. For the association of continuous ADAMTS13 activity (per SD decrease) and stroke, we estimated whether the risks were influenced by outliers of ADAMTS13 activity by excluding the lowest and highest 1% and 2.5%. These data showed comparable risk estimates as shown in table 3 suggesting that there is not an important influence of outliers. High VWF:Ag levels have shown to be associated with the risk of stroke in previous studies [5-7]. To investigate whether stroke is also dependent upon the interaction between VWF and ADAMTS13, we analyzed groups based on the combination of ADAMTS13 activity and VWF:Ag levels. Individuals with low ADAMTS13 activity and high VWF levels had the highest risk of ischemic stroke, suggesting that ADAMTS13 and VWF are independent risk factors. This finding is similar to that of two recent case-control studies in patients with cardiovascular disease [12,13].We observed no association between VWF:Ag levels and ADAMTS13 activity, which is consistent with previous reports [12,13,25,26]. This might be explained by the fact that the ADAMTS13 activity levels measured in our study participants are apparently high enough for cleavage of plasma VWF. However, locally at the site of vessel damage the reduced ADAMTS13 activity may lead to reduced cleavage

of secreted large multimers of VWF and thereby contribute to local thrombus formation [13].
A causal role of ADAMTS13 in the outcome of stroke has recently been suggested in patients with a congenital deficiency of ADAMTS13 (Upshaw Schulman syndrome) and in animal studies. These patients without ADAMTS13 also suffer from ischemic stroke events [27,28]. In addition, two independent groups reported larger infarctions in ADAMTS13-deficient mice in whom ischemic stroke was induced experimentally[9-11]. Additionally, abolishment of the ADAMTS13 gene strongly accelerates atherosclerosis in a murine model [29,30], suggesting a role of ADAMTS13 in the progression of atherosclerosis. Infusion of recombinant human ADAMTS13 in wild-type mice with cerebral ischemia before reperfusion, reduced the infarct size and improved the functional outcome[9]. These studies suggest that recombinant human ADAMTS13, may have a role as therapeutic agent in ischemic stroke patients [31], however this should be addressed in future clinical studies.
The measurement of ADAMTS13 in a large population based cohort of nearly 6000 individuals allowed us to study the association between ADAMTS13 activity and several baseline characteristics and provides more insight in the pathophysiology of ADAMTS13. We observed a strong age dependency of ADAMTS13, indicating lower ADAMTS13 activity in the elderly population. In our population of individuals above 55 years, the mean ADAMTS13 activity was 91.9%. ADAMTS13 activity was significantly lower in individuals of increasing age which is consistent with other smaller studies[19,26,32,33]. Although both age and ADAMTS13 activity are associated with the risk of stroke, we found that ADAMTS13 activity was associated with an increased risk of stroke also independent of age. Interestingly other studies have revealed that even in children, low levels of ADAMTS13 are associated with stroke[15]. In addition, we found an association between ADAMTS13 activity and sex. ADAMTS13 activity was higher in women than in men, as previously reported[19,33]. We also observed that patients with diabetes had a higher activity of ADAMTS13. This is in contrast with a previous study that showed lower ADAMTS13 activity in patients with diabetes mellitus compared with controls [34]. However, in that study a positive association between glycemic control, measured as HbA1c, and ADAMTS13 activity was reported. The authors suggested that this might be explained in these patients by the higher level of metabolic stress that increases the hepatic production and release of ADAMTS13 as a compensatory mechanism[34]. We observed also a weak association between use of lipid reducing agents and ADAMTS13. In addition, we found a positive association with total cholesterol, which confirms a previous study[26]. Although ADAMTS13 activity was associated with multiple characteristics and drugs, the association between ADAMTS13 activity and ischemic stroke found in our study was independent of these variables.
The initial step in evaluation of a new marker is to examine if the marker could predict development of future events. While providing a significant evidence of association is

necessary, the key aspect is to show that a new risk marker improves predictions over the standard clinical risk assessment tools. When added to the traditional risk factors (age, sex, systolic blood pressure, treatment for hypertension, total and high density lipoprotein cholesterol, smoking, and diabetes)[23], ADAMTS13 improved the ischemic stroke risk predictions, as measured by an increase in model discrimination and moderate reclassification in the overall population and in persons categorized as having a risk between 5% to 7.5% by traditional cardiovascular risk factors, as depicted in table 5. This is of importance because in these patients preventive treatment is suggested to be considered[24]. To allow for a more precise and clinically relevant interpretation of the contribution of ADAMTS13 activity to stroke risk prediction, we repeated the analysis for different traditional risk factors including systolic blood pressure, total and HDL cholesterol, smoking, and diabetes. We added each traditional risk factor to the model already containing other traditional risk factors and assessed improvements in model fit, discrimination and reclassification for each traditional risk factor (data not shown). Our results confirmed that the contribution of ADAMTS13 to stroke risk prediction is comparable to those of systolic blood pressure, HDL cholesterol and smoking.

Addition of VWF to the model did not improve the risk prediction. This might be explained by the fact that ADAMTS13 has a more important role in the prediction of stroke than VWF:Ag levels. We previously already published that the role of VWF:Ag in predicting stroke in this elderly population study was not very strong [5].

The strength of our study is that this is a prospective study with nearly 6000 individuals on the role of ADAMTS13 in ischemic stroke with a long follow-up of 10.7 years in a population that is representative for the Dutch population. A limitation of our study is that 23% of the incident strokes had to be classified as unspecified due to the lack of cerebral imaging. These strokes mainly occurred in the early phase of the study when patients were often not referred to the hospital. As 80-90% of all strokes are ischemic [35,36], most of the unspecified strokes in our study will also be of ischemic origin. Another limitation is that ischemic stroke could only be subclassified in etiologic subgroups in a limited number of ischemic stroke patients. This limited our ability to study the importance of ADAMTS13 activity as a risk factor of various subtypes of ischemic stroke. We measured ADAMTS13 activity only at baseline as this is an observational study, and it would be of interest how ADAMTS13 levels change over time. Furthermore, a marker of stroke severity like the modified Rankin Score, was not determined in our study. Therefore we could not investigate whether ADAMTS13 activity is associated with stroke severity. However, a TIA is considered to be a less severe stroke and we found similar risk estimates of the association between ADAMTS13 activity and TIA or ischemic stroke. This suggests that there is no association between ADAMTS13 activity and stroke severity. Our study was performed in a population of predominantly Caucasians of 55 years and older that live in a middle income district of Rotterdam, which limits the generalizability of our results. We did not take into account any competing

morbidity and mortality in the analyses or clinical variables during follow-up. Maybe we could have obtained more insight on the association between ADAMTS13 and stroke if multiple measurements over time would have been available.

In conclusion, low ADAMTS13 activity was significantly associated with the risk of ischemic stroke, independently of age, sex, and established cardiovascular risk factors over a median of 10.7 years of follow-up. Addition of ADAMTS13 activity improved the accuracy of risk predictions for ischemic stroke beyond the traditional risk factors.

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SUPPLEMENTARY MATERIAL

Stroke was defined according to WHO criteria as a syndrome of rapidly developing clinical signs of focal (or global) disturbance of cerebral function, with symptoms lasting 24 h or longer or leading to death, with no apparent cause other than of vascular origin. (see also *Wieberdink et al. 2012* Eur J Epidemiol. 2012 Apr;27(4):287-95 and *Hatano S. et al. Bull World Health Organ. 1976;54(5):541-53*).

Transient ischemic attacks were attacks of sudden neurological symptoms that completely resolved within 24 hours, with no clear evidence for the diagnosis of migraine, epilepsy, Ménière disease, hyperventilation, cardiac syncope, hypoglycaemia, or orthostatic hypotension (Bos et al. JAMA 2007 Dec 26;298(24):2877-85).

Table 6. Previous findings on markers and stroke in the Rotterdam Study

Marker	Findings	References
Age	Stroke incidence increases with age	Hollander M. et al. J Neurol Neurosurg. 2003. Wieberdink R.G. et al. Eur J Epidemiol. 20120
Sex	Stroke incidence is higher in men	Hollander M. et al. J Neurol Neurosurg. 2003.
Total cholesterol	Total cholesterol is not significantly associated with an increased risk of stroke	Hollander M. et al. Circulation, 2002.
HDL cholesterol	HDL cholesterol is not significantly associated with an increased risk of stroke	Hollander M. et al. Circulation, 2002. Bos M.J. et al. PLoS Med. 2014.
Diabetes Mellitus	Diabetes Mellitus is associated with an increased risk of stroke	Hollander M. et al. Circulation, 2002. Bos M.J. et al. PLoS Med. 2014.
Hypertension	Hypertension is associated with an increased risk of stroke	Bos M.J. et al. PLoS Med. 2014. Vermeer S.E. et al. Stroke, 2002.
Smoking	Smoking is associated with an increased risk of stroke	Bos M.J. et al. PLoS Med. 2014. Vermeer S.E. et al. Stroke, 2002.
BMI	BMI is not significantly associated with an increased risk of stroke	Bos M.J. et al. PLoS Med. 2014.
Atrial fibrillation	Atrial fibrillation is associated with an increased risk of stroke	Hollander M. et al. Circulation, 2002. Bos M.J. et al. PLoS Med. 2014.
Left ventricular hypertrophy	LVH is associated with an increased risk of stroke	Bots M.L. et al. J Epidemiol Community Health, 2002.

Table 7. Cox proportional hazard regression analysis between ADAMTS13 quartiles and stroke additionally adjusted for prevalent coronary heart disease

ADAMTS13 level	Number of cases / Total number at risk	HR (95% CI)
All strokes (N= 461)		
Quartile 1	163 / 1486	1.52 (1.14 – 2.03)
Quartile 2	108 / 1485	1.08 (0.80 – 1.46)
Quartile 3	111 / 1485	1.17 (0.87 – 1.57)
Quartile 4	79 / 1485	1 (ref)
Per SD decrease		1.13 (1.02 – 1.25)
Ischemic strokes (N= 306)		
Quartile 1	109 / 1486	1.67 (1.19 – 2.36)
Quartile 2	70 / 1485	1.08 (0.75 – 1.55)
Quartile 3	71 / 1485	1.13 (0.79 – 1.62)
Quartile 4	56 / 1485	1 (ref)
Per SD decrease		1.20 (1.06 – 1.35)
TIA (N= 315)		
Quartile 1	101 / 1486	1.65 (1.17 – 2.32)
Quartile 2	78 / 1485	1.26 (0.90 – 1.78)
Quartile 3	77 / 1485	1.21 (0.86 – 1.71)
Quartile 4	59 / 1485	1 (ref)
Per SD decrease		1.19 (1.05 – 1.34)
Any cerebrovascular event (N= 776)		
Quartile 1	264 / 1486	1.57 (1.26 – 1.96)
Quartile 2	186 / 1485	1.15 (0.92 – 1.45)
Quartile 3	188 / 1485	1.19 (0.95 – 1.48)
Quartile 4	138 / 1485	1 (ref)
Per SD decrease		1.15 (1.07 – 1.25)

Adjusted for age, sex, antithrombotic medication, antihypertensive drugs, diabetes mellitus, lipid reducing agents, BMI, smoking, total cholesterol, HDL cholesterol, systolic blood pressure, diastolic blood pressure, and prevalent coronary heart disease.
All strokes indicates ischemic, hemorrhagic and unspecified strokes.
Any cerebrovascular event indicates all strokes and TIA.
Cut-off points (%) for quartiles were: ≤80.73%, 80.74 – 91.44%, 91.45 – 102.26%, and ≥ 102.27%

Table 8. Cox proportional hazard regression analysis between ADAMTS13 and VWF and stroke additionally adjusted for prevalent coronary heart disease

ADAMTS13 and VWF:Ag level	Number of cases / Total number at risk	HR (95% CI)
All strokes (N= 461)		
VWF < p75 and ADAMTS13 > p25	223 / 3395	1 (ref)
VWF ≥ p75 and ADAMTS13 > p25	76 / 1055	0.91 (0.70 – 1.18)
VWF < p75 and ADAMTS13 ≤ p25	101 / 1032	1.26 (0.99 – 1.61)
VWF ≥ p75 and ADAMTS13 ≤ p25	61 / 451	1.51 (1.12 – 2.04)
Ischemic strokes (N= 306)		
VWF < p75 and ADAMTS13 > p25	153 / 3395	1 (ref)
VWF ≥ p75 and ADAMTS13 > p25	44 / 1055	0.81 (0.58 – 1.14)
VWF < p75 and ADAMTS13 ≤ p25	68 / 1032	1.36 (1.01 – 1.82)
VWF ≥ p75 and ADAMTS13 ≤ p25	41 / 451	1.73 (1.20 – 2.48)
TIA (N= 315)		
VWF < p75 and ADAMTS13 > p25	156 / 3395	1 (ref)
VWF ≥ p75 and ADAMTS13 > p25	58 / 1055	1.06 (0.78 – 1.43)
VWF < p75 and ADAMTS13 ≤ p25	72 / 1032	1.50 (1.12 – 2.00)
VWF ≥ p75 and ADAMTS13 ≤ p25	29 / 451	1.26 (0.83 – 1.91)
Any cerebrovascular event (N= 776)		
VWF < p75 and ADAMTS13 > p25	379 / 3395	1 (ref)
VWF ≥ p75 and ADAMTS13 > p25	134 / 1055	0.96 (0.79 – 1.18)
VWF < p75 and ADAMTS13 ≤ p25	173 / 1032	1.35 (1.12 – 1.63)
VWF ≥ p75 and ADAMTS13 ≤ p25	890 / 451	1.43 (1.12 – 1.82)

Adjusted for age, sex, antithrombotic medication, antihypertensive drugs, diabetes mellitus, lipid reducing agents, BMI, smoking, total cholesterol, HDL cholesterol, systolic blood pressure, diastolic blood pressure, and prevalent coronary heart disease.
All strokes indicates ischemic, hemorrhagic and unspecified strokes.
Any cerebrovascular event indicates all strokes and TIA.
ADAMTS13 ≤ 25 percentile represents ≤ 80.72%, VWF ≥ 75 percentile ≥ 1.58 IU/ml.

Table 9. Cox proportional hazard regression analysis between low ADAMTS13, high VWF and stroke

ADAMTS13 and VWF:Ag level	Number of cases / Total number at risk	Absolute risk (%)	Model 1 HR (95% CI)	Model 2 HR (95% CI)
All strokes (N= 461)				
VWF ≤ p25 and ADAMTS13 ≥ p75	14 / 430	3.3	1 (ref)	1 (ref)
VWF ≥ p75 and ADAMTS13 ≤ p25	61 / 451	13.5	2.76 (1.42 – 5.38)	2.94 (1.49 – 5.78)
Ischemic strokes (N= 306)				
VWF ≤ p25 and ADAMTS13 ≥ p75	11 / 430	2.6	1 (ref)	1 (ref)
VWF ≥ p75 and ADAMTS13 ≤ p25	41 / 451	9.1	3.04 (1.41 – 6.56)	3.51 (1.60 – 7.70)
TIA (N= 315)				
VWF ≤ p25 and ADAMTS13 ≥ p75	19 / 430	4.4	1 (ref)	1 (ref)
VWF ≥ p75 and ADAMTS13 ≤ p25	29 / 451	6.4	1.59 (0.79 – 3.19)	1.55 (0.76 – 3.14)
Any cerebrovascular event (N= 776)				
VWF ≤ p25 and ADAMTS13 ≥ p75	33 / 430	7.7	1 (ref)	1 (ref)
VWF ≥ p75 and ADAMTS13 ≤ p25	90 / 451	20.0	2.10 (1.31 – 3.36)	2.16 (1.34 – 3.49)

Model 1 adjusted for age and sex
Model 2 additionally adjusted for antithrombotic medication, antihypertensive drugs, diabetes mellitus, lipid reducing agents, BMI, smoking, total cholesterol, HDL cholesterol, systolic blood pressure and diastolic blood pressure
All strokes indicates ischemic, hemorrhagic and unspecified strokes.
Any cerebrovascular event indicates all strokes and TIA.
ADAMTS13 ≤ 25 percentile represents ≤ 80.72%, VWF ≥ 75 percentile ≥ 1.58 IU/ml.

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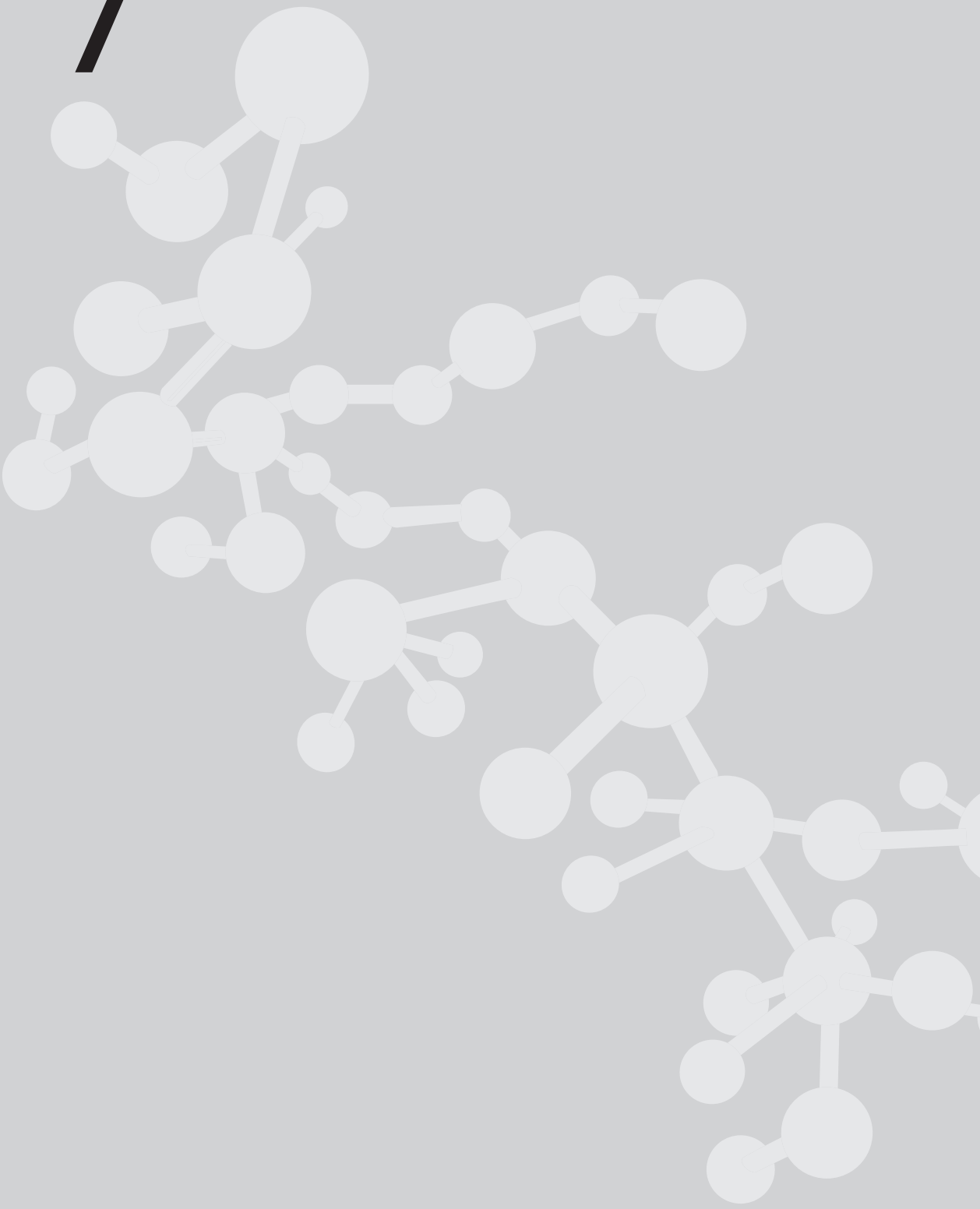

LOW ADAMTS13 ACTIVITY AND THE RISK OF CORONARY HEART DISEASE: A PROSPECTIVE COHORT STUDY – THE ROTTERDAM STUDY

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SUMMARY

Background

The metalloprotease ADAMTS13 cleaves high molecular weight von Willebrand factor multimers into smaller, less procoagulant forms. Low ADAMTS13 activity is associated with an increased risk of ischemic stroke but its pathogenic role in coronary heart disease is unclear.

We aimed to determine the association between ADAMTS13 activity and the risk of coronary heart disease in a large prospective population-based cohort study.

Methods

5688 participants of the Rotterdam Study, a population-based cohort study among individuals ≥ 55 years without a history of coronary heart disease (CHD) were included. ADAMTS13 activity was measured by the FRETS-VWF73 assay and VWF:Ag levels by ELISA. We assessed the association between ADAMTS13 activity, VWF:Ag levels and coronary heart disease using Cox proportional hazard regression analysis, adjusting for cardiovascular risk factors.

Results

Over a median follow-up time of 9.7 years, 456 individuals suffered from coronary heart disease. A low ADAMTS13 activity (quartile 1) was associated with an increased CHD risk (HR 1.42, 95%CI 1.07 – 1.89) compared with the reference highest quartile.

Conclusions

Low ADAMTS13 activity is associated with an increased risk of coronary heart disease in the elderly, independently of VWF and established cardiovascular risk factors.

INTRODUCTION

ADAMTS13, A Disintegrin and Metalloprotease with ThromboSpondin motif repeats 13(1-3), cleaves large Von Willebrand Factor (VWF) multimers into smaller, less procoagulant forms (4). The important physiological function of ADAMTS13 is evident from patients with thrombotic thrombocytopenic purpura (TTP), characterized by a complete deficiency of ADAMTS13. A deficiency of ADAMTS13 results into reduced cleavage of the most active high molecular weight (HMW) multimers, resulting in increased microthrombi formation. Patients with TTP suffer from microthrombi that may lead to focal microangiopathy, renal insufficiency, cerebral ischemia and myocardial infarction (5).

Recently, we have shown the association between low ADAMTS13 activity and increased risk of ischemic stroke (6). As ischemic stroke and coronary heart disease (CHD) have multiple shared risk factors and as in both diseases atherosclerosis plays a role, we hypothesized that there is also an association between ADAMTS13 activity and CHD. This hypothesis is supported by the observation that ADAMTS13-deficient mice have larger myocardial infarctions upon induction of myocardial ischemia, compared with wild type mice (7-9). In addition, small case-control studies have suggested that ADAMTS13 may also play a role in CHD (10, 11). This suggests a role for ADAMTS13 in myocardial ischemia. Prospective cohort studies on the association between ADAMTS13 and CHD are lacking. Therefore, we investigated prospectively the association between ADAMTS13 activity and the risk of coronary heart disease in a large population cohort (Rotterdam Study) among individuals 55 years and older.

MATERIALS AND METHODS

Study design and study population

Participants were included from the Rotterdam Study (RS), a population-based cohort study among individuals of 55 years and older living in a suburb of Rotterdam, the Netherlands (12). In 1990, the study started with 7,983 individuals (out of 10,215 invitees) (RS-I) and was extended in 1999 with an additional 3,011 individuals (out of 4,472 invitees) (RS-II), who moved into the study district or became 55 years since the start of the study. Every 3 to 4 years, participants visited the research center for detailed evaluations including the assessment of established cardiovascular risk factors. For this study, we used data obtained from participants at the third examination of the original cohort (RS-I-3, 1997-1999) and the first examination of the extended cohort (RS-II-1, 2000-2001). We included all participants of whom blood was sampled (N= 6452) and excluded participants with a history of CHD or stroke at the moment of blood sampling (1997 – 2001) (N= 764). The Rotterdam Study has been approved by the medical ethics committee according to the Population Study Act Rotterdam Study, executed by the Ministry of Health, Welfare and Sports of the Netherlands. Written informed consent was obtained of all participants.

ADAMTS13 activity and VWF:Ag levels measurements

ADAMTS13 activity was measured in a kinetic assay using the Fluorescence Resonance Energy Transfer Substrate VWF 73 (FRETS-VWF73) as previously described (13). Samples were measured against a reference curve of serial dilutions of pooled normal human plasma (George King Biomedical Inc) defined to have an ADAMTS13 activity of 1 U/ml.

VWF antigen (VWF:Ag) levels were determined with an in-house ELISA, using polyclonal rabbit anti-human VWF antibodies (DakoCytomation, Glostrup, Denmark) for catching and tagging.

Assessment of coronary heart disease

History of CHD and stroke in all participants was determined during the baseline interview and verified in medical records. After enrollment in the Rotterdam Study, participants were continuously monitored for incident CHD through automated linkage of the study database with files from general practitioners, the municipality, and nursing home physicians. Additional information was obtained from hospital records. Incident coronary heart disease was defined as myocardial infarction (MI) and CHD mortality. Myocardial infarction was defined as pathology findings of an acute MI within 28 days of death, or a rise/fall in cardiac biomarkers and/or objective indicative ECG changes, and preferably the presence of symptoms or signs (e.g. cardiac pain, cardiogenic shock). CHD mortality was defined as definite fatal MI, definite fatal CHD and possible fatal CHD (14).

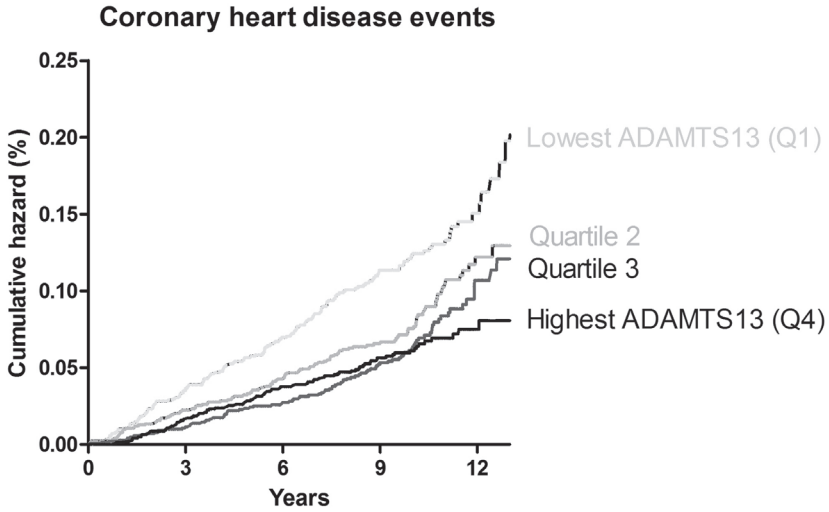
Other measurements

At inclusion in the study (RS-I-3 and RS-II-1), a detailed interview was taken from all participants, as well as an extensive set of examinations, including a physical examination and blood sampling. All determinants at inclusion of the study (1997 – 2001) were used as baseline characteristics. Blood pressure was calculated as the mean of two measurements using a random-zero sphygmomanometer at the right brachial artery while the individual was in a sitting position. Antithrombotic medication was defined as the use of vitamin K antagonists, platelet aggregation inhibitors, and direct thrombin Xa inhibitors. Antihypertensive drugs was defined as the use of antihypertensive medication indicated for the treatment of high blood pressure (≥ grade 1 hypertension according to World Health Organization criteria (15)). Diabetes mellitus was defined as the use of blood glucose-lowering medication and/or a fasting serum glucose level ≥ 7.0 mmol/L. Lipid reducing agents was defined as the use of statins. Total cholesterol and high-density lipoprotein cholesterol were measured using an automated enzymatic procedure. Body mass index was calculated as the weight (in kilograms) divided by the square of the height (in meters). Smoking status was defined as current, former and no smoking. Blood group antigen phenotypes were reconstructed by haplotypes analysis of 4 single nucleotide polymorphisms, rs687289, rs507666, rs8176704, and rs8176749, which collectively serve as tagging SNPs for the O, A1, A2, and B allele. Previous studies have shown an association between cardiovascular risk factors and coronary heart disease in the Rotterdam Study (16-18).

Statistical analysis

Data on baseline characteristics are shown as mean and standard deviation for continuous variables and as counts and percentages for categorical variables. The association between ADAMTS13 and CHD events was calculated using Cox proportional hazards regression analysis using ADAMTS13 quartiles and additionally per 1 SD of ADAMTS13 decrease. Individuals who developed a stroke before the CHD event, were censored for stroke. All participants were followed from inclusion to MI, death, last health status update where they were known to be free of CHD, or January 1, 2012, whichever came first. To assess proportional hazards assumption, we tested the log minus log plots. A Kaplan Meier cumulative hazard curve was assessed for each of the ADAMTS13 quartiles and compared by using the log-rank test. For the association between ADAMTS13 activity, VWF:Ag levels and the risk of CHD events, combinations of the ADAMTS13 and VWF quartiles were made. Participants with an ADAMTS13 activity above the 25th percentile and a VWF:Ag level below the 75th percentile were used as the reference. Participants with an ADAMTS13 activity below the 25th percentile and a VWF:Ag level above the 75th percentile were supposed to have the highest risk of CHD. ADAMTS13 activity was multiplied by VWF:Ag levels to use

as an interaction term and added to the multivariate cox regression including ADAMTS13 activity and VWF:Ag levels. We also performed a 'Youden Index' analysis. The 'Youden Index' is a summary measure of the ROC curve and gives a cut-off by a trade-off between sensitivity and specificity of ADAMTS13 in prediction of CHD risk. We adjusted all analyses for age and sex, and additionally for antithrombotic agents, antihypertensive drugs, diabetes mellitus, lipid reducing agents, smoking, total cholesterol, HDL cholesterol, and systolic blood pressure. Missing values of these covariates (0-4.9%) were imputed five times using a multiple imputation method including all covariates. Statistical analyses were performed on all five datasets and pooled into one final result using SPSS software. Data were analyzed with SPSS version 21 (SPSS 21.0. IBM, Somers, NY, USA). All statistical tests were two-tailed and p-values <0.05 were considered statistically significant.



No. at risk					
Quartile 1	1425	1257	1048	733	134
Quartile 2	1419	1310	1180	906	171
Quartile 3	1421	1342	1234	960	193
Quartile 4	1422	1348	1248	941	167

Figure 1. Kaplan-Meier curve
Kaplan-Meier curve for the cumulative hazard of CHD events per quartile of ADAMTS13 activity. Cut-off points (%) for quartiles were: ≤ 80.92%, 80.93- 91.44%, 91.45- 102.21%, and ≥ 102.22%

RESULTS

In total, we included 5688 individuals who were free from coronary heart disease and stroke at baseline. Over a median follow-up time of 9.7 years, 456 individuals (8.0%) experienced a cardiovascular event of whom 230 individuals died due to CHD and 226 individuals experienced a myocardial infarction. The mean age of all individuals was 68.9 years (± 8.2) and 59.7% was female (Table 1). The mean ADAMTS13 activity in the total population was 91.9%(± 17.6). There was no relevant correlation between ADAMTS13 activity and VWF:Ag levels (R -0.10, P<0.001).

Table 1. Baseline characteristics

N = 5688	Total cohort N (%) or mean ± SD
Age (years)	68.9 ± 8.2
Female sex	3398 (59.7%)
Smoking	
Current	1000 (17.8%)
Former	2687 (47.7%)
BMI (kg/m²)	27.0 ± 4.1
Antithrombotic medication	752 (13.9%)
Antihypertensive drugs	1250 (23.1%)
Lipid reducing agents	535 (9.9%)
Diabetes Mellitus	545 (9.7%)
Total cholesterol (mmol/l)	5.9 ± 1.0
HDL cholesterol (mmol/l)	1.4 ± 0.4
Systolic blood pressure (mmHg)	143 ± 21
Diastolic blood pressure (mmHg)	77 ± 11
Blood group O	2189 (45.5%)
ADAMTS13 activity (%)	91.93 ± 17.64
VWF:Ag (IU/ml)	1.31 ± 0.58

Data are presented as N (%) or mean ± SD

Individuals in the lowest quartile of ADAMTS13 had a 42% higher risk of CHD compared with individuals in the highest quartile of ADAMTS13 (HR 1.42, 95%CI 1.07 – 1.89; Table 2), independent of cardiovascular risk factors. Also in Kaplan Meier survival analysis, individuals in the lowest quartile of ADAMTS13 had the highest hazard compared with the other quartiles and this increased risk was consistent over time (Figure 1). The risk of CHD increased with 1.12 fold per decrease of 1 SD of ADAMTS13 activity, when ADAMTS13 was entered as continuous variable in the model (per SD decrease HR 1.12, 95%CI 1.01 – 1.24).

Individuals with a combination of the highest VWF:Ag level and the lowest ADAMTS13 activity, had an increased risk of developing CHD compared with individuals with lower VWF:Ag levels and higher ADAMTS13 activity (HR 1.43, 95%CI 1.04 - 1.96, Table 3). Individuals with a low ADAMTS13 activity and a VWF:Ag level below the 75th percentile had an almost similar risk estimate as individuals with a low ADAMTS13 and a high VWF:Ag level (above the 75th percentile; HR 1.32, 95% CI 1.03 - 1.68). After additionally adjustment for blood group in these associations, the risk estimates changed slightly (Table 3). There was no interaction between ADAMTS13 activity and VWF:Ag levels in the association with CHD events (P=0.95).

Table 2. Cox proportional hazard regression analysis between ADAMTS13 quartiles and CHD

ADAMTS13 activity	Mean ADAMTS13 activity (95% CI)	Number of cases / total number at risk	Model 1 HR (95% CI)	Model 2 HR (95% CI)
CHD event (N= 456)				
Quartile 1	70.5 (70.1 – 71.0)	156 / 1425	1.30 (0.98 – 1.72)	1.42 (1.07 – 1.89)
Quartile 2	86.4 (86.2 – 86.5)	116 / 1419	1.05 (0.79 – 1.39)	1.10 (0.83 – 1.47)
Quartile 3	96.6 (96.4 – 96.7)	99 / 1421	0.96 (0.72 – 1.29)	1.00 (0.75 – 1.34)
Quartile 4	114.3 (113.7 – 114.9)	85 / 1422	1 (ref)	1 (ref)
Per SD decrease			1.08 (0.98 – 1.20)	1.12 (1.01 – 1.24)

Model 1 adjusted for age and sex
Model 2 additionally adjusted for antithrombotic medication, antihypertensive drugs, diabetes mellitus, lipid reducing agents, smoking, total cholesterol, HDL cholesterol, and systolic blood pressure.

Table 3. Cox proportional hazard regression analysis between ADAMTS13 and VWF and CHD

ADAMTS13 and VWF:Ag level	Number of cases / total number at risk	Model 1 HR (95% CI)	Model 2 HR (95% CI)	Model 3 HR (95% CI)
CHD event (N= 453)				
VWF < p75 and ADAMTS13 > p25	221 / 3240	1 (ref)	1 (ref)	1 (ref)
VWF ≥ p75 and ADAMTS13 > p25	77 / 1015	0.97 (0.74 – 1.26)	0.98 (0.76 – 1.28)	0.95 (0.71 – 1.27)
VWF < p75 and ADAMTS13 ≤ p25	103 / 998	1.22 (0.96 – 1.55)	1.32 (1.03 – 1.68)	1.40 (1.08 – 1.81)
VWF ≥ p75 and ADAMTS13 ≤ p25	52 / 425	1.41 (1.03 – 1.93)	1.43 (1.04 – 1.96)	1.31 (0.92 – 1.87)

Model 1 adjusted for age and sex
Model 2 additionally adjusted for antithrombotic agents, antihypertensive drugs, diabetes mellitus, lipid reducing agents, smoking, total cholesterol, HDL cholesterol, and systolic blood pressure.
Model 3 additionally adjusted for blood group.

DISCUSSION

We have shown in this large prospective population cohort study that individuals with low ADAMTS13 activity have an increased risk of coronary heart disease events.

Our finding provides clarity on the existence of the relationship between ADAMTS13 and CHD. This association is likely to be explained by less cleavage of high molecular weight multimers of VWF, which are the most procoagulant forms. This results in more (ultra-)large procoagulant VWF multimers and consequently a prothrombotic state leading to thrombus formation at sites of endothelial damage and high shear stress.

The available literature was confusing, since previous case-control studies have shown conflicting results on the association between ADAMTS13 and CHD (19-23). This can be explained by the fact that most of these studies included a small number of patients. In addition, our recently published meta-analysis showed an increased but not statistically significant risk of CHD in individuals with a low ADAMTS13 (10). Maino et al. performed a meta-analysis based on individual patient data and found that in individuals with ADAMTS13 levels <5% of normal, there was an increased risk of acute myocardial infarction (11). These data may suggest that there is more a low level threshold rather than a clear dose association of ADAMTS13 and CHD, as was also suggested for ischemic stroke (6). Nearly all individuals in our study in the lowest ADAMTS13 quartile had ADAMTS13 activity in the normal range (50 – 150%) (24). Nonetheless, our study clearly shows that moderately reduced ADAMTS13 activity is associated with an increased risk of thrombotic complications, even though the levels are not as low as characteristic for TTP patients. However, in our study we showed a graded response effect from quartile 4 to quartile 1 suggesting that with decreasing ADAMTS13 activity the CHD risk is increasing. Additionally, we performed a Youden Index analysis showing the highest sensitivity with activity levels of ADAMTS13 of around 69.7, which is almost equal to the mean levels of ADAMTS13 activity in the first quartile (Table 2: 70.5%). Accordingly, in the 'Youden Index' analysis, the first quartile of ADAMTS13 appeared to carry the highest sensitivity in the association between ADAMTS13 and CHD risk.

The important role of ADAMTS13 in cardiovascular disease has previously been shown in experimental animal studies showing larger myocardial infarctions in ADAMTS13 deficient mice compared with wild type mice (7-9). These studies suggest a major role of ADAMTS13 in myocardial infarction. These studies also showed that infusion of recombinant human ADAMTS13, reduced the infarct size in wild type mice with myocardial ischemia before reperfusion (7, 9). This suggests that ADAMTS13 may also have a therapeutic role in CHD patients. However, future large prospective cohort studies are needed to determine the exact role of ADAMTS13 in CHD.

When we compared the association of ADAMTS13 with CHD, with the association we previously reported with stroke, it is suggested that the influence of ADAMTS13 is more prominent in ischemic stroke than in CHD (HR 1.65, 95% CI 1.16 - 2.32; HR 1.42, 95%CI 1.07 – 1.89; respectively) (6, 10). A major difference between stroke and CHD is that there

is wide variety in etiology of ischemic stroke (25) with thromboembolism being the most frequent cause, while in CHD the major cause is atherosclerosis. A previous review on hypercoagulability also found that hypercoagulability is a stronger risk factor for ischemic stroke than myocardial infarction (26). This suggests a general more pronounced role for coagulation-related risk factors for ischemic stroke than CHD.

Previously, we found an association between high VWF levels and CHD in the Rotterdam (27). To investigate whether VWF and ADAMTS13 interact in determining CHD risk, we analyzed the risk of CHD using groups based on a combination of ADAMTS13 activity and VWF levels. In individuals with a low ADAMTS13 activity and a high VWF level, the risk of CHD was increased compared with the reference group and we found no interaction between VWF levels and ADAMTS13 activity. Thereby this study suggests that ADAMTS13 and VWF are both independent risk factors, which has also been shown in previous studies (6, 11, 19). This might be explained by the fact that ADAMTS13 activity levels measured in our study participants are apparently high enough for cleavage of plasma VWF. This has also been shown previously in ADAMTS13 heterozygote individuals that they had normal VWF multimer patterns in plasma. However, locally at sites of endothelial damage the reduced ADAMTS13 activity may lead to reduced cleavage of secreted large multimers of VWF and thereby contribute to local thrombus formation. Individuals with a low ADAMTS13 activity and a VWF:Ag level below the 75th percentile had an almost similar risk estimate as individuals with a low ADAMTS13 and a high VWF:Ag level (above the 75th percentile). This suggests that ADAMTS13 could be a more prominent risk factor for CHD than VWF, however further studies are needed to evaluate the independent roles and interaction between ADAMTS13 and VWF. As it is known that individuals with blood group O have 25% lower VWF levels (28), we additionally adjusted for blood group in these associations. We found similar risk estimates, although the association between low ADAMTS13 and high VWF:Ag level with the risk of CHD was not statistically significant after additional adjustment.

We have shown an association between low ADAMTS13 and an increased risk of CHD in a large prospective cohort study with a long follow-up (median 9.7 years). A limitation of our study is that only individuals of 55 years and older were included and therefore whether these results can be extended to younger populations remains unclear. In addition, we measured ADAMTS13 activity at baseline to determine the subsequent CHD risk, to understand more about underlying mechanisms future studies should perform multiple measurements of ADAMTS13 over time. Although we adjusted for all cardiovascular risk factors, the possibility of residual confounding cannot be ruled out and thereby influencing the results.

In conclusion, this large prospective cohort study shows that low ADAMTS13 activity is associated with an increased risk of coronary heart disease among individuals of 55 years and older, independently of VWF and established cardiovascular risk factors.

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VON WILLEBRAND FACTOR, ADAMTS13 AND THE RISK OF MORTALITY: THE ROTTERDAM STUDY

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Submitted

ABSTRACT

Background

Von Willebrand Factor (VWF) is a plasma protein that plays a major role in platelet adhesion and aggregation. Large VWF multimers are cleaved into smaller, less coagulant forms by the metalloprotease ADAMTS13. Previous studies have shown that high VWF and low ADAMTS13 levels are associated with cardiovascular disease, but whether these factors are associated with mortality is unclear. Our aim is to establish the association between VWF antigen (VWF:Ag) levels, ADAMTS13 activity, and mortality.

Methods

We included 6130 participants of the Rotterdam Study, a population-based cohort study among individuals ≥ 55 years. We determined the association between ADAMTS13 activity, VWF:Ag levels and all-cause mortality and cardiovascular mortality by Cox proportional hazard regression analysis.

Results

Over a median follow-up time of 11.3 years and a total of 90635 person years, 1868 of the 6130 individuals died (30.5%), of whom 442 due to cardiovascular disease (23.7%). In individuals with low ADAMTS13 activity, the risk of cardiovascular mortality (HR 1.46, 95%CI 1.09 -1.96) was higher than in individuals with high ADAMTS13 activity. The risk of cardiovascular mortality (HR 1.29, 95%CI 0.98 – 1.70) was higher in individuals with the highest VWF:Ag levels than in those with the lowest. In individuals with both low ADAMTS13 activity and high VWF:Ag levels, the risk of cardiovascular mortality (HR 1.73 95%CI 1.28 – 2.35) was also higher.

Conclusions

In this large prospective cohort study, ADAMTS13 activity and VWF:Ag levels are both associated with an increased risk of all-cause and cardiovascular mortality.

INTRODUCTION

VWF is a multimeric glycoprotein that plays a major role in platelet adhesion and aggregation (1). Large VWF multimers are highly procoagulant and are cleaved by its metalloprotease ADAMTS13 (*A Disintegrin And Metalloprotease with ThromboSpondin motif repeats 13*) in smaller, less procoagulant forms (2). In case of a deficiency of ADAMTS13, large VWF multimers are not cleaved, resulting in more high molecular VWF multimers in the circulation. This is characteristic for thrombotic thrombocytopenic purpura (TTP) (3) in which patients suffer from microangiopathies, kidney failure and neurological symptoms due to the formation of microthrombus in the circulation.

Cardiovascular disease is one of the leading causes of death in the Western world (4). We recently showed in the Rotterdam study that high Von Willebrand Factor (VWF) and low ADAMTS13 levels are associated with an increased risk of cardiovascular disease, including coronary heart disease and ischemic stroke (5-8). Although VWF and ADAMTS13 are associated with cardiovascular disease, it is not clear yet whether these factors are associated with mortality.

Some prospective studies have shown an association between VWF levels and mortality (9-13). Most of these studies have shown a significant association between high VWF levels and a worse cardiovascular health (9, 10, 12, 13), but almost all studies have focused on the cardiac specific mortality (9-11) and only two studies have focused on the total cardiovascular mortality due to coronary heart disease or stroke (12, 13). Studies on the association between ADAMTS13 and all-cause mortality are lacking.

We aimed to investigate the association between VWF levels, ADAMTS13 activity and all-cause or cause-specific mortality, especially cardiovascular mortality.

MATERIALS AND METHODS

Study design and study population

This study is part of the Rotterdam Study, a prospective population-based cohort study among individuals of 55 years and older who are all living in Ommoord, a suburb of Rotterdam, The Netherlands (14). Of the 10,215 eligible individuals, 7,983 agreed to participate at the start of the study in 1990 (RS-1). In 1999 the study was extended with 3,011 individuals (out of 4,472 invitees) who moved into the study district or became 55 years since the start of the study. All participants were asked to visit the study center every 3 to 4 years to assess established cardiovascular risk factors. The Rotterdam Study has been approved by the Medical Ethics Committee of the Erasmus MC and by the Ministry of Health, Welfare and Sport of the Netherlands, implementing the Population Studies Act: Rotterdam Study. Written informed consent was obtained from all participants. For this study, we used the data of the participants in the third examination of the original cohort (RS-I-3) and the first examination of the extended cohort (RS-II-1). We included all participants who were alive at baseline and of whom blood was sampled (N= 6511). Patients who died within 3 years after blood sampling were excluded from the analyses (N= 381) as an already existing disease at moment of blood sampling may have influenced ADAMTS13 activity and VWF levels.

Von Willebrand Factor and ADAMTS13 measurements

VWF antigen (VWF:Ag) levels were determined with an in-house ELISA. Polyclonal rabbit anti-human VWF antibodies were used for catching and tagging (DakoCytomation, Glostrup, Denmark). ADAMTS13 activity was measured in a kinetic assay with the previously described Fluorescence Resonance Energy Transfer Substrate VWF 73 (FRETS-VWF73) (15). All samples were measured against a reference curve of serial dilutions of normal human plasma (Cryocheck normal reference plasma, Presizion Biologic, Dartmouth, USA).

Assessment of mortality

Information on mortality was continuously reported through automatic linkage of general practitioner files. Additionally, municipal records were checked on a monthly basis. Information on cause of death was obtained from general practitioners and hospital records. Research physicians reviewed all information and coded all events according to the *International Classification of Diseases, 10th edition (ICD-10).* Deaths due to cardiovascular disease were coded as I21 or I64. A consensus panel adjudicated the final cause of death according to ICD-10 codes using standardized definitions (16). If the cause of death was coded as C01-C97, the cause of death was labeled as cancer related mortality. Chronic Obstructive Pulmonary Disease (COPD) related mortality was coded as J43-J44. Participants were followed from date of entry in the study until date of death, lost to follow-up or January 1st, 2012, whichever came first.

Baseline characteristics

Of all participants, data was collected by structured interviews and physical examination. In a subset of participants blood was sampled. Blood pressure was measured as the mean of two readings using a random-zero sphygmomanometer in sitting position. The use of antihypertensive drugs was defined as the use of antihypertensive medication indicated for the treatment of high blood pressure (≥ grade 1 hypertension according to World Health Organization Criteria (17)). Antithrombotic medication was defined as the use of vitamin K antagonists, platelet aggregation inhibitors, and direct thrombin inhibitors. Diabetes mellitus was defined as fasting serum glucose level ≥ 7.0 mml/L and/or the use of blood glucose lowering medication (18). Lipid lowering agents was defined as the use of statins. Total cholesterol and high-density lipoprotein cholesterol were measured using an automated enzymatic procedure. Body mass index was calculated as the weight (in kilograms) divided by the square of the height (in meters). Smoking status was defined as current, former or no smoking at baseline.

Statistical analysis

Mean and standard deviation or counts and percentages were used to describe baseline characteristics. Log minus Log plots were drawn to determine proportional hazards assumption. Cox proportional hazard regression analysis was used to assess the association between ADAMTS13, VWF and mortality. For these analyses quartiles of ADAMTS13 activity and VWF:Ag levels were used. A Kaplan Meier curve was constructed using ADAMTS13 and VWF quartiles adjusted for age and sex. For the combination of ADAMTS13 activity and VWF levels, we used the 25 percentile for ADAMTS13 and the 75 percentile as cut-off for VWF. All analyses were adjusted for age and sex and additionally for use of antithrombotic agents, antihypertensives, diabetes mellitus, lipid reducing agents, BMI, current smoking, total cholesterol, HDL cholesterol, systolic blood pressure and diastolic blood pressure and finally additionally adjusted for prevalent cardiovascular disease. Missing values of all these covariates (0-4.7%) were imputed five times using a multiple imputation method including all covariates. Statistical analyses were performed on all five datasets and pooled into one final result using SPSS software. Data were analyzed using SPSS version 21 (SPSS 21.0 IBM, Somers, NY, USA). All statistical tests were two-tailed and a p value of <0.05 was considered statistically significant.

RESULTS

In total, 6130 individuals were included in this study. The mean age of the included individuals was 69 ± 8.0 years and 57% was female. The median VWF:Ag level was 1.19 [IQR 0.92 – 1.58] IU/ml and the mean ADAMTS13 activity was 91.8 ± 17.6%. During the median follow-up time of 11.3 years, 1868 (30.5%) individuals died, 442 of them (23.7%) due to cardiovascular disease, 518 (27.7%) due to cancer, and 59 (3.2%) due to COPD.

After adjusting for confounders, the risk of all-cause mortality was significantly higher in individuals in the lowest ADAMTS13 quartile than in individuals with the highest ADAMTS13 (HR 1.46, 95%CI 1.26 – 1.69, Table 2). The risk of cardiovascular mortality was also increased in individuals with the lowest ADAMTS13 activity (HR 1.46, 95%CI 1.09 – 1.96). The risks of cancer related mortality and COPD related mortality were 1.30 and 1.91, but not statistically significant (HR 1.30, 95%CI 0.99 – 1.71; HR 1.91, 95%CI 0.69 – 5.32, respectively).

Table 1. Baseline characteristics

N = 6130	Total cohort N (%) or mean ± SD
Age (years)	68.9 ± 8.0
Female sex	3520 (57.4%)
Smoking	
Current	1057 (17.4%)
Former	3019 (49.6%)
BMI (kg/m^2)	27.0 ± 4.1
Antithrombotic medication	1194 (20.4%)
Hypertension	1406 (24.1%)
Lipid reducing agents	785 (13.4%)
Diabetes Mellitus	623 (10.3%)
Total cholesterol (mmol/l)	5.8 ± 1.0
HDL cholesterol (mmol/l)	1.4 ± 0.4
Systolic blood pressure (mmHg)	143 ± 21
Diastolic blood pressure (mmHg)	77 ± 11
Blood group O	2360(45.3%)
ADAMTS13 activity (%)	91.76 ± 17.59
VWF:Ag level (IU/ml), median [IQR]	1.19 [0.92-1.58]

In individuals in the highest VWF quartile, the risk of all-cause mortality was increased compared with individuals in the lowest quartile of VWF (HR 1.21, 95%CI 1.06 – 1.38, Table 3). When we analyzed the different mortality causes, the risk estimate of cardiovascular related mortality (HR 1.29, 95%CI 0.98 – 1.70) was similar to the risk of all-cause mortality.

Table 2. Cox proportional hazard regression analysis between ADAMTS13 quartiles and mortality

ADAMTS13 activity	Number of cases / total number at risk	Model 1 HR (95% CI)	Model 2 HR (95% CI)	Model 3 HR (95% CI)
All-cause mortality (N= 1868)				
Quartile 1	679 / 1530	1.50 (1.30 – 1.73)	1.46 (1.26 – 1.69)	1.46 (1.26 – 1.69)
Quartile 2	490 / 1531	1.21 (1.04 – 1.40)	1.18 (1.02 – 1.38)	1.20 (1.03 – 1.39)
Quartile 3	412 / 1530	1.13 (0.97 – 1.32)	1.12 (0.96 – 1.31)	1.13 (0.97 – 1.31)
Quartile 4	287 / 1530	1 (ref)	1 (ref)	1 (ref)
Cardiovascular mortality (N= 442)				
Quartile 1	171 / 1514	1.41 (1.06 – 1.89)	1.46 (1.09 – 1.96)	1.46 (1.09 – 1.96)
Quartile 2	98 / 1525	0.92 (0.68 – 1.25)	0.93 (0.68 – 1.27)	0.95 (0.69 – 1.29)
Quartile 3	100 / 1525	1.05 (0.78 – 1.43)	1.07 (0.79 – 1.45)	1.07 (0.79 – 1.46)
Quartile 4	73 / 1523	1 (ref)	1 (ref)	1 (ref)

Model 1 adjusted for age and sex
Model 2 additionally adjusted for antithrombotic medication, hypertension, diabetes mellitus, lipid reducing agents, BMI, smoking, total cholesterol, HDL cholesterol, systolic blood pressure and diastolic blood pressure
Model 3 additionally adjusted for prevalent cardiovascular disease

No association was observed between VWF:Ag levels and COPD mortality (HR 1.07, 95%CI 0.83 – 1.37) and cancer mortality (HR 1.02, 95%CI 0.80 – 1.30).
The lowest ADAMTS13 and the highest VWF quartiles were both associated with the lowest event-free survival after adjustment for age and sex (Figure 1).

In individuals with both high VWF:Ag and low ADAMTS13 activity (N=448), the risk of all-cause mortality was strongly increased compared to individuals with both low VWF:Ag and high ADAMTS13 activity (Table 4). We additionally investigated whether individuals with both high VWF:Ag levels and low ADAMTS13 activity had an even stronger risk of mortality. Of these 448 individuals 247 individuals died (55.1%) during follow-up compared to 22.6% in the group individuals with low VWF:Ag levels and high ADAMTS13 activity. Individuals with both high VWF and low ADAMTS13 had a 58% increased risk of all-cause mortality (HR 1.58, 95%CI 1.36 – 1.84). Also, the risk of cardiovascular mortality was increased most strongly in individuals with both low ADAMTS13 and high VWF (HR 1.73, 95%CI 1.28 – 2.35). The risks of cancer related mortality and COPD related mortality were both not increased with the combination of low ADAMTS13 and high VWF (HR 1.16, 95%CI 0.84 – 1.60; HR 1.32 95%CI 0.54 – 3.22).

Table 3. Cox proportional hazard regression analysis between VWF quartiles and mortality

VWF:Ag levels	Number of cases / total number at risk	Model 1 HR (95% CI)	Model 2 HR (95% CI)	Model 3 HR (95% CI)
All-cause mortality (N= 1863)				
Quartile 1	366 / 1558	1 (ref)	1 (ref)	1 (ref)
Quartile 2	414 / 1537	0.99 (0.86 – 1.14)	0.99 (0.86 – 1.14)	0.99 (0.86 – 1.14)
Quartile 3	451 / 1495	0.96 (0.84 – 1.11)	0.97 (0.84 – 1.11)	0.97 (0.84 – 1.11)
Quartile 4	632 / 1530	1.21 (1.06 – 1.38)	1.21 (1.06 – 1.38)	1.21 (1.06 – 1.38)
Cardiovascular mortality (N= 439)				
Quartile 1	80 / 1551	1 (ref)	1 (ref)	1 (ref)
Quartile 2	95 / 1526	1.03 (0.77 – 1.39)	1.04 (0.77 – 1.41)	1.03 (0.76 – 1.38)
Quartile 3	109 / 1486	1.04 (0.77 – 1.39)	1.04 (0.78 - 1.39)	1.05 (0.78 – 1.40)
Quartile 4	155 / 1523	1.30 (0.99 – 1.71)	1.29 (0.98 – 1.70)	1.29 (0.98 - 1.70)

Model 1 adjusted for age and sex
Model 2 additionally adjusted for antithrombotic medication, hypertension, diabetes mellitus, lipid reducing agents, BMI, smoking, total cholesterol, HDL cholesterol, systolic blood pressure and diastolic blood pressure
Model 3 additionally adjusted for prevalent cardiovascular disease

Table 4. Cox proportional hazard regression analysis between ADAMTS13 and VWF and mortality

ADAMTS13 and VWF:Ag level	Number of cases / total number at risk	Model 1 HR (95% CI)	Model 2 HR (95% CI)	Model 3 HR (95% CI)
Mortality (N = 1862)				
VWF < p75 and ADAMTS13 > p25	798 / 3502	1 (ref)	1 (ref)	1 (ref)
VWF ≥ p75 and ADAMTS13 > p25	385 / 1080	1.23 (1.09 – 1.39)	1.24 (1.10 – 1.41)	1.24 (1.10 – 1.40)
VWF < p75 and ADAMTS13 ≤ p25	432 / 1081	1.33 (1.18 – 1.50)	1.31 (1.16 – 1.48)	1.31 (1.16 – 1.48)
VWF ≥ p75 and ADAMTS13 ≤ p25	247 / 448	1.60 (1.38 – 1.86)	1.57 (1.35 – 1.82)	1.58 (1.36 – 1.84)
Cardiovascular mortality (N= 439)				
VWF < p75 and ADAMTS13 > p25	174 / 3486	1 (ref)	1 (ref)	1 (ref)
VWF ≥ p75 and ADAMTS13 > p25	95 / 1078	1.35 (1.05 – 1.74)	1.35 (1.05 – 1.74)	1.33 (1.03 – 1.72)
VWF < p75 and ADAMTS13 ≤ p25	110 / 1070	1.51 (1.18 – 1.94)	1.57 (1.22 – 2.01)	1.54 (1.20 – 1.97)
VWF ≥ p75 and ADAMTS13 ≤ p25	60 / 443	1.73 (1.28 – 2.34)	1.72 (1.27 – 2.33)	1.73 (1.28 – 2.35)

Model 1 adjusted for age and sex
Model 2 additionally adjusted for antithrombotic agents, antihypertensives, diabetes mellitus, lipid reducing agents, BMI, current smoking, total cholesterol, HDL cholesterol, systolic blood pressure and diastolic blood pressure
Model 3 additionally adjusted for prevalent cardiovascular disease

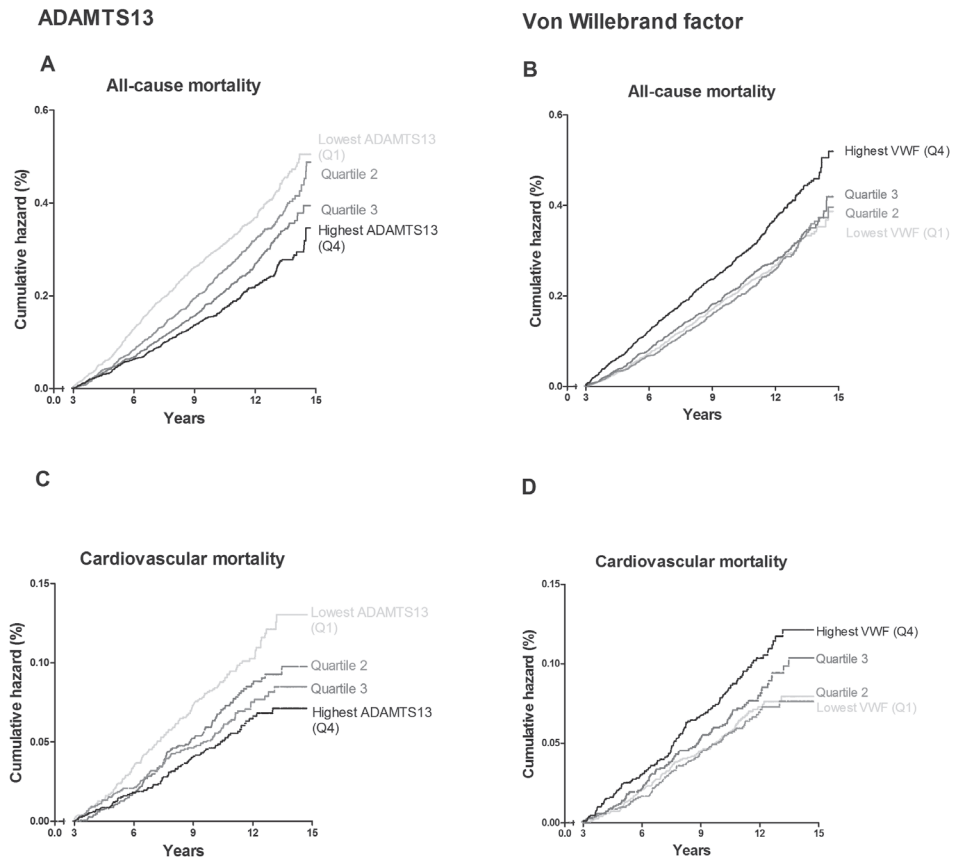

Figure 1. Kaplan meier curves for VWF:Ag levels and ADAMTS13 activity
Kaplan Meier curve for the cumulative survival for all-cause mortality per quartile of ADAMTS13 activity (A) and VWF:Ag levels (B) adjusted for age and sex. Kaplan Meier curve for the cumulative survival for cardiovascular mortality per quartile of ADAMTS13 activity (C) and for VWF:Ag levels (D) adjusted for age and sex. Cut-points for ADAMTS13 quartiles were: ≤70.99%, 71.00 – 90.80%, 90.81 – 111.51%, and ≥111.52%. Cut-points for VWF quartiles were: ≤0.56, 0.57 – 1.09, 1.10 – 1.82, and 1.83 IU/L

DISCUSSION

In this large prospective cohort study with a median follow-up time of 11.3 years and a total of 90635 person years, ADAMTS13 activity and VWF:Ag levels were associated with an increased risk of mortality and specifically cardiovascular mortality. These risks were independent of prevalent cardiovascular disease and established cardiovascular risk factors. We found an association between low ADAMTS13 activity and the risk of all-cause and cardiovascular mortality. Recently, we have shown that low ADAMTS13 activity is associated with an increased risk of ischemic stroke and coronary heart disease (8, 19), but no studies on the association with mortality have been performed to date. We found that individuals with low ADAMTS13 activity (< 80.7%) had a 1.46 fold higher risk of mortality than individuals with high ADAMTS13 activity. In individuals with the lowest ADAMTS13, the risk estimates of all-cause and cardiovascular mortality (HR 1.46 for both) were similar, suggesting that the risk of all-cause mortality might be driven by an increased risk of cardiovascular disease.

We found an increased risk of all-cause mortality in individuals with the highest VWF:Ag levels. In addition, there was a borderline significant association between cardiovascular mortality and high VWF:Ag levels, although the risk estimate was higher compared with all-cause mortality. This might be explained by the smaller number of cases in this subgroup. These results indicate that ADAMTS13 activity may play a more prominent role in cardiovascular disease in our elderly study population. Previous studies using the Rotterdam Study have already found a higher cardiovascular risk in individuals with a low ADAMTS13 activity than with a high VWF:Ag level (5, 8, 20). The association between VWF levels and cardiovascular mortality has been reported before (9-13). However, these studies only investigated cardiac specific or cardiovascular mortality and no studies are performed yet on the association with all-cause mortality and cause specific mortality.

In a model with both low VWF levels and high ADAMTS13 activity, we found that individuals with a high VWF:Ag level and low ADAMTS13 activity have an increased risk of all-cause and cardiovascular mortality up to 1.73 fold, although this was only a small subgroup of individuals (24.1 and 7.3%, respectively). This finding suggests that VWF and ADAMTS13 could be independent risk factors and might have an additive effect, which was also shown before (8).

The risks of cancer and COPD related mortality were not increased in individuals with either high VWF:Ag levels or low ADAMTS13 activity, although the risk of cancer related mortality was borderline significant in individuals with the lowest ADAMTS13 activity. Cancer and COPD are both common causes of mortality. Previous studies have shown a relation between high VWF levels and cancer (21-24), COPD (25, 26), or inflammation (27) and also an association between ADAMTS13 and inflammation was shown before (28-30). This would suggest that there may be an association between high VWF levels, low ADAMTS13 and cancer or COPD related mortality. However, the lack of this association suggests that ADAMTS13 and VWF

are influenced by other mechanisms such as inflammation in patients with COPD or cancer, and their effect might be explained by alternative factors.

So far, this is the first study that investigated the association between VWF:Ag levels, ADAMTS13 activity and the risk of all-cause and cardiovascular mortality in a population that is representative for the Dutch population. We had a long follow-up time of 11.3 years in which many individuals died (30.5%). However, only 59 participants died due to COPD related mortality which was too small to have sufficient power to evaluate the association. Moreover, VWF and ADAMTS13 could have been influenced by an already existing disease at the moment of blood sampling. Therefore, we excluded all individuals who died within the first 3 years after blood sampling to reduce this influence. Our study was performed in a population of predominantly Caucasians of 55 years and older that live in a middle income district of Rotterdam. This limits the generalizability of our results, especially for younger individuals.

In conclusion, we observed in this large prospective cohort study with a long follow-up time, an association between high VWF levels, low ADAMTS13 activity and all-cause or cardiovascular mortality. This risk increased up to 1.73 fold when using both high VWF and low ADAMTS13.

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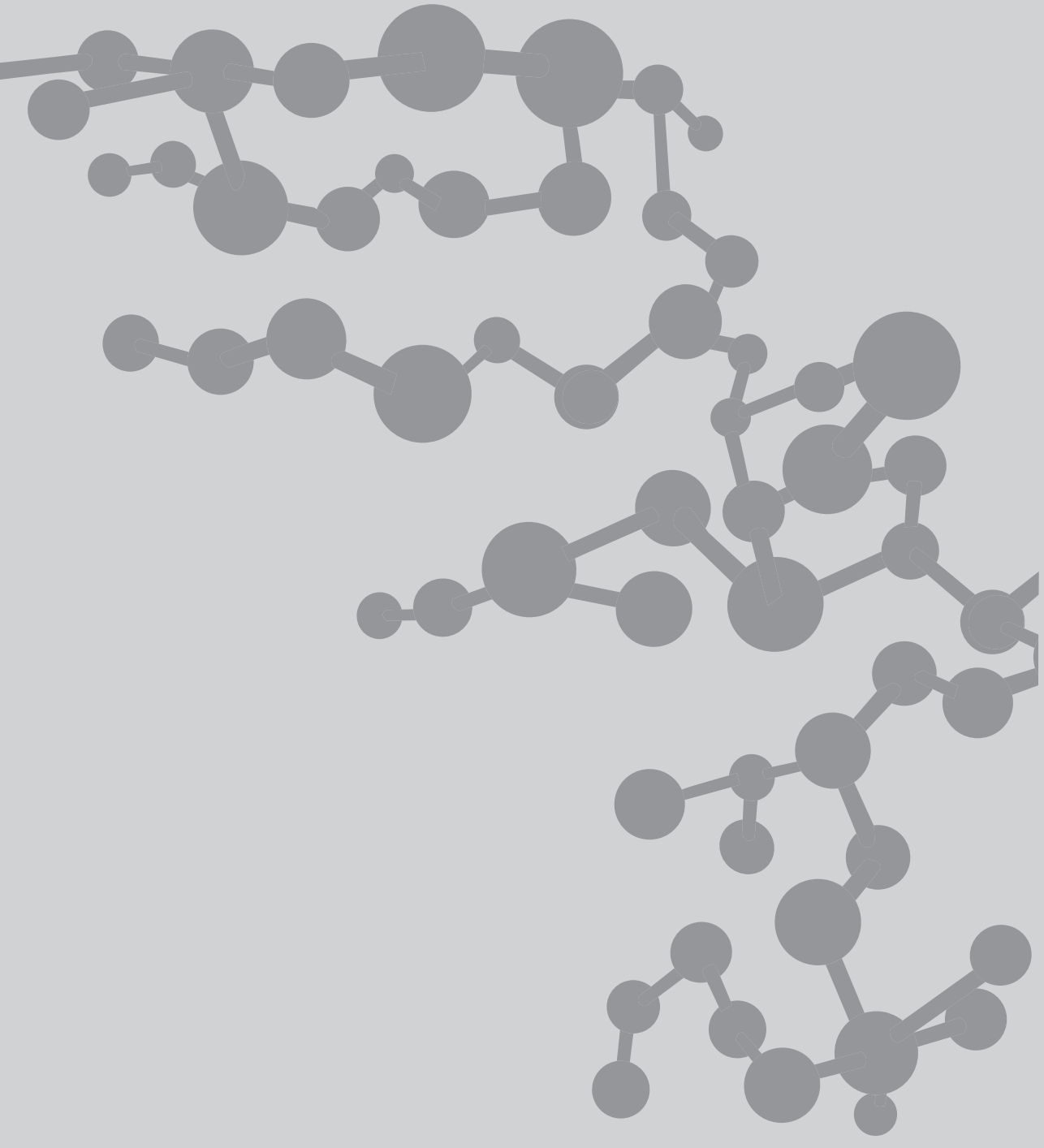

PERFORMANCE RELATED FACTORS ARE THE MAIN DETERMINANTS OF THE VON WILLEBRAND FACTOR RESPONSE TO EXHAUSTIVE PHYSICAL EXERCISE

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ABSTRACT.

Background

Physical stress triggers the endothelium to release von Willebrand Factor (VWF) from the Weibel Palade bodies. Since VWF is a risk factor for arterial thrombosis, it is of great interest to discover determinants of VWF response to physical stress. We aimed to determine the main mediators of the VWF increase by exhaustive physical exercise.

Methods

105 healthy individuals (18-35 years) were included in this study. Each participant performed an incremental exhaustive exercise test on a cycle ergometer. Respiratory gas exchange measurements were obtained while cardiac function was continuously monitored. Blood was collected at baseline and directly after exhaustion. VWF antigen (VWF:Ag) levels, VWF collagen binding (VWF:CB) levels, ADAMTS13 activity and common variations in Syntaxin Binding Protein-5 (*STXBP5,* rs1039084 and rs9399599), Syntaxin-2 (*STX2,* rs7978987) and VWF (promoter, rs7965413) were determined.

Results

The median VWF:Ag level at baseline was 0.94 IU/mL [IQR 0.8-1.1] and increased with 47% [IQR 25-73] after exhaustive exercise to a median maximum VWF:Ag of 1.38 IU/mL [IQR 1.1-1.8] ($p<0.0001$). VWF:CB levels and ADAMTS13 activity both also increased after exhaustive exercise (median increase 43% and 12%, both $p<0.0001$). The strongest determinants of the VWF:Ag level increase are performance related ($p<0.0001$). We observed a gender difference in VWF:Ag response to exercise (females 1.2 IU/mL; males 1.7 IU/mL, $p=0.001$), which was associated by a difference in performance. Genetic variations in *STXBP5*, *STX2* and the VWF promoter were not associated with VWF:Ag levels at baseline nor with the VWF:Ag increase.

Conclusions

VWF:Ag levels strongly increase upon exhaustive exercise and this increase is strongly determined by physical fitness level and the intensity of the exercise, while there is no clear effect of genetic variation in *STXBP5*, *STX2* and the VWF promoter.

INTRODUCTION

Blood coagulation changes in response to physical exercise (1-6). One of the major players in blood coagulation is von Willebrand factor (VWF), a multifunctional glycoprotein that initiates primary haemostasis. Ultralarge very active VWF multimers are cleaved by A *Disintegrin and Metalloprotease with ThromboSpondin motif repeats 13* (ADAMTS13) into smaller, less prothrombotic forms. It is well known that levels of VWF increase steeply upon intense physical exercise (7). To date it is not fully understood which mediators, both non-genetic and genetic, affect VWF response to stress. However, it is of great interest to discover new determinants of the excretion mechanism of VWF molecules, since high VWF levels have been associated with venous thrombosis (8) and arterial thrombosis (9-11).

VWF is mainly synthesized by endothelial cells and marks endothelial cell activation (12, 13). The majority of the freshly synthesized VWF molecules are constitutively released into the circulation. A small part of especially large VWF multimers - harbouring the greatest haemostatic potential - is stored in Weibel Palade Bodies of endothelial cells (14-17). Numerous agonists initiate the release from these storage granules, including hypoxia, epinephrine, histamine, thrombin, fibrin, and vasopressin (18, 19).

Plasma VWF levels have a wide biological variation, since numerous lifestyle factors, environmental factors, and genetic factors continuously influence VWF levels in the circulation (19). Previous studies among human twins have demonstrated that more than half of the variability in VWF levels is caused by genetic variations in the genome (20, 21). The most important genetic determinant is ABO blood group (22). In addition, recently six new genetic loci have been discovered using a hypothesis-free approach with genome-wide association studies (23). Two of the newly identified genetic loci, Syntaxin Binding Protein-5 (*STXBP5*) and Syntaxin-2 (*STX2*), are of specific interest, since their encoding proteins interact with SNARE complex proteins, such as SNAP23 and syntaxin-4, which have been shown to be involved in Weibel Palade Body (WPB) exocytosis, a well-known mechanism for the secretion of VWF molecules by endothelial cells (24). Another genetic modulator is the VWF promoter in which four single nucleotide polymorphisms (SNPs) have been identified, that are associated with VWF levels (25, 26).

We aimed to identify important mediators, including lifestyle factors, environmental factors, and common genetic variations in the *STXBP5*, *STX2* and VWF promoter genes, of VWF response to incremental exhaustive exercise in a large group of young healthy individuals.

MATERIAL AND METHODS

Ethics Statement

The study was approved by the medical ethical committee at Erasmus University Medical Center and written informed consent was obtained from all participants at inclusion.

Study participants

For the "RESPOnse" (Role of SNARE protein genes in the regulation of von Willebrand Factor concentration and other coagulation factors) study, we included 105 healthy individuals, who were between 18 to 35 years of age and of North-European ancestry. Exclusion criteria were known cardiovascular risk factors, including hypertension, hypercholesterolemia, diabetes, obesity (BMI > 30 kg/m^2), and a positive family history of cardiovascular disease. In addition, participants never had a thrombotic event or coagulation disorder, were non-smokers, had no known malignancies, no liver or renal dysfunction, did not use medication that may influence VWF levels and were not pregnant. Oral contraceptives use was allowed in this study. Subjects were requested to abstain from caffeinated and alcoholic beverages twelve hours prior to the test and to avoid heavy or high-intensity physical exercise and sports activities on the day of the test.

Baseline measurements

At baseline, all patients received a questionnaire on current health status and physical condition. We measured weight using a calibrated digital scale (SECA GmbH & co, model 861) and height using a wall mounted telescopic height rod (SECA GmbH & co, model 220). Blood pressure was measured in an upright sitting position with a calibrated sphygmanometer (Welch Allyn, model Maxi-Stabil 3) and left upper-arm adjusted cuff size (WelchAllyn, FlexiPort reusable blood pressure cuff). Also, before the start of the cycle ergometer test, we performed a rest electrocardiogram (ECG) to exclude abnormalities in electric conduction through the heart, arrhythmias etc. All participants declared to be in good health and none of them had medical contra-indications for participation in the study.

Cycle ergometer maximal test

Each participant performed an incremental exercise test until exhaustion, performed on a cycle ergometer (Ergoline, ER800, Lode, the Netherlands) using a linearly increasing (12 or 18.5 W min-1) ramp protocol. These slopes were chosen to achieve exhaustion within 8-12 minutes as recommended by Zhang et al (27). Participants started with a warming-up phase of 4 minutes without resistance. They were instructed to pedal at a frequency between 60 and 80 rotations per minute (rpm). The loaded phase was terminated when pedalling frequency dropped below 60 rpm and was followed by cooling-down for at least 2 minutes. Cardiac function was monitored using a 12-lead electrocardiogram with heart rate (HR)

being recorded continuously. Respiratory gas exchange measurements were performed continuously by using a computerized metabolic cart (Oxycon Pro, Carefusion, the Netherlands) that was calibrated before each test. Maximal whole-body oxygen uptake capacity was defined in the present study as VO_2 remaining unchanged or increasing less than 1 ml/min/kg for 30 sec or more despite an increment in work load (28). VO_2 is the capacity to transport and use oxygen during exercise and is a measure for physical fitness. The ventilatory threshold (VT1) was determined by an increase in ventilation (Ve)/VO_2 but without a concomitant increase in Ve/VCO_2. The ventilatory threshold represents the moment at which metabolism changes from aerobic to anaerobic. Two experienced exercise physiologists reviewed the plots averaged over 30 sec of the Ve/VO_2 and Ve/VCO_2 and determined VT1 values. In case of disagreement, the opinion of a third investigator was sought. The metabolic equivalent (METs) score is calculated by the VO2 max and VO2 VT1 values (1 MET = 3.5 ml/min/kg VO2). A METs score (calculated by the VO2 max) above 10 represents activities associated with significant exertion. A METs score calculated by the VO2 VT1 represents the intensity to sustain activities for 1 hour and is a measurement of the physical fitness of an individual. The power output (W) was assessed to establish the workload capacity of the participant. Power output is dependent on physical fitness, but also on talent. Finally, maximum respiratory exchange ratio (RER) at peak power output was determined. The RER is a ratio between the amount of carbon dioxide (CO_2) exhaled and the amount of oxygen (O2) inhaled per breath. Together with the maximum achieved heart rate as percentage of age-predicted heart rate (i.e. 208-0.7*age) (29), the maximum RER is a measure for the intensity of the test.

Blood sampling

Venous blood was drawn from the forearm before and directly after exhaustion (within 1 minute) via a cannula in the antecubital vein using a Vacutainer system (Becton-Dickinson, Plymouth, UK). The first 2 ml of blood was discarded at every time point. Blood for coagulation measurements was collected in 3.2% trisodium citrate (9:1 vol/vol). Citrated blood was centrifuged within 1 hour at 3500 rpm for 10 min at 4°C. Plasma was additionally centrifuged at 14 000 rpm at room temperature and stored in aliquots at -80°C. For DNA isolation we stored the buffy coats of the remaining citrated blood at -20°C until use. Genomic DNA was isolated according to standard salting-out procedures and stored at 4°C for genetic analysis.

Laboratory measurements

VWF antigen (VWF:Ag) was determined with an in-house ELISA with polyclonal rabbit anti-human VWF antibodies and horseradish peroxidase conjugated anti-human VWF antibodies

(DakoCytomation, Glostrop, Denmark) for catching and tagging, respectively.
VWF collagen binding (VWF:CB) was determined with an in-house ELISA using collagen bovine antibodies and horseradish peroxidase conjugated anti-human VWF antibodies (DakoCytomation, Glostrop, Denmark) for catching and tagging, respectively.
ADAMTS13 activity was measured by a Fluorescence Resonance Energy Transfer Substrate (FRETS) assay using a fluorescent VWF peptide consisting of 73 amino acids (FRETS-VWF73). Plates were read with BioTek's microplate reader (BioTek). The intra-assay coefficients of variation for VWF:Ag, VWF:CB and ADAMTS13 were 1.9%, 4.5% and 3.6%, respectively. The inter-assay coefficients of variation for VWF:Ag, VWF:CB and ADAMTS13 were 8.3%, 7.9% and 7.5%, respectively.

Genotyping
The STXBP5 gene spans 182 kbps and is located in the q24 region of chromosome 6. Initially, we obtained data from the International HapMap project (phase II November 2008 http://www.hapmap.org) on the linkage disequilibrium (LD) pattern and selected haplotype-tagging single-nucleotide polymorphisms (ht-SNPs) using Haploview software (version 3.11; www.broad.mit.edu/mpg/haploview/index/php). For the *STXBP5* and *STX2* genes blocks of haplotypes with a frequency of ≥ 3% were defined in order to select these ht-SNPs. We took potential functionality into consideration by preferentially selecting non-synonymous ht-SNPs or SNPs that are located in known regulatory elements. We considered only SNPs that were present in a Caucasian population. Of these ht-SNPs, three were significantly associated with VWF:Ag levels in our previous study among young patients with arterial thrombosis and healthy controls (30). Therefore, we selected and genotyped only these three SNPs in *STXBP5* (rs1039084 and rs9399599) and in *STX2* (rs7978987) for our current study. The polymorphisms in *STXBP5*, rs1039084 and rs9399599, are in high linkage disequilibrium with rs9390459, which had the highest genome wide significance level for VWF plasma levels in the meta-analysis of the CHARGE consortium (D' = 1.00, R^2 = 0.87 for rs9399599 and D'= 0.96, R^2 = 0.86 for rs1039084) (phase II November 2008 http://www.hapmap.org). Also, rs7978987 in *STX2* had a highly significant P value of 3.82×10^{-11} in this meta-analysis(23).
The gene encoding VWF is approximately 180 kb in length, contains 52 exons and is located on chromosome 12. Four SNPs in this gene have been identified, which are in strong linkage disequilibrium and segregate as two haplotypes (25, 31). We have selected and genotyped one of these SNP's (rs7965413).
Genotyping was done using Custom TaqMan Genotyping Assays (Applied Biosystems, Foster City, CA, USA). Endpoint fluorescence was measured on the ABI 7900HT instrument (Applied Biosystems, Foster City, CA, USA) and clustered according to genotype using SDS 2.1 software (Applied Biosystems, Foster City, CA, USA). Genotyping was successful for each SNP in on average 97% of all subjects.

Statistical Analysis

Data on baseline characteristics are presented as means and standard deviations for continuous variables and as counts and percentages for categorical data. Since VWF:Ag levels were skewed, these data were natural logarithmically transformed (lnVWF:Ag) and presented as median and interquartile range (IQR). Mann-Whitney tests were used for unpaired two-group comparisons of non-parametric data.

The association between baseline characteristics and performance related determinants with lnVWF:Ag increase were assessed with linear regression models. Logistic regression was used to assess the relationship between VWF:Ag response and baseline characteristics, using VWF:Ag response as categorical variable. VWF:Ag increase was divided into two categories: low response with a VWF:Ag increase below the median (<0.40 IU/mL) and high response with a VWF:Ag increase above the median (≥ 0.40 IU/mL). Gender differences in performance-related determinants were assessed with univariate analysis of variance (ANOVA). The power output and the VO_2 were adjusted for weight and presented as the value per kg.

Allele frequencies of the VWF polymorphisms were calculated by genotype counting. For each SNP, the deviation from the Hardy-Weinberg equilibrium was tested by means of a Chi-squared test with one degree of freedom. We used linear regression analyses with additive genetic models to determine the association between genetic variations in VWF and lnVWF:Ag levels. We had a power of 0.80 to detect a difference of 0.2 between carriers of the minor allele and carriers of the common allele, assuming a minor allele frequency of 0.40. Beta-coefficients represent the increase in lnVWF:Ag levels per coded allele. Statistical analyses were performed with SPSS for Windows, version 20.0 (SPSS Inc, Chicago, USA). A two-sided value of $p < 0.05$ was considered statistically significant.

RESULTS

In the current study, 105 healthy individuals were included. Baseline characteristics are shown in table 1. The mean age was 24 years and 61% were female. Blood pressure (mean 114/70 mmHg) and BMI (mean 23 kg/m2) were within the normal range. Of all subjects, 86 (82%) used alcoholic beverages. Of all female participants, 33 women (52%) used oral contraceptives, seven women (11%) had an intra-uterine device (MirenaÒ), and two women (3%) had a Nuva ringÒ.

The median VWF:Ag level at baseline was 0.94 IU/mL and increased with 47% after exhaustive exercise to a median maximum VWF:Ag of 1.38 IU/mL ($p < 0.0001$) (table 1, figure 1). The median VWF:CB level at baseline was comparable with the VWF:Ag level, 0.93 IU/mL, and increased with 43% to a median maximum of 1.36 IU/mL ($p < 0.0001$). The absolute increase in VWF:Ag and VWF:CB levels was highly correlated (R 0.81, $p < 0.0001$). The VWF:CB / VWF:Ag ratio was 1.00 at baseline and was decreasing to 0.96 at maximum exercise. ADAMTS13 levels also increased after exhaustive exercise with 12% (Table 1, Figure 1). There was no correlation between the absolute increase in VWF:Ag level and ADAMTS13 (R 0.06, $p = 0.57$).

Baseline characteristics were not associated with baseline VWF:Ag levels (table 2). As expected, blood group non-O was associated with higher baseline VWF:Ag levels (geometric mean of VWF:Ag for blood group O: 0.82 ± 0.03 IU/mL and for non-O: 1.08 ± 0.05 IU/mL). Sex, systolic blood pressure, and diastolic blood pressure were significantly associated with the VWF:Ag level response (table 2 and figure 2). Alcohol consumption, defined as any amount of glasses per week, was borderline significantly associated with a lower VWF:Ag response ($p = 0.06$). The strongest determinant was sex. In a multivariate model including sex, blood pressure, and alcohol consumption, only sex remained significantly associated with VWF:Ag levels (beta-coefficient for males 0.79 [95% CI 0.52;1.06], $p < 0.0001$).

At baseline, we observed no difference in VWF:Ag levels between males and females (median [IQR]: females 0.95 IU/mL [0.78;1.12] and males 0.91 IU/mL [0.74;1.11], $p = 0.50$). At exhaustion, males had significantly higher VWF:Ag levels than females (females 1.22 IU/mL [1.06;1.58] and males 1.66 IU/mL [1.24;2.05], $p = 0.001$). OAC use was not associated with the VWF:Ag level response to exercise.

Next, we investigated the association between performance related determinants and the VWF:Ag response (table 3A). All performance-related determinants were significantly associated with the increase in VWF:Ag levels, but not with the baseline VWF:Ag levels. The strongest performance related determinants ($p < 0.0001$) were the peak power output per kg bodyweight, the ratio between the power output at the ventilatory threshold and the peak power output in Watt, VO_2 peak per kg and the maximum RER at peak power output. Physical fitness, as represented by the VO_2 VT1/max and power output VT1/max, was negatively associated with the VWF:Ag increase. In addition, a multivariate regression

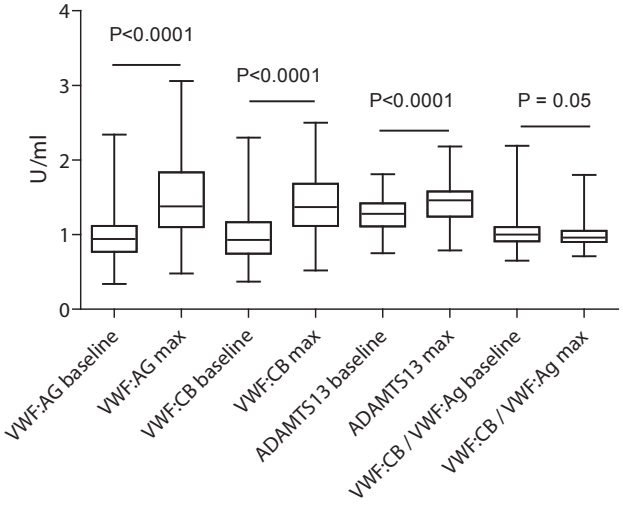

Figure 1. Levels (U/ml) of VWF:Ag, VWF:CB, ADAMTS13 and the ratio VWF:CB / VWF:Ag at baseline and maximum. P values represents the difference between baseline and maximum levels.

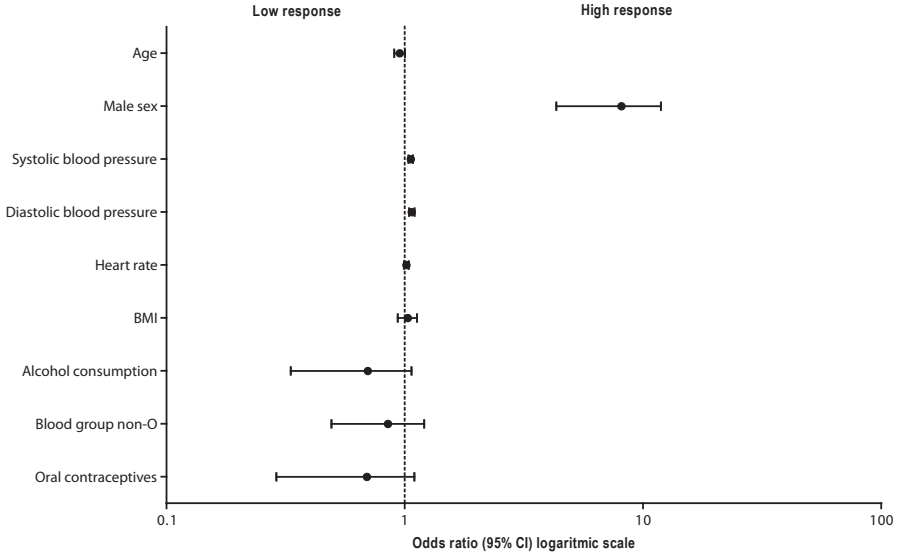

Figure 2. Association between baseline characteristics and VWF:Ag response defined by the Odds ratio. VWF:Ag increase divided into two categories: low response with a VWF:Ag increase below the median (<0.40 IU/ml) and high response with a VWF:Ag increase above the median (≥0.40 IU/ml).

Table 1. Baseline characteristics

	N = 105
Age (years)	24.3 ± 4.4
Female sex, N (%)	64 (61%)
Systolic blood pressure (mmHg)	114 ± 12
Diastolic blood pressure (mmHg)	70 ± 9
Heart rate (bpm)	76 ± 10
BMI (kg/m^2)	23 ± 2
Oral contraceptives (% of total women)	33 (52%)
Blood group O, N (%)	54 (51%)
METs score (max)	12.4 ± 2.4
METs score (VT1)	8.6 ± 1.9
VWF:Ag baseline (IU/mL), median (IQR)	0.94 (0.77-1.12)
VWF:Ag maximum (IU/mL), median (IQR)	1.38 (1.10-1.84)
VWF:Ag absolute increase (IU/mL), median (IQR)	0.40 (0.23-0.70)
VWF:Ag relative increase (%), median (IQR)	47 (25-73)
VWF:CB baseline (IU/mL), median (IQR)	0.93 (0.75-1.17)
VWF:CB maximum (IU/mL), median (IQR)	1.36 (1.11-1.68)
VWF:CB absolute increase (IU/mL), median (IQR)	0.37 (0.17-0.67)
VWF:CB relative increase (%), median (IQR)	43 (19-69)
ADAMTS13 baseline (U/ml), median (IQR)	1.28 (1.11-1.42)
ADAMTS13 maximum (U/ml), median (IQR)	1.46 (1.24-1.58)
ADAMTS13 absolute increase (U/ml), median (IQR)	0.15 (0.07-0.24)
ADAMTS13 relative increase (%), median (IQR)	12 (6-19)
VWF:CB / VWF:Ag ratio baseline, median (IQR)	1.00 (0.91-1.10)
VWF:CB / VWF:Ag ratio maximum, median (IQR)	0.96 (0.89-1.05)

Summary statistics for continuous variables are presented as mean ± standard deviation. Categorical data are summarized as percentages. VWF:Ag, VWF:CB and ADAMTS13 levels and the VWF:CB / VWF:Ag ratio are presented as median and interquartile range (IQR).

was performed. However, strong multicolinearity was observed between all performance-related variables. Therefore, we selected variables that showed the least correlation with each other for the multivariate model. To this end, we used two models with different combinations of variables. Nevertheless, multicolinearity could not be excluded completely, whereby the results of the multivariate regression analysis should still be interpreted with care. Next, we added sex to model I and model II, which resulted in the loss of the significant

association between sex and VWF:Ag increase (Model I: β = 0.27 [95% CI -0.04;0.58], p = 0.09; model II: β = 0.23 [95% CI -0.84;0.54], p = 0.15). Additionally, when stratifying for sex the association between performance related determinants and the increase in VWF:Ag levels remained. In table 3B the performance-related values are presented for males and females separately. Physical fitness (VO_2 VT1/max and power output VT1/max) was slightly higher in females than in males. The intensity of the test and the endurance of the participant, as represented by the VO2 peak, peak power output, and the maximum RER, were higher in males than in females. There was no difference in test duration between males and females. When excluding women with oral contraceptives the results of the study did not change.
Finally, we investigated the association between genetic variations in *STXBP5*, *STX2* and VWF promoter on VWF:Ag response to exercise (table 4). There was no significant association between genetic variations and VWF:Ag levels at baseline, at exhaustion, nor an association with the absolute VWF:Ag increase. Also, ABO blood group, the most important genetic determinant of VWF:Ag levels, was not associated with the VWF:Ag level response to exercise. There was also no significant difference in the association between VWF increase and genetic variation between individuals with blood group O and individuals with blood group non-O (data not shown).

Table 2. Association between baseline characteristics and VWF:Ag levels

	Baseline VWF:Ag levels Beta-coefficient [95% CI]	**P-value**	**Absolute VWF:Ag increase Beta-coefficient [95% CI]**	**P-value**
Age	0.01 [-0.004; 0.02]	0.18	-0.03 [-0.06;0.01]	0.14
Male sex	-0.02 [-0.14;0.10]	0.73	0.79 [0.54;1.04]	< 0.0001
Systolic blood pressure	0.001 [-0.01;0.01]	0.93	0.02 [0.003;0.03]	0.02
Diastolic blood pressure	-0.004 [-0.11;0.002]	0.21	0.02 [0.001;0.03]	0.04
BMI	-0.002 [-0.03;0.02]	0.90	0.02 [-0.05;0.08]	0.60
Alcohol consumption	-0.10 [-0.26;0.05]	0.18	-0.36 [-0.74;0.01]	0.06
Blood group non-O	0.28 [0.17;0.39]	< 0.0001	-0.10 [-0.39;0.19]	0.48
Oral contraceptives	-0.04 [-0.21;0.14]	0.67	-0.23 [-0.59;0.14]	0.22

Univariate linear regression analysis, beta-coefficient represents the increase of lnVWF:Ag levels with 95% confidence interval per unit increase of the selected variable.

Table 3. Performance-related determinants of VWF:Ag increase and mean differences between males and females

	A Univariate β [95% CI]	Multivariate Model I β [95% CI]	Multivariate Model II β [95% CI]	B Males N = 41	Females N = 64	P-value
Peak Power Output per kg	0.57 [0.42;0.72]*		0.51 [0.36;0.65]*	4.4 ± 0.1	3.4 ± 0.1	< 0.0001
Watts per kg VT1	0.42 [0.17;0.66]†			2.8 ± 0.1	2.3 ± 0.1	< 0.0001
Watts VT1/ Watts max	-2.57 [-3.95;-1.20]*	-1.02 [-2.36;0.33]		63 ± 1	67 ± 1	0.04
VO_2 peak per kg	0.05 [0.03;0.06]*	0.04 [0.03;0.06]*		50.2 ± 1.0	39.2 ± 0.8	< 0.0001
VO_2 VT1 per kg	0.03 [0.01;0.05]†			33.9 ± 0.9	27.9 ± 0.7	< 0.0001
VO_2 VT1 / VO_2 peak	-2.14 [-3.55;-0.72]†		-0.41 [-1.66;0.83]	68 ± 2	72 ± 1	0.051
Max Respiratory Exchange Ratio	3.75 [2.01;5.49]*	2.52 [0.79;4.25]†	2.49 [0.90;4.07]†	1.18 ± 0.01	1.13 ± 0.01	0.001
Test duration	0.09 [0.03-0.15]†			18.1 ± 0.3	18.2 ± 0.3	0.90

*Linear regression analysis with natural log VWF:Ag as dependent. Beta-coefficient represents the increase in lnVWF:Ag per unit increase of the selected variable. * $p < 0.0001$, † $p < 0.01$.*

A Performance related determinants of VWF:Ag increase in the total population

B Gender differences in performance-related determinants

Table 4. VWF:Ag levels per genotype of polymorphisms in *STXBP5*, STX2 and VWF promoter

	N	Baseline (IU/mL)	Exhaustion (IU/mL)	Absolute difference (IU/mL)
rs1039084 (STXBP5)				
GG	33	0.94 ± 0.06	1.45 ± 0.08	0.52 ± 0.06
AG	44	1.02 ± 0.05	1.56 ± 0.07	0.54 ± 0.05
AA	25	0.96 ± 0.06	1.42 ± 0.10	0.46 ± 0.06
P for trend		0.44	0.44	0.67
rs9399599 (STXBP5)				
AA	32	0.89 ± 0.06	1.40 ± 0.09	0.51 ± 0.06
AT	45	1.05 ± 0.05	1.59 ± 0.07	0.54 ± 0.05
TT	25	0.96 ± 0.06	1.41 ± 0.10	0.45± 0.06
P for trend		0.11	0.18	0.56
rs7978987 (STX2)				
GG	48	0.98 ± 0.05	1.43 ± 0.07	0.45 ± 0.05
AG	45	0.98 ± 0.05	1.55 ± 0.07	0.57 ± 0.05
AA	12	0.94 ± 0.09	1.42 ± 0.14	0.47 ± 0.09
P for trend		0.94	0.43	0.18
rs7965413 (VWF promoter)				
AA	47	1.01 ± 0.05	1.48 ± 0.07	0.47 ± 0.05
AG	40	0.94 ± 0.05	1.43 ± 0.08	0.49 ± 0.05
GG	13	1.03 ± 0.09	1.61 ± 0.13	0.58 ± 0.09
P for trend		0.55	0.50	0.55

DISCUSSION

In this study among 105 healthy young subjects, VWF:Ag levels increased significantly upon incremental exhaustive exercise (mean METs score (max) >10) with a median increase of 47%. The VWF:Ag response was highly variable and was strongly dependent on performance-related and physical fitness-related determinants. Neither baseline characteristics nor the studied genetic determinants of VWF:Ag levels affected the extent of VWF:Ag response to physical exercise.

VWF:CB levels increased after exhaustive exercise in the same proportion as VWF:Ag levels. ADAMTS13 levels at baseline were in the high normal range. This study included young and healthy individuals and a previous study also showed higher levels in young individuals (3). ADAMTS13 levels increased after exhaustion, although it was to a lower extent than VWF levels. A previous study showed similar results as ADAMTS13 increased after exercise in patients with von Willebrand disease type 2B (3). However, in another study in healthy individuals there was no difference in ADAMTS13 between baseline and immediately after exercise (3, 5). In addition, ADAMTS13 decreased after infusion of desmopressin, which induces a release of large VWF multimers into the circulation, in healthy individuals (32). However, all these studies included only low number of patients.

As the average maximal achieved heart rate as a percentage of age-predicted maximum heart rate was 97 ± 6%, together with a plateauing of oxygen uptake and a mean RER of 1.15 ± 0.08, our test protocol can be considered as an exhaustive cycle ergometer test in our subjects (33, 34).

In our study, the VWF:Ag levels increase upon exercise was the highest in individuals with the lowest physical fitness, although this was not significant after multivariate analysis, and in individuals with the most intensive exercise. Intensive physical exercise is associated with more recruitment of capillaries in the muscle and additionally an increase in vascular conductance in muscle composed predominantly of fast-twitch oxidative (type IIa) and fast-twitch glycolytic (type IIb) fibers (37). This results in more endothelial exposure to shear stress and adrenergic stimulation, which may explain the increased release of VWF upon exercise. The highest VWF:Ag response in individuals with the lowest physical fitness underlines the hypothesis that frequent physical exercise has a positive effect on cardiovascular risk. This is in contrast with a previous study which showed no difference in VWF:Ag increase upon exercise between resistance trained and untrained individuals (35). However, in this study only a small number of individuals were included (N=20). For a long time it has been anticipated that regular physical exercise has a favourable effect on many biological mechanisms, thereby improving health and fitness. Regular physical exercise is associated with a decreased all-cause mortality and with a reduced cardiovascular risk (38). The positive effects of physical exercise on cardiovascular disease development may be induced by alterations in haemostasis that lead to a hypocoagulable state. This hypocoagulable

state is achieved by a compensatory exhaustion of platelets in physically active individuals and underlines the beneficial effects of exercise on long-term prevention of cardiovascular disease (39). In our study, the baseline VWF:Ag levels were not associated with physical fitness or intensity of the test. This observation is in line with previous findings that showed that levels of coagulation factor VII (FVII), VWF, and FVIII at rest were similar in professional athletes and controls (40). There are multiple factors which could have influenced our results, including the type of sport and the training status of the participants, which are known to influence e.g. capillary density and in vivo endothelial function in skeletal muscle. However, the majority of our subjects participated in recreational type of sports activities and can be regarded as a representative sample of healthy active individuals.

STXBP5 and *STX2* are two novel genetic loci that have been associated with VWF:Ag levels in the general population (23). In addition, genetic variation within these genes affects VWF:Ag levels in young patients with a first event of arterial thrombosis (30). Considering the involvement of the *STXBP5* and *STX2* encoding proteins in the regulated secretion of VWF molecules, our hypothesis was that genetic variants within these genes would affect the release of VWF molecules, but not the steady state levels, which are determined by the constitutive pathway. To this end, we included young individuals below the age of 35 years to exclude the presence of extensive atherosclerosis, which is related to endothelial dysfunction and consequent higher VWF:Ag levels. Their baseline VWF:Ag levels therefore represent a steady state situation. To provoke release of VWF molecules from Weibel Palade Bodies in our study, all participants performed exhaustive physical exercise, which induces beta-adrenergic receptor activation (41) and subsequent endothelial cell activation. Genetic variation in *STXBP5* and *STX2* was not associated with VWF:Ag levels at baseline, though we had sufficient power to detect the previously observed effect of these genetic variants. The VWF:Ag increase upon exercise was also not affected by genetic variation in *STXBP5* and *STX2* . This finding was in contrast to our hypothesis.

In addition, we genotyped a common variation in the VWF promoter gene. Previous studies have shown an association between this genetic variation and VWF:Ag levels at baseline (25, 31). However, other studies have failed to show an effect of this polymorphism on VWF levels under normal conditions (42-44). Despite the fact that the influence of genetic variation in the VWF promoter on VWF levels has never been studied in arterial thrombosis or at stress before, we hypothesized that a genetic variation in this gene would affect the expression of VWF at physical exercise resulting in a difference in VWF plasma levels. In our study, we could not find a significant association between baseline VWF:Ag levels and genetic variations in the VWF promoter gene, probably caused by a relatively small study population. Furthermore, the VWF:Ag increase upon exercise was also not associated with the VWF promoter. This suggests that, in contrast to our hypothesis, the VWF release is not

depending on genetic variations in the VWF promoter at exhaustive physical exercise.
The negative results of the influence of genetic variation on VWF increase may be caused by the fact that genetic variations in *STXBP5* and *STX2* may not be involved in WPB exocytosis. In addition, VWF is also stored in alpha-granules of platelets and the contribution of platelet VWF to plasma levels upon exercise is not known (45). Therefore, VWF release from platelet alpha-granules during exercise cannot be excluded and might explain the negative results of the role of genetic variations involved in Weibel Palade Body exocytosis. Furthermore, we observed that the VWF:Ag increase was highly variable and strongly dependent on physical fitness and the intensity of the exercise performed. Consequently, the effect of environmental factors may have been stronger than the genetic effect.
Another important genetic determinant of VWF:Ag levels is blood group (46). Individuals with blood group O have 25% lower VWF:Ag levels than individuals with blood group non-O, because the presence of blood group A and B antigens on VWF molecules leads to a decreased clearance of VWF molecules (47). Furthermore, blood group non-O has been associated with an increased risk of CHD (48-50). Ribeiro et al. observed that males with blood group non-O (N = 8) had higher post-exercise VWF:Ag levels than males with blood group O (N = 8), although the rise of VWF:Ag was not statistically significantly different between the groups (51). In our study, subjects with blood group non-O had higher levels at exhaustion (median [IQR], 1.60 IU/mL [1.2-2.0]), than subjects with blood group O (1.29 IU/mL [1.1-1.6], p = 0.03). However, blood group was not associated with the VWF:Ag increase upon exercise (0.49 IU/mL [0.2-0.8] in non-O versus 0.46 [0.2-0.7] in O, p = 0.34). Assuming that exercise induces the release of VWF molecules from its storage granules and the clearance does not change during exercise, it was expected that subjects with blood group O and subjects with blood group non-O had a similar increase after exercise. In this study, we only included young healthy individuals and therefore the results of the study cannot be extrapolated to the older population. However, those individuals were included to exclude the presence of extensive atherosclerosis which represents a steady state situation.
In conclusion, we have shown in a large and homogeneous group of young healthy individuals that VWF:Ag levels increase strongly upon exhaustive physical exercise and is primarily dependent on physical fitness and the intensity of the exercise performed. Genetic variations in *STXBP5*, *STX2* and VWF promoter that have previously been identified as important genetic determinants of VWF:Ag levels, were not associated with the VWF:Ag response to physical exercise. Also, ABO blood group, the most important genetic determinant of VWF:Ag levels was not associated with the VWF:Ag increase. These findings suggest that environmental factors may be more important than genetic factors in determining the VWF:Ag response to stress.

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10

GENERAL DISCUSSION

GENERAL DISCUSSION

The aim of this thesis was to study the role of Von Willebrand Factor (VWF) and A Disintegrin And Metalloprotease with ThromboSpondin motif repeats 13 (ADAMTS13) in the pathogenesis of cardiovascular disease and to obtain more insight in the underlying mechanisms. Firstly, we investigated the association between VWF and atherosclerosis. Secondly, we studied the role of ADAMTS13 in cardiovascular disease and thirdly we studied the role of ADAMTS13 and VWF as determinants of cardiovascular mortality.

Von Willebrand Factor, atherosclerosis and atherothrombosis

Previous studies have shown that high VWF levels are associated with an increased risk of arterial thrombosis, including acute coronary syndrome and ischemic stroke (1-3). It is of importance to discover the underlying mechanism of this association in order to understand the pathophysiologic role of VWF in cardiovascular disease and to determine whether VWF can play a role in the development of arterial thrombosis. The question remains whether VWF increases the risk of arterial thrombosis by having a pathogenetic role in the development of atherosclerosis or by promoting platelet aggregation and subsequent thrombus formation on preexisting atherosclerotic lesions, or that both play a role. Several studies suggest that VWF is involved in the development of atherosclerosis (4, 5). VWF is synthesized in endothelial cells and released at moments of endothelial dysfunction or damage. VWF is therefore an established marker of endothelial dysfunction (4, 6). Endothelial dysfunction is one of the first stages of the development of atherosclerosis (7). VWF may therefore also be a marker of atherosclerosis, especially in the first and stable stage of atherosclerosis.

Studies on association between VWF and atherosclerosis

Animal studies have shown less atherosclerosis in VWF deficient animals compared to wild type animals (8-10). Besides that this suggests an association between atherosclerosis and VWF levels, it also suggests a causal role for VWF in the development of atherosclerosis. However, other animal studies showed contradictory results (11, 12). It has become evident that reduced atherosclerosis is not caused by VWF deficiency but rather by a polymorphism in Apolipoprotein B100 (5, 13). This polymorphism has a major role in determining the severity of diet-induced hypercholesterolemia and thereby influences the degree of atherosclerosis. Studies in von Willebrand disease (VWD) type 3 patients, who completely lack VWF, found similar extent of atherosclerosis compared with controls (14-16). This suggests that in humans there is no causal role for VWF in the development of atherosclerosis. Recently, Sanders et al showed that VWD patients with moderate or severe VWD, have a lower prevalence of arterial thrombosis compared to the general population (17). These studies in VWF deficient individuals suggest that VWF has a more prominent role in atherothrombosis than in atherosclerosis.

VWF and atherosclerosis

We investigated the association between VWF levels and the extent of atherosclerosis in three different studies. All studies had a cross-sectional design and included different study populations and had different markers of atherosclerosis.

VWF and calcification volume

Firstly, we measured in two studies the calcification volume determined using multidetector CT angiography, which correlates with the extent of atherosclerosis. Calcifications may be present at early stages of atherosclerosis, but also in advanced atherosclerotic lesions which consist of a necrotic lipid rich core. In our Erasmus stroke study among 925 ischemic stroke and TIA patients, we found a positive association between VWF levels and calcification volume measured in the carotid and aortic artery (18). This indicates that there is an association between the extent of atherosclerosis measured at these sites and VWF levels. However, in the Parisk study among 158 ischemic stroke and TIA patients in which the calcification volume was measured in the carotid artery we did not find this association (*chapter 5*). Also a previous cohort study found no association between coronary calcium score and VWF levels in healthy individuals (19). These controversial results can be explained by a difference in study populations, sample size and type of arteries which were used to quantify atherosclerosis. In the Parisk study we found a higher median calcification volume, due to the fact that only 10% of patients had no calcification which was lower than in the ESS in which we found no calcification in 42% of the patients. This can be explained by the fact that we only included patients with a mild to moderate stenosis in the Parisk study. Secondly, in the Parisk study we included a relatively small number of patients and therefore the study may not have had enough power to detect an association. In addition, the previously mentioned cohort study by Folsom et al. included also only a small number of 215 healthy individuals. Thirdly, in the Parisk study we had a median time of 46 days between event and blood sampling for VWF compared with a median of 5 days in the ESS study, which will influence the VWF:Ag levels as VWF levels are known to be increased in the acute phase after an event due to an acute phase response (4, 20). There may be a difference in the results between the three previous mentioned studies as different groups with various extents of atherosclerosis are included. A recent review has shown that symptomatic plaques in the carotid artery have a lower calcification percentage compared with asymptomatic plaques, suggesting that the calcification percentage is a parameter for plaque stability (21). In addition, the absence of calcifications in the coronary artery does not mean that there is no atherosclerosis (22). Therefore it would be of interest to use other markers of atherosclerosis and to study their association with VWF levels.

VWF and plaque burden

To further investigate whether VWF levels are associated with the type and extent of

atherosclerosis, we measured plaque burden as a marker of atherosclerosis by intravascular ultrasound (IVUS) and by multidetector CT angiography (MDCTA) in two different study populations of patients with coronary heart disease patients (23) and ischemic stroke patients (*chapter 5*). We found that plaque burden was positively associated with VWF:Ag levels in stable angina pectoris (SAP) patients, but not in acute coronary syndrome (ACS) patients. This difference may be explained by the strongly increased VWF:Ag levels due to the acute phase response in ACS patients, which we did not observe in SAP patients (4, 20). A recent ACS may lead to such a strong increase of VWF levels that the variability due to difference in extent of atherosclerosis cannot be detected. In the Parisk study, plaque burden was defined as the degree of stenosis by the European Carotid Surgery Trial (ESCT). We found a mean carotic stenosis of 55% (95% CI 30-69%) and plaque burden in the carotid artery was not significantly associated with VWF levels. The difference in findings between these two studies may be explained by a different study population in which atherosclerosis was measured in two different arteries and also the imaging technique of which IVUS is a more accurate technique using virtual histology. Additionally, plaque burden was differently defined. In the study using IVUS plaque burden was defined as the plaque and media cross-sectional area divided by the external elastic membrane cross-sectional area. In the Parisk study, degree of stenosis was used which has been shown to be less correlated with plaque burden (22). Furthermore, In the study using IVUS we measured the plaque burden in a non-culprit coronary artery in contrast to the Parisk study in which the culprit carotid artery was used. Measurement in a non-culprit artery may even have led to an underestimation of the association between plaque burden in the overall coronary tree and VWF:Ag levels. Another explanation might be that only the most severe stenosis in the artery was used for the analysis in both studies. This could have led to an overestimation of the amount of atherosclerosis.

VWF and high risk lesions

As high risk lesions are vulnerable to rupture and therefore associated with an increased risk of MACE, we also investigated whether VWF levels are decreased in the presence of high risk lesions and via this route lead to an increased risk of arterial thrombosis (24). We found no association between high risk lesions and VWF levels (23)(*chapter 5*). This surprised us, since VWF levels are associated with the extent of atherosclerosis. A possible explanation is that local VWF levels may be higher at the site of high risk lesions and that this is not reflected by systemic high VWF plasma levels. A previous mice study using molecular imaging showed that activated VWF was found in atherosclerotic disease with high risk features (25). In this study locally activated VWF was measured and in our studies we measured circulating VWF:Ag plasma levels. In addition, a difference in the pathophysiologic mechanism of destabilising the plaque between mice and humans could also explain the conflicting results between these studies (26-29).

VWF an atherothrombosis

The question still remains whether VWF is associated with atherosclerosis or with atherothrombosis and thereby with arterial thrombosis. VWF mediates platelet adhesion and aggregation and thereby the formation of a thrombus. Rupture of an unstable atherosclerotic plaque triggers coagulation and leads to platelet aggregation and thrombus formation (30). VWF might therefore also have an influence in this stage of arterial thrombosis. A previous histochemical study showed that VWF was present at sites of platelet accumulation in coronary thrombi of patients with an acute myocardial infarction (31). In addition, two animal studies showed that there is an association between VWF plasma levels and platelet aggregation at atherosclerotic sites in the carotid and aortic arteries (32, 33). This suggests that there is a role of VWF in atherothrombosis at atherosclerotic plaques, and therefore it may be possible that VWF plays a role at a later stage of platelet thrombi without playing a major role in the formation of atherosclerosis.

In concusion, our results suggest that VWF may be associated with the extent of atherosclerosis, and not with the phenotypic more vulnerable atherosclerotic lesions. This suggests that VWF might be more involved in stable atherosclerosis or with the total extent of atherosclerosis. These studies suggest that VWF is more a marker of atherosclerosis than a causal factor in the development of atherosclerosis.

ADAMTS13 activity and cardiovascular disease

ADAMTS13 cleaves high molecular weight VWF multimers into smaller, less coagulant forms (34, 35). Low levels of ADAMTS13 result into higher molecular weight VWF multimers and a prothrombotic state as observed in Thrombotic Thrombocytopenic Pupura (TTP) which is characterized by a severe deficiency of ADAMTS13. Untreated, TTP patients suffer from thrombotic complications like ischemic stroke and myocardial infarction and the disease is fatal in the majority of untreated patients (36). It has been hypothesized that more subtle reduced levels of ADAMTS13 may also lead to more active VWF and therefore be a risk factor of thrombosis. This has first been studied in animal models. In these studies reduced atherosclerosis was found in ADAMTS13 deficient mice compared to wild type mice (37, 38), suggesting that ADAMTS13 is involved in the pathogenesis of atherosclerosis. Additionally, ADAMTS13 deficient mice in whom myocardial or cerebral ischemia was induced showed a reduced infarct volume compared with wild type mice (39-44). In conclusion, the animal studies showed an association between reduced levels of ADAMTS13, atherosclerosis and arterial thrombosis. More recently several case-control studies have investigated the association between ADAMTS13 levels and arterial thrombosis and indeed these studies suggest that individuals with low ADAMTS13 levels had an increased risk of arterial thrombosis (3, 45-47).

We have performed a meta-analysis of these case-control studies and established that low ADAMTS13 is associated with an increased risk of ischemic stroke (48). The association with coronary heart disease is less clear. Recently, these case-control studies were combined in

an individual patient data meta-analysis. This meta-analysis included all individual patient data of five different studies and found that only extremely low ADAMTS13 levels (<5% of normal) were associated with an increased myocardial infarction risk (49).However, these studies were limited by their case-control design. Therefore, there was a need for large prospective population based cohort studies to further establish the role of ADAMTS13 in arterial thrombosis. In the Rotterdam Study, a prospective cohort study including nearly 6000 individuals over a median of 10.7 years of follow-up, we found an association between low ADAMTS13 activity and an increased risk of both ischemic stroke and coronary heart disease (50). This strengthens the hypothesis that low ADAMTS13 is a risk factor for cardiovascular disease. We found that individuals in the lowest quartile of ADAMTS13 activity had an increased risk of arterial thrombosis. Nearly all individuals in this lowest quartile had an ADAMTS13 activity (95% CI 69.8 - 70.8%) in the normal range (50 - 150%)(51). Even though the ADAMTS13 levels are not as low as characteristic for TTP patients (<10%), we found an increased risk of thrombotic complications in these individuals. There was no graded pattern from quartile 1 to quartile 4 in both studies, as was also observed in other studies (45, 52, 53). An explanation might be that there is a threshold level for ADAMTS13 rather than a clear plasma level dose association, indicating that individuals with an ADAMTS13 activity below a certain level have a higher risk of arterial thrombosis, as was also suggested in the individual patient data meta-analysis using case-control studies (49). Interestingly, recent follow-up data of TTP survivors indicates that the risk of stroke is significantly higher in TTP survivors with a persistent low ADAMTS13 activity compared with controls, despite the fact that they are in remission and do not have signs of microangiopathy, thrombocytopenia, and atherosclerosis (54). This also points towards an important role of ADAMTS13 in arterial thrombosis, especially in individuals with the lowest level of ADAMTS13 activity (55).

In the Rotterdam Study, we observed a higher risk of ischemic stroke than of coronary heart disease in individuals with low ADAMTS13 activity, although it is difficult to compare these diseases. Atherosclerosis is a common underlying cause of a myocardial infarction, whereas in ischemic stroke thromboembolism may be more prevalent and many other patients have stroke of undetermined etiology. Embolisation of a thrombus may be caused by a less stable thrombus and the activity of ADAMTS13 may be of more importance in these patients. We found significantly higher VWF:Ag levels in ischemic stroke and TIA patients with a cardioembolic origin of stroke compared with small vessel occlusion patients (18). This indicates a different role of VWF and of ADAMTS13 in different etiologies of stroke. In individuals with an undetermined etiology of ischemic stroke, hypercoagulability may also be a risk factor for stroke as has also recently been suggested in a review by Maino et al (56). This review included 70 studies in which they compared the effect of multiple coagulation factors, as measure of hypercoagulability on the risk of ischemic stroke and myocardial infarction. They concluded that there is a more important role for hypercoagulability in ischemic stroke than in myocardial infarction.

Interaction between ADAMTS13 and Von Willebrand Factor

ADAMTS13 cleaves VWF into smaller forms leading to less high molecular weight multimers. Low ADAMTS13 activity will therefore result into high VWF activity and increase of (ultra) large VWF multimers. We found a very weak association between VWF:Ag levels and ADAMTS13 activity in our large cohort. This has been previously described (3, 47) and might be explained by the fact that mainly the activity of VWF is influenced by ADAMTS13 due to proteolysis. As VWF antigen levels represent the VWF plasma concentration this may not be influenced by the ADAMTS13 activity.

Reduced ADAMTS13 levels may lead to increased VWF locally at sites of high shear stress or plaque rupture. VWF unfolds at sites of vessel damage and low ADAMTS13 activity will lead to reduced cleavage of secreted large multimers of VWF. This finally results in the recruitment of platelets and the formation of a thrombus. By cleaving unfolded VWF, ADAMTS13 regulates the formation of a platelet plug and thrombus growth. Concluding, there may be an association between VWF levels and ADAMTS13 activity locally, but this may not be reflected in the VWF:Ag plasma levels in the circulation.

ADAMTS13, Von Willebrand Factor and outcome of cardiovascular disease

High VWF levels are known to be prothrombotic and it has been hypothesized that high levels may lead to more recurrent events or a worse outcome (57-61). To investigate whether high VWF levels or low ADAMTS13 activity are associated with an unfavorable outcome after event, we performed three different studies. First, in the Rotterdam Study we investigated the association between ADAMTS13, VWF and all-cause and cause specific mortality. We showed that both low ADAMTS13 activity and high VWF:Ag levels measured in a group of elderly (≥ 55 years) individuals of the general population, were associated with an increased risk of cardiovascular and all-cause mortality. This is in line with previous studies in which an association between high VWF levels and cardiac specific mortality was found (57, 58, 62). However, only two of these studies have focused on CHD and stroke related mortality (63, 64). One study, including 1057 individuals with proven coronary artery disease, found a significant association (HR 1.29, 95% CI 1.15 - 1.43) (63). None of these studies investigated the association between VWF levels and all-cause mortality. Additionally, no studies on the association between ADAMTS13 and mortality had been performed yet. We found that ADAMTS13 activity are associated with all-cause mortality, and that the risk estimates for cardiovascular mortality were comparable with all-cause mortality suggesting that this is mainly driven by cardiovascular mortality. No relationship between cancer related mortality and COPD related mortality and ADAMTS13 and VWF levels was observed.

In the study in patients with stable angina pectoris (SAP) and acute coronary syndrome (ACS), we also found an association between VWF:Ag levels and cardiovascular outcome (MACE) in ACS patients after 1 year. High VWF levels measured within hours till days after the event are associated with a worse outcome after ACS (23). Despite VWF levels are

known to be increased in the acute phase of an event (4, 20), our study suggest that the VWF levels measured in ACS patients, the most severe CAD patients, shortly after the event predict MACE at follow-up.

Lastly, we found an association between high VWF:Ag levels and an unfavorable outcome in ischemic stroke and TIA patients as assessed by the modified Rankin Score (mRS) and the NIHSS in the ESS (18). This is in agreement with a previous study in hemorrhagic and ischemic stroke patients in which high VWF levels were associated with a poor mRS (65).

In conclusion, these studies suggests that VWF levels and ADAMTS13 activity may be determinants of an unfavorable outcome and mortality and that treatment strategies may be tailored based on these measurements. Our findings may indicate that patients with ischemic stroke or ACS with high VWF:Ag levels or low ADAMTS13 activity may benefit from more intensive anticoagulant therapy or monitoring. However, this should be investigated in prospective studies.

Methodological considerations

The association between VWF:Ag levels and markers of atherosclerosis was determined in three different cross-sectional studies. A disadvantage of these studies was that atherosclerosis and VWF levels were determined at almost the same moment (within weeks). This limits the ability to determine the exact role of VWF in atherosclerosis, whether it is causally involved or more a marker of atherosclerosis. In addition, we have shown that VWF levels increase at moments of exercise, stress and in the acute phase of an event, and therefore blood sampling shortly after an event should be reconsidered (66). Measurement of these parameters over time may even better predict outcome in these patient groups.

In the ESS including ischemic stroke and TIA patients, we found that patients with blood group O had 30% lower VWF:Ag levels compared with patients with blood group non-O, as is also observed in healthy individuals. Interestingly, patients with blood group O had a similar calcification volume, a measurement of the extent of atherosclerosis, compared with patients with non-O. If VWF would have an important role in the development of atherosclerosis, genetic variations which determines VWF levels, like blood group, may have influenced the extent of atherosclerosis. The fact that we did not find a difference in the calcification volume also suggests that VWF is more a marker of atherosclerosis. Previously we already showed that genetic variations that are associated with VWF levels did not influence the risk of ischemic stroke (67). Overall these findings suggests that increased levels of VWF do not play a major role in the pathogenesis of atherosclerosis and are more a marker of atherosclerosis.

We investigated the role of ADAMTS13 in cardiovascular disease using the Rotterdam Study, which is a prospective population based cohort study. The advantage of this prospective study is that we were able to longitudinally follow patients to determine if and when they become diseased and whether the exposure status at baseline changed the outcome. We

were able to determine the role of ADAMTS13 activity at baseline with cardiovascular incidence during nearly 10 year follow-up. However, this study design also has some disadvantages. The Rotterdam Study included individuals of 55 years and older. Therefore, the results of this study cannot automatically be generalized to a younger population. Additionally, it is an observational study and therefore no conclusions can be made upon causality of the association.

Cardiovascular disease, including coronary heart disease and ischemic stroke, is a multifactorial disease in which several underlying factors play a role. Since multiple determinants are known and are associated with each other, it is difficult to distinguish whether a determinant is causally involved. Determinants may be associated with outcome due to the association with other factors, called confounding. On the other hand, some factors may also be a consequence of the outcome. This makes observational studies difficult in proving causality. We also included a risk prediction model, in our study on the role of ADAMTS13 activity in ischemic stroke. Risk prediction serves as the basis for clinical decision making for initiation of therapy, motivating adherence to lifestyle changes, and treatment and raising awareness of the disease. In cardiovascular disease various classical risk factors are included in the model which generates a risk score and this can estimate a probability to develop a cardiovascular event within a certain time period. Several studies have investigated whether newer risk markers can add in this risk prediction (68-71). A previous sub study of the Rotterdam Study indicated that VWF was not an additional risk predictor in coronary heart disease (68). We found however that ADAMTS13 activity has an additive value in the risk prediction of ischemic stroke. Among subjects in the intermediate risk category for ischemic stroke, the Net Reclassification Index (NRI) was 0.212 (95% CI 0.048 - 0.376). This may be explained by the fact that ADAMTS13 might have a more prominent role in arterial thrombosis than VWF, as has been shown before (72, 73). To evaluate the clinical value of a risk marker, it is important to consider whether the marker changes the risk prediction sufficiently to change recommended therapy. We quantified this by calculating the percentage of individuals that are correctly reclassified into risk categories with the addition of a new risk marker by the NRI. In this respect, the intermediate risk category is the most interesting group as in individuals in this group treatment should be considered. In the highest risk category all individuals already receive preventive treatment. The NRI was significant in the intermediate risk category, which suggests that ADAMTS13 activity may be of value in ischemic stroke risk prediction in this subgroup, independent of other risk factors. Those individuals with low ADAMTS13 activity in the intermediate group have a higher risk of stroke and are therefore candidates for more intensive preventive measurements.

Clinical implications

The findings of this thesis provide new insights in the role of VWF and ADAMTS13 in risk prediction of cardiovascular disease and outcome. However, so far these insights do not have direct clinical implications. One of our goals was to determine the association between ADAMTS13 activity and cardiovascular disease as this was never studied in prospective studies so far. Our findings should be validated in other population based cohort studies. Future studies are necessary to further establish the role of ADAMTS13 in cardiovascular disease and to see whether ADAMTS13 may be a target for therapy. ADAMTS13 activity was found to improve the risk prediction of ischemic stroke independent of classical risk factors. This suggests that ADAMTS13 can be used as risk marker of ischemic stroke and CHD. However, additional studies need to be determine the role of ADAMTS13 in cardiovascular disease risk prediction.

Recently, several animal studies have shown that rhADAMTS13 infusion may be a treatment option in ischemic stroke and myocardial infarction. Wild type mice in which cerebral or myocardial ischemia was induced were treated with rhADAMTS13 just before reperfusion. These mice developed less extended infarctions compared with wild type mice without treatment (39, 42-44). It is not yet known whether increasing ADAMTS13 levels in humans by infusion of rhADAMTS13 may be beneficial in the outcome of arterial thrombosis. However, this hypothesis first has to be proven in animal studies with cerebral or myocardial ischemia. Ultimately, this may result in a new treatment strategy for patients with stroke, of which the outcome is still poor.

In addition, we have found that VWF levels are associated with atherosclerosis in two large studies. VWF:Ag levels could maybe be used as a marker of atherosclerosis. Measurements of atherosclerosis using CTA or MRI are expensive and additionally CTA exposes individuals to radiation which is associated with an increased risk of developing malignancies. Instead of this, VWF:Ag measurements are not expensive and can easily be determined. In addition, VWF levels were also associated with an unfavorable outcome in ischemic stroke determined by a disability score, and also with major adverse cardiovascular events in ACS patients and with mortality in elderly individuals. However, future studies have to be performed to determine whether VWF is specific enough as a marker.

Recommendations for future studies

The ultimate goal is to find new risk markers and therapeutic agents for cardiovascular disease. We studied for the first time the association between ADAMTS13 activity and the risk of cardiovascular disease in a prospective design. Although we found a significant association in a large cohort with a long follow-up, additional prospective studies are needed to confirm these associations. The ultimate study design would be a prospective cohort study with multiple ADAMTS13 activity measurements and determination of clinical characteristics during follow-up.

Nowadays ischemic stroke patients are treated with antiplatelet agents and with thrombolysis with recombinant tissue plasminogen activator (rt-PA). Recently, studies have shown improvement of outcome when patients were treated additionally with thrombectomy. However, thrombolysis and thrombectomy have only shown to be effective within 4-6 hours after onset of symptoms and still 30-40% of patients have no benefit of this therapy (74-76). Therefore, new therapeutic agents are needed. In this thesis we have shown an association between ADAMTS13 activity and cardiovascular disease and an additive predictive value of ADAMTS13 activity in ischemic stroke risk prediction. Animal studies showed a beneficial effect of rhADAMTS13 infusion in experimental models (39, 42, 43). Currently, only mice studies have shown reduced infarct volumes in rhADAMTS13 treated mice compared with wild type mice (39, 42, 43). This was not associated with an increase of bleeding symptoms. Taken together, ADAMTS13 may be a promising new treatment option in cardiovascular disease. In the future, phase 1 and 2 studies on feasibility and safety and if successful randomized-controlled trials with rhADAMTS13 in humans are necessary to study this drug. First, ischemic stroke patients not responding to thrombolysis and thrombectomy could be treated with rhADAMTS13. Furthermore, an option for a future study may be more intensive prevention of arterial thrombosis in individuals with a 'lower ADAMTS13 activity' to reduce their risk of developing arterial thrombosis. However, first the threshold of low ADAMTS13 activity needs to be determined and strategies to increase ADAMTS13 need to be identified. We found in our study that ADAMTS13 activity was higher in individuals using statins compared to non statin users. A previous in vitro study showed that simvastatin upregulated the ADAMTS13 expression in mice podocytes (77). If ADAMTS13 levels do increase after statin treatment this may be an option for primary prevention in individuals with low ADAMTS13 activity.

In addition, it would be of interest to study the association between ADAMTS13 levels with the outcome after stroke determined by clinical outcome, stroke severity measured as the modified Rankin score (mRS) or the NIHSS and the infarct size. If ADAMTS13 is associated with a worse outcome, it would be worthwhile to study ADAMTS13 as a treatment strategy. Based on our findings of the predictive role of ADAMTS13 activity in cardiovascular disease, studies should be performed to identify determinants of ADAMTS13 levels. We have recently performed a Genome Wide Association Study (GWAS) to identify common genetic variations that determine ADAMTS13 levels. We found that genetic variations in the ADAMTS13 and SUPT3H genes were associated with ADAMTS13 activity (78). Future studies can focus on the association between these genetic variations and cardiovascular disease. Genetic variations that influences the ADAMTS13 level and are associated with the risk of cardiovascular disease may provide additional evidence that ADAMTS13 is involved in the development of cardiovascular disease.

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11

SUMMARY / SAMENVATTING

SUMMARY

Von Willebrand Factor (VWF) is an important player in primary hemostasis by mediating platelet adhesion and aggregation leading to clot formation. High molecular weight VWF multimers are the most procoagulant forms and are cleaved by ADAMTS13 (A Disintegrin And Metalloprotease with ThromboSpondin motif repeats 13) into smaller, less coagulant, forms.

Recent studies have shown an association between both high VWF:Ag levels, low ADAMTS13 levels and arterial thrombosis, including ischemic stroke and acute coronary syndrome. In this thesis several studies on VWF, ADAMTS13 and arterial thrombosis have been presented.

Chapter 1 is a general introduction on the background on the function of VWF and ADAMTS13 and the pathogenesis of atherosclerosis and cardiovascular disease.

In **chapter 2** we reviewed the available literature on the association between both VWF and ADAMTS13 and arterial thrombosis. Additionally, we performed meta-analyses of the reported studies. One of our main observations was that high VWF levels are associated with both an increased risk of coronary heart disease and ischemic stroke. An association between low ADAMTS13 and arterial thrombosis has only been reported in a few small case-control studies, therefore it remains unclear whether ADAMTS13 is an independent risk factor for arterial thrombosis. We found an increased risk of coronary heart disease with low ADAMTS13 levels (lowest tertile or quartile), although this did not reach statistically significance. In contrast, we observed a significant association between low ADAMTS13 and ischemic stroke (OR 2.72, 95%CI 1.52 – 4.86). Although these studies differed in study design and some included only a small number of cases, these studies suggests that both VWF and ADAMTS13 are associated with arterial thrombosis. However, the mechanism of the association between high VWF levels and arterial thrombosis is still unknown. Therefore, in order to obtain more insight in these findings we investigated whether VWF was associated with atherosclerosis using three different studies.

In **chapter 3** we determined the association between VWF levels and atherosclerosis in patients with ischemic stroke or transient ischemic attack (TIA). In this study we measured the extent of atherosclerosis by the calcification volume in both the aortic arch and the carotid arteries using a recently developed technique by CT angiography (CTA). We found a strong positive association between the calcification volume and VWF:Ag levels in these patients. In addition, VWF:Ag levels were significantly higher in patients with a large artery atherosclerosis subtype of ischemic stroke compared with other stroke subtypes. The study also suggests that VWF may have prognostic value in patients with ischemic stroke, as high VWF levels were associated with an unfavorable outcome.

Next, we investigated the association between atherosclerosis and VWF levels in patients with an acute coronary syndrome (ACS) or stable angina pectoris (SAP) by measuring the coronary plaque burden using intravascular ultrasound virtual histology (IVUS-VH) (**chapter 4**).

High coronary plaque burden was associated with higher VWF:Ag levels in SAP patients, but not in ACS patients. This might be explained by the time of blood sampling in ACS patients during the acute phase, which may lead to increased VWF levels. High risk lesions which are vulnerable to rupture were not associated with VWF levels. Furthermore, we found that high VWF:Ag levels have a predictive role for adverse cardiovascular outcome in SAP and ACS patients, and also for major adverse cardiac events (MACE) in ACS patients.

In **chapter 5** we investigated the association between atherosclerosis, VWF:Ag levels and ADAMTS13 activity in patients with ischemic stroke with mild to moderate carotid artery stenosis. Atherosclerosis was measured by different imaging biomarkers of atherosclerosis including calcification volume and plaque ulceration. We found no association between the imaging markers of atherosclerosis and VWF:Ag levels and ADAMTS13 activity. This is in contrast with the other two previous studies. This might be explained by the specific selection of patients in this study since only patients with a mild to moderate carotid artery stenosis were included. Our results suggest that VWF is more important in patients with advanced atherosclerosis, as we also found the highest VWF:Ag levels in patients with a large artery atherosclerosis type of ischemic stroke (chapter 3). On the other hand, VWF and ADAMTS13 seem to be associated with the extent of atherosclerosis and not with the specific characteristics of atherosclerosis.

The role of ADAMTS13 in arterial thrombosis was so far only studied in small case-control studies and prospective cohort studies are needed. Therefore, we investigated the association between ADAMTS13 and ischemic stroke in a large population-based cohort study the Rotterdam Study, among individuals of 55 years and older (**chapter 6**). ADAMTS13 activity was associated with age, sex and several cardiovascular risk factors in this study. We measured VWF levels and ADAMTS13 activity in a total of 5941 individuals. The median follow-up time of these individuals was 10.7 years. We showed that low ADAMTS13 activity was significantly associated with an increased risk of ischemic stroke (HR 1.65, 95%CI 1.16 – 2.32) and all strokes (HR 1.49, 95%CI 1.12 – 2.00). In addition, we found that ADAMTS13 activity improved the accuracy of risk prediction for ischemic stroke beyond the traditional risk factors.

In the same study population as described in chapter 6, we investigated the association between low ADAMTS13 activity and coronary heart disease in the Rotterdam Study (**chapter 7**). In total 453 individuals suffered from coronary heart disease. In individuals with the lowest quartile of ADAMTS13 activity, the risk of CHD was significantly increased compared with individuals with the highest quartile of ADAMTS13 activity (HR 1.42, 95%CI 1.07 – 1.89). Individuals with low ADAMTS13 activity and high VWF:Ag levels also had an increased risk of CHD.

To study whether ADAMTS13 activity and VWF levels were associated with outcome of cardiovascular disease we investigated the role of VWF levels and ADAMTS13 activity on the risk of all-cause and cardiovascular mortality in the Rotterdam Study (**chapter 8**). We found

that high VWF:Ag levels and low ADAMTS13 activity were associated with an increased risk of all-cause mortality and cardiovascular mortality. We did not find an association between VWF, ADAMTS13 and cancer-related mortality or COPD related mortality.

Physical stress triggers the endothelium to release VWF from Weibel Palade Bodies (WPB). Since VWF is a risk factor for arterial thrombosis, it is of great interest to discover determinants of VWF response to physical stress. In a study among 105 healthy young individuals, VWF:Ag levels increased significantly upon incremental exhaustive exercise (**chapter 9**). The VWF:Ag response was highly variable and was strongly dependent on performance-related and physical fitness-related determinants. Genetic variations in *STXBP5*, *STX2* and VWF promoter that have previously been identified as genetic determinants of VWF:Ag levels, were not associated with the VWF:Ag response to physical exercise. This suggests that environmental factors may be more important than genetic factors in determining the VWF:Ag reponse to stress.

In the last chapter (**chapter 10**) the findings of our studies are discussed. We conclude that the extent of atherosclerosis is strongly associated with VWF levels. Furthermore, VWF might be a marker of an unfavorable outcome in coronary heart disease and ischemic stroke patients. Plaque characteristics, such as high risk lesions, do not seem to be associated with VWF levels. Lastly, low ADAMTS13 is strongly associated with an increased risk of arterial thrombosis, including ischemic stroke and coronary heart disease. Overall, the studies in this thesis provide a better understanding of the role of VWF and ADAMTS13 in arterial thrombosis.

NEDERLANDSE SAMENVATTING

Von Willebrand factor (VWF) speelt een belangrijke rol in de bloedstolling door het binden en plakken van bloedplaatjes. Uiteindelijk leidt dit tot het vormen van een bloedstolsel. Hoog moleculair gewichts VWF multimeren zijn de meest actieve vormen die leiden tot meer stolselvorming. Deze multimeren worden geknipt door ADAMTS13 (A Disintegrin And Metalloprotease with ThromboSpondin motif repeats 13) in kleinere en minder actieve vormen. Recentelijk hebben verschillende studies een associatie gevonden tussen hoge VWF antigen (VWF:Ag) spiegels, lage ADAMTS13 spiegels en hart- en vaatziekten, waaronder het hart- en herseninfarct. In dit proefschrift worden meerdere studies gericht op VWF, ADAMTS13 en hart- en vaatziekten gepresenteerd.

Hoofdstuk 1 is een introductie van de achtergrond van de functies van VWF en ADAMTS13 en daarnaast de ontstaanswijze van atherosclerose en hart- en vaatziekten.

In **hoofdstuk 2** hebben we de relevante informatie over de associatie tussen zowel VWF als ADAMTS13 en hart- en vaatziekten samengevat. Hierin hebben we tevens een meta-analyse verricht van de beschikbare studies. Eén van de belangrijkste bevindingen was dat VWF spiegels geassocieerd zijn met zowel een verhoogd risico op het acuut coronair syndroom als het herseninfarct. Tevens werd in een aantal kleine case-control studies een associatie tussen ADAMTS13 en hart- en vaatziekten gevonden dus of ADAMTS13 een onafhankelijke risicofactor voor hart- en vaatziekten is blijft nog onduidelijk. Wij vonden in de meta-analyse dat lage ADAMTS13 spiegels (laagste tertiel of kwartiel) geassocieerd waren met een verhoogd risico op het acuut coronair syndroom, ook al was dit niet statistisch significant. Daar tegenover vonden we een significante associatie tussen lage ADAMTS13 spiegels en het herseninfarct (OR 2.72, 95%CI 1.52 - 4.86). Ondanks dat deze studies allemaal een andere opzet hadden en sommige studies maar een kleine groep mensen geïncludeerd hadden, lijkt het er op dat VWF en ADAMTS13 beide geassocieerd zijn met hart- en vaatziekten. Het mechanisme tussen hoge VWF spiegels en hart- en vaatziekten is tot op heden nog onduidelijk. Daarom wilden wij onderzoeken of VWF geassocieerd is met atherosclerose, aderverkalking, om meer inzicht te krijgen in deze bevindingen en hebben we hiervoor drie verschillende studies gebruikt.

In **hoofdstuk 3** hebben we gekeken naar de associatie tussen VWF spiegels en atherosclerose in patiënten met een herseninfarct of een TIA (Transient Ischemic Attack). Hierin hebben we het kalkvolume in zowel de aortaboog als de arterie carotis gemeten als maat van de uitgebreidheid van atherosclerose middels een nieuw ontwikkelde techniek met behulp van CT angiografie (CTA). We vonden in deze patiëntengroep een sterk positieve associatie tussen het kalkvolume en VWF:Ag spiegels. Daarnaast waren VWF:Ag spiegels significant hoger in patiënten met uitgebreide atherosclerose ten opzichte van patiënten met een herseninfarct met een andere oorzaak. In de studie tonen we aan dat VWF mogelijk ook een prognostische waarde kan hebben in patiënten met een herseninfarct, omdat hoge VWF spiegels geassocieerd waren met een slechtere uitkomst na het infarct.

Tevens hebben we gekeken naar de associatie tussen atherosclerose en VWF spiegels in patiënten met een acuut coronair syndroom (ACS) of stabiele angina pectoris (SAP) waarbij we de totale plaque in de coronair arterieën hebben gemeten middels intravascular ultrasound virtual histology (IVUS-VH) (**hoofdstuk 4**). Grote plaques in de coronairen waren geassocieerd met hogere VWF:Ag spiegels in SAP patiënten, maar niet in ACS patiënten. Dit wordt mogelijk verklaard doordat het moment van bloedafname in ACS patiënten acuut na het ontstaan van de klachten is, wat waarschijnlijk heeft geleid tot verhoogde VWF spiegels. Hoog risico laesies, welke makkelijk kunnen ruptureren en kunnen leiden tot een infarct, waren niet geassocieerd met VWF spiegels. Daarnaast vonden we dat hoge VWF:Ag spiegels een voorspellende waarde hebben voor een slechtere cardiovasculaire uitkomst in SAP en ACS patiënten. In ACS patiënten was dit ook het geval voor een recidief cardiovasculaire aandoening (major adverse cardiac event).

In **hoofdstuk 5** hebben we in patiënten met een herseninfarct met een milde tot matige stenose van de arterie carotis, de associatie tussen atherosclerose, VWF:Ag spiegels en ADAMTS13 activiteit bekeken. Atherosclerose was in deze studie gemeten door middel van verschillende beeldvormende biomarkers van atherosclerose waaronder kalkvolume en plaque ulceratie. In deze studie vonden we geen associatie tussen de verschillende biomarkers en zowel VWF:Ag spiegels als ADAMTS13 activiteit. Dit is in tegenstelling tot de andere twee studies. Mogelijk wordt dit verklaard door de specifieke selectie van patiënten in deze studie, omdat alleen patiënten met een milde tot matige stenose werden geïncludeerd. Concluderend suggereren onze resultaten dat VWF meer geassocieerd is in patiënten met een ernstige atherosclerose, omdat we ook vonden dat patiënten met een uitgebreide atherosclerose de hoogste VWF:Ag spiegels hadden (hoofdstuk 3). Aan de andere kant lijken VWF en ADAMTS13 geassocieerd met de uitgebreidheid van atherosclerose en niet met de specifieke karakteristieken van atherosclerose.

De rol van ADAMTS13 in hart- en vaatziekten was tot nu toe alleen in kleine case-control studies onderzocht en daarom waren prospectieve cohort studies nodig. Hierom hebben wij de associatie tussen een ADAMTS13 activiteit en het herseninfarct onderzocht in een grote populatie gebaseerde cohort studie, de Rotterdam studie, waarin mensen van 55 jaar en ouder werden geïncludeerd (**hoofdstuk 6**). ADAMTS13 activiteit was geassocieerd met leeftijd, geslacht en meerdere andere cardiovasculaire risicofactoren. We bepaalden ADAMTS13 activiteit en VWF spiegels in een totale groep van 5941 mensen. De mediane follow-up tijd in deze individuen was 10.7 jaar. We vonden dat een lage ADAMTS13 activiteit significant geassocieerd was met een verhoogd risico op het herseninfarct (HR 1.65, 95%CI 1.16 - 2.32) en tevens met alle beroertes inclusief hersenbloedingen (HR 1.49, 95%CI 1.12 - 2.00). Daarnaast vonden we dat ADAMTS13 activiteit de risico voorspelling op het herseninfarct verbeterde, bovenop de traditionele risicofactoren.

In dezelfde studie populatie zoals beschreven in hoofdstuk 6, hebben we de associatie tussen een lage ADAMTS13 activiteit en coronair hartlijden in de Rotterdam studie onderzocht

(**Hoofdstuk 7**). In totaal 453 individuen ontwikkelde coronair hartlijden in deze studie. In mensen met de laagste ADAMTS13 activiteit (laagste 25%) was het risico op coronair hartlijden verhoogd ten opzichte van mensen met de hoogste ADAMTS13 activiteit (hoogste 25%) (HR 1.42, 95%CI 1.07 - 1.89). Individuen met de laagste ADAMTS13 activiteit en de hoogste VWF:Ag spiegels hadden ook een verhoogd risico op het ontwikkelen van coronair hartlijden.

Om te onderzoeken of ADAMTS13 activiteit en VWF spiegels geassocieerd zijn met de uitkomst van hart- en vaatziekten, hebben we de rol van VWF spiegels en ADAMTS13 activiteit op het risico op overlijden aan alle ziekten en specifiek cardiovasculair overlijden onderzocht in de Rotterdam studie (**hoofdstuk 8**). Hierbij vonden we dat hoge VWF:Ag spiegels en een lage ADAMTS13 activiteit geassocieerd waren met een verhoogd risico op overlijden, algemeen en cardiovasculair geassocieerde mortaliteit. We vonden geen associatie tussen VWF, ADAMTS13 en kanker gerelateerde mortaliteit of COPD (chronic obstructive pulmonary disease) gerelateerde mortaliteit.

Fysieke stress stimuleert het endotheel zodat VWF vrij komt uit Weibel Palade Bodies (WPB). Omdat VWF een risicofactor is voor hart- en vaatziekten, is het interessant om te kijken naar de factoren die VWF beïnvloeden op momenten van vrijkomen tijdens fysieke stress. In een studie waarbij we 105 gezonde jonge mensen includeerden, vonden we dat VWF:Ag spiegels significant stegen bij uitputtende inspanning (**hoofdstuk 9**). De VWF:Ag respons was erg variabel en was sterk afhankelijk van uitvoerings-gerelateerde en fysieke fitheids-gerelateerde factoren. Genetische variaties in *STXBP5*, *STX2* en de VWF promoter, die eerder geïdentificeerd waren als genetische factoren van VWF:Ag spiegels, waren niet geassocieerd met de VWF:Ag respons op fysieke inspanning. Dit suggereert dat omgevingsfactoren mogelijk een belangrijkere invloed hebben dan genetische factoren in de VWF:Ag respons op stress.

In het laatste hoofdstuk (**hoofdstuk 10**) worden de bevindingen van al onze studies beschreven. Concluderend is er een sterke associatie tussen de uitgebreidheid van atherosclerose en VWF spiegels. Daarnaast is VWF een marker van een ongunstige uitkomst in patiënten met coronair hartlijden en een herseninfarct. Plaque karakteristieken, zoals hoog risico laesies, lijken niet geassocieerd met VWF spiegels. Tevens is een lage ADAMTS13 activiteit sterk geassocieerd met een verhoogd risico op hart- en vaatziekten, inclusief coronair hartlijden en het herseninfarct. De studies in dit proefschrift geven een beter inzicht in de rol van VWF en ADAMTS13 in hart- en vaatziekten.

A

APPENDICES

PUBLICATION LIST

Sonneveld MAH, de Maat MPM and Leebeek FWG.
Von Willebrand factor and ADAMTS13 in arterial thrombosis: a systematic review and meta-analysis. *Blood Reviews*. 2014;28:167-78.

Sonneveld MAH, van Dijk AC, van den Herik EG, van Loon JE, de Lau LML, van der Lugt A, Koudstaal PJ, de Maat MPM and Leebeek FWG.
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Von Willebrand factor in relation to coronary plaque characteristics and cardiovascular outcome. Results of the ATHEROREMO-IVUS study. *Thrombosis Haemostasis*. 2015;113: 577-84.
* Contributed equally as first author

Sonneveld MAH, de Maat MPM, Portegies ML, Kavousi M, Hofman A, Turecek PL, Rottensteiner H, Scheiflinger F, Koudstaal PJ, Ikram MA and Leebeek FWG.
Low ADAMTS13 activity is associated with an increased risk of ischemic stroke. *Blood*. 2015;126:2739-46.

van Loon JE*, **Sonneveld MAH***, Praet SFE, de Maat MPM and Leebeek FWG.
Performance Related Factors Are the Main Determinants of the von Willebrand Factor Response to Exhaustive Physical Exercise. *PloS one*. 2014;9.
* Contributed equally as first author

de Vries PS, Boender J, **Sonneveld MAH**, Rivadeneira F, Ikram MA, Rottensteiner H, Hofman A, Uitterlinden AG, Leebeek FWG, Franco OH, Dehghan A, de Maat MPM.
Genetic variants in the ADAMTS13 and SUPT3H genes are associated with ADAMTS13 activity. Blood. 2015;125:3949-55.

Sedaghat S, de Vries PS, Boender J, **Sonneveld MAH**, Hoorn EJ, Hofman A, de Maat MPM, Franco OH, Ikram MA, Leebeek FWG, Dehghan A. von Willebrand Factor, ADAMTS13 activity, and decline in kidney function: A population-based cohort study. American Journal of Kidney Diseases. 2016

AWARDS AND PRIZES

2014 **Scientific Excellence Award**
Abstract award, annual symposium Dutch Society of Thrombosis and Haemostasis (NVTH)

2014 **ASH Abstract Achievement Award**
56th American Society of Haematology Annual Meeting

2015 **CSL Behring Prof. Heimburger Award 2015**

2015 **Young Investigator Award**
XXVth Congress of the International Society on Thrombosis and Haemostasis

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CURRICULUM VITAE

Michelle Sonneveld werd geboren op 31 december 1985 te Rotterdam. Zij volgde het voortgezet onderwijs aan het Sint Laurenscollege te Rotterdam, waar zij in 2004 haar VWO diploma behaalde. In 2005 behaalde zij haar propedeuse voor de studie gezondheidswetenschappen, beleid en management gezondheidszorg aan de Erasmus Universiteit Rotterdam. In datzelfde jaar startte zij met de studie Geneeskunde aan de Erasmus Universiteit Rotterdam. Op de afdeling neurologie deed zij in 2011 keuzeonderzoek onder leiding van Prof. dr. P.J. Koudstaal en dr. E.G. van den Herik, in samenwerking met Prof. dr. F.W.G. Leebeek en dr. M.P.M. de Maat. Dit onderzoek was de basis voor dit proefschrift. In 2011 behaalde zij haar artsexamen, waarna zij in november 2011 startte met haar promotieonderzoek op de afdeling hematologie. Per 1 mei 2015 is zij begonnen met de opleiding Interne Geneeskunde in het Erasmus MC (opleider dr. S.C.E. Klein Nagelvoort – Schuit). Momenteel volgt zij haar vooropleiding in het Ikazia Ziekenhuis te Rotterdam (opleider dr. A. Zandbergen).

PHD PORTFOLIO SUMMARY

Name PhD student: M.A.H. Sonneveld	PhD period: November 2011 – January 2015
Erasmus MC Department: Haematology	Promotor: Prof. Dr. F.W.G. Leebeek
Research School: COEUR	Supervisors: Dr. M.P.M. de Maat; Dr. M.A. Ikram

1. PHD TRAINING	**Year**	**Workload (Hours/ECTS)**
General academic skills		
• Biomedical English Writing and Communication	2014	4.0
• Research Integrity	2014	0.3
Research skills		
• SNP's and human disease	2011	2.0
• Introduction to clinical research	2012	0.9
• Biostatistics for clinicians	2012	1.0
• Regression analysis for clinicians	2012	1.9
In-depth courses (e.g. Research school, Medical Training)		
• 5x COEUR courses on cardiovascular medicine, pathophysiology of ischemic heart disease, cardiovascular imaging and diagnostics, clinical cardiovascular epidemiology, and atherosclerotic and aneurysmal disease	2011-2014	7.5
• Molmed presenting skills	2012	1.0
• 3x NVTH AIO course on thrombosis and hemostasis	2012-2014	3.0
Presentations		
• 2x oral presentation at Nederlandse Vereniging Trombose en Hemostase	2013-2014	1.6
• 3x E-poster oral presentations at International Society of Thrombosis and Haemostasis	2013	0.9
• Oral presentation at COEUR PhD day	2014	0.8
• Poster presentation at Bari International Conference	2014	0.3
• Oral presentation at American Society of Hematology annual meeting	2014	0.8
• Oral presentation Prof. Heimburger award conference	2015	0.8
• 2x oral presentation at International Society of Thrombosis and Haemostasis	2015	1.6
International conferences		
• Van Creveld symposium	2011	0.3
• 3x NVTH symposium	2012-2014	1.6
• 2x International Society of Thrombosis and Haemostasis Congress	2013-2015	3.6
• Bari International Conference	2014	0.9
• American Society of Haematology annual meeting	2014	1.2
• Prof. Heimburger award conference	2015	0.3
Seminars and workshops		
9x COEUR research seminar	2012-2013	3.6
2x COEUR PhD day	2012-2013	0.8
3x NVTH PhD day	2012-2014	1.2
2. TEACHING ACTIVITIES		
Lecturing		
• 10x coagulation lecture for nurses	2012-2014	1.0
• 2x lecture arterial thrombosis 2nd year medical students	2013-2014	0.4
Supervising Master's thesis		
• Supervision of M.F. Chohan (keuzeonderzoek)	2014	1.0
• Supervision of W. Klaasen (HLO student)	2014	0.9
	Total	45.2