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Association between fibrinogen and fibrinogen γ' and atherosclerotic plaque morphology and composition in symptomatic carotid artery stenosis: Plaque-At-RISK study

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ABSTRACT

Introduction: Von Willebrand Factor (VWF), ADAMTS13, fibrinogen and fibrinogen γ' are associated with an increased risk of ischemic stroke. Carotid atherosclerosis is an important risk factor for ischemic stroke. Characteristics of the vulnerable plaque; intraplaque hemorrhage (IPH), plaque ulceration and lipid-rich necrotic core (LRNC) can be visualized with imaging techniques. Since atherosclerosis might attribute to the association between coagulation factors and ischemic stroke risk, the aim of this study is to investigate the association between coagulation factors and atherosclerotic plaque characteristics in more detail.

Materials and methods: In 182 patients of the Plaque-At-RISK study (prospective multicenter cohort study) with a recent transient ischemic attack (TIA) or ischemic stroke and a symptomatic mild-to-moderate carotid artery stenosis, we measured VWF antigen (VWF:Ag), ADAMTS13 activity, fibrinogen (Clauss), and fibrinogen γ'. Presence of plaque ulceration, IPH volume and LRNC volume were determined by Multidetector-Row Computed Tomography (MDCTA, $n = 160$) and Magnetic Resonance Imaging (MRI, $n = 172$). Linear regression analysis was used to assess the association between imaging biomarkers and coagulation factors.

Results: VWF:Ag or ADAMTS13 levels were not significantly associated with plaque ulceration, IPH and LRNC. We found an inverse association between fibrinogen and fibrinogen γ' and IPH volume (B = $-23.40\,mm^3$/g/L, $p = 0.01$ and B = $-161.73\,mm^3$/g/L, p = 0.01) and between fibrinogen and fibrinogen γ' and LRNC volume (B = $-38.89\,mm^3$ g/L, $p < 0.01$ and B = $-227.06\,mm^3$ g/L, p = 0.01). Additional adjustments for C-reactive protein (CRP) did not change the results.

Conclusions: Fibrinogen and fibrinogen γ' are inversely associated with IPH volume and LRNC volume, independent of inflammation.

Clinical Trial Registration: clinicaltrials.gov NCT01208025

1. Introduction

Atherosclerosis of the carotid arteries is an important risk factor for the development of ischemic stroke [1]. Presence of plaque ulceration, intraplaque hemorrhage (IPH) and a lipid-rich necrotic core (LRNC) are important characteristics of the vulnerable plaque, a plaque more prone

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to rupture and causing an ischemic event [2]. Presence of IPH has been associated with a ~6-fold higher risk for ischemic events (stroke, transient ischemic attack or amaurosis fugax) [3]. IPH and LRNC can be assessed and quantified by Magnetic Resonance Imaging (MRI). Rupture of the atherosclerotic plaque can be visible as plaque ulceration on Multidetector-row Computed Tomography (MDCTA) [4]. Rupture of the plaque leads to activation of primary and secondary hemostasis and formation of a thrombus which can eventually lead to occlusion of an artery of the brain and ischemic stroke or transient ischemic attack (TIA).

VWF has an important function in primary hemostasis via its role in platelet adhesion and aggregation. Endothelial damage, for instance in plaque ulceration, leads to increased plasma levels of VWF. *A Disintegrin And Metalloprotease with ThromboSpondin motif repeats 13* (ADAMTS13) cleaves large VWF multimers into smaller and less prothrombotic forms [5]. High VWF and low ADAMTS13 levels are associated with an increased risk of ischemic stroke and myocardial infarction [6,7]. One candidate mechanism underlying the association between VWF and ischemic stroke may be atherosclerosis. Previous studies suggest that atherosclerosis is a determinant of VWF levels [8]. In vitro and in vivo studies also suggest that VWF might contribute to the pathogenesis of atherosclerosis, where VWF deficiency showed to be protective against atherosclerosis [9–11]. When taking all these studies together, the data on the relation between VWF and atherosclerosis are inconclusive.

Fibrinogen is a key element of secondary hemostasis and also acts as an acute phase reactant in inflammation. Several isoforms of fibrinogen are present in the blood; circulating fibrinogen consists for 8–15% of fibrinogen γ' [12]. Fibrinogen γ' is formed as a result of alternative messenger RNA processing, and as a result, high affinity binding sites for thrombin are formed as well as a disrupted binding site for platelet integrin α_{IIB} β_3[12]. High fibrinogen and fibrinogen γ' levels are associated with cardiovascular diseases (CVD), including ischemic stroke [13,14]. However, some studies did not show that fibrinogen γ' levels are associated with the incidence of CVD [15,16]. One of the mechanisms underlying this association may be atherosclerosis. It remains unclear whether fibrinogen and fibrinogen γ' levels are mainly related to the extent of the inflammatory process of atherosclerotic plaque formation, or whether fibrinogen plays a role in the formation and/or progression of the atherosclerotic plaque and especially plaque ulceration and IPH.

There is still a lot of inconsistency about the association between VWF, ADAMTS13, fibrinogen and fibrinogen γ' and atherosclerosis. With this study we aim to gain more knowledge about these associations. Since plaque ulceration, IPH and LRNC are important characteristics of the vulnerable plaque, we investigated the association between these blood biomarkers and plaque ulceration, IPH and LRNC in patients with a recent TIA or ischemic stroke and ipsilateral mild-to-moderate carotid artery stenosis.

2. Materials and methods

2.1. Study population

This study was embedded in the PARISK-study (Plaque-At-RISK; clinical trials.gov NCT01208025); 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 [17]. All included patients had a recent TIA, including amaurosis fugax or minor stroke in the carotid artery territory prior to inclusion. TIA was defined as an episode of temporary and focal cerebral dysfunction of vascular origin, lasting for a maximum of 24 h, leaving no persistent neurologic deficits. Minor stroke was defined as an episode of temporary and focal cerebral dysfunction of vascular origin, lasting for > 24 h or a nondisabling stroke with a modified Rankin Scale score of ≤3. Amaurosis fugax was defined as a sudden loss of vision of presumed vascular origin and confined to one eye. Degree of stenosis is determined with clinically obtained Doppler ultrasound or MDCTA. The upper cutoff value of 70% is based on the NASCET criteria. The lower cutoff value is an atherosclerotic plaque with a thickness of at least 2–3 mm, which corresponds to a European Carotid Surgery Trial (ECST) stenosis of 30% [18]. Exclusion criteria are a probable cardiac source of embolism, a clotting disorder, inability to visit the hospital and undergo the study procedures due to severe comorbidity, standard contra-indications for MRI, a documented allergy for MRI or CT contrast agent or a renal clearance of < 30 ml/min. 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; 182 patients had either a MDCTA (n = 160) or MRI (n = 172) of the carotid arteries, and had an available blood sample.

2.2. Cardiovascular risk factors

Clinical baseline data such as age, sex, body mass index, type of stroke, medication use, medical history and cardiovascular risk factors were collected. Hypercholesterolemia was defined as fasting total cholesterol of > 5 mmol/L or the use of cholesterol-lowering medication at the time of the TIA or ischemic stroke. We defined hypertension as systolic blood pressure of > 140 mm Hg or a diastolic blood pressure of > 90 mm Hg during 2 episodes of at least 15 min of continuous noninvasive blood pressure measurement or treatment with antihypertensive medication. Diabetes mellitus was defined as a fasting serum glucose level of > 6.9 mmol/L, 2-hour postload glucose level of > 11.0 mmol/L, or the use of antidiabetic medication. We assessed smoking status at the time of the TIA or ischemic stroke and dichotomized it into current smoker or no current smoker. History of CVD was defined as history of TIA or ischemic stroke, history of ischemic heart disease and/or history of peripheral artery disease.

2.3. MDCTA and 3 T MRI data acquisition and analysis

Standardized, previously described, contrast-enhanced MDCTA and multi-sequence contrast-enhanced MRI protocols were used [17]. All imaging studies were evaluated by trained readers blinded for clinical data and other imaging tests [19].

MDCTA images were reviewed using dedicated 3D analysis software (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) [19]. Secondly, presence of plaque ulceration was assessed. We defined plaque ulceration as an extension of contrast material of > 1 mm into the atherosclerotic plaque on at least 2 orthogonal planes [20,21]. In addition, 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 [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 [22].

MR images were evaluated with dedicated vessel wall analysis software (Vesselmass, Department of Radiology, Leiden University Medical Center, Netherlands). Information of all five MRI carotid artery sequences was used. Image quality was rated on a 5-point scale; poor and not eligible for analysis (1) to good and eligible for analysis (5) [19]. 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 (IPH, lipid-rich necrotic core, calcifications) were manually segmented. Fifteen transverse adjoining slices of 2 mm

each covering the entire plaque were annotated. Additionally to plaque component volumes, maximum vessel wall area and other plaque component volumes of the symptomatic carotid artery could be derived from these annotations.

2.4. Blood sampling, VWF levels, ADAMTS13 activity, fibrinogen and fibrinogen γ' measurements

Citrated blood was centrifuged at 2000*g* for 10 min; then the plasma was centrifuged at 14,000*g* for 10 min and stored in aliquots at −80 °C. 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) [23]. Total fibrinogen levels were measured according to von Clauss on a fully-automated coagulation analyzer (Sysmex CS-5100 system, Siemens Healthcare Diagnostics, Breda, the Netherlands). Fibrinogen γ' antigen levels were measured with an enzyme-linked immunosorbent assay as described previously using anti-γ' fibrinogen antibodies for catching and HRP-labeled rabbit anti-fibrinogen antibodies for tagging [24].

2.5. Statistical analysis

Baseline clinical characteristics and blood measurements are listed for all patients. All available imaging characteristics, including plaque ulceration and IPH, are shown for the symptomatic carotid artery. Data are presented as mean ± standard deviation (SD), median [25th–75th percentile] or number of patients (%). Differences between the two groups were evaluated by using the student *t*-test or Mann-Whitney *U* test for continuous data and the Chi-squared test for categorical data, respectively. In this cross-sectional analysis, linear regression models were used to investigate the association between clinical characteristics (independent variables) and coagulation factors (dependent variables). Additionally, we investigated the association between coagulation factors (independent variables) and plaque characteristics of the symptomatic carotid artery, including plaque ulceration, IPH volume and LRNC volume (dependent variables). CRP levels were not normally distributed and therefore log-transformed. Adjustments were made for age, sex, hypercholesterolemia, hypertension, diabetes mellitus, current smoking, BMI and cardiovascular history. In the analyses with VWF:Ag, we also adjusted for blood group. All analyses were repeated after adding interval between index event and blood withdrawal and – separately – ECST stenosis to the covariates. Finally, in the analyses with fibrinogen and fibrinogen γ', we additionally adjusted for C-reactive protein (CRP). Subgroup analyses for significant associations between plaque ulceration, IPH and LRNC with coagulation factors were performed in patients with and without a history of CVD and in patients with and without use of a statin or an anti-platelet agent separately. Additionally, interaction terms were created and added to the regression model in order to investigate the effect of interactions between possible confounding variables. All analyses were repeated for the additional imaging biomarkers (degree of stenosis, calcification volume, maximum vessel wall area and lipid-rich necrotic core volume). Statistical analyses were performed using STATA software (version 13.1, StataCorp, College Station, Texas) and SPSS software (version 24, IBM). $P < 0.05$ was considered statistically significant.

3. Results

Baseline clinical characteristics, imaging characteristics and blood measurements are shown in Table 1. In our patients, the mean age was 67 ± 9 years and 74% were male. Hypercholesterolemia was present in 78% of the patients and hypertension was present in 71% of the patients. A history of CVD before the index event was highly prevalent amongst patients (49%). Prevalence of plaque ulceration on MDCTA was 28%. Prevalence of IPH in the symptomatic plaque on MRI was 39%. Blood measurements showed a median VWF:Ag level of 1.45 [1.10–1.81] IU/mL, ADAMTS13 activity of 98.6 ± 22.8%, mean fibrinogen of 3.68 ± 1.02 g/L, and a mean fibrinogen γ' of 0.36 ± 0.14 g/L.

Table 1
Clinical characteristics, imaging biomarkers and blood measurements.

Clinical characteristic (*n* = 182)	
Age (years)	67 ± 9
Male	135 (74%)
Classification event	
• TIA	77 (42%)
• Stroke	82 (45%)
• Amaurosis fugax	23 (13%)
Hypercholesterolemia	142 (78%)
Hypertension	129 (71%)
Diabetes Mellitus	44 (24%)
Current smoking	39 (21%)
BMI	26.7 ± 4.4
History of CVD	89 (49%)
Blood group non-O	106 (59%)
Medication use prior to event	
• Statins	92 (51%)
• Antihypertensives	111 (61%)
• Antidiabetic drugs	33 (18%)
• Antiplatelet drugs	79 (43%)
• Anticoagulants	5 (3%)
Imaging biomarkers (symptomatic artery)	
Degree of stenosis (ECST) (%)[a]	55 ± 16
MDCTA (n = 160)	
Interval event–MDCTA (days)	32 [12–52]
Presence plaque ulceration	44 (28%)
Presence calcifications	144 (90%)
Calcification volume (mm^3)	27.9 [5.1–84.2]
MRI (n = 172)	
Interval event–MRI (days)	47 [30–67]
Presence IPH	67 (39%)
IPH volume (mm^3)	0.0 [0.0–54.6]
Presence lipid-rich necrotic core	108 (63%)
Lipid-rich necrotic core volume (mm^3)	26.3 [0.0–150.0]
Maximum vessel wall area (mm^2)	73.1 [57.2–90.2]
Blood measurements (n = 182)	
Interval event-blood withdrawal (days)	46 [31–67]
VWF:Ag (IU/mL)	1.45 [1.10–1.81]
ADAMTS13 activity (%)	98.6 ± 22.8
Fibrinogen (g/L)	3.68 ± 1.02
Fibrinogen γ' (g/L)	0.36 ± 0.14
γ'/total fibrinogen ratio	0.10 [0.08–0.12]
CRP (mg/L)	1.23 [0.60–3.46]

Data are presented as mean ± SD, absolute numbers of patients (%), or median [25th–75th percentile]. TIA, transient ischemic attack; BMI, body mass index; CVD, cardiovascular disease; ECST, European Carotid Surgery Trial; MDCTA, Multidetector–Row Computed Tomography; MRI, Magnetic Resonance Imaging; IPH, intraplaque hemorrhage; VWF:Ag, Von Willebrand Factor antigen; CRP, C-Reactive Protein.

[a] If Multidetector–Row Computed Tomography (MDCTA) was absent, degree of stenosis was assessed at MRI (*n* = 20).

3.1. Primary hemostasis: VWF:Ag levels and ADAMTS13 activity

Focusing on factors of primary hemostasis, we found that increasing age was associated with higher VWF:Ag levels (B = 0.12 IU/mL/10 years [95% CI 0.03;0.21], $p = 0.01$). Individuals with blood group non-O had higher VWF:Ag levels compared with individuals with blood group O (B = 0.22 IU/mL [95% CI 0.10;0.33], $p < 0.001$). Age was inversely associated with ADAMTS13 activity (B = −6.22%/10 years

Table 2
Association between VWF:Ag levels, ADAMTS13 activity, fibrinogen or fibrinogen γ' and plaque ulceration, intraplaque hemorrhage and lipid-rich necrotic core.

Characteristic	Primary hemostasis				Secondary hemostasis			
	VWF:Ag[a] (IU/mL)		ADAMTS13[a] (%)		Fibrinogen[a] (g/L)		Fibrinogen γ'[a] (g/L)	
	B [95% CI]	P	B [95% CI]	P	B [95% CI]	P	B [95% CI]	P
Plaque ulceration	−0.08 [−0.23;0.06]	0.25	0.00 [−0.00;0.00]	0.57	0.00 [−0.07;0.07]	0.98	−0.21 [−0.69;0.28]	0.40
IPH volume (mm^3)	−17.13 [−52.22;17.95]	0.34	0.11 [−0.81;1.03]	0.82	−23.40 [−41.09;−5.70]	0.01	−161.73 [−285.76;−37.70]	0.01
Adjustment for CRP[†]					−23.90 [−46.99;−0.81]	0.04	−144.41 [−277.48;−11.35]	0.03
LRNC volume (mm^3)	−18.38 [−66.94;30.18]	0.46	−0.38 [−1.66;0.89]	0.55	−38.89 [−63.12;−14.65]	0.00	−227.06 [−398.91;−55.21]	0.01
Adjustment for CRP[†]					−40.08 [−71.71;−8.46]	0.01	−192.01 [−376.00;−8.01]	0.04

VWF:Ag, Von Willebrand Factor antigen; CRP; C-Reactive Protein; IPH, intraplaque hemorrhage; LRNC, lipid-rich necrotic core.
[a] Adjusted for age and sex, smoking, body mass index, hypertension, hypercholesterolemia, diabetes mellitus, history of cardiovascular disease and peripheral arterial disease; additionally for blood group non-O in case of VWF.
[†] Associations with $p \leq 0.1$ were additionally adjusted for CRP.

[95% CI −9.94;−2.51], $p = 0.001$). We also found an inverse association between the time from index event to blood withdrawal and ADAMTS13 activity (B = −0.13%/day [95% CI -0.23;-0.03], $p = 0.008$). None of the other clinical characteristics were associated with VWF:Ag levels or ADAMTS13 activity.

We found no significant associations between VWF:Ag levels or ADAMTS13 activity and plaque ulceration, IPH volume and LRNC volume (Table 2). Additional adjustment for interval event–blood withdrawal or ECST stenosis did not change the results. Also no significant association between VWF:Ag levels or ADAMTS13 activity and the additional imaging biomarkers was found (Table 3).

3.2. Secondary hemostasis; fibrinogen and fibrinogen γ'

Focusing on factors of secondary hemostasis, we found that age was inversely associated with fibrinogen γ' (B = −0.03 g/L/10 years [95% CI −0.05;0.00], $p = 0.02$). Fibrinogen and fibrinogen γ' were both associated with C-reactive protein (CRP) (B = 0.42 g/L/mg/L [95% CI 0.32;0.51], $p < 0.001$ and B = 0.04 g/L/mg/L [95% CI 0.03;0.06], $p < 0.001$ respectively). None of the other clinical characteristics were associated with fibrinogen or fibrinogen γ'.

A significant association was found between fibrinogen and IPH volume (B = −23.40 mm^3/g/L [95% CI −41.09;−5.70], $p = 0.01$) and between fibrinogen γ' and IPH volume (B = −161.73 mm^3/g/L [95% CI −285.76;−37.70], p = 0.01), regardless of adjustment for CRP (B = −23.90 mm^3/g/L [95% CI −46.99;−0.81], $p = 0.04$ and B = −144.41 mm^3/g/L [95% CI −277.48;−11.35], $p = 0.03$, respectively) (Table 2). Patients with higher fibrinogen and fibrinogen γ' levels showed significantly lower IPH volume compared to patients with a lower fibrinogen and fibrinogen γ' levels. Furthermore, in case of fibrinogen, additional adjustment for interval index event–blood withdrawal and degree of stenosis (ECST) did not change the results (B = −25.24 mm^3/g/L [95% CI −47.88;−2.60], p = 0.03). However, in case of fibrinogen γ', there was no longer an association (B = −118.93 mm^3/g/L [95% CI −254.88;17.02], $p = 0.09$). Adjustment for medication use prior to the event did not influence the results. Subgroup analysis of patients with and without a statin and patients with and without anti-platelet drugs, showed that there was no association between fibrinogen and fibrinogen γ' and IPH volume in any subgroup. Interaction terms of fibrinogen or fibrinogen γ' with statin use and fibrinogen or fibrinogen γ' with anti-platelet drugs were not significant.

When focusing on patients with and without a history of CVD, we found as expected significantly more use of cardiovascular medication (statins, antihypertensive drugs, antidiabetics and antiplatelet agents) in the group with a history of CVD. There was no difference in presence of IPH. Subgroup analysis of patients with and without a history of CVD, showed no association between fibrinogen and fibrinogen γ' and IPH volume in any subgroup. Interaction terms of fibrinogen or fibrinogen γ' with a history of CVD were not significant (respectively $p = 0.28$ and $p = 0.45$), indicating that there was no interaction between fibrinogen and fibrinogen γ' with a history of CVD.

Additionally, we found an inverse association between fibrinogen and LRNC volume and between fibrinogen γ' and LRNC volume, also after additional adjustment for CRP (B = −40.08 mm^3/g/L [95% CI −71.71;−8.46], $p = 0.01$ and B = −192.01 mm^3/g/L [95% CI −376.00;−8.01], $p = 0.04$, respectively) (Table 2). Subgroup analyses of patients with and without a statin, patients with and without anti-platelet drugs and patients with and without a history of CVD showed no association between fibrinogen and fibrinogen γ' and LRNC volume in any subgroup. Also interaction terms were not significant.

4. Discussion

This study shows a significant inverse association between biomarkers of secondary hemostasis; fibrinogen and fibrinogen γ' and IPH volume and LRNC volume, in patients with a recent TIA or ischemic stroke and a symptomatic mild-to-moderate carotid artery stenosis. No

Table 3
VWF:Ag levels, ADAMTS13 activity, fibrinogen or fibrinogen γ' and additional imaging biomarkers.

Characteristic	Primary hemostasis				Secondary hemostasis			
	VWF:Ag[a] (IU/mL)		ADAMTS13[a] (%)		Fibrinogen[a] (g/L)		Fibrinogen γ'[a] (g/L)	
	B [95% CI]	P	B [95% CI]	P	B [95% CI]	P	B [95% CI]	P
Degree of stenosis (%, ECST)	−0.96 [−5.93;4.02]	0.71	0.00 [−0.12;0.12]	0.96	−0.62 [−3.06;1.83]	0.62	−12.08 [−29.13;4.97]	0.16
Calcification volume (mm^3)	−11.10 [−43.79;21.59]	0.50	−0.27 [−1.06;0.52]	0.50	−0.84 [−17.15;-15.47]	0.92	−14.60 [−127.89;98.69]	0.80
Maximum vessel wall area (mm^2)	−0.47 [−7.88;6.95]	0.90	−0.01 [−0.21;0.19]	0.91	2.25 [−1.62;6.12]	0.25	−4.79 [−32.27;22.69]	0.73

[a] Adjusted for age and sex, smoking, body mass index, hypertension, hypercholesterolemia, diabetes mellitus, history of cardiovascular disease and peripheral arterial disease; additionally for blood group non-O in case of VWF. VWF:Ag, Von Willebrand Factor antigen; ECST, European Carotid Surgery Trial; CRP; C-Reactive Protein.

associations were found between markers of primary hemostasis, VWF:Ag levels or ADAMTS13 activity and plaque ulceration, IPH volume and LRNC volume. To the best of our knowledge, this study is the first to investigate the relationship between the specific vulnerable plaque characteristics plaque ulceration and intraplaque hemorrhage with markers of primary and secondary hemostasis.

4.1. Primary hemostasis; VWF:Ag levels and ADAMTS13 activity

In the current study, no associations were found between VWF and ADAMTS13 and plaque ulceration, IPH, LRNC or additional plaque characteristics. 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, in this previous study we also found significantly higher VWF levels in patients with large artery atherosclerosis compared to other etiological subtypes of TIA or stroke [25,26]. It might be that VWF levels are differently associated with plaque burden measurements than with vulnerable plaque characteristics like plaque ulceration, IPH and LRNC. For example, in acute coronary syndrome patients, the presence of atherosclerosis measured by intravascular ultrasound (IVUS) was associated with VWF:Ag levels, but high risk, prone-to-rupture atherosclerotic lesions were not associated with VWF:Ag levels [27]. However, due to the known 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 might be that damage of the endothelial layer due to widespread atherosclerotic disease, causes a change in VWF levels. On these terms, blood coagulation markers are markers of a widespread atherosclerotic disease and not a risk marker for secondary prevention. However, a complex role of VWF and ADAMTS13 in atherosclerotic plaque development cannot be ruled out.

4.2. Secondary hemostasis; fibrinogen and fibrinogen γ'

Our current finding of an inverse association between fibrinogen and fibrinogen γ' and IPH volume and LRNC volume suggests a protective role of fibrinogen and fibrinogen γ' in the development of a vulnerable plaque. This association was independent of CRP and in case of fibrinogen also independent of degree of stenosis. Higher levels of plasma fibrinogen can induce clot formation more rapidly, possibly resulting in confinement of IPH and a smaller IPH volume and thereby a smaller LRNC volume. The presence of IPH has been shown to enlarge the LRNC [28]. In this study IPH was assessed as part of the LRNC, therefore LRNC and IPH volume were highly correlated.

Some studies on the incidence of CVD and fibrinogen γ' levels did not find significant associations [15,16]. It might be that fibrinogen γ' independently does not predict the occurrence of CVD, but that low levels might play a role in the progression of an atherosclerotic plaque due to a larger IPH or LRNC volume.

IPH is assumed to be the result of blood leakage within the core due to leakage of immature intraplaque neovessels [29,30]. These neovessels are formed due to hypoxia inside the atherosclerotic plaque [31]. Another described pathophysiological mechanism of IPH is repeated plaque fissuring followed by the formation of a non-occlusive luminal thrombus that gets incorporated into the atherosclerotic plaque [32]. Membranes of red blood cells contain a high load of unesterified cholesterol, leading to enlargement of the LRNC, and eventually a reduced plaque stability. IPH was observed to increase the risk of subsequent ischemic cerebrovascular events by five times [33].

In this study we found no association between fibrinogen levels and IPH volume or LRNC volume in patients with or without a history of CVD. When adding an interaction term of fibrinogen or fibrinogen γ' with a history CVD to the regression model, we found that these variables were not significantly interacting. IPH increases the risk of an ischemic event but not all IPH leads directly to a plaque rupture. IPH can occur at all time points during the progression of an atherosclerotic plaque, also in patients with a history of CVD. As was found in a study of Spagnoli et al. where fresh IPH was observed in carotid plaques from carotid endarterectomy samples of patients up to 24 months after an ischemic stroke [34].

Medication use in patients with and without a history of CVD, including the use of statins and anti-platelet drugs, are important possible confounders to consider. For instance, previous studies show that statins are associated with an increased stabilization of the atherosclerotic plaque, as shown by increased plaque echogenicity [35]. Furthermore, statins have shown to influence levels of several coagulation factors in plasma, including fibrinogen, thereby inducing an anticoagulant effect [36]. A recent study in 1740 participants of a population-based cohort study with carotid atherosclerosis found a positive trend with current or past use of anti-platelet agents and higher presence of IPH [37]. In the present study we found no association between fibrinogen and fibrinogen γ' with IPH volume and LRNC volume in subgroup analysis of patients with or without a statin or anti-platelet drugs. We also found that there was no interaction between both drugs and these coagulation factors. It might be that both statins and anti-platelet agents are associated with the presence of IPH and a LRNC rather than IPH volume and LRNC volume.

Previous studies on inflammatory biomarkers in atherosclerotic disease highlight a role of CRP and fibrinogen in patients with early stages of atherosclerosis and progression of the atherosclerotic plaque [38]. The CRP levels in our study were relatively low and were no longer affected by acute inflammation during the index event (median interval between event and blood withdrawal was 47 days). In contrast to our results, Buljubasic et al. found a significant association between high fibrinogen levels and coronary plaque burden, but not with plaque composition in patients with acute coronary syndrome or stable angina pectoris as measured by IVUS [39]. However, blood samples were collected before the procedure at a moment of acute inflammation. Fibrinogen is known to be elevated during a state of inflammation; after adjustment for CRP, significance was no longer reached. In addition, IVUS is unable to measure IPH volume, whereas MRI is a sensitive and specific imaging modality suitable for detecting IPH [40]. Sabeti et al. also found an association between high fibrinogen levels at baseline and progression of the atherosclerotic plaque during follow-up, as measured with carotid ultrasound. They found a hazard ratio for progression of atherosclerosis of 2.45 ($p = 0.002$) between de highest and lowest quartile of baseline fibrinogen [41]. However, also in their study there was no longer a significant association after adjusting for CRP.

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 index event and blood sampling/imaging, which might have influenced the association via a change in plaque composition and levels of coagulation factors [42]. VWF and fibrinogen are known to be increased in the acute phase of an event [13,43]. However, imaging and blood sampling were performed at the same moment and we found no change in results after additional adjustment for the interval event-blood withdrawal. Moreover, due to the lack of acute phase, levels of blood biomarkers may resemble the levels before the index event.

5. Summary

Fibrinogen and fibrinogen γ' were inversely associated with IPH volume and LRNC volume in patients with a recent TIA or ischemic stroke and a symptomatic mild-to-moderate carotid artery stenosis. No association was found between VWF:Ag levels and ADAMTS13 activity and vulnerable plaque characteristics. The inverse association between fibrinogen and fibrinogen γ' and IPH volume and LRNC volume appears to be independent of inflammation and suggests a protective role of

fibrinogen and fibrinogen γ' in relation to vulnerable plaque characteristics.

Conflict of interest

FL reports unrestricted research grants from CSL Behring and Shire and is a consultant to UniQure, Shire and NovoNordisk.

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