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Increased endothelial activation in recently symptomatic versus asymptomatic carotid artery stenosis and in cerebral microembolic-signal-negative patient subgroups

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Background and purpose: von Willebrand factor propeptide (VWF:Ag II) is potentially a more sensitive marker of acute endothelial activation than von Willebrand factor antigen (VWF:Ag). These biomarkers have not been simultaneously assessed in asymptomatic versus symptomatic carotid stenosis patients. The relationship between endothelial activation and cerebral microembolic signals (MESs) detected on transcranial Doppler ultrasound is unknown.

Methods: In this multicentre observational analytical study, plasma VWF:Ag and VWF:Ag II levels in patients with ≥50% asymptomatic carotid stenosis were compared with those from patients with ≥50% symptomatic carotid stenosis in the 'early' (≤4 weeks) and 'late' (≥3 months) phases after transient ischaemic attack or ischaemic stroke. Endothelial activation was also longitudinally assessed in symptomatic patients during follow-up. Transcranial Doppler ultrasound monitoring classified patients as MES-positive or MES-negative.

Results: Data from 31 asymptomatic patients were compared with those from 46 early symptomatic and 35 late phase symptomatic carotid stenosis patients, 23 of whom had undergone carotid intervention. VWF:Ag II levels were higher in early (12.8 μg/ml; $P < 0.001$), late (10.6 μg/ml; $P = 0.01$) and late post-intervention (10.6 μg/ml; $P = 0.038$) symptomatic patients than asymptomatic patients (8.9 μg/ml). VWF:Ag levels decreased in symptomatic patients followed up from the early to late phase after symptom onset ($P = 0.048$). Early symptomatic MES-negative patients had higher VWF:Ag II levels (13.3 vs. 9.0 μg/ml; $P < 0.001$) than asymptomatic MES-negative patients.

Conclusions: Endothelial activation is enhanced in symptomatic versus asymptomatic carotid stenosis patients, in early symptomatic versus asymptomatic MES-negative patients, and decreases over time in symptomatic patients. VWF:Ag II levels are a more sensitive marker of endothelial activation than VWF:Ag levels in carotid stenosis. The potential value of endothelial biomarkers and concurrent cerebral MES detection at predicting stroke risk in carotid stenosis warrants further study.

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Introduction

von Willebrand factor (VWF:Ag) is an adhesive multimeric plasma glycoprotein synthesized in vascular endothelial cells and megakaryocytes [1,2]. von Willebrand factor propeptide (VWF:Ag II) is produced by cleavage of pro-VWF into VWF:Ag and VWF:Ag II [3,4]. Endothelial cells secrete VWF into circulating blood or the subendothelial matrix, or may store VWF in endothelial Weibel–Palade bodies [5]. VWF:Ag II undergoes endoproteolytic cleavage to form VWF:Ag in endothelial cells and is also stored in Weibel–Palade bodies until released during endothelial activation [3]. VWF:Ag II is believed to be a more sensitive marker of acute endothelial cell activation than VWF:Ag because VWF:Ag II has a shorter plasma half-life and returns to baseline concentration briskly after the stimulus to endothelial activation is removed [3,4]. The mechanisms responsible for the higher risk of stroke in patients with recently symptomatic than asymptomatic carotid stenosis are not fully understood [6–9] but could relate to differences in platelet activation or endothelial activation status between groups [10–12]. Prior to the conduct of this study, there were limited data on VWF in transient ischaemic attack (TIA) and ischaemic stroke due to carotid stenosis [13,14]. Some studies have demonstrated elevated plasma VWF levels in the early [1,14–17] and late [16,18] phases following TIA or ischaemic stroke versus healthy controls. Very few studies have investigated plasma VWF levels specifically in patients with asymptomatic or symptomatic carotid stenosis [19–22].

Previous studies have illustrated the potential role of microembolic signals (MESs) detected on transcranial Doppler ultrasound (TCD) or carotid plaque imaging in identifying asymptomatic and symptomatic carotid stenosis patients who may benefit most from optimal medical or surgical therapy to prevent TIA or stroke [6,23–26]. To our knowledge, simultaneous quantification of plasma levels of VWF:Ag and VWF:Ag II or the VWF:Ag/VWF:Ag II ratio has not been performed in patients with asymptomatic versus symptomatic carotid stenosis, in conjunction with simultaneous quantification of cerebral MESs.

The aims of this pilot study were to determine whether there were differences in endothelial activation *ex vivo* between patients with asymptomatic and recently symptomatic moderate or severe carotid stenosis, and whether endothelial activation decreased over time in recently symptomatic carotid stenosis patients. A comparison was also made of endothelial activation in asymptomatic and symptomatic subgroups who were MES-positive and MES-negative. It was hypothesized that endothelial activation would be increased in recently symptomatic compared with asymptomatic carotid stenosis, and that endothelial activation would decrease during longitudinal follow-up in recently symptomatic patients. It was also hypothesized that endothelial activation data would be informative in certain patient subgroups stratified according to MES status [12].

Methods

Consecutive eligible patients >18 years old with asymptomatic or symptomatic moderate or severe carotid artery stenosis or carotid occlusion, identified on colour Doppler ultrasound using standardized velocity criteria [27,28], were recruited from the Rapid Access Stroke Prevention Service, general neurology and vascular surgery clinics, stroke service, and neurology and vascular surgery wards at two secondary and tertiary referral university teaching hospitals between August 2007 and February 2010 [12].

Patients were included in the asymptomatic carotid stenosis group if they were incidentally noted to have moderate (50%–69%) or severe (≥70%) carotid stenosis on colour Doppler ultrasound, e.g. during vascular work-up in a patient with a carotid bruit or coronary artery disease [12]. Asymptomatic patients had no history of TIA or stroke, or had not had a TIA or stroke in the ipsilateral carotid or any other cerebrovascular territory within the preceding 3 years. Patients were included in the symptomatic carotid stenosis group if they recently had a TIA or ischaemic stroke in the vascular territory supplied by a moderate or severe carotid stenosis or carotid occlusion within the preceding 4 weeks (early phase), with symptoms attributed to the stenosed carotid artery of interest. Patients with carotid occlusion were only included if no other cause for stroke or TIA was identified.

Exclusion criteria for asymptomatic and symptomatic patients included active infection, inflammation or neoplasia; platelet count <120 or >450 $\times$ 10^9/l; myocardial infarction, pulmonary embolism, deep vein thrombosis or major surgery within the preceding 3 months; prior primary intracerebral haemorrhage; known bleeding or clotting diathesis; ongoing unstable coronary or peripheral arterial disease; renal impairment (urea > 10 mM); or non-steroidal anti-inflammatory drug intake other than prescribed aspirin within the preceding 2 weeks [12]. Patients were subsequently excluded from the symptomatic group if a potential cardioembolic source of embolism was detected within 3 months of recruitment, or if they had symptoms, signs or subsequent neuro-imaging evidence of acute cerebral ischaemia outside the vascular territory supplied by the stenosed carotid artery of interest (see below).

All patients underwent detailed neurovascular assessment by a neurology research registrar/resident (JAK or WOT) or experienced consultant vascular neurologist (DJHM) to confirm that asymptomatic patients met inclusion criteria and to confirm a diagnosis of large artery atherosclerotic TIA or stroke in the symptomatic cohort. TIA and stroke work-up was performed according to European Stroke Organization guidelines [29]. Information regarding vascular risk factors including hypertension, prior TIA or stroke, ischaemic heart disease, atrial fibrillation, valvular heart disease, diabetes mellitus, hyperlipidaemia, peripheral vascular disease, migraine, family history of stroke, medication intake (including anti-thrombotic therapy), smoking status, alcohol intake, and the method of detection of carotid stenosis was collected prospectively. If antiplatelet therapy was altered by their treating physician in the early phase after presentation, patients were invited to undergo repeat blood testing approximately 14 ± 7 days later if they had not undergone carotid intervention by that stage. All symptomatic patients who attended for follow-up were clinically reassessed at each follow-up visit.

In our main cross-sectional study, data from asymptomatic patients were compared with those from early and late phase symptomatic carotid stenosis patients. In our nested longitudinal study in the symptomatic cohort, data from early phase symptomatic patients were compared with those who had prospective follow-up data ≥3 months later.

Blood sampling and laboratory tests

All subjects were rested for at least 20 min, and careful atraumatic venepuncture was performed from a free-flowing vein using a 21G butterfly needle and a Vacutainer system (Becton Dickinson, Oxford) with a luer adaptor [16,30,31]. After taking an ethylenediaminetetraacetic acid sample and seven further 3 ml 3.2% citrate-anticoagulated whole blood samples, double spun platelet poor plasma (PPP) was obtained from the second to the sixth citrate-anticoagulated tubes by centrifugation and stored at −80°C within 1 h of venepuncture. Samples were thawed once at 37°C for 20 min before analysis in a VWF:Ag enzyme-linked immunosorbent assay (ELISA). Samples were then refrozen, stored at −80°C, and thawed once more for the VWF:Ag II ELISAs. The concentrations of VWF:Ag and VWF:Ag II in each PPP sample were quantified as described previously to assess endothelial activation status [32,33]. In brief, polyclonal rabbit anti-human VWF antibody (DAKO) was used as coating antibody and polyclonal rabbit anti-human VWF/HRP antibody (DAKO) as detection antibody for the VWF:Ag ELISA. M193902 CLB-Pro 35 coating antibody (Plesmanlaan 125) and M103904HRP CLB-Pro 14.3 detection antibody (Plesmanlaan 125) were used for VWF:Ag II quantification. The ELISA result was measured by spectrophotometry at 490 nm, using a VERSA Max Tuneable Microplate Reader. VWF:Ag and VWF:Ag II levels were recorded as micrograms per millilitre.

Transcranial Doppler ultrasound

Bilateral simultaneous 1 h TCD recordings of the middle cerebral artery were performed by one of two highly experienced operators (JAK or WOT) with a Viassys Pioneer TC8080, as described previously [12]. As reported previously, inter-observer agreement regarding the presence or absence of MESs between the first author and an experienced independent observer (MS) blinded to clinical details, symptomatic status and recorded MES status of the study subjects was found to be 'excellent' (93% concordance; Cohen's unweighted kappa statistic 0.89) [12,34]. Therefore, all remaining TCD data analysis was performed locally by the first author. The study was approved by the research ethics committee at AMNCH/St James's Hospital (Project/REC Reference 2007/03/01). Written informed consent, or assent from a relative where appropriate, was obtained from all participants.

Statistical methods

Paired or unpaired *t* tests were used for comparison of paired and unpaired parametric variables, respectively. The Wilcoxon signed rank and the Wilcoxon rank sum tests were used for comparison of paired and unpaired non-parametric variables, respectively, and the Kruskal-Wallis rank sum test for comparison of multiple non-parametric variables, where appropriate. Differences in proportions between groups were assessed with Chi-squared or Fisher exact tests, where appropriate. Multiple linear regression analysis was performed to examine the potential influence of independent demographic or vascular risk factors on any observed differences between groups, where appropriate. Correlation between variables was assessed with the Pearson product moment coefficient (r). Subgroup analyses in symptomatic and asymptomatic patients on aspirin monotherapy and patients with severe (≥70%) carotid stenosis were also planned. Because simultaneous measurements of VWF:Ag and VWF:Ag II had not previously been performed in asymptomatic versus symptomatic carotid stenosis, power calculations for this study were not possible. The number of subjects chosen for this novel pilot study was based on power

calculations for a study powered to detect differences in platelet activation status between these patient groups [12]. $P < 0.05$ was considered statistically significant. All statistical calculations were performed with R version 2.15.2 [35].

Results

Thirty-one patients with asymptomatic carotid stenosis and 61 early phase symptomatic carotid stenosis patients were initially recruited [12]. Fifteen early phase symptomatic patients were subsequently excluded [12], leaving data from 46 patients for analysis, six of whom had symptomatic internal carotid artery occlusion. Of the remaining 40 symptomatic patients in whom intervention could be considered, 28 underwent carotid endarterectomy, one had carotid stenting, six declined surgical intervention and the remaining five chose optimal medical management based on advice from their physician. Thirty-five symptomatic patients attended for late phase follow-up. Two developed 50%–69% carotid restenosis, and one developed >70% restenosis on repeat colour Doppler ultrasound at least 3 months after endarterectomy. Two patients had a 'perioperative' ischaemic stroke following carotid endarterectomy: one awoke following endarterectomy with a new ischaemic stroke, and one developed symptoms 48 hours post-operatively. Major surgery within the preceding 3 months was an exclusion criterion for 'laboratory reassessment' during follow-up according to our pre-planned study protocol because platelet activation results could potentially be influenced by the effects of the carotid intervention itself. Therefore, although these events were captured during our data collection period, the 'late phase' clinical and laboratory study reassessment in these cases had to be deferred for at least 3 months after carotid intervention. No patients experienced a recurrent 'non-perioperative' TIA or stroke during follow-up to the late phase at least 3 months after symptom onset or intervention. Eleven symptomatic patients did not have available late phase laboratory data: six declined follow-up, one moved and could not re-attend, one developed active severe symptomatic peripheral vascular disease, and another ongoing active inflammatory gouty arthropathy precluding laboratory reassessment according to our study protocol; two died from unrelated causes (respiratory sepsis and cholecystitis).

There was a higher prevalence of current smokers but a lower prevalence of hypertension amongst symptomatic patients, and a lower prevalence of statin use amongst early symptomatic than asymptomatic patients (Table 1). Otherwise, demographic and vascular risk profiles were similar in asymptomatic and symptomatic patients.

Table 1 Demographic and vascular risk factor profile of patients with endothelial activation data

Parameter	Asymptomatic carotid stenosis ($n = 31$)	Early symptomatic carotid stenosis ($n = 46$)	Late symptomatic carotid stenosis ($n = 35$)
Mean age (years)	68.2 (±7.95)	65.0 (±9.58)	65.0 (±9.9)
P value		0.78	0.78
Sex (M; %)	18 (58%)	28 (61%)	20 (57%)
P value		0.8	0.94
Median interval from symptom onset (days; range)	N/A	7.5 (0–27)	175 (99–360)
Degree of stenosis			
Moderate: ≥50%–69%	11 (35%)	7 (15%)	15 (43%)
P value		**0.039**	0.54
Severe: ≥70%–99%	20 (65%)	33 (72%)	9 (26%)
P value		0.50	**0.0015**
Occlusion	0	6 (13%)	4 (11%)
P value		**0.04**	0.07
Antiplatelet therapy			
Aspirin monotherapy	22 (71%)	35 (76%)	15 (43%)
P value		0.62	**0.02**
Aspirin/dipyridamole combination	2 (6%)	4 (9%)	11 (31%)
P value		0.54	**0.01**
Clopidogrel monotherapy	5 (16%)	2 (4%)	6 (17%)
P value		0.09	0.6
Aspirin/clopidogrel combination	2 (6%)	5 (11%)	3 (9%)
P value		0.4	0.6
Ischaemic heart disease	7 (23%)	10 (22%)	7 (20%)
P value		0.93	0.8
Hypertension	27 (87%)	29 (63%)	23 (66%)
P value		**0.02**	**0.04**
Diabetes mellitus	7 (23%)	8 (17%)	6 (17%)
P value		0.57	0.58
Prior TIA/stroke before index event	8 (26%)	7 (15%)	6 (17%)
P value		0.25	0.39
Prior venous thromboembolism	1 (3%)	0	0
P value		0.4	0.5
Peripheral vascular disease	5 (16%)	5 (11%)	6 (17%)
P value		0.5	0.91
Migraine (with or without aura)	6 (19%)	5 (11%)	5 (14%)
P value		0.3	0.58
Family history stroke	9 (29%)	16 (35%)	12 (34%)
P value		0.6	0.65

(continued)

Table 1 *(Continued)*

Parameter	Asymptomatic carotid stenosis (n = 31)	Early symptomatic carotid stenosis (n = 46)	Late symptomatic carotid stenosis (n = 35)
Current smokers	5 (16%)	21 (46%)	14 (40%)
P value		**0.007**	**0.03**
Ex-smoker	22 (71%)	17 (37%)	13 (37%)
P value		**0.003**	**0.006**
Never smoker	4 (13%)	8 (17%)	8 (23%)
P value		0.59	0.3
Statin therapy	28 (90%)	33 (72%)	27 (77%)
P value		**0.043**	0.13

TIA, transient ischaemic attack. P values relate to chi-squared or Fisher exact testing between asymptomatic and symptomatic carotid stenosis groups. Values are means (± standard deviation) or absolute values. Significant P values are highlighted in bold.

There were no significant differences in unadjusted VWF:Ag levels or in the VWF:Ag/VWF:AgII ratio between the overall population of asymptomatic patients and symptomatic patients at any stage after symptom onset or intervention ($P \geq 0.056$). However, VWF:Ag II levels were higher in early (12.8 μg/ml; $P < 0.001$), late (10.6 μg/ml; $P = 0.01$) and late post-intervention (10.6 μg/ml; $P = 0.038$) symptomatic patients than asymptomatic carotid stenosis patients (8.9 μg/ml) (Table 2). Within the symptomatic patient group with longitudinal follow-up data, VWF:Ag levels significantly decreased over time from the early to late phase after symptom onset (16.5 vs. 14.8 μg/ml, $P = 0.048$; Table 3). Otherwise, there were no differences in endothelial activation markers between early symptomatic and late phase symptomatic patients, regardless of whether they underwent intervention (Tables 3 and 4). Having controlled for the independent influence of smoking status, hypertension and statin use with multiple linear regression, adjusted VWF:Ag II levels remained significantly higher in early symptomatic ($P = 0.0007$) and late symptomatic ($P = 0.035$) than asymptomatic patients. Furthermore, differences in adjusted VWF:Ag levels between early symptomatic and asymptomatic patients became significant ($P = 0.029$).

Pre-planned subgroup analyses

Because differences in prescribed antiplatelet regimens could potentially have influenced observed differences between asymptomatic and symptomatic patients (Table 1), pre-planned subgroup analysis was performed in patients on aspirin monotherapy. VWF:Ag levels (15.4 vs. 12.1 μg/ml; $P = 0.045$) and VWF:Ag II levels (12.8 vs. 8.7 μg/ml; $P = 0.003$) were higher in early symptomatic than asymptomatic patients on aspirin (Table 5). Otherwise, there were no other differences between symptomatic and asymptomatic carotid stenosis subjects on aspirin monotherapy, but it must be emphasized that smaller numbers of subjects were included in this subgroup analysis. VWF:Ag II levels were also elevated in early (12.6 μg/ml; $P = 0.004$) and late (10.9 μg/ml; $P = 0.023$) symptomatic severe carotid stenosis (≥70%) versus asymptomatic severe carotid stenosis (8.8 μg/ml) (Table 6). There were no other differences between symptomatic and asymptomatic severe carotid stenosis patients.

Endothelial activation status in MES-positive and MES-negative subgroups

Twenty-five asymptomatic, 31 early symptomatic and 27 late symptomatic patients had TCD data available for analysis (Table S1) [12]; 12% asymptomatic vs. 32% early symptomatic ($P = 0.02$) and 19% late symptomatic ($P = 0.2$) patients were MES-positive (Table S1) [12].

There were no significant differences in endothelial activation markers between early or late symptomatic versus asymptomatic MES-positive subjects. However, VWF:AgII levels were higher (13.3 vs. 9.0 μg/ml; $P < 0.001$) and the VWF:Ag/VWF:Ag II ratio was lower (0.95 vs. 1.6; $P = 0.006$) in early symptomatic

Table 2 Comparison of von Willebrand factor (VWF:Ag), von Willebrand factor propeptide (VWF:Ag II) and the VWF:Ag/VWF:Ag II ratio in asymptomatic versus early symptomatic, late phase symptomatic and post-intervention late symptomatic patients

Marker	Asymptomatic (n = 31)	Early symptomatic (n = 46)	Late symptomatic (n = 35)	Late symptomatic post-intervention (n = 23)
VWF:Ag (μg/ml)	13.3 (9.2–16.1)	15.4 (11.9–21.0)	12.8 (9.9–19.9)	12.4 (9.9–17.7)
P value		0.056	0.6	0.97
VWF:Ag II (μg/ml)	8.9 (3.4–10.4)	12.8 (8.6–17.0)	10.6 (7.2–17.0)	10.6 (7.1–17.0)
P value		**< 0.001**	**0.01**	**0.038**
VWF:Ag/VWF:Ag II ratio	1.6 (1.2–3.3)	1.3 (0.8–2.3)	1.3 (1.1–1.9)	1.2 (0.9–2.1)
P value		0.096	0.5	0.07

Values are medians (25th−75th percentile). Significant P values are highlighted in bold.

Table 3 Endothelial activation data in early versus late symptomatic carotid stenosis patients who had data at each time point

	Early symptomatic (n = 35)	Late symptomatic (n = 35)	P value
VWF:Ag (μg/ml)	16.5 (±6.4)	14.8 (±6.4)	**0.048**
VWF:Ag II (μg/ml)	13.0 (±6.0)	11.7 (±6.0)	0.33
VWF:Ag/VWF:Ag II ratio	1.2 (0.8–1.8)	1.3 (1.1–1.9)	1.0

Values are either means (±SD) or medians (25th−75th percentile). Significant P values are highlighted in bold.

Table 4 Endothelial activation markers in early symptomatic pre-intervention versus late symptomatic post-intervention carotid stenosis patients

	Early symptomatic (n = 23)	Late symptomatic (n = 23)	P value
VWF:Ag (μg/ml)	16.0 (±6.7)	13.7 (±5.7)	0.054
VWF:Ag II (μg/ml)	11.9 (±5.5)	11.2 (±5.8)	0.67
VWF:Ag/VWF:Ag II ratio	1.4 (0.9–2.1)	1.2 (0.9–2.1)	0.28

Values are means (±SD) or medians (25th−75th percentile).

than asymptomatic MES-negative patients (Table S2). VWF:Ag II levels were also higher in late symptomatic than asymptomatic MES-negative patients (10.7 vs. 9.0 μg/ml; $P = 0.01$; Table S2). Having controlled for differences in smoking prevalence, statin use and hypertension between early symptomatic, late phase symptomatic and asymptomatic MES-negative patients, all above differences in VWF:Ag II levels and the VWF:Ag/VWF:Ag II ratio persisted between relevant subgroups ($P < 0.05$).

Discussion

This novel longitudinal study has shown convincing evidence of excessive endothelial activation, as evidenced by elevated VWF:Ag II levels in early, late and late post-intervention symptomatic patients and elevated adjusted VWF:Ag levels in early symptomatic versus asymptomatic carotid stenosis patients. Because VWF:Ag II is a marker of acute rather than chronic endothelial cell activation [3,4] the persistent changes during follow-up indicate that the findings in early symptomatic patients are not simply reflective of an acute phase response to recent ocular or cerebral ischaemia or infarction. Furthermore, our data confirm that VWF:Ag II is a more sensitive marker of acute endothelial activation than VWF:Ag in studies comparing patients with symptomatic and asymptomatic carotid stenosis.

VWF:Ag and VWF:Ag II levels were higher in early symptomatic than asymptomatic patients on aspirin monotherapy. These findings indicate that endothelial activation is increased in early symptomatic versus asymptomatic patients despite treatment with low to medium dose aspirin therapy, and that differences in endothelial activation status are not simply explained by differences in antiplatelet regimens between groups. However, because of the relatively small numbers of subjects included in our subgroup analyses, the effects of different antiplatelet regimens on endothelial activation in carotid stenosis deserves further formal study. VWF:Ag II levels were also elevated in early and late symptomatic versus asymptomatic severe carotid stenosis indicating ongoing excessive endothelial activation levels in this higher risk symptomatic subgroup.

Our longitudinal study in symptomatic patients revealed that endothelial activation status (VWF:Ag) decreased as patients were followed up from the early to the late stage after symptom onset or carotid intervention. These findings may partly reflect resolution of the acute phase response over time, the effects of successful removal of the stenosing atherosclerotic plaque in 66% of patients, and perhaps alteration of antiplatelet therapy in some symptomatic patients by the late stage of follow-up.

Prior to this study, published data supported the concept that MESs predict subsequent TIA or ischaemic stroke risk in patients with >60% [6,23–25] and ≥70% asymptomatic carotid artery stenosis [26,36].

Table 5 Comparison of VWF:Ag, VWF:Ag II and the VWF:Ag/VWF:Ag II ratio in asymptomatic versus early symptomatic, late phase symptomatic and post-intervention late phase symptomatic patients on aspirin monotherapy

Marker	Asymptomatic (n = 22)	Early symptomatic (n = 30)	Late symptomatic (n = 15)	Late symptomatic post-intervention (n = 10)
VWF:Ag (μg/ml)	12.1 (9.1–16.5)	15.4 (12.2–20.2)	10.9 (8.9–17.2)	10.7 (7.9–14.7)
P value		**0.045**	0.8	0.52
VWF:Ag II (μg/mL)	8.7 (3.5–10.5)	12.8 (8.6–17.6)	9.4 (6.6–15.7)	10.6 (6.6–18.2)
P value		**0.003**	0.38	0.35
VWF:Ag/VWF:Ag II ratio	2.0 (1.1–3.5)	1.2 (0.8–1.8)	1.4 (1.1–1.8)	1.1 (0.8–1.9)
P value		0.07	0.28	0.21

Values are means (±SD) or medians (25th−75th percentile). Significant P values are highlighted in bold.

Table 6 Comparison of endothelial activation in asymptomatic versus early symptomatic and late phase symptomatic subjects with severe (≥70%) carotid stenosis

Marker	Asymptomatic (n = 20)	Early symptomatic (n = 33)	Late symptomatic (n = 9)
VWF:Ag (μg/mL)	13.8 (9.4–16.9)	15.1 (10.2–19.3)	12.6 (10.5–15.9)
P value		0.31	0.84
VWF:Ag II (μg/mL)	8.8 (3.2–10.0)	12.6 (7.8–16.9)	10.9 (7.7–18.2)
P value		**0.004**	**0.023**
VWF:Ag/ VWF:Ag II ratio	1.8 (1.4–3.9)	1.2 (0.8–1.9)	1.3 (1.1–1.8)
P value		0.052	0.055

Values are medians (25th−75th percentile).

However, to our knowledge, no studies compared both endothelial activation status and MESs in asymptomatic versus early and late symptomatic ≥50% carotid stenosis or symptomatic patients followed up to the late phase after symptom onset or intervention. The finding of higher VWF:Ag II levels in early and late phase symptomatic than in asymptomatic MES-negative patients suggests persistent excessive endothelial activation in this important subgroup of symptomatic patients who do not have detectable emboli on TCD, despite treatment with antithrombotic and other secondary preventive therapy. However, one must accept that the number of subjects included in the different subgroups in this analysis that was stratified according to MES status was limited; therefore, these data must be interpreted with caution and need to be confirmed in further studies with much larger numbers of subjects.

Spence *et al*. previously reported that plasma total homocysteine levels were higher in asymptomatic carotid stenosis patients who were MES-positive [6,23]. Although these levels would have been very interesting to assess in our study, dedicated funding was not available to measure plasma total homocysteine levels in all of our patients. However, simultaneous quantification of homocysteine, VWF:Ag and VWF:Ag II levels and MESs definitely warrants further study.

We cannot make any definitive conclusions at present about the value of these biomarkers in predicting the risk of recurrent vascular events in patients with carotid stenosis because none of our patients had 'non-perioperative' recurrent vascular events during medium-term follow up in this study. These data enhance our understanding of the importance of endothelial activation in carotid stenosis and of the cellular mechanisms potentially responsible for the disparity in stroke risk between symptomatic and asymptomatic carotid stenosis patients. Therapeutic agents that specifically inhibit the VWF pathway have the potential to subsequently reduce endothelial activation and platelet activation or reactivity, and hence the risk of recurrent vascular events in this important patient population with moderate to severe carotid stenosis [37].

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Disclosure of conflicts of interest

The authors declare no financial or other conflicts of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Demographic data and risk factor profiles of study patients with available TCD data.
Table S2. Comparison of endothelial activation in asymptomatic versus early symptomatic and late phase symptomatic carotid stenosis patients who were MES-negative.

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