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Pregnancy-Specific Glycoproteins Bind Integrin αIIbβ3 and Inhibit the Platelet-Fibrinogen Interaction.

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Increased platelet counts and platelet activation in early symptomatic versus asymptomatic carotid stenosis and relationship with microembolic status: Results from the Platelets And Carotid Stenosis (PACS) Study

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Abstract

Background: Cerebral microembolic signals (MES) may predict increased stroke risk in carotid stenosis. However, the relationship between platelet counts or platelet activation status and MES in symptomatic versus asymptomatic carotid stenosis has not been comprehensively assessed.

Setting: University teaching hospitals.

Methods: This prospective, pilot observational study assessed platelet counts and platelet activation status, and the relationship between platelet activation and MES in asymptomatic versus early (≤4 weeks after TIA/stroke) and late phase (≥3 months) symptomatic moderate or severe (≥50%) carotid stenosis patients. Full blood count measurements were performed, and whole blood flow cytometry was used to quantify platelet surface activation marker expression (CD62P and CD63) and circulating leucocyte-platelet complexes. Bilateral simultaneous transcranial Doppler ultrasound monitoring of the middle cerebral arteries was performed for 1 hour to classify patients as MES-positive or MES-negative.

Results: Data from 31 asymptomatic patients were compared with 46 symptomatic patients in the early phase, and 35 of these patients followed up to the late phase after symptom onset. The median platelet count (211 *vs*. 200 x 10^9/L; p=0.03) and the median % lymphocyte-platelet complexes were higher in early symptomatic than asymptomatic patients (2.8 *vs*. 2.4%, p=0.001). The % lymphocyte-platelet complexes was higher in early symptomatic than asymptomatic patients with ≥70% carotid stenosis (p=0.0005), and in symptomatic patients recruited within 7 days of symptom onset (p=0.028). Complete TCD data were available in 25 asymptomatic and 31 early phase symptomatic, and 27 late phase symptomatic patients. 12% of asymptomatic versus 32% of early phase symptomatic (p=0.02) and 19% of late phase symptomatic patients (p=0.2) were MES-positive. Early symptomatic MES-negative patients had a

higher % lymphocyte-platelet complexes than asymptomatic MES-negative patients (2.8 *vs*. 2.3%; p=0.0085).

Discussion: Recently symptomatic carotid stenosis patients have higher platelet counts (potentially reflecting increased platelet production, mobilisation or reduced clearance) and platelet activation status than asymptomatic patients. MES were more frequently detected in early symptomatic than asymptomatic patients, but the differences between late symptomatic and asymptomatic groups were not significant. Increased lymphocyte-platelet complex formation in recently symptomatic *vs*. asymptomatic MES-negative patients indicates enhanced platelet activation in this early symptomatic subgroup. Platelet biomarkers, in combination with TCD, have the potential to aid risk-stratification in asymptomatic and symptomatic carotid stenosis patients.

Introduction

The two-year risk of stroke in the territory supplied by a severely stenosed (≥70%) internal carotid artery has decreased over the years from 4% [1] to 0.7-1.7% in >50% asymptomatic patients treated with best medical therapy,[2-4] but is reported to be as high as 26% in symptomatic patients with recent TIA or stroke treated with best medical therapy alone.[5] This disparity in risk may relate to differences in morphology and/or stability of the atherosclerotic plaque,[6] different degrees of endothelial and/or platelet activation, or differences in thrombogenicity of the circulating blood.[7] Excessive platelet activation could predispose to carotid plaque 'activation' or thromboembolism from an activated plaque, which could contribute to the high risk of recurrent stroke in recently symptomatic patients; however, there are only limited pilot data available on this topic.[6;8;9;10].

Prior studies have illustrated the potential role of microembolic signals (MES) detected on transcranial Doppler ultrasound (TCD), and carotid plaque imaging in identifying asymptomatic and symptomatic carotid stenosis patients who may benefit from best medical rather than surgical therapy.[11-14] The ACES study found that MES could be used to identify patients with ≥70% asymptomatic carotid stenosis at highest risk of TIA or stroke.[15] TCD has also been used to assess the impact of different antiplatelet regimens on MES in patients with extracranial or intracranial arterial stenosis.[16;17] The CARESS study demonstrated that short-term aspirin and clopidogrel combination therapy reduced the incidence of MES on TCD compared with aspirin alone in patients with recently symptomatic ≥50% carotid stenosis (relative risk reduction 39.8%; p=0.0046).[17] These studies illustrate that TCD may identify asymptomatic carotid stenosis patients who warrant intervention, and the limited available data suggest that TCD has the potential to predict the response to

antiplatelet regimens in the clinical setting. Other studies have also demonstrated the potential importance of statin therapy in reducing the risk of cerebrovascular events in patients with carotid stenosis [18,19], but the impact of statins on platelet activation status and MES is unknown.

The primary aims of this component of the Platelets And Carotid Stenosis (PACS) study were to determine whether platelet counts or platelet activation markers were increased in patients with recently symptomatic *vs*. asymptomatic carotid stenosis, to assess whether recently symptomatic carotid patients had more MES on TCD than those with asymptomatic carotid stenosis, and to assess the impact of time from symptom onset and carotid intervention on platelet activation and MES status. We hypothesised that platelet counts or platelet activation markers, and the incidence of MES would be higher in symptomatic than asymptomatic patients, and that these parameters would decrease over time from symptom onset or carotid intervention.

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 (CDUS) using standardised velocity criteria,[20;21] were invited to participate between August 2007 and February 2010. Patients were recruited from the Rapid Access Stroke Prevention clinics, vascular surgery and general neurology clinics, stroke service, and neurology and vascular surgery wards. The study was approved by the St James Hospital / AMNCH local research ethics committee. Written informed consent (or assent) was obtained in all cases.

Inclusion/Exclusion Criteria

Patients were **included** in the '**asymptomatic carotid stenosis'** group if incidentally noted to have moderate (50-69%) or severe (≥70%) carotid stenosis on screening CDUS e.g. following auscultation of a carotid bruit, during pre-operative work-up for another cause, or during vascular work-up in the vascular surgery or other clinics. Patients were deemed asymptomatic if they had never had a prior TIA or stroke, or had not had a TIA or stroke in the ipsilateral carotid or any other cerebrovascular territory within the preceding three years.

Patients were **included** in the '**symptomatic carotid stenosis'** group if they had a TIA or ischaemic stroke in the vascular territory supplied by a moderate or severe carotid stenosis or occlusion within the preceding 4 weeks (**early phase**), with symptoms attributed to the carotid artery of interest according to the TOAST classification. Patients with carotid occlusion were only included if no other cause for TIA or stroke was identified. These patients were prospectively reassessed ≥3 months after symptom onset or after surgical or endovascular intervention (**late phase**).

Exclusion criteria for all asymptomatic and symptomatic patients included active infection, inflammation or neoplasia; platelet count <120 or >450 x 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 mmol/l); or non-steroidal anti-inflammatory drug (NSAID) intake other than prescribed aspirin within the preceding 2 weeks. Patients were subsequently excluded from the symptomatic group if a potential cardioembolic source of embolism was detected within 3 months of recruitment. Patients were also excluded from the symptomatic group 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.

Clinical Assessment

All patients underwent detailed clinical and neurovascular assessment by a neurology research resident (JAK or WOT) or supervising consultant vascular neurologist (DJHM) to confirm that all inclusion/exclusion criteria were satisfied. Information regarding vascular risk factors, 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 14 +/- 7 days later if they had not undergone carotid intervention by that stage. CT of brain and/or MRI of brain was performed in all symptomatic patients, and magnetic resonance angiography (MRA) or CT angiography (CTA) was performed

where deemed appropriate by the treating physician to establish concordance between CDUS and another non-invasive imaging modality.

Blood sampling and platelet studies

All subjects were rested for ≥20 minutes, and venepuncture performed from a free-flowing vein using a 21G butterfly needle and a Vacutainer® system with a luer adaptor, as previously described.[22] After drawing an initial EDTA-anticoagulated sample, seven further 3ml samples were collected into 3.2% citrate-anticoagulated Vacutainer tubes. The first 3ml citrate-anticoagulated sample was used for whole blood flow cytometric analysis on a Beckman Coulter XL MCL flow cytometer. Platelets were distinguished from white and red cells by their characteristic forward and side scatter patterns, and by ensuring that >95% of cells expressed GpIbα (CD42b).[22] Platelet activation was assessed by quantifying platelet surface CD62P and CD63 expression [7;8;22] ≤ 90 minutes of venepuncture,[22] and the percentages of circulating neutrophil–platelet, monocyte–platelet, and lymphocyte–platelet complexes ≤ 3 hours of venepuncture.[7;22] using previously described [22] and validated methodology.[6;22] The next 5 tubes were used to prepare platelet poor plasma (stored at -80^{o}C for prospective studies). The seventh citrate-anticoagulated sample was processed to measure platelet count, MPV and PDW in citrate-anticoagulated blood between 2 and 4 hours after venepuncture on a Sysmex XE-2100 haematology analyser (Sysmex UK Ltd.).

Transcranial Doppler ultrasound

TCD was performed by one of two highly-experienced operators (JAK or WOT) with a Viassys Pioneer TC8080. Each recording was performed with a 2-MHz probe, and

usually included a sample volume (SV) of 5-8mm, although in some instances, a SV >8mm was used. We used a constant sweep speed of 5.1 seconds, and 128-point fast-Fourier transform (FFT) spectral analysis, giving an overlap of >50%. Depth of insonation was 50-60 mm. Spontaneous MES detected in each middle cerebral artery (MCA) were recorded and quantified during a 1-hour period [23;24] according to published criteria.[25;26] The Doppler audio signal was recorded on the hard drive and recordings subsequently analysed 'off-line' by one experienced investigator blinded to patient details (JAK). MES were identified by their typical appearance within the spectral display and their characteristic high-pitched, chirping sound, using an intensity threshold of > 7dB.[25;26] Symptomatic patients were classified as 'MES-positive' if they had **≥ 1** MES detected ipsilateral to the stenosed carotid artery of interest. Asymptomatic patients were considered 'MES-positive' if **≥ 1** MES was detected ipsilateral to an asymptomatic ≥50% carotid stenosis. Other patients were deemed 'MES-negative'. If symptomatic patients did not undergo surgical or endovascular intervention, they were reassessed >3 months after symptom onset. Any patients who underwent intervention were reassessed >3 months following intervention. Full blood count (FBC) and platelet activation measurements were performed within 24 hours of TCD recordings.[7;22]

Statistical Analysis

Paired or unpaired t-tests were used for comparison of paired and unpaired parametric variables, respectively, the Wilcoxon signed rank test and the Wilcoxon rank sum test for comparison of paired and unpaired non-parametric variables, and the Kruskal-Wallis rank sum test for comparison of multiple non-parametric variables, where appropriate. Chi-squared or Fisher exact tests compared proportions between groups.

Multiple linear regression analysis was performed to investigate the potential influence of independent demographic or vascular risk factors on any observed differences between groups, where appropriate. Approximately 10% of recordings were also analysed by an experienced, independent observer (MS) blinded to clinical details, symptomatic status, and recorded MES status of the study subjects, to validate the accuracy of TCD data collected at our centre. Inter-observer agreement between JK and MS in the assessment of 'emboli-positive' patients (those with ≥1 MES) was calculated with Cohen's unweighted kappa statistic (κ) [27]. $P < 0.05$ was considered statistically significant. All statistical calculations were performed with R version 2.9.1.[28]

Results

31 asymptomatic and 61 recently symptomatic carotid stenosis patients initially had platelet activation data available for analysis. Figure 1 outlines details of symptomatic patients subsequently excluded from analysis; therefore, we included data from 46 early phase symptomatic patients, and from 35 of these patients followed up to the late phase after symptoms, 23 of whom had undergone carotid intervention (Table 1). 22 late symptomatic patients had undergone carotid endarterectomy, one had carotid stenting, 7 declined surgical intervention or chose optimal medical management and 5 had carotid occlusion. Two of the post-intervention group developed 50–69% carotid restenosis, and one developed >70% restenosis on repeat CDUS at least 3 months after endarterectomy, but none experienced recurrent symptoms before follow-up. Of the 23 early symptomatic patients who presented with stroke, the median NIHSS score among this group was 1 (range: 0 – 17).

Platelet activation status and platelet parameters:

The median platelet count in citrate-anticoagulated whole blood was higher in early symptomatic than asymptomatic patients (211 *vs*. 200 x 10^9/L; p=0.03, Table 2). There were no differences in CD62P or CD63 expression, or % neutrophil-platelet or monocyte-platelet complexes between asymptomatic and symptomatic patients (Table 2). However, median % lymphocyte-platelet complexes was higher in early symptomatic than asymptomatic patients (2.8 *vs*. 2.4%; p=0.001, Table 2). After controlling for differences in the prevalence of hypertension, statin use and smoking between groups, the median platelet count (p=0.045) and median % lymphocyte-

platelet complexes (p=0.037) remained higher in early symptomatic than asymptomatic patients.

Twenty-five asymptomatic patients, and 31 early and 27 late symptomatic patients had TCD data available for analysis (Table 3a). Twenty-two symptomatic patients had longitudinal TCD data available at both early and late phases. Inter-observer agreement in assessing the presence or absence of MES between JK and MS was excellent (93% concordance, $\kappa = 0.89$), strongly supporting the validity of our remaining data analysis performed on site.

Three (12%) asymptomatic patients *vs*. 32% early symptomatic (p = 0.02) and 19% late symptomatic patients (p = 0.2) were MES-positive (Table 3a). Amongst the 3 asymptomatic MES-positive patients, 2 had >50% stenosis, and the third had complete carotid occlusion with contralateral severe stenosis with MES detected distal to the occlusion. There were no statistically significant differences in the incidence of MES between asymptomatic patients (12%) and subgroups of late phase symptomatic patients who did (3/19 [16%], p=0.35) and did not undergo carotid intervention (2/8 [25%], p=0.19). Amongst symptomatic patients with longitudinal data at both phases after symptom onset, there was a non-significant trend towards a reduction in the incidence of MES between the early and late phases (36% *vs*. 23%; p=0.17).

Platelet count and activation status in MES-positive and MES-negative subgroups

There were no significant differences in platelet counts or activation markers between early or late symptomatic and asymptomatic MES-positive subjects. However, the median platelet count (191 *vs.* 172 x 10^9/L; p=0.013) and % lymphocyte-platelet complexes (2.78 *vs*. 2.29%; p=**0.0085**) were higher in early symptomatic than

asymptomatic **MES-negative** patients. Having controlled for differences in smoking prevalence between early symptomatic MES-negative and asymptomatic MES-negative patients, differences in platelet counts (p=0.035) and % lymphocyte-platelet complexes (p=0.02) persisted between subgroups.

Pre-planned subgroup analyses:

Because different antiplatelet regimens could potentially have influenced observed differences in platelet activation status between asymptomatic and symptomatic patients (Table 1), pre-planned subgroup analysis was performed in patients on aspirin monotherapy. The % lymphocyte-platelet complexes remained higher in early symptomatic than asymptomatic patients on aspirin monotherapy (p=0.013). CD63 expression was slightly lower in late symptomatic than asymptomatic patients on aspirin (p=0.048).

Median % lymphocyte-platelet complexes was also elevated in early symptomatic (n=33) versus asymptomatic **severe** (≥70%) carotid stenosis (n=20; 2.86% *vs*. 2.43%, p=0.0005). Otherwise, there were no other significant differences in platelet activation between early or late symptomatic versus asymptomatic **severe** carotid stenosis patients (p≥0.1).

% lymphocyte-platelet complexes was also higher in early symptomatic patients who had TIA or stroke ≤7 days of study inclusion (n=23) than asymptomatic patients (2.8 *vs*. 2.4%; p=0.028).

There were no changes in platelet count, MPV, PDW or platelet activation over time, or after carotid intervention in the symptomatic patient subgroup with follow-up data

at each timepoint ($P > 0.22$). However, the number of subjects included in these subgroup analyses was limited.

Discussion

The finding of a higher platelet count in early symptomatic versus asymptomatic moderate or severe carotid stenosis may indicate enhanced platelet production, platelet secretion or reduced clearance early after TIA or stroke, and is in keeping with the findings of a smaller pilot study in a similar population with severe carotid stenosis.[7] The lack of significant differences in platelet counts between late phase symptomatic and asymptomatic patients could reflect resolution of the acute phase response during prolonged clinical follow-up or following intervention, or could reflect a type II error because only 35/46 symptomatic patients had late phase data, and overall study numbers were relatively small. Our longitudinal subgroup data suggest that this study was probably underpowered to detect changes between late symptomatic (n=35) and asymptomatic patients (n=31) because the platelet count and platelet activation status did not decrease significantly during follow-up in the subgroup of symptomatic patients assessed in both the early and late phases after TIA or stroke onset, or before and after carotid intervention.

Elevated leucocyte–platelet complexes have previously been reported in a pilot study in **severe** symptomatic versus asymptomatic carotid stenosis.[7] Our subgroup analysis that revealed elevated lymphocyte-platelet complexes in early symptomatic versus asymptomatic severe carotid stenosis is consistent with these data.[7] The concurrent findings of increased platelet counts and lymphocyte-platelet complexes may reflect pre-existing excessive platelet production/secretion and activation, or could have arisen secondary to acute ocular or cerebral ischaemia/infarction early after TIA or stroke in patients with recently symptomatic carotid stenosis compared with their asymptomatic counterparts. Platelets and leucocytes may have excitatory effects on one another, with leucocyte-platelet complex formation facilitating

interaction between the two cell types.[29;30] The formation of leucocyte-complexes may increase the risk of thrombus formation or plaque inflammation, and predispose to recurrent cerebrovascular events due to embolism from the thrombotic milieu of an activated plaque in symptomatic patients.

Our study suggests that measurement of % leucocyte-platelet complexes with unstimulated whole blood flow cytometry is a more sensitive method of assessing platelet activation status than measurement of %CD62P or CD63 expression. However, a cross-sectional and case-control study found elevated soluble P-selectin, and surface CD62P and CD63 expression in symptomatic >70% compared with asymptomatic >50% carotid stenosis patients ($p < 0.05$).[9] The apparent disparity in results between this study and ours may reflect differences in demographic profiles, antithrombotic therapy, time from symptom onset and stenosis severity in the symptomatic groups, and differences in methodology employed to quantify leucocyte-platelet complexes. However, each study provides important evidence of enhanced platelet activation in symptomatic *vs*. asymptomatic carotid stenosis.

Only 50% of early symptomatic patients experienced symptoms within one week of enrolment, and the remainder were recruited over the subsequent 3 weeks after TIA or stroke. The % lymphocyte-platelet complexes may have been predominantly increased due to binding of activated platelets to more 'chronic inflammatory' leucocytes i.e. lymphocytes, with less pronounced binding to more 'hyperacute-phase' leucocytes, such as neutrophils or monocytes during the initial 4 week recruitment period, but this hypothesis is speculative. There are no data available on the precise half-life of individual leucocyte-platelet complexes *in vivo* in humans. Marquardt *et al.* assessed leucocyte-platelet complexes in 45 patients within 24 hours of ischaemic

stroke and compared these data with 30 healthy age- and sex-matched controls.[31] Neutrophil-platelet complexes were elevated from days 1 – 10, and monocyte-platelet complexes on day 2 following stroke in patients versus controls. Lymphocyte-platelet complexes were not elevated at any time point up to 90 days after symptoms in patients versus controls.

Prior to this study, published data supported the concept that MES predict subsequent TIA or ischaemic stroke risk in patients with >60% [11-14] and ≥70% asymptomatic carotid artery stenosis.[15;32] However, to our knowledge, no studies compared both platelet counts, platelet activation status and MES in asymptomatic versus recently symptomatic ≥50% carotid stenosis, and symptomatic patients followed up to the late phase after symptoms or intervention. There are no data, to our knowledge, comparing MES status in late phase symptomatic versus asymptomatic carotid stenosis patients.

Ritter *et al*. found higher soluble P-selectin levels in patients with >50% asymptomatic carotid stenosis with MES versus those without MES (p=0.0007).[33] However, platelet surface CD62P expression was actually lower in MES-positive than MES-negative patients with recent atherothrombotic stroke (p=0.004). In our study, MES were significantly more common in early symptomatic than asymptomatic patients, but the difference between late symptomatic and asymptomatic patients was non-significant. These early phase data are in keeping with the subgroup analysis in an earlier study in asymptomatic versus symptomatic patients within 3 months of TIA or stroke onset.[14] Our longitudinal, late-phase data may reflect successful treatment of the stenosing atheromatous plaque in 21/31 (68%) symptomatic patients during follow-up, with alteration of antiplatelet therapy [17;34;35] or ‘plaque healing’ in others. At the discretion of the treating physicians who referred patients for

recruitment to this study, 44% of late symptomatic patients were treated with aspirin monotherapy, whereas others were treated with aspirin-dipyridamole combination therapy (30%), clopidogrel monotherapy (19%), and two patients were on aspirin-clopidogrel combination therapy (Table 1). Because the principal researchers did not alter treatment in patients who were not directly under our care, we could not formally assess the impact of altering antiplatelet regimens on MES status.

The absolute percentage of MES-positive patients was slightly lower in late symptomatic patients who underwent carotid intervention (16%) than those who did not (25%), but differences between these symptomatic subgroups were not significant. However, the number of subjects included in this *post hoc* analysis was far too small to make any definitive conclusions about the impact of endarterectomy on MES status. There is some evidence that 'diffuse inflammation' may occur in the vascular system, perhaps secondary to atherosclerotic plaque activation associated with a systemic inflammatory response,[36] and in patients with symptomatic carotid stenosis with contralateral <50% or >50% 'clinically asymptomatic' stenosis.[37] Activation of these 'apparently clinically-silent plaques' could lead to distal embolisation and detection of contralateral MES in some subjects.

There were no significant differences in lymphocyte-platelet complexes between early symptomatic and asymptomatic MES-positive patients, perhaps reflecting the fact that these patients may have similarly elevated levels of platelet activation secondary to MES formation. Platelet counts and lymphocyte-platelet complex formation were higher in early symptomatic than asymptomatic MES-negative patients, also potentially indicative of increased platelet production, secretion or reduced clearance,

and increased platelet activation in this early symptomatic subgroup. These data confirm that platelets may be excessively activated in **subgroups of symptomatic patients** who do not have detectable emboli on TCD.

Further prospective studies are warranted to determine whether a multimodal risk-stratification model, including MES detection and concurrent platelet count/activation assessment, especially with quantification of leucocyte-platelet complexes, has the potential to identify carotid stenosis patients at highest risk of recurrent events who need urgent medical or surgical intervention.

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All named collaborators qualify for authorship and contributed to the manuscript as follows:

Study design; collection, analysis and interpretation of data; manuscript writing: Kinsella JA, Tobin WO, McCabe DJH.

Study design, Data collection and interpretation; critical revision of the manuscript for important intellectual content: Hamilton G.

Data collection and interpretation; critical revision of the manuscript for important intellectual content: Tierney S, Feeley TM, Egan B, Collins DR, Coughlan T, O'Neill D, Harbison JA, Madhavan P, Moore DJ, O'Neill SM, Colgan MP, Saqqur M, Doherty CP, Moran N, Murphy RP.

Conflict of interest statement:

None of the authors report any conflicts of interest or other disclosures. None of the above charities or funding bodies had any influence on design or conduct of this study, or had any influence on the decision to submit the final manuscript for publication. All authors have read and approved the submitted manuscript, and the manuscript has not been submitted elsewhere nor published elsewhere in whole or in part, except as an abstract.

Table 1: Demographic and vascular risk factor profile of patients with platelet activation data. 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. TIA = Transient Ischaemic Attack; PE = Pulmonary embolism; DVT = Deep vein thrombosis.

Parameter	Asymptomatic (n = 31)	Early Symptomatic (n=46)	Late Symptomatic (n=35)
Mean age (years)	68.2 [± 7.95]	65.0 [± 9.58]	65.0 [±9.9]
P-value		0.78	0.78
Sex (Male; %)	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
Ischemic 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	8 (26%)	7 (15%)	6 (17%)
P-value		0.25	0.39
Prior DVT/PE	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 of Stroke	9 (29%)	16 (35%)	12 (34%)
P-value		0.6	0.65
Current smoker	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

Table 2: Platelet activation markers and platelet count in asymptomatic versus early and late symptomatic carotid stenosis subjects. Values are medians (25th - 75th percentile). P values refer to comparison between asymptomatic and symptomatic groups.

Marker	Asymptomatic (n=31)	Early Symptomatic (n=46)	Late Symptomatic (n=35)
%CD62P	1.97 (1.19 – 2.44)	1.88 (1.29 – 2.5)	1.87 (1.31 – 2.76)
p		0.88	0.46
%CD63	11.0 (8.86 – 15.6)	9.78 (7.86 – 15.6)	10.1 (7.6 – 12.7)
p		0.64	0.1
% Neutrophil-Platelet Complexes	2.72 (2.4 – 3.1)	2.9 (2.5 – 3.4)	2.76 (2.4 – 3.4)
p		0.22	0.53
% Monocyte-Platelet Complexes	4.6 (4.3 – 5.6)	5.5 (4.0 – 6.8)	5.0 (4.1 – 5.9)
p		0.3	0.69
% Lymphocyte-Platelet Complexes	2.4 (1.8 – 2.7)	2.8 (2.4 – 3.3)	2.5 (2.2 – 3.0)
p		**0.001**	0.21
Platelet Count ($x10^9$/L) (Citrate)	200 (179 – 217)	211 (187 – 273)	219 (167 – 255)
p		**0.03**	0.10

Table 3a: Demographic data and risk factor profiles of study patients with TCD data. P values relate to chi-squared or Fisher exact tests between asymptomatic versus early or late symptomatic carotid stenosis patients. Values are means (±Standard deviation) or absolute counts, with percentages in square parentheses, where appropriate. MES = Microembolic Signals; TIA = Transient Ischaemic Attack; PE = Pulmonary embolism; DVT = Deep vein thrombosis.

Parameter	Asymptomatic (n = 25)	Early Symptomatic (n=31)	Late Symptomatic (n=27)
Mean age (years)	69 (±7.8)	65.6 (±9.8)	64.9 (±10.4)
P-Value		0.2	0.14
Sex (Male [%])	17 (68%)	23 (74%)	19 (70%)
P-Value		0.61	0.85
Degree of Stenosis:			
...Moderate, 50–69%	8 (32%)	4 (13%)	5 (19%)
P-Value		0.11	0.34
...Severe, 70 – 99%	17 (68%)	24 (77%)	2 (7%)
P-Value		0.43	**< 0.001**
*…**Occlusion***	0	3 (10%)	3 (11%)
P-Value		0.25	0.24
No of patients who were MES+ (%)			
…Ipsilateral to carotid stenosis of interest	3 (12%)	10 (32%)	5 (19%)
P-Value		**0.02**	0.2
<50% stenosis	2 (8%)	0	3 (11%)
P-Value		0.2	1.0
Moderate stenosis ≥50–69%	0	0	1 (4%)
P-Value		1.0	1.0
Severe stenosis ≥ 70–99%	0	9 (29%)	0
P-Value		**0.003**	1.0
Occlusion	1 (4%)	1 (3%)	1 (4%)
P-Value		1	1.0
Contralateral to carotid stenosis of interest	0	5 (16%)	3 (11%)
P-Value		0.058	0.24
-Bilateral MES	0	3 (10%)	1 (4%)
P-Value		0.11	0.33
Antiplatelet therapy			
- Aspirin monotherapy	16 (64%)	22 (71%)	12 (44%)
P-Value		0.58	0.16
- Aspirin-Dipyridamole combination Rx	2 (8%)	4 (13%)	8 (30%)
P-Value		0.56	**0.048**
- Clopidogrel monotherapy	5 (20%)	1 (3%)	5 (19%)
P-Value		**0.044**	0.89
- Aspirin-Clopidogrel combination Rx	2 (8%)	1 (3%)	2 (7%)
P-Value		0.43	0.94
Ischemic Heart Disease	6 (24%)	7 (23%)	5 (19%)
P-Value		0.9	0.63
Hypertension	22 (88%)	19 (61%)	17 (63%)
P-Value		**0.025**	**0.037**
Diabetes Mellitus	5 (20%)	5 (16%)	3 (11%)
P-Value		0.71	0.37
Prior TIA/Stroke	8 (32%)	5 (16%)	5 (19%)
P-Value		0.16	0.26
Prior DVT/PE	0	0	0
P-Value		1.0	1.0
Peripheral Vascular Disease	4 (16%)	6 (19%)	5 (19%)
P-Value		0.74	0.81
Migraine (with or without aura)	5 (20%)	4 (13%)	3 (11%)
P-Value		0.49	0.46
Family History of Stroke	8 (32%)	8 26%)	10 (37%)

P-Value		0.61	0.7
Current smoker	4 (16%)	15 (48%)	5 (19%)
P-Value		**0.013**	1.0
Ex-smoker	18 (72%)	12 (39%)	18 (67%)
P-Value		**0.013**	0.68
Never smoker	3 (12%)	4 (13%)	4 (15%)
P-Value		0.92	0.77
Statin therapy	24 (96%)	22 (71%)	26 (96%)
P-Value		**0.015**	0.96

Table 3b: Prescribed antiplatelet and statin therapy in MES-positive asymptomatic and early and late symptomatic carotid stenosis patients. Results are actual numbers with percentages in parentheses.

Medication	Asymptomatic (n=3)	Early Symptomatic (n=10)	Late Symptomatic (n=5)
Aspirin monotherapy (75-300mg/d)	3 (100%)	9 (90%)	4 (80%)
Aspirin-Dipyridamole combination	0	0	1 (20%)
Clopidogrel monotherapy (75mg/d)	0	0	0
Aspirin-Clopidogrel combination	0	0	0
Statin Therapy (10-30mg)	2 (67%)	3 (30%)	5 (100%)
(40mg)	1 (33%)	2 (20%)	0
(80mg)	0	1 (10%)	0

Table 3c: Prescribed antiplatelet and statin therapy in MES-negative asymptomatic and early and late symptomatic carotid stenosis patients. Results are actual numbers with percentages in parentheses.

Medication	Asymptomatic (n=22)	Early Symptomatic (n=21)	Late Symptomatic (n=22)
Aspirin monotherapy (75-300mg/d)	13 (59%)	14 (67%)	8 (36%)
Aspirin-Dipyridamole combination	2 (9%)	4 (19%)	7 (32%)
Clopidogrel monotherapy (75mg/d)	5 (23%)	2 (10%)	5 (23%)
Aspirin-Clopidogrel combination	2 (9%)	0	2 (9%)
Statin Therapy (10-20mg)	11 (50%)	5 (24%)	11 (50%)
(40-50mg)	9 (41%)	8 (38%)	7 (32%)
(80mg)	1 (5%)	3 (14%)	3 (14%)

Table 3d: Median markers of platelet activation and platelet count in early symptomatic versus asymptomatic MES-negative carotid stenosis patients

	Early Symptomatic MES-negative (n=21)	Asymptomatic MES-negative (n=22)	P value
CD62P %	1.87 (1.57 – 2.25)	1.81 (1.05 – 2.31)	0.47
CD63 %	14.5 (8.84 – 15.7)	10.8 (8.54 – 13.93)	0.14
% Neutrophil–platelet complexes	3.11 (2.26 – 3.54)	2.7 (2.4 – 3.1)	0.26
% Monocyte–platelet complexes	4.95 (3.84 – 6.55)	4.6 (4.4 – 5.5)	0.99
% Lymphocyte-platelet complexes	2.78 (2.49 – 3.36)	2.29 (1.8 – 2.6)	**0.0085**
Platelet Count ($x10^9$/L) (Citrate)	191 (173 – 234)	172 (159 – 190)	**0.013**

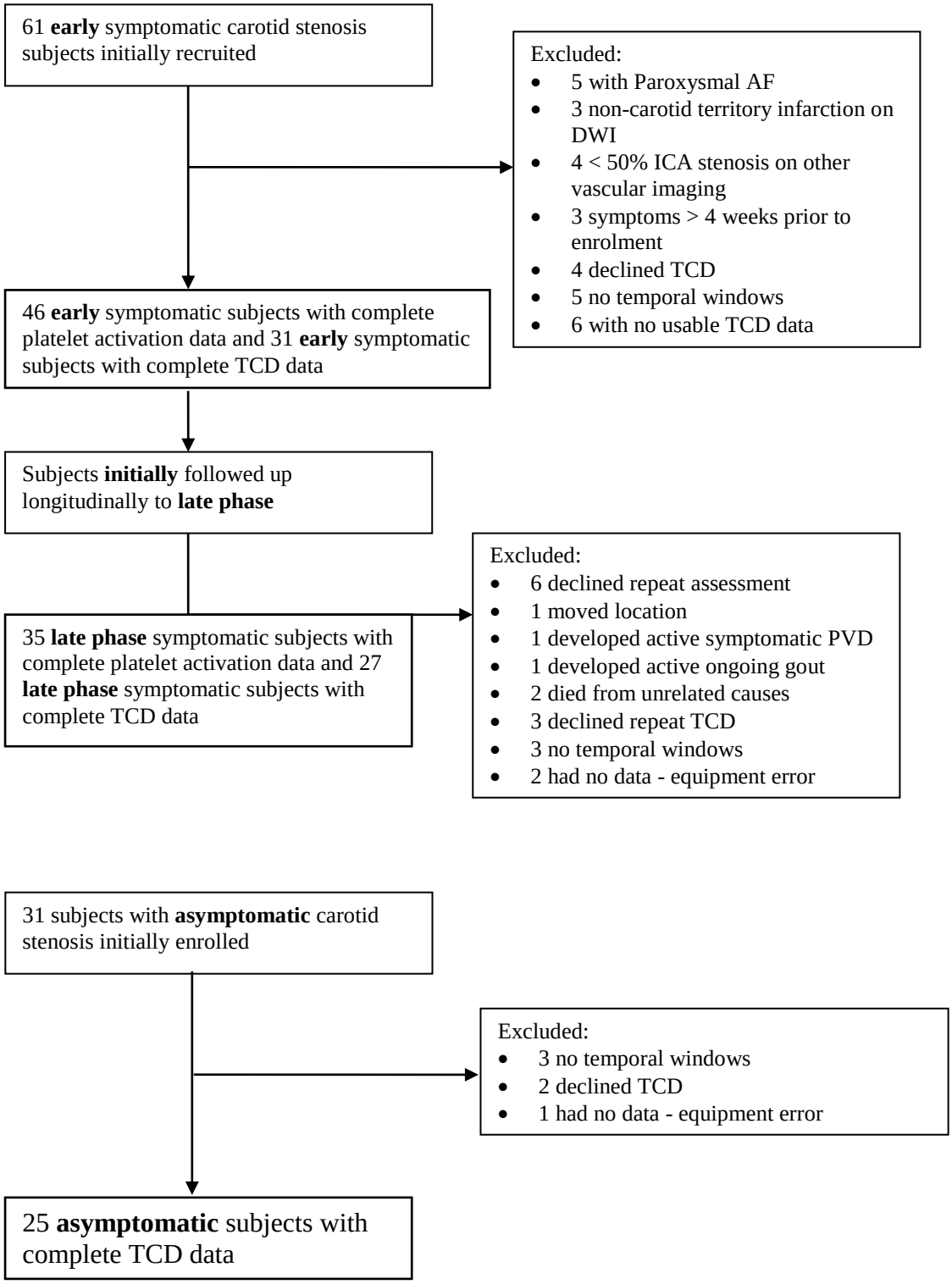


Figure 1: Algorithm of asymptomatic and symptomatic carotid stenosis patients screened and subsequently excluded from the study

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