# Thrombin generation parameters, relationship with cerebral micro-embolic signal status, and a systematic review of the literature on platelet biomarkers in patients with symptomatic and asymptomatic carotid stenosis

A dissertation submitted for a Master in Science (Clinical Medicine),

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# Declaration

I performed all of the studies described in this MSc thesis with the advice and supervision of my colleagues listed in the acknowledgements section below. All of the patients whose samples were analysed to quantify thrombin generation potential for this thesis were recruited and clinically assessed by my predecessors, Dr. Stephen Murphy and Dr. Soon-Tjin Lim, under the close supervision of Professor Dominick McCabe during the **H**a**E**mostasis **I**n carotid **ST**enosis (**HEIST**) study. They performed venepuncture on all study participants and prepared and bio-banked the plasma samples for later analysis. Dr. Murphy also performed transcranial Doppler ultrasound on all eligible patients.

This work partly represents a pre-planned innovative sub-study of the HEIST study. I analysed the bio-banked samples to assess thrombin generation potential in patients with symptomatic and asymptomatic carotid stenosis, with assistance in the performance and interpretation of these assays by my colleagues acknowledged below. I also assessed the relationship between these biomarkers and micro-embolic signal status based on transcranial Doppler ultrasound examination of eligible patients in the HEIST study patient population. I entered all of the data onto a database and performed the statistical analysis of the data with the help and expert supervision of Prof. McCabe.

I also conducted a comprehensive, updated systematic review of the literature on platelet biomarkers in patients with symptomatic and asymptomatic carotid stenosis in collaboration with colleagues.

I declare that this thesis has not been submitted as an exercise for a degree at this or any other University and that it is entirely my own work.

On acceptance, I agree to deposit this thesis in the University's open access institutional repository or allow the library to do so on my behalf, subject to Irish copyright legislation and Trinity College Dublin's library conditions of use and acknowledgement.

*Confirmed electronically by Dr Arun Subramanian on 25th May 2022*

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## Copyright Statement

# Summary

**Background:** Activation of platelets and the coagulation system are integrally involved in acute thrombus formation and distal thrombo-embolism. A better understanding of these complex thrombotic and haemostatic pathways could improve primary and secondary prevention in patients with atherosclerotic carotid stenosis.

**Aims I:** One of the main aims of this thesis was to perform innovative preliminary experiments to assess coagulation system potential in patients with moderate or severe asymptomatic and symptomatic carotid artery stenosis and to assess the relationship between these biomarkers and cerebral micro-embolic signals (MES).

**Aims II:** The co-primary aim of this thesis was to conduct a systematic review to enhance our understanding of the role of platelet biomarkers in the pathogenesis of vascular events and risk stratification in patients with asymptomatic and symptomatic atherosclerotic carotid stenosis.

**Methods I:** An observational analytical study, with longitudinal follow-up in the symptomatic cohort, was performed to assess thrombin generation potential parameters in patients with moderate or severe (≥50-99%) asymptomatic *vs*. early (≤4 weeks) and late phase symptomatic (≥3 months after TIA or stroke) carotid stenosis. Coagulation system/thrombin generation potential was assessed in platelet poor plasma in patients who had been recruited to the **Ha**a**E**mostasis **I**n carotid **ST**enosis (**HEIST**) study. Transcranial Doppler ultrasound was performed to evaluate for the presence of cerebral MES in both study groups.

**Methods II:** A Systematic review was conducted in accordance with the current Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement to collate all available data from 1975-2020 on *ex vivo* platelet activation and platelet function/reactivity in patients with atherosclerotic carotid stenosis.

**Results I:** Data from 34 asymptomatic, 39 'early symptomatic' and 31 'late symptomatic' patients were analysed. Peak thrombin production (365.1 *vs*. 327.2 nM, P = 0.003) and endogenous thrombin potential ETP (2242.6 *vs*. 2078.4 nM*min, P = 0.048) significantly decreased between the early and late phase after symptom onset in symptomatic patients with 'matched data' at each time point, regardless of whether or not they underwent carotid interventional treatment. Of note, peak thrombin levels only decreased in the symptomatic subgroup who underwent carotid intervention between the early phase and late post-intervention phase (P = 0.007) but did not change significantly over time in the symptomatic subgroup who did not have carotid interventional treatment and who were treated with best medical therapy alone. There were no differences in any other thrombin generation parameters over time within subgroups of symptomatic patients who did or did not undergo carotid intervention. Transcranial Doppler ultrasound data were available in 28 asymptomatic, 30 early symptomatic, and 26 late symptomatic patients. In our nested longitudinal study, peak thrombin generation also significantly decreased between the early and late phase after symptom onset in the subgroup of MES-ve symptomatic patients who had matched data at each time point (376.8nM *vs*. 325.4nM; P = 0.029)

**Results II:** For our systematic review, 43 manuscripts met inclusion criteria; the majority included patients on antiplatelet therapy. Five studies showed increased platelet biomarkers in patients with ≥30% asymptomatic carotid stenosis *vs*. controls, with one neutral study. Preliminary data from one study suggest that quantification of 'coated platelets' in combination with stenosis severity may aid risk stratification in patients with ≥50-99% asymptomatic stenosis. Platelets are excessively activated in patients with ≥30% symptomatic carotid stenosis compared with controls (≥11 positive studies and one neutral study). Antiplatelet-High on-Treatment Platelet Reactivity (HTPR), previously called 'antiplatelet-resistance,' was observed in 23 -

57% on aspirin (N = 7/30 - 17/30), with clopidogrel-HTPR in 25-100% (N= 1/4 - 4/4) of patients with ≥50-99% asymptomatic stenosis in the literature. Aspirin-HTPR was noted in 9.5-64% (N = 4/42 - 7/11) and clopidogrel-HTPR in 0-83% (N = 0/6 - 5/6) of patients with ≥50% symptomatic stenosis. Platelets are excessively activated (N=5), with increased platelet counts (N=3) in recently symptomatic *vs*. asymptomatic carotid stenosis patients, including those without micro-emboli on transcranial Doppler monitoring (N=2). Most available studies (7/13) showed that platelets might become more reactive or activated following carotid endarterectomy or stenting, either as an acute phase response to intervention or peri-procedural treatment.

**Discussion and Conclusions:** Thrombin generation potential decreased over time following TIA or stroke associated with recently symptomatic carotid stenosis, especially in patients who underwent intervention and in the subgroup who were MES-ve. These data enhance our understanding of the haemostatic/thrombotic biomarker profiles in patients with moderate-severe carotid stenosis.

Our systematic review has shown that platelets are excessively activated in carotid stenosis patients compared with controls, recently symptomatic compared with asymptomatic carotid stenosis patients, and may become activated/hyper-reactive following carotid intervention despite commonly-prescribed antiplatelet regimens. However, the use of *ex vivo* platelet function/reactivity testing to tailor antiplatelet therapy is not currently recommended outside of a research setting.

Further prospective multicentre studies are required to determine whether models combining clinical, neurovascular-imaging, and thrombin generation/platelet biomarker data can facilitate optimised antithrombotic therapy in individual patients with carotid stenosis.

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# Abbreviations

| | |
|---|---|
| **ACES** | Asymptomatic Carotid Emboli Study |
| **ACS** | Asymptomatic Carotid Stenosis |
| **ADP** | Adenosine Diphosphate |
| **AF** | Atrial Fibrillation |
| **APTT** | Activated Partial Thromboplastin time |
| **ARU** | Aspirin Reaction Units |
| **CAS** | Carotid artery stenting |
| **CCA** | Common Carotid Artery |
| **CD** | Cluster Differentiation |
| **CDUS** | Colour Doppler Ultrasound |
| **CEA** | Carotid Endarterectomy |
| **C-EPI** | Collagen-Epinephrine |
| $\chi^2$ | Chi-Squared statistic |
| **CI** | Confidence Interval |
| **CT** | Computerised Tomography |
| **CTA** | Computerised Tomography Angiography |
| **CVD** | Cerebrovascular Disease |
| **DWI** | Diffusion Weighted Imaging |
| **ETP** | Endogenous Thrombin Potential |
| **FBC** | Full Blood Count |
| **HEIST** | **H**a**E**mostasis **I**n carotid **ST**enosis Study |
| **HITS** | High Intensity Transient Signals |
| **HTPR** | High On-Treatment Platelet Reactivity |

| | |
|---|---|
| **ICA** | Internal Carotid Artery |
| **LMWH** | Low Molecular Weight Heparin |
| **MCA** | Middle Cerebral Artery |
| **MES** | Micro-embolic Signals |
| **MPV** | Mean Platelet Volume |
| **MR** | Modified Release |
| **MRA** | Magnetic resonance angiography |
| **MRI** | Magnetic resonance imaging |
| **NASCET** | North American Symptomatic Carotid Endarterectomy Trial |
| **NSAID** | Non-steroidal Anti-inflammatory Drug |
| **OR** | Odds Ratio |
| **PACS** | Platelets and Carotid Stenosis Study |
| **PAD** | Peripheral Arterial Disease |
| **PFA-100®** | Platelet Function Analyser-100® |
| **PPP** | Platelet Poor Plasma |
| **PRP** | Platelet Rich Plasma |
| **PRU** | P2Y12 Reaction Units |
| **PT** | Prothrombin Time |
| **RP** | Reticulated platelets |
| **SCS** | Symptomatic Carotid Stenosis |
| **SD** | Standard deviation |
| **TCD** | Transcranial Doppler |
| **TIA** | Transient Ischaemic Attack |
| **TNF-α** | Tumour Necrosis Factor-α |
| **TOAST** | Trial of ORG 10172 in Acute Stroke Treatment |
| **tPA** | tissue-type Plasminogen Activator |

# 1. Assessment of thrombin generation parameters and their relationship with micro-embolic signal status in patients with carotid stenosis

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## 1.1. Introduction

### 1.1.1. Overview of Transient Ischaemic Attack (TIA) and Ischaemic Stroke

Stroke is the 2nd leading cause of death (GBD 2016 Stroke Collaborators, 2019) and the most common cause of acquired physical disability in adults worldwide (Gorelick, 2019). All transient ischaemic attacks (TIAs) by definition and the majority (85%) of strokes (Kapila *et al.,* 2019) are ischaemic in origin and are caused by ischaemia or infarction of the brain, eye, or spinal cord. Moderate-severe (50-99%) symptomatic atherosclerotic carotid stenosis is responsible for 10-20 % of all transient ischaemic attacks (TIAs) or acute ischaemic strokes, most commonly due to thromboembolism rather than haemodynamic cerebral ischaemia (Kapila *et al.,* 2019). Previous studies have shown that approximately 4.1% of the population aged between 50-80 years have an asymptomatic moderate (50-69%) stenosis of at least one carotid artery on ultrasound screening, with severe asymptomatic stenosis (≥ 70%) identified in 1.7% (de Weerd et al., 2010). As outlined in section 2.1, the average annual risk of ipsilateral ischaemic stroke was 0.34%. The average annual risk of ipsilateral TIA was 1.78% in the vascular territory supplied by an asymptomatic ≥ 50% carotid stenosis in a population-based study of patients treated with 'best medical therapy'

alone for a TIA or minor ischaemic stroke in another vascular territory (Marquardt et al., 2010). The annual risk of ipsilateral stroke in hospital-based series varied between 0.7% in patients with asymptomatic ≥ 70% carotid stenosis (Markus et al. ACES study, 2010) to 1.4% in patients with asymptomatic ≥ 60% carotid stenosis (Spence *et al.,* 2010) without micro-embolic signals (MES) on transcranial Doppler ultrasound. The risk of recurrent ipsilateral stroke has previously been shown to be as high as approximately 17% in the first year after randomisation in patients with symptomatic 70-99% carotid stenosis in the North American Symptomatic Carotid Endarterectomy Trial (NASCET) treated with best medical therapy alone (NASCET, 1991).

Over the past two decades, translational research studies have improved our understanding of some of the potential mechanisms responsible for this disparity in the risk of cerebrovascular events between patients with symptomatic and asymptomatic moderate-severe carotid stenosis. There are differences in the composition of the atherosclerotic plaques (Golledge *et al.,* 2000; Rothwell *et al.,* 2004), with increased endothelial activation (Kinsella *et al.* 2014), and platelet production, turnover, or activation (McCabe DJ *et al.,* 2005; Kinsella JA *et al.,* 2013; Murphy SJX *et al.,* 2018; Murphy SJX *et al.,* 2019) in symptomatic compared with asymptomatic moderate-severe carotid stenosis. However, there are very limited data on thrombin generation potential in patients with symptomatic *vs*. asymptomatic moderate-severe carotid stenosis (Kinsella JA et al., 2015), and some patients with carotid stenosis have recurrent vascular events despite 'optimal' evidence-based antiplatelet therapy, which is the standard of care in this patient cohort (Naylor AR et al., 2018; Murphy et al., 2019; Naylor AR & McCabe DJ 2020).

### 1.1.2. Endogenous Thrombin Generation

The processes of platelet and coagulation system activation are integrally involved in acute thrombus formation, propagation, and stabilisation, as well as distal thrombo-embolism (Hoffman & Monroe, 2007). Activation of platelets and the coagulation system may occur simultaneously or consecutively and may be initiated by the same agonists in some instances. A better understanding of the complex interaction between activated platelets, the coagulation system, and the endothelium could facilitate improvements in preventing vascular events in patients with ischaemic cerebrovascular disease (CVD) overall, including in the important subgroup of patients with atherosclerotic carotid stenosis.

Tissue Factor (TF) is a transmembrane protein that is present on activated platelets, some white cells (especially monocytes), endothelial cells (Grover *et al.,* 2018), vascular smooth muscle cells, adventitial fibroblasts, pericytes, and atherosclerotic plaques (Grover *et al.,* 2020). Tissue factor acts as a high-affinity receptor for factor VII (Grover *et al.,* 2018) and may form a complex with and activate factor VII to form factor VIIa; the TF-factor VIIa complex can then directly activate factor X to form factor Xa via the extrinsic (tissue factor) coagulation pathway, or can indirectly activate factor X via initial activation of factor IX (Göbel *et al.,* 2018). Factor Xa then catalyses the cleavage of prothrombin (factor II) to form thrombin (factor IIa) (Göbel *et al.,* 2018), but *in vivo*, thrombin may be formed from prothrombin as a result of activation of either the intrinsic (contact) or extrinsic pathways of the coagulation cascade (Al Dieri *et al.*, 2012). Activated factor Xa in complex with its co-factor Factor Va forms the 'prothrombinase complex,' which also catalyses the conversion of prothrombin to thrombin. Thrombin is an active protease enzyme that plays a central role in blood coagulation and pro-atherogenic pathways (Figure 1.1)

and catalyses the cleavage of fibrinogen to form fibrin monomers, which upon polymerisation, form the fibrin component of a blood clot (Göbel *et al.,* 2018). Once created, thrombin stimulates its own generation through activation of factor XI in the intrinsic pathway and via activation of other co-factors (factor V and factor VIII). Thrombin also attenuates its own formation via activation of protein C to form activated protein C (APC) (Figure 1.1 - adapted with permission from Kinsella JA, Ph.D. Thesis, TCD, 2012).

Platelet activation is increased in patients in both the early and late phases after TIA or ischaemic stroke (McCabe *et al.*, 2004). Elevation in cytosolic calcium during platelet activation induces procoagulant phosphatidylserine (PS) exposure on the platelet surface, thus promoting assembly of tenase and prothrombinase complexes and subsequent accelerated thrombin generation (van der Meijden *et al.*, 2005). Thrombin is also a potent platelet agonist, and during thrombus formation, it activates platelets by binding to the GpIb-IX-V complex (Berndt *et al.*, 2001). However, ADP-induced activation of the P2Y12 ADP receptor on platelets increases the time taken to achieve peak thrombin formation. It may serve as a type of 'negative feedback mechanism' to decrease the rate of thrombin generation (van der Meijden *et al.*, 2005). A sub-population of activated platelets may also separate from the propagating thrombus, bind to circulating platelet microparticles via interactions between CD62P and P-selectin glycoprotein 1 (PSGL-1), and subsequently increase the concentration of microparticles expressing monocyte-derived tissue factor at the thrombus surface (Kappelmayer & Nagy, 2017). Activated platelets and endothelial cells may also release protein disulfide isomerase at the site of vascular injury, which catalyses the 'decryption' of platelet microparticle-bound tissue factor, facilitating further activation of the coagulation system via the tissue factor pathway. Insight into these

interactions could improve our understanding of why some patients are not protected from additional vascular events and why other patients experience bleeding complications on specific antiplatelet regimens.

Elevated endogenous thrombin potential (ETP) and peak thrombin levels have been observed in patients with venous thromboembolism (VTE) and acute coronary syndromes (Schöchl et al., 2014). Tripodi *et al*. reported differences in thrombin generation kinetics between these disease states, elevated ETP and peak thrombin generation in patients with VTE, and high lag time and time to peak thrombin generation in patients with ischaemic heart disease (Tripodi *et al.*, 2008).

Further clarification of the profile of thrombin generation parameters in patients with carotid stenosis could assist with determining whether there is a role for treatment with, e.g., direct thrombin inhibitors or other anticoagulation regimens in selected patients with asymptomatic or symptomatic moderate-severe carotid stenosis (Balogun *et al.*, 2016).

### *1.1.2A. Background Literature on Thrombin Generation Parameters in Ischaemic Cerebrovascular Disease*

To our knowledge, only four studies have investigated thrombin generation potential in the early and/or late phases after TIA or ischaemic stroke overall (Faber *et al.*, 2003; van der Meijden *et al.*, 2005; Balogun *et al.*, 2016), with only one study assessing ETP in **both** the early and late phases after TIA and ischaemic stroke (Tobin *et al.*, 2013). Faber *et al*. compared endogenous thrombin potential (ETP), platelet-derived procoagulant activity, VWF Ag levels, plasma fibrinogen levels, and antithrombin activity in 41 patients < 50 years old at least three months after a non-cardioembolic

ischaemic stroke with 70 age- and gender-matched healthy controls (Faber *et al.*, 2003). Prescribed antiplatelet medications at the time of study entry were not reported. ETP data were reported as a percentage of 'pooled normal plasma' for the platelet-poor plasma (PPP) samples, but as a percentage of the 'age- and gender-matched control' samples for results in platelet-rich plasma (PRP). There was no significant increase in ETP in non-cardioembolic stroke patients compared with pooled normal PPP samples, but stroke patients had a higher median ETP than age-and gender-matched controls in PRP ($P < 0.01$). The authors suggested that the finding of increased coagulation system potential (i.e., ETP) in this patient cohort in PRP alone might have been mediated by platelet surface-derived procoagulant factors in PRP. Subsequently, Van der Meijden *et al*. investigated peak thrombin, time-to-peak thrombin, and ETP in PRP from 11 'younger' (Age: 32 - 51 years) and nine older stroke patients (Age: 60- 74 years) within 3-12 months of an ischaemic stroke, and also in 12 patients with type-II diabetes mellitus (26 - 73 years) and 11 healthy controls whose age was not outlined in the manuscript (van der Meijden *et al.*, 2005). The nine older stroke patients were also re-assessed two weeks after introducing antiplatelet monotherapy with 75mg of clopidogrel daily. The authors did not specify the proportion of patients on aspirin or the prescribed aspirin dose at the study entry. Peak thrombin generation was higher in PRP in the older stroke and type II diabetic patients than in healthy controls ($P \leq 0.05$). The authors commented that platelets in PRP might have contributed to elevated thrombin generation potential in patients compared with measurements from PPP samples. There were no significant differences in any thrombin generation potential markers between young stroke patients and controls. However, the number of subjects in each subgroup was far too small to make any definite conclusions.

The pilot, longitudinal study by Tobin *et al.* was the first study to prospectively quantify thrombin generation potential in patients who had started or changed antiplatelet therapy following a TIA or ischaemic stroke (Tobin *et al.*, 2013). The authors found that the addition of dipyridamole to aspirin may reduce peak thrombin and total thrombin generation *ex vivo* following TIA or ischaemic stroke overall. However, there were no consistent effects of commencing aspirin or changing from aspirin to clopidogrel on thrombin generation potential during follow-up. The potential clinical relevance of these findings needs to be assessed in a larger, longitudinal study to determine whether there is a subgroup of patients in whom thrombin generation is inhibited who are at a lower risk of recurrent vascular events than those in whom thrombin generation potential is not altered. In addition, further longitudinal studies are needed to investigate whether patients in whom thrombin generation is markedly inhibited are at a higher risk of bleeding complications during long-term follow-up.

Therefore, the limited data available prior to this thesis suggested that endogenous thrombin potential in PRP may be elevated in younger (Faber *et al.*, 2003) or older patients (van der Meijden *et al.*, 2005) following ischaemic stroke. However, the findings in younger patients have not been replicated to date. Furthermore, the available pilot data suggest that thrombin generation potential may be influenced by the antithrombotic regimens prescribed for these patients (Tobin *et al.*, 2012).

### *1.1.2B. Background Literature on Thrombin Generation in Subgroups of Patients*

With Noto *et al*. performed a case-control study to assess the relationship between carotid plaque morphology on ultrasound and 'indirect' markers of thrombin

generation in 128 patients with ≥ 35% carotid stenosis and 136 age- and sex-matched controls without carotid stenosis (With Noto et al., 2008). Thrombin-antithrombin complexes (TAT) which are formed by the binding of thrombin to antithrombin following thrombin generation, and prothrombin fragments 1 and 2 (F1 and F2), which are generated during the conversion of prothrombin to thrombin (Fareed *et al.*, 1998), were measured. The authors did not provide information regarding prescribed antiplatelet regimens. Patients with echogenic plaques [N = 63] had higher levels of TAT (5.24 μg/l) than those with echolucent plaques (3.44 μg/l, N = 65; P < 0.001) and controls (3.33 μg/l; N = 136; P < 0.001). Furthermore, F1 and F2 levels were higher in patients with echogenic plaques (2.14 nM) than in those with echolucent plaques (1.54 nM; P < 0.001) or controls (1.49 nM; P < 0.001). TAT (P = 0.002) and F1 and F2 levels (P = 0.001) increased in association with more marked plaque echogenicity, independent of the degree of stenosis. These findings are open to being interpreted as suggesting that patients with echogenic carotid plaques, which are often considered to be more stable and less likely to undergo rupture and cause symptoms (Gupta *et al.*, 2015), are more likely to have increased thrombin generation than patients with 'echolucent plaques.' However, some recent studies have not found clear evidence of increased plaque echolucency in patients with recently symptomatic compared with asymptomatic carotid stenosis (Murphy et al., 2019; Kolkert et al., 2015), so one cannot simply dichotomise patients with carotid stenosis into higher or lower risk groups based on plaque echogenicity. Furthermore, because a threshold of ≥ 35% carotid stenosis was chosen to select patients for this study, many of these patients may have had mild carotid stenosis, which is less likely to be relevant to the aetiology of TIA or ischaemic stroke in many cases.

One cross-sectional case-case/control study compared plasma markers of 'haemostasis or fibrinolysis' in consecutive patients with symptomatic *vs*. 'asymptomatic' severe (≥70% NASCET) carotid stenosis (Soinne *et al.*, 2005). Fifty-four symptomatic patients were assessed within a median of 41 days after TIA or stroke onset in the vascular territory supplied by a severely stenosed carotid artery of interest, 20 patients had symptoms in another vascular territory, and the plaque of interest was deemed to be 'asymptomatic' (median duration from symptom onset of 106 days), and 18 patients were 'truly asymptomatic.' In the 'symptomatic plaque group' (N = 54), 19 patients were on anticoagulation (mainly warfarin). One patient who had a TIA or stroke in another vascular territory was also on anticoagulation and was included in the overall 'asymptomatic group.' Plasma levels of prothrombin fragments F1 and F2, TAT, plasminogen activator inhibitor-1 (PAI-1), and tissue-type plasminogen activator (tPA) antigen and activity were measured. Plasma TAT levels were reduced in the 54 symptomatic compared with the 18 truly asymptomatic carotid stenosis subjects overall (2.2 *vs*. 4.0 μg/l; P = 0.006). However, when the authors confined the analysis to the subset of symptomatic patients who were not on anticoagulants (N = 35), the difference in plasma TAT levels between symptomatic and asymptomatic carotid stenosis patients was no longer statistically significant (2.6 *vs*. 4.0 μg/l; P = 0.05). There were no other differences in plasma markers between the symptomatic and asymptomatic groups. The indications for anticoagulation were unclear because patients with a cardioembolic source of embolism were excluded from the study. This patient population is not representative of currently-treated cohorts of patients with severe carotid stenosis. Evidence-based practice now recommends optimal antiplatelet therapy rather than anticoagulation with warfarin (Naylor et al., 2018; Murphy et al., 2018; Naylor AR & McCabe DJH, 2020). Therefore, one should not conclude that indirect markers of thrombin

generation are reduced in the subacute phase after symptom onset in patients with severe carotid stenosis compared with their asymptomatic counterparts.

A subsequent study assessed the concentration of TAT complexes and antithrombin (AT) in carotid plaques with an ELISA technique and also quantified levels of these biomarkers in platelet-poor plasma (PPP) from patients with symptomatic (N = 20) and asymptomatic (N = 18) carotid stenosis (mean stenosis severity of 75%; range: 50-95%) (Migdalski *et al.*, 2004). Symptomatic plaques contained a higher concentration of TAT complexes than asymptomatic plaques (20.35 *vs*. 11.08 ng/mg of protein; $P < 0.04$). There was no difference in plasma TAT concentration between symptomatic and asymptomatic patients ($P > 0.05$). These findings may indicate that plaque rupture, and perhaps local thrombus formation may lead to thrombin generation in the focal milieu of a recently symptomatic plaque. That plasma assays of TAT are not a sensitive method of detecting differences in thrombin generation potential between symptomatic and asymptomatic patients.

Cote *et al*. prospectively measured plasma TAT and F1 and F2 levels in 82 patients within seven days of TIA onset, 41% of whom had ≥50% recently symptomatic carotid stenosis; the authors did not provide any further details re the degree of carotid stenosis. They compared their data with 157 asymptomatic patients with a cervical bruit (56% of whom had ≥ 50% carotid stenosis) and 65 healthy controls (Cote *et al.*, 2000). Detailed information regarding prescribed antiplatelet regimens or the same dose was not provided aside from mentioning that a 'substantial proportion' of patients at baseline took aspirin. There were no differences in TAT or F1 and F2 levels between symptomatic and asymptomatic patients or controls ($P \geq 0.09$). However, F1 and F2 levels were found to be independent predictors of subsequent cerebral or cardiac ischaemic events in asymptomatic patients with a cervical bruit

(relative risk (RR) = 1.70; 95% CI: 1.14-2.53), and of MI, ischaemic stroke or vascular death in patients following TIA (RR = 2.36; 95% CI: 1.19-4.68) over a mean follow-up period of 2.83 years. These data suggest that quantifying plasma TAT does not improve our understanding of the mechanisms responsible for a TIA or stroke in patients with symptomatic *vs*. asymptomatic carotid stenosis. However, interpretation is limited by the fact that identifying a cervical bruit is neither a sensitive or specific method of detecting carotid stenosis (Cote *et al.*, 2000), and the majority of 'symptomatic patients' in this study had < 50% stenosis.

One pilot study by our research group assessed thrombin generation potential in both the early and late phases after TIA or ischaemic stroke in patients with 50-100% symptomatic *vs*. 50-99% asymptomatic carotid stenosis (Kinsella *et al.,* 2015). Thrombin generation parameters were longitudinally assessed in symptomatic patients with data at each time point. Bilateral transcranial Doppler ultrasound monitoring of the middle cerebral arteries was performed whenever possible to classify patients as micro-embolic signal (MES)-positive or MES-negative. This study showed that thrombin generation potential (peak thrombin and ETP) was enhanced in a clinically well-defined cohort of patients with recently symptomatic compared with asymptomatic moderate-severe carotid stenosis, as well as in the subgroup with severe recently symptomatic versus severe asymptomatic stenosis. Furthermore, peak thrombin production decreased in symptomatic patients followed up from the early to late phase after TIA or stroke (P = 0.02), with some evidence of a more procoagulant state in recently symptomatic MES-positive compared with asymptomatic MES-positive patients (shorter 'time-to-peak thrombin,' P = 0.04).

Overall, these data suggest a potentially important role of the coagulation system in the pathogenesis of vascular events in some patients with TIA or ischaemic stroke,

including those with carotid stenosis, but further studies were warranted in patients with carotid stenosis without ICA occlusion.

Therefore, the HEIST study was designed to address this issue in an independent cohort of patients with recently symptomatic *vs*. asymptomatic ≥50-99% ICA stenosis and in symptomatic patients who were followed up from the early to late phase after symptom onset or intervention.

### 1.1.3. Transcranial Doppler (TCD)

As previously described by my *post-doc* collaborator for this thesis, Dr. Stephen Murphy, transcranial Doppler ultrasound (TCD) is a non-invasive imaging technique that visualises blood flow in the cerebral circulation by insonating via low density 'bone windows' which are naturally present in the skull (Murphy, 2016). Micro-emboli entering the cerebral circulation can be detected by their characteristic acoustic signature and are referred to as high-intensity transient signals (HITS). HITS are used to categorise patients as micro-emboli positive or negative (MES +ve / MES -ve). To detect emboli arising from the carotid artery, the middle cerebral artery (MCA) can be insonated via a trans-temporal bone window (Mackinnon et al., 2005). A prior systematic review has shown that MES can be detected in up to 43% of patients with symptomatic carotid stenosis and up to 10% with asymptomatic >50% carotid stenosis (Ritter *et al.,* 2009), usually based on one-hour TCD recordings of the MCA. Furthermore, a meta-analysis has shown that the presence of MES predicted both stroke alone (OR: 9.57; $P = 0.02$) and stroke or TIA (OR: 6.36; $P < 0.0001$) in patients with symptomatic carotid stenosis (King and Markus, 2009). MES also predicted stroke alone (OR: 7.46; $P = 0.001$) and stroke or TIA (OR: 12.00; $P = 0.002$) in patients with asymptomatic carotid stenosis. A 'high frequency' of MES immediately after carotid endarterectomy also predicted recurrent stroke alone (OR: 24.54; $P < 0.0001$) and stroke or TIA (OR: 32.04; $P < 0.0001$) (King and Markus, 2009). The multicentre Asymptomatic Carotid Emboli Study (ACES) subsequently found that the presence of MES on TCD in patients with ≥ 70% asymptomatic carotid stenosis identified those at a higher risk of stroke or TIA (Markus et al. 2010). The hazard ratio for the risk of ipsilateral stroke and TIA from baseline to 2 years in MES +ve compared with MES negative patients was 2.54 (95% CI: 1.20–5.36; $P = 0.015$),

thus indicating that assessment of MES status on TCD might be useful in selecting patients with asymptomatic carotid stenosis who are likely to benefit most from carotid intervention or alteration of their medical therapy. Other data also support the concept that MES predict subsequent TIA or ischaemic stroke risk in patients with >60% (Spence *et al.,* 2010; Spence *et al.,* 2005; Madani *et al.,* 2011; Molloy *et al.,* 1999) and ≥70% asymptomatic carotid artery stenosis (Markus *et al.,* 2010; Topakian *et al.,* 2011).

To our knowledge, prior to the conduct of this thesis, simultaneous quantification of thrombin generation potential and cerebral MES on TCD had only been performed in one study by our research group in patients with symptomatic versus asymptomatic carotid stenosis (Kinsella, 2015). Therefore, prospective studies in carefully phenotyped patients with recently symptomatic compared with asymptomatic moderate-severe carotid stenosis, in whom surgical or endovascular intervention might be considered, were warranted to address this topic further.

### 1.1.4. Aims

The **aims** of this aspect of the HEIST study were to determine whether:

**1.** Thrombin generation parameters differ between patients with recently symptomatic versus asymptomatic moderate-severe carotid stenosis;

**2.** We could confirm prior pilot data from our research group that thrombin generation parameters decrease over time in patients with recently symptomatic moderate-severe carotid stenosis followed up from the early to the late phase after symptom onset or carotid intervention;

**3.** There are observed differences in profiles of thrombin generation parameters in asymptomatic and symptomatic moderate-severe carotid stenosis subgroups who are classified as MES+ve or MES-ve on TCD.

### 1.1.5. Hypotheses

We **hypothesised** that:

**1.** Patients with recently symptomatic carotid stenosis would have some markers of increased thrombin generation potential compared with patients with asymptomatic carotid stenosis;

**2.** Thrombin generation potential would decrease over time in patients with recently symptomatic carotid stenosis who were followed up from the early to the late phase after symptom onset on 'best medical therapy' or following carotid interventional treatment.

**3**. Based on pilot data from our research group, thrombin generation potential data might be informative in symptomatic patient subgroups stratified according to MES status.

## 1.2. Methodology

The general methodology pertaining to clinical screening, recruitment, and assessment of patients for this aspect of the HEIST study has been described in detail by my *post-doc* collaborator, Dr. Stephen Murphy, with whom I closely collaborated to write the methodology section of this thesis. Much of this information has previously been published in detail in Dr. Murphy's Ph.D. thesis entitled 'Markers of Platelet Activation and Function and their relationship to Cerebral Micro-Embolic Signals in Symptomatic and Asymptomatic Carotid Stenosis' (Murphy, Ph.D., TCD 2016). Further information is outlined in Dr. Murphy's MSc thesis entitled: 'Profiles of von Willebrand factor antigen, von Willebrand factor propeptide and ADAMTS13 activity in patients with carotid stenosis and their relationship with cerebral micro-embolic signal status' (Murphy, MSc, TCD 2020). However, I will summarise the relevant clinical methodology below and outline the details of the laboratory assays I used to assess thrombin generation parameters on the HEIST patient cohort. I will also describe the transcranial Doppler ultrasound (TCD) methodology employed by Dr. Murphy on the patients recruited to the HEIST study.

### 1.2.1. Study Subjects

#### *1.2.1A. Ethical approval*

The study was fully approved by the St. James's Hospital / AMNCH-Tallaght University Hospital Joint Research Ethics Committee (JREC) [Project/REC References: 2011/31/02; 2017-07 List 25 (12); 2019-07 List 25 (14)]. Written informed consent was obtained in all cases following detailed discussions regarding the nature of the study and procedures, and after all, patients were given detailed study information sheets to read.

#### *1.2.1B. Inclusion criteria*

***Symptomatic moderate or severe carotid artery stenosis***

Consecutive, eligible patients > 18 years old were recruited to the '**symptomatic carotid stenosis group'** if they had had a TIA or ischaemic stroke in the vascular territory supplied by a moderate (≥ 50-69%) or severe (≥ 70-99%) ipsilateral carotid artery stenosis within the preceding four weeks (**early phase**). Most patients had their stenosis initially identified on colour Doppler ultrasound using standardised velocity criteria (Grant et al., 2003; Sidhu and Allan, 1997). Patients were classified as having 50-69% internal carotid artery stenosis on colour Doppler ultrasound if the peak systolic velocity in the internal carotid artery ($PSV_{ICA}$) was ≥ 130 cm/s but < 230 cm/s, the end-diastolic velocity in the internal carotid artery ($EDV_{ICA}$) was < 110 cm/s, and the ratio of the $PSV_{ICA}$ to the PSV in the common carotid artery ($PSV_{CCA}$) was < 4.0. The ICA stenosis severity was considered ≥ 70% if the $PSV_{ICA}$ was ≥ 230 cm/s, the $EDV_{ICA}$ was ≥ 110 cm/s, or the $PSV_{ICA}$: $PSV_{CCA}$ ratio was ≥ 4.0 (Sidhu and Allan 1997). The degree of stenosis was confirmed on extracranial MRA or CTA as part of routine clinical workup in all cases. Symptomatic patients were followed up and re-

assessed in the '**late phase'** ≥ 3 months after symptom onset if treated with the best medical therapy alone or ≥ three months after surgical/endovascular intervention (Murphy, 2020).

***Asymptomatic moderate or severe carotid artery stenosis***

Consecutive, eligible patients > 18 years old with 'non-occluded moderate (≥ 50-69%) or severe (≥ 70-99%) asymptomatic carotid artery stenosis, incidentally identified on colour Doppler ultrasound using standardised velocity criteria outlined above (Grant et al. 2003; Sidhu and Allan 1997), were recruited to this study (Murphy, 2020). Patients were classified as 'asymptomatic' if they had never had symptoms in the vascular territory of the stenosed carotid artery of interest or had no history of TIA or stroke in the preceding 3 years in the carotid or any other cerebrovascular territory. Some asymptomatic patients were identified after referral to investigate an incidental carotid bruit, during routine external screening, or, e.g., during routine vascular workup before coronary artery bypass surgery. The degree of carotid stenosis was confirmed on extracranial MRA or CTA in 32 out of the 34 (94%) recruited asymptomatic patients (Murphy, 2020).

### *1.2.1C. Exclusion criteria for patients with asymptomatic or symptomatic carotid stenosis (Murphy, 2020)*

- Ipsilateral extracranial carotid occlusion in symptomatic patients, and bilateral carotid occlusion in asymptomatic patients
- Known bleeding or clotting diathesis, including known platelet-related bleeding disorders
- Platelet count < 120 x $10^9$/L or > 450 x $10^9$/L
- Myocardial infarction, PE or DVT within the preceding 3 months

- Prior history of primary intracerebral haemorrhage
- Ongoing unstable angina or unstable symptomatic peripheral vascular disease
- Active vasculitis, active neoplasia or other inflammatory conditions
- Major surgery within the preceding 3 months
- Systemic haemorrhage within the preceding three months (Haemoglobin drop of >1g/dl in one day, or requiring transfusion)
- Current active infection (Clinical signs of infection, white cell count > 11 x $10^9$/L)
- NSAID intake other than aspirin within the previous 14 days
- Renal impairment e.g. urea > 10mmol/l or GFR < 30ml/min
- Prior history of paroxysmal atrial fibrillation
- Patients were subsequently excluded from the symptomatic group if there was evidence of a potential cardioembolic source of embolism causing their TIA or stroke that emerged in the 3 months after recruitment e.g. paroxysmal atrial fibrillation on a subsequent ECG or 24 hour Holter monitoring.

### *1.2.1D. Recruitment Source*

Patients were recruited from the Rapid Access Stroke Prevention Clinic, Neurology, Age-Related Health Care or Vascular Surgery clinics, and Acute Stroke Unit and inpatient Neurology, Age-Related Health Care or Vascular Surgery services TUH-AMNCH. Other patients were recruited from the Medicine for the Elderly / Stroke Service and Vascular Surgery outpatient and inpatient services at St James's Hospital (Murphy, 2020).

### *1.2.1E. Baseline Clinical Assessment*

Prior to study inclusion, all other potential 'non-carotid sources' of TIA and ischaemic stroke were excluded by comprehensive neurovascular workup, coordinated by the patients' treating physician according to prior ESO guidelines (European Stroke Organisation Executive Committee 2008), (Murphy, 2020). As described previously, all participants also underwent a specific research study assessment by one of three examiners before study recruitment [Dr. Stephen Murphy (SJM), Dr. Soon-Tjin Lim (STL), and/or Prof Dominick J H McCabe (DJHM)] using a standardised study proforma. 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, smoking status, alcohol intake, illicit substance intake, and the method of detection of carotid stenosis was collected prospectively. Details regarding medication intake, including anti-thrombotic therapy regimens, dose, and duration of therapy, were recorded. If their treating physician altered antiplatelet treatment in the early phase after presentation, patients were invited to undergo repeat blood testing approximately 14 days later if they had not undergone carotid intervention by that stage. Results of available routine haematological (FBC, ESR,

B12, and serum folate), coagulation (PT/APTT), and biochemical profiles (renal and liver profiles, fasting blood glucose, and lipid profile testing, HbA1C, TFTs) were collected prospectively. Homocysteine levels were checked in all symptomatic patients in the latter part of the study. CT of the brain and/or MRI of the brain (with FLAIR, T2-weighted, T1-weighted, DWI, and T2* sequences, unless MRI was contraindicated), colour Doppler ultrasound of carotid and vertebral arteries, and extracranial CTA or MRA, chest radiograph, electrocardiograph (ECG), 24 hour ECG/Holter monitoring, transthoracic echocardiography (TTE) or trans-oesophageal echocardiography (TOE) with bubble and Valsalva were performed in all symptomatic patients, as deemed appropriate by the treating physician. All symptomatic patients met diagnostic criteria for inclusion in the 'large artery atherosclerotic' TIA and ischaemic stroke TOAST subgroup, as confirmed in all cases by an experienced Research SpR in Vascular Neurology (SJM or STL), and/or by an experienced Consultant Vascular Neurologist (DJHM). Any patients in whom there was an initial concern about the precise underlying TIA or stroke subtyping were discussed and finally subtyped with Prof. McCabe; these patients were only included in the study if they fulfilled all criteria for classification as a large artery atherosclerotic TIA or ischaemic stroke in association with ipsilateral 50-99% carotid stenosis (Murphy, 2020).

### 1.2.2. Study design

(I) ***Case:Case/Control Study:*** Data from patients with asymptomatic ≥ 50-99% carotid stenosis were compared with those from patients with early and late phase symptomatic ≥ 50-99% carotid stenosis.

(II) ***Nested Longitudinal Study in Symptomatic Patients:*** Matched data were compared from the same symptomatic patients who were assessed in both the early (within 4 weeks of symptom onset) and late phases (> 3 months) after TIA or ischaemic stroke onset or carotid intervention.

### 1.2.3. Sample collection and separation

#### *1.2.3A. Sample collection*

After resting for at least 20-30 minutes to minimise platelet activation *in vivo*, careful venepuncture was performed in all patients using a standardised protocol, as described previously (Murphy, 2020). Blood was taken from a free-flowing vein using a sterile 21G Butterfly® needle (Venisystems™, Abbott, Ireland) and a Vacutainer® system with a Luer adaptor (Becton Dickinson Vacutainer® Systems, U.K.). A tourniquet was applied to the arm and was released during collection of the first 3 ml of blood that was drawn into a 3 ml sterile Vacutainer® tube containing 3.2% (0.105M) buffered sodium citrate and subsequently discarded. Three other 3.2% citrate-anticoagulated samples drawn during the venepuncture procedure were used to prepare platelet-poor-plasma (PPP), which was later used for the thrombin generation potential assays outlined in this thesis (see below).

#### *1.2.3B. Sample separation*

Separation of plasma samples was performed in all subjects within 60 minutes of venepuncture, and the samples were frozen at -70oC to -80°C within 90 minutes of venepuncture, unless stated otherwise (Murphy, 2020).

#### *1.2.3C. Platelet poor plasma (PPP)*

PPP was prepared from three 0.105M (3.2%) buffered sodium citrate anticoagulated blood samples within one hour of venepuncture (Murphy, 2020; Murphy, 2016). The samples were centrifuged at 2250 G for 15 minutes at room temperature. A plastic Pasteur pipette was used to carefully aspirate the upper two-thirds of each sample into 12 x 75 mm polypropylene sample tubes. These were then centrifuged again at 2250

G for 15 minutes. The double-spun PPP was recovered from the upper two-thirds of these samples and aliquoted into three polypropylene tubes (Sarstedt®, Germany) which were immediately frozen at -70oC to -80°C for later analysis of thrombin generation potential. The bottom third of each sample was also stored, but this sample was **not** considered 'double-spun PPP.'

### 1.2.4. Thrombin Generation Potential

#### *1.2.4A. General principle*

As stated in the introduction to this thesis, platelet activation is also intricately linked to activation of the coagulation system (Monroe *et al.*, 2002). In this thesis, thrombin generation potential was assessed as a marker of 'coagulation system potential.'

#### *1.2.4B. Laboratory Methods*

***Thrombin Generation Parameters:***

This assay was based on the methodology described by Dr Justin Kinsella when he was a PhD student with Prof. McCabe's Vascular Neurology Research Group at AMNCH-TUH/TCD and collaborated with Prof. James's O'Donnell's group at St James's Hospital (Kinsella, 2012).

***Reagents***

- 5pM PPP-Reagent, containing a mixture of phospholipids and tissue factor (Thrombinoscope BV, Netherlands)
- Thrombin Calibrator (Thrombinoscope BV, Netherlands)
- Fluo-Substrate, containing the fluorogenic substrate solubilised in dimethyl sulfoxide (DMSO) (Thrombinoscope BV, Netherlands).
- Fluo-Buffer, containing Hepes (pH 7.35) and Calcium Chloride. (Thrombinoscope BV, Netherlands)
- Fluroskan Ascent® microplate fluorometer with Thrombinoscope™ software (Thrombinoscope BV, Netherlands)

***Thrombin Generation Potential Assay***

As described in the Introduction, thrombin generation potential was measured as previously described (Faber *et al.,* 2003; Kinsella, 2012). 20 µl of 5 pM PPP-Reagent, containing a mixture of phospholipids and Tissue Factor (Thrombinoscope BV, Netherlands), was added to 80 µl of platelet-poor plasma in a polystyrene 96-well plate, in triplicate. In the fourth well for each patient, 20 µl of Thrombin Calibrator (Thrombinoscope BV, Netherlands) was added to 80 µl of platelet-poor plasma from the same patient. No thrombin production took place in this well, and it served as an internal control from which thrombin production in the other three wells was calculated. The 96-well plate was pre-heated to 37°C for 5 min in a Fluroskan Ascent® microplate fluorometer with ThrombinoscopeTM software (Thrombinoscope BV, Netherlands). Fluo-Buffer (Thrombinoscope BV, Netherlands), containing Hepes (pH 7.35) and Calcium Chloride, was warmed in a water bath at 37oC before the experiment. Fluo-Substrate (Thrombinoscope BV, Netherlands), comprising the fluorogenic substrate solubilised in DMSO, was added to the warmed Fluo-Buffer shortly before the experiment and loaded onto the Fluroskan Ascent® microplate fluorometer. The device automatically dispensed pre-mixed and warmed Fluo-Buffer and Fluo-Substrate into each of the wells. This mixture re-calcified the citrated plasma, and thrombin generation commenced after that. After the addition of 20 µL FluCa, the final reaction mixture contained 5 pM Tissue Factor and 4 µM phospholipids. The fluorescent signal, which corresponds to the degree of thrombin generation, was automatically measured every 20 seconds. Thrombin generation curves were calculated for each of the three wells per patient, using the internal control to correct for patient-to-patient differences in colour of plasma, inner filter effect, and substrate consumption. Thrombin generation curve values were averaged

over the three tested wells for each patient. ‘Lag time,’ ‘thrombin spark’ (min), ‘peak thrombin’ generated (nM), ‘time to peak thrombin’ (ttPeak; min), ‘start-tail’ time to end of thrombin generation (min), and endogenous thrombin potential (ETP, nM*min) were quantified for each patient in plasma (Figure 1.2 – pasted below, with permission, from Dr. Justin A Kinsella’s Ph.D. thesis). Lag time represents the time to initiate thrombin generation and happens to correspond to the clotting time because a clot appears when roughly 1% of thrombin is formed; a shorter lag time corresponds with a more pro-coagulant sample. Peak thrombin represents the maximum concentration of thrombin that a given sample is capable of generating. The time to peak thrombin is a measure of how quickly maximal thrombin generation can be reached; a shorter time to peak thrombin represents a more pro-coagulant sample. The start-tail represents the total time taken to generate thrombin; longer start-tail times indicate that a more persistent stimulus to thrombin generation is present. Therefore, samples with longer start-tail times are believed to be more pro-coagulant. Endogenous thrombin generation potential corresponds to the total amount of thrombin produced (Kinsella, 2012).

### 1.2.5. Transcranial Doppler Ultrasound Detection of Cerebral Micro-embolic signals

#### *1.2.5A. Transcranial Doppler Ultrasound (TCD)*

All TCD recordings were performed on-site by my predecessor, Dr. Stephen Murphy, with a single machine (Viassys Pioneer TC8080) using the same operational settings and emboli-detection thresholds for the entire study duration. The technique employed by Dr. Murphy has already been described in detail in his MSc thesis (Murphy, 2020) and other recent papers (Murphy *et al.,* 2020; Murphy *et al.,* 2021) but will be described again below for the readers of this thesis. One-hour bilateral, simultaneous TCD recordings of the middle cerebral arteries (MCAs) were planned to detect high-intensity transient signals (HITS) indicative of micro-emboli (Kaczynski et al. 2018; Molloy and Markus 1999). The arteries were insonated through the temporal windows at 45-60mm, per published criteria (Ringelstein *et al.,* 1998). The 2 MHz probes were coated with conducting gel and fixed in place using a 3-axis clamp with the patient in a supine position. A standardised sample volume range of 5-12 mm was used, with a constant sweep speed of 5.1 seconds. An automated 128-point fast-Fourier transform (FFT) spectral analysis was performed, giving an overlap of > 50%. One full hour of simultaneous recordings was planned and performed in most cases. However, patient discomfort from the Viassys headband necessitated the termination of two recordings after approximately 45 minutes. However, these data were still included in the analyses of the '1-hour' recordings in this study. The spectral waveform and MES data were recorded on the system's hard drive. The full recordings were analysed offline at TUH-AMNCH by Dr. Stephen Murphy, who was fully trained to perform and analyse TCD data by our research group, with external validation of his findings (see below).

### *1.2.5B. Definition of MES+ve and MES–ve patients and inter-operator standardisation (Murphy, 2020),*

HITS were classified according to standardised international criteria by their:

**1.** Typical visual appearance and orientation within the spectral waveform display;

**2.** Characteristic high pitched 'chirping' sound;

**3.** Signal intensity threshold of ≥ 7 decibels (Markus *et al*. 2010; Ringelstein *et al*. 1998; Yeo and Sharma 2010).

Symptomatic patients were described as MES+ve if they had ≥ 1 signal fulfilling all of these criteria for HITS on the side ipsilateral to a clinically symptomatic 50-99% carotid stenosis (Figure 1.3). Patients with bilateral HITS were also considered to be MES+ve for the purpose of this analysis because they also had ipsilateral HITS by definition. Contralateral HITS were also recorded prospectively for descriptive purposes in all patients, using the same internationally accepted criteria for HITS outlined above (Murphy, 2020). However, in accordance with an established methodology by our research group (Kinsella et al. 2014), patients who only had contralateral HITS were designated as MES-ve for this analysis. Asymptomatic patients were considered MES+ve if they had ≥ 1 signal fulfilling all of the criteria for HITS ipsilateral to a 50-99% clinically asymptomatic carotid artery stenosis. All other patients were designated as MES -ve.

Inter-observer agreement regarding the presence of MES versus artefact between Dr. Stephen Murphy and an experienced independent observer (Dr. Justin Kinsella), blinded to clinical details, symptomatic status, and recorded MES status of the study subjects, was found to be 'excellent' (97.4% concordance; Cohen's unweighted kappa

statistic 0.95). Therefore, all remaining TCD data analysis was performed locally at TUH-AMNCH by Dr. Stephen Murphy (Murphy, 2020).

### 1.2.6. Statistical Methods

The primary analysis focused on unmatched comparisons between asymptomatic and early and late phase symptomatic carotid stenosis patients. Subgroup analyses were pre-planned for patients on aspirin monotherapy, for patients with severe (≥70-99%) carotid stenosis, for patients with moderate (50-69%) *vs*. severe (≥70-99%) carotid stenosis, and for patients stratified according to MES status. We also performed paired, longitudinal comparisons in symptomatic patients followed up from the early to late phase after symptom onset or carotid intervention. Paired or unpaired t-tests were used for the comparison of parametric variables. The Wilcoxon signed-rank and Wilcoxon rank-sum tests were used to compare 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 were used to compare proportions between groups. Spearman rank correlation analysis assessed the correlation between non-parametric variables. Multiple linear regression analysis was performed to examine the potential influence of relevant independent variables on any observed differences between groups or associations between variables. $P < 0.05$ was considered statistically significant. All calculations were performed with IBM® Statistical Package for the Social Sciences version 26® (IBM SPSS®, 2019).

## 1.3. Results

### 1.3.1. Demographic and Vascular Risk Profiles of Study Participants

Thirty-six asymptomatic and 51 recently symptomatic carotid stenosis patients were initially recruited (Figure 1.4). Of the 36 asymptomatic patients recruited, only 34 were enrolled in the study because two patients had < 50% internal carotid stenosis on another non-invasive imaging modality (MR or CT angiography) after their CDUS suggested ≥ 50% stenosis. The following symptomatic patients were subsequently excluded: two with paroxysmal atrial fibrillation detected on subsequent ECG monitoring within one month of enrolment; one with acute infarction on diffusion-weighted MR brain imaging outside the vascular territory of the stenosed carotid artery of interest; 3 with < 50% internal carotid stenosis on another non-invasive imaging modality (MR or CT angiography) after their CDUS suggested ≥ 50% stenosis; 2 on non-steroidal anti-inflammatory medication; 3 did not have sufficient samples for analysis; and one patient's sample had to be discarded due to a laboratory device error during initial sample analysis. Therefore, data from 39 early phase symptomatic patients were analysed. Of these 39 symptomatic patients in whom intervention could be considered, 29 (74%) underwent carotid endarterectomy, one was deemed 'anatomically unsuitable' for endarterectomy at exploration due to a very high carotid bifurcation and proceeded to subsequent uncomplicated endovascular treatment and stenting (3%); the remaining 9 (23%) chose best medical management based on advice from their treating physician (Murphy et al. 2018; Murphy et al. 2019). The mean duration of follow-up in symptomatic patients after TIA or stroke onset was 101±15.5 days (range 87–155 days). One medically treated patient experienced a further left MCA territory TIA distal to a 60% left ICA stenosis during follow-up. No other patients experienced recurrent cerebrovascular, cardiovascular, or

venous thrombotic outcome events during the peri-procedural period or during follow-up (Murphy et al. 2018; Murphy et al. 2019).

Thirty-one symptomatic patients had late phase follow-up data. Eight symptomatic patients did not have late phase data: 4 declined follow-up, 1 patient could not travel from residential care, 1 had a severely disabling index stroke and could not attend for follow-up, and 2 patients' samples had to be discarded because of a laboratory device error during initial sample analysis.

Early and late phase symptomatic patients were significantly younger than the asymptomatic patients, and there was a higher prevalence of current smokers amongst symptomatic than asymptomatic patients (Table 1.1 and Table 1.6). Otherwise, demographic and vascular risk profiles were similar in the symptomatic and asymptomatic patient groups (Table 1.1 and Table 1.6). The median daily dose of aspirin in the overall study patient population who were prescribed aspirin monotherapy or combination therapy with dipyridamole or clopidogrel was significantly higher in early symptomatic (225 mg, $P<0.001$), but not in late symptomatic (75 mg, $P = 0.16$) *vs*. asymptomatic patients (75 mg); all prescribed aspirin doses were within the range of 75–300 mg daily.

### 1.3.2. Thrombin Generation Parameters

#### *1.3.2A. Case:Case/Control Data*

The numerically higher mean peak thrombin and ETP values in early symptomatic than asymptomatic patients did not reach statistical significance ($P \geq 0.17$), with no other significant differences in thrombin generation parameters between symptomatic and asymptomatic groups (Table 1.2). When multiple linear regression analysis was performed to control for the potential independent influence of age and smoking status on the comparisons between groups, there were still no statistically significant differences between the early symptomatic and asymptomatic patients ($P > 0.05$) (Table 1.2).

#### *1.3.2B. Nested Longitudinal Study in Symptomatic Patients*

Peak thrombin production (365.1 *vs*. 327.2 nM, P = 0.003) and ETP (2242.6 *vs*. 2078.4 nM*min, P = 0.048) significantly decreased between the early and late phase after symptom onset in symptomatic patients with 'matched data' at each timepoint, regardless of whether or not they underwent carotid interventional treatment (Table 1.3). Lag time and time to peak thrombin did not change significantly during follow-up in symptomatic patients overall who had matched longitudinal data (Table 1.3). Of note, peak thrombin levels significantly decreased in the symptomatic subgroup who underwent carotid intervention and who had data in both the early phase and late post-intervention phases (P = 0.007) (Table 1.4). However, thrombin generation parameters did not change significantly over time in the symptomatic subgroup who did not have carotid interventional treatment and who were treated with best medical therapy alone (N = 5) (Table 1.4). There were no differences in any other thrombin

generation parameters over time within subgroups of symptomatic patients who did or did not undergo carotid intervention (Table 1.4).

### 1.3.3. Pre-planned Subgroup Analyses

#### *1.3.3A. Aspirin monotherapy*

In the subgroup of patients on **aspirin monotherapy**, there were no differences in thrombin generation biomarkers between patients with early symptomatic (N = 23; median daily dose: 225 mg; range = 75-300 mg daily) or late symptomatic (N =13; median daily dose: 75 mg; range = 75-300 mg daily) versus asymptomatic carotid stenosis (N = 23; median daily dose: 75 mg; range = 75-300 mg daily) ($P > 0.05$).

#### *1.3.3B. Severe (≥ 70%) Carotid Stenosis*

There were no significant differences in thrombin generation parameters between early or late symptomatic subgroups compared with asymptomatic severe carotid stenosis patients (Table 1.5).

#### *1.3.3C. Moderate (≥ 50–69%) versus Severe (≥ 70–99%) Carotid Stenosis Subgroups*

There were no significant differences in any thrombin generation parameters between moderate *vs*. severe early symptomatic carotid stenosis subgroups ($P > 0.05$). Within the asymptomatic cohort, there were no differences in the expression of these biomarkers between moderate and severe asymptomatic carotid stenosis subgroups ($P > 0.05$).

### *1.3.3D. Thrombin Generation Parameters in MES+ve and MES-ve subgroups*

Twenty-eight of 34 asymptomatic patients, 30 of 39 early symptomatic, and 26 of 31 late symptomatic patients had complete TCD data available for analysis in this component of the HEIST study (Table 1.6). Two of 28 (7%) asymptomatic patients *vs*. 8/30 (27%) early symptomatic patients (P = 0.05) and 1 (4%) late symptomatic patient (P = 0.39) were MES+ve (Table 1.6). The median number (25th–75th centile) of MES was 3 (1-5) in the asymptomatic, 2 (1–5) in the early symptomatic, and 2 (1–3) in the late symptomatic groups.

There were no significant differences in thrombin generation parameters between early or late symptomatic *vs*. asymptomatic MES-ve patient subgroups or between early symptomatic and asymptomatic MES+ve patient subgroups (P > 0.05). Because only one late symptomatic patient and two asymptomatic patients were MES+ve, it was not possible to formally compare median or mean data between these subgroups.

In our nested longitudinal study, peak thrombin generation also significantly decreased between the early and late phase after symptom onset in the subgroup of MES-negative symptomatic patients who had matched data at each time point (376.8nM *vs*. 325.4nM; P = 0.029) (Table 1.7).

## 1.4. Discussion

To our knowledge, this is only the second longitudinal, observational study to compare thrombin generation parameters in patients with early and late phase symptomatic versus asymptomatic carotid stenosis, with nested longitudinal follow-up in symptomatic patients, in conjunction with TCD for MES detection in the majority.

Our **case-case/control study** in this independent, carefully-phenotyped patient population has not confirmed prior pilot data from our research group that peak thrombin and ETP levels were significantly increased in patients with recently symptomatic compared with asymptomatic carotid stenosis overall (Kinsella *et al.,* 2014). These findings may reflect a type II error because the absolute levels of these two biomarkers were also numerically higher in recently symptomatic compared with asymptomatic carotid stenosis patients in the HEIST study (Table 1.2). The number of symptomatic patients included in this component of the HEIST study (N = 39) was slightly lower than in the PACS study (N = 46) (Kinsella *et al.,* 2014). Our case:case/control data on peak thrombin and ETP in the patient subgroups on aspirin monotherapy and those with severe carotid stenosis might also be reflective of a type II error, with prior data showing that these biomarkers were significantly higher in severe early symptomatic *vs*. severe asymptomatic carotid stenosis (Kinsella *et al.,* 2015). There were no significant differences in demographic or vascular risk factors on *post hoc* analysis between participants in the HEIST and PACS studies to account for this apparent disparity in these findings between the two studies.

However, our **nested longitudinal study** has confirmed that thrombin generation parameters (peak thrombin and ETP) decreased during follow-up at least 3 months after following symptom onset in a clinically well-phenotyped cohort of recently symptomatic moderate-severe carotid stenosis patients overall (Kinsella *et al.,* 2014). In contrast to the PACS study, the decrease in peak thrombin production during follow-up in recently symptomatic patients was statistically significant in those undergoing carotid intervention in HEIST, but not in those treated with best medical therapy alone (Table 1.4); we did not observe any decrease in ETP levels over time in patients treated with best medical therapy alone in HEIST. One must interpret these latter data with caution because the number of patients who did not undergo intervention and who had longitudinal follow-up data was limited in HEIST (N = 5) and PACS (N = 12). These subgroup analyses were clearly prone to type II errors or type I errors, respectively (Kinsella *et al.,* 2014). Therefore, larger longitudinal studies are warranted to assess further the impact of carotid intervention or medical treatment alone on thrombin generation potential over time in patients with recently symptomatic carotid stenosis.

We also observed a significant reduction in peak thrombin levels during follow-up from the early to late phase after TIA/stroke onset in the subgroup of symptomatic patients who were MES-ve. These MES-ve patients have been considered to be at a lower risk of having recurrent symptoms than those who are MES+ve (Markus *et al.,* 2005). However, they are still at risk of having recurrent vascular events after their initial TIA or stroke (Murphy *et al.,* 2019). Increased peak thrombin levels may partly drive following symptom onset. Other thrombin generation parameters did not change significantly in symptomatic patients over time, again indicating that peak thrombin and ETP appear to be more sensitive markers of thrombin generation

potential overall than lag time or time to peak thrombin in patients with carotid stenosis (Kinsella *et al.,* 2015).

The findings in our nested longitudinal study in the symptomatic group could either partly reflect the resolution of the acute phase response to recent carotid plaque activation or ocular/cerebral ischaemia or infarction over time, or successful carotid interventional treatment of the stenosing atherosclerotic plaque in the majority (84%) of symptomatic patients included in this subgroup analysis. It is also possible that changes in antiplatelet regimens might have impacted thrombin generation parameters in matched symptomatic patients over time because there was an increase in the proportion of symptomatic patients included in this subgroup analysis who were treated with aspirin-dipyridamole combination therapy between the early (13%) *vs.* late phases (32%) after symptom onset. Our research group has previously shown that there was no consistent effect of commencing aspirin monotherapy on thrombin generation parameters, but that the addition of dipyridamole modified-release (MR) to aspirin may reduce thrombin generation over time in an overall patient population with TIA or stroke (Tobin *et al.,* 2013). However, this component of the HEIST study was not designed or powered to address the impact of adding dipyridamole MR to aspirin on thrombin generation in patients with carotid stenosis; this issue warrants assessment in a larger patient population.

Increased platelet and endothelial activation have also previously been noted in a similar population of recently symptomatic carotid stenosis (Kinsella *et al.,* 2015; Murphy *et al.,* 2019; Murphy *et al.,* 2021). Therefore, our study cannot determine whether enhanced coagulation system potential was a 'co-primary phenomenon' that could have led to thrombosis/thromboembolism in patients with recently symptomatic

carotid stenosis or a 'secondary phenomenon' which arose the following platelet and/or endothelial activation.

Our study had some **limitations**. This pilot observational study cannot make any definitive conclusions re the role played by thrombin generation biomarkers in the aetiology of TIA or stroke in patients with ≥50-99% ICA stenosis *per se*. This study was mainly designed to look at associations and the profile of thrombin generation parameters in ACS *vs*. early and late phase SCS patients in our case: case/control study, and the profile of thrombin generation parameters over time in our nested longitudinal study in SCS patients followed up from the early to late phase after symptom onset. We cannot make any conclusions about the value of thrombin generation biomarkers in predicting the risk of recurrent vascular events in patients with carotid stenosis because only one patient had a 'non-perioperative' recurrent TIA during medium-term follow-up in this study. This study was not designed to assess the immediate peri-procedural thrombin generation profile in patients with symptomatic carotid stenosis because we prospectively planned to reassess these biomarkers at least 3 months after symptom onset or intervention when the acute phase response from surgery/stenting had settled to some degree (Murphy *et al.,* 2021). We could not reliably address the effects of different aspirin doses on the results obtained. To explore this issue, one would need to quantify thrombin generation parameters in patients who were initially on 75 mg of aspirin daily for at least 11 days, and then retest them when they had been on 150 mg of aspirin daily for at least 11 days, and then retest them again after increasing to 300 mg of aspirin daily for at least 11 days. This was also beyond the scope of this aspect of the HEIST study, and would have been unreliable in any case in our SCS population because most SCS patients underwent urgent successful revascularisation which could have triggered an

acute phase response, would have confounded the data, and limited the interpretation of these results. This pilot study may have been prone to type II errors and occasional type I errors, as clearly acknowledged above. However, the analyses are still informative and were performed in a very carefully phenotyped cohort of patients with asymptomatic and symptomatic carotid stenosis. As outlined before (Kinsella *et al.,* 2015), our research group's goal was not to perform simultaneous histological carotid plaque and biomarker analyses in this study, so we cannot comment on the relationship between thrombin generation parameters and histologically-defined plaque morphology in patients with carotid stenosis. Similar to the PACS study, the HEIST study was not designed to simultaneously measure thrombin generation parameters, thrombin anti-thrombin (TAT) complexes (Migdalski *et al.,* 2005), or F1 and F2 prothrombin fragments (produced during the generation of thrombin from prothrombin) (Cote *et al.,* 2000; Soinne *et al.,* 2005). However, the thrombin generation assay employed during the HEIST study provides a comprehensive and reproducible measurement of thrombin generation/coagulation system potential in patients with carotid stenosis (Kinsella *et al.,* 2015).

## 1.5. Conclusions

Thrombin generation potential decreased over time following TIA or stroke associated with recently symptomatic moderate-severe carotid stenosis, especially in patients who underwent revascularisation and in the subgroup who were MES-ve. These data may reflect the successful removal of the carotid plaque, combined with the effects of active secondary preventive medical treatment during follow-up in the majority of patients, as well as resolution of the acute phase response. This study improves our understanding of the haemostatic/thrombotic biomarker profiles in patients with moderate-severe asymptomatic and symptomatic moderate-severe carotid stenosis. It should prompt the design of future studies to assess the prognostic value of thrombin generation biomarkers at predicting the risk of first or subsequent TIA or stroke in this patient population. These studies would need to involve larger numbers of participants from a new, wider multi-centre consortium, or a meta-analysis of individual patient data on thrombin generation parameters derived from experiments at separate centres using identical laboratory methodology. One could assess thrombin generation parameters in both ACS or SCS patients at baseline, and perform long-term prospective follow-up to construct receiver operating characteristic curves to determine whether one could identify 'threshold levels' of peak thrombin or ETP with the highest sensitivity, specificity, positive and negative predictive values at identifying patients with ICA stenosis at highest risk of vascular events over time. If expression of peak thrombin or ETP above a certain threshold were proven to predict outcomes, then one could potentially target such patients if they were only on aspirin monotherapy with additional treatment with dipyridamole which has been shown to reduce peak thrombin generation (Tobin *et al.,* 2013), and potentially expedite revascularisation in those deemed to be at highest risk.

**Table 1.1: Demographics and Vascular Risk Profiles of Patients at the time of initial study recruitment. P values relate to $\chi^2$ or Fisher exact testing between asymptomatic and symptomatic carotid stenosis groups. Values are means (± SD) or absolute values (%). Statistically significant P values in bold**

| Parameter | Asymptomatic Carotid Stenosis (N=34) | Early Symptomatic Carotid Stenosis (N=39) | Late Symptomatic Carotid Stenosis (N=31) |
|---|---|---|---|
| **Mean age** (years)<br>P Value | 71.1 (±8.9) | 65.46 (±8.6)<br>**0.005** | 65.35 (±8.8)<br>**0.023** |
| **Male Sex** (%)<br>P Value | 24 (71%) | 27 (69%)<br>0.24 | 24 (77%)<br>0.21 |
| **Median Interval from symptom onset** (Days) | N/A | 5 (1-28) | 96 (84-179) |
| **Degree of Stenosis:** | | | |
| Moderate (≥50 - 69%)<br>P Value | 18 (53%) | 15 (38.5%)<br>0.11 | 10 (32%)<br>0.08 |
| Severe (≥70 - 99%)<br>P Value | 16 (47%) | 24 (61.5%)<br>0.12 | 21 (68%)<br>0.07 |
| **Antiplatelet Therapy:** | | | |
| Aspirin monotherapy (75–300 mg daily)<br>P Value | 23 (68%) | 23 (59%)<br>0.12 | 13 (42%)<br>0.19 |
| Aspirin (75–300 mg daily)/ Dipyridamole MR (200 mg twice daily) combination therapy, P Value | 5 (15%) | 10 (25.5%)<br>0.11 | 10 (32%)<br>0.18 |
| Clopidogrel monotherapy (75 mg daily)<br>P Value | 2 (6%) | 1 (2.5%)<br>0.39 | 1 (3%)<br>0.43 |
| Aspirin (75 mg daily) / Clopidogrel (75 mg daily) combination therapy<br>P Value | 3 (9%) | 5 (13%)<br>0.31 | 7 (23%)<br>0.12 |
| **Ischaemic heart disease**<br>P Value | 10 (29.5%) | 13 (33%)<br>0.21 | 13 (42%)<br>0.14 |
| **Hypertension**<br>P Value | 29 (85%) | 29 (74.5%)<br>0.16 | 21 (68%)<br>0.07 |
| **Diabetes mellitus**<br>P Value | 7 (20.5%) | 7 (18%)<br>0.25 | 5 (16%)<br>0.25 |
| **Prior TIA/stroke before index event**<br>P Value | 9 (26.5%) | 6 (15.5%)<br>0.15 | 4 (13%)<br>0.45 |
| **Hyperlipidaemia**<br>P Value | 32 (94%) | 29 (74.5%)<br>0.05 | 22 (71%)<br>0.05 |
| **Peripheral vascular disease**<br>P Value | 3 (9%) | 1 (2.5%)<br>0.24 | 1 (3%)<br>0.30 |
| **Prior Venous Thromboembolism**<br>P Value | 1 (3%) | 2 (5%)<br>0.44 | 1 (3%)<br>0.53 |
| **Migraine (with or without aura)**<br>P Value | 3 (9%) | 2 (5%)<br>0.32 | 2 (6.5%)<br>0.37 |
| **Family history of stroke**<br>P Value | 11 (32.5%) | 15 (38.5%)<br>0.19 | 11 (35.5%)<br>0.23 |
| ***Smoking Status*** | | | |
| Current smoker<br>P Value | 4 (12%) | 14 (36%)<br>**0.016** | 6 (19.5%)<br>**0.02** |
| Ex-smoker<br>P Value | 21 (62%) | 19 (49%)<br>0.13 | 21 (68%)<br>0.11 |
| Never smoker<br>P Value | 9 (26.5%) | 6 (15.5%)<br>0.15 | 4 (13%)<br>0.14 |
| **Statin therapy**<br>P Value | 30 (88%) | 32 (82%)<br>0.24 | 26 (84%)<br>0.29 |

**Table 1.2: Comparison of thrombin generation parameters in asymptomatic *vs*. early symptomatic, in asymptomatic *vs*. late phase symptomatic patients overall, and in asymptomatic *vs*. late phase symptomatic post-intervention patients**

| Marker | Asymptomatic (N = 34) | Early Symptomatic (N = 39) | Late Symptomatic (N=31) | Late Symptomatic Post-Intervention (N=26) |
|---|---|---|---|---|
| Lag time (min) | 5.51(±1.33) | 5.71 (±1.24) | 5.41 (±0.99) | 5.16 (±0.80) |
| P Value | | 0.50 | 0.74 | 0.84 |
| Peak thrombin (nM) | 344.7 (±85.1) | 372.7 (±90.1) | 327.2 (±63.4) | 329.8 (±62.9) |
| P Value | | 0.18 | 0.36 | 0.43 |
| Time to peak thrombin (min) | 9.14 (±1.89) | 9.19 (±1.55) | 9.14 (±1.29) | 8.86 (±1.04) |
| P Value | | 0.89 | 0.99 | 0.89 |
| ETP (nM*min) | 2113.8 (±481) | 2300.3 (±633.7) | 2078.4 (±471.4) | 2057.6 (±438.1) |
| P Value | | 0.17 | 0.77 | 0.82 |

Values are means (±SD). ETP = Endogenous Thrombin Potential

**Table 1.3: Comparison of thrombin generation data in early versus late symptomatic carotid stenosis patients with matched longitudinal data at each time point**

| Marker | Early Symptomatic (N=31) | Late Symptomatic (N=31) | P Value |
|---|---|---|---|
| Lag time (min) | 5.69 (±1.32) | 5.41 (±0.99) | 0.11 |
| Peak thrombin (nM) | 365.1 (±83.0) | 327.2 (±63.4) | **0.003** |
| Time to peak thrombin (min) | 9.2 (±1.71) | 9.14 (±1.29) | 0.82 |
| ETP (nM*min) | 2242.6 (±596.7) | 2078.4 (±471.4) | **0.048** |

Values are means (±SD) Statistically significant P values highlighted in bold.

**Table 1.4: Comparison of matched longitudinal thrombin generation data in early symptomatic versus late symptomatic post-intervention patients, and early symptomatic versus late symptomatic patients who did not undergo carotid intervention**

| **Marker** | **Early Symptomatic (N=26)** | **Late Symptomatic Post-Intervention (N=26)** | **P Value** |
|---|---|---|---|
| Lag time (min) | 5.56 (±1.31) | 5.29 (±0.87) | 0.16 |
| Peak thrombin (nM) | 362.5 (±84.8) | 317.5 (±71.8) | **0.007** |
| Time to peak thrombin (min) | 8.98 (±1.60) | 9.30 (±1.94) | 0.43 |
| ETP (nM*min) | 2132.4 (±527.5) | 1983.5 (±448.5) | 0.12 |
| | **Early Symptomatic (N=5)** | **Late Symptomatic without Intervention (N=5)** | **P Value** |
| Lag time (min) | 6.62 (±0.27) | 6.28 (±1.01) | 0.67 |
| Peak thrombin (nM) | 333.6 (±57.9) | 283.8 (±82.7) | 0.19 |
| Time to peak thrombin (min) | 10.73 (±0.54) | 10.01 (±1.69) | 0.63 |
| ETP (nM*min) | 2402.5 (±889.2) | 1786.7 (±395.1) | 0.24 |

Values in means (±SD) Statistically significant p values in bold.

**Table 1.5: Comparison of thrombin generation data in asymptomatic versus early symptomatic and late phase symptomatic patients with severe (≥70%) carotid stenosis**

| Marker | Asymptomatic (N = 21) | Early Symptomatic (N = 25) | Late Symptomatic (N = 22) |
|---|---|---|---|
| Lag time (min) | 5.48 (±1.28) | 5.67(±1.42) | 5.13(±0.92) |
| P Value | | 0.65 | 0.31 |
| Peak thrombin (nM) | 354.5 (±89.8) | 374.8(±87.5) | 331.5(±54.9) |
| P Value | | 0.44 | 0.32 |
| Time to peak thrombin (min) | 9.02 (±1.79) | 9.11(±1.84) | 8.84(±1.28) |
| P Value | | 0.88 | 0.70 |
| ETP (nM*min) | 2168.8 (±555.4) | 2328.3(±650.2) | 2119.1(±485.9) |
| P Value | | 0.38 | 0.76 |

Values are means (±SD).

| Table 1.6: Demographic and risk factor profiles of study patients with available TCD data | | | |
|---|---|---|---|
| **Parameter** | **Asymptomatic Carotid Stenosis (N=28)** | **Early Symptomatic Carotid Stenosis (N=30)** | **Late Symptomatic Carotid Stenosis (N=26)** |
| ***Mean age (years)*** | 70.1 (±9.0) | 64.2 (±7.8) | 63.7 (±9.1) |
| *P Value* | | **0.004** | **0.023** |
| ***Male Sex (%)*** | 22 (79%) | 24 (80%) | 23 (88.5%) |
| *P Value* | | 0.28 | 0.21 |
| ***Median Interval from Symptom Onset (Days)*** | | 5 (1-28) | 96 (84-179) |
| ***Degree of Stenosis:*** | | | |
| Moderate (≥50 - 69%) | 17 (61%) | 12 (40%) | 10 (38.5%) |
| P Value | | 0.08 | 0.06 |
| Severe (≥70 - 99%) | 11 (39%) | 18 (60%) | 16 (61.5%) |
| P Value | | 0.09 | 0.07 |
| ***No of Patients who were MES+ (%)*** | 2 (7%) | 8 (27%) | 1 (4%) |
| | | 0.05 | 0.39 |
| ***No of Patients who were MES- (%)*** | 26 (93%) | 22 (73%) | 25 (96%) |
| | | 0.05 | 0.42 |
| ***Antiplatelet Therapy:*** | | | |
| Aspirin monotherapy (75–300 mg daily) | 21 (75%) | 18 (60%) | 14 (54%) |
| P Value | | 0.12 | 0.16 |
| Aspirin (75–300 mg daily)/ Dipyridamole MR (200 mg twice daily) combination therapy | 4 (14%) | 7 (23%) | 6 (23%) |
| P Value | | 0.21 | 0.21 |
| Clopidogrel monotherapy (75 mg daily) | 1 (4%) | 1 (3%) | 1 (4%) |
| P Value | | 0.53 | 0.52 |
| Aspirin (75 mg daily)/Clopidogrel (75 mg daily) combination therapy | 2 (7%) | 4 (13%) | 5 (19%) |
| P Value | | 0.28 | 0.16 |
| ***Ischaemic heart disease*** | 11 (39%) | 13 (43%) | 6 (23%) |
| P *Value* | | 0.24 | 0.12 |
| ***Hypertension*** | 23 (82%) | 25 (83%) | 15 (58%) |
| P *Value* | | 0.06 | 0.06 |
| ***Diabetes mellitus*** | 4 (14%) | 9 (30%) | 2 (8%) |
| P Value | | 0.11 | 0.28 |
| ***Prior TIA/stroke before index event*** | 8 (29%) | 2 (7%) | 4 (15.5%) |
| P Value | | 0.28 | 0.15 |
| ***Hyperlipidaemia*** | 26 (93%) | 22 (73%) | 18 (69.5%) |
| P Value | | 0.07 | 0.29 |
| ***Peripheral vascular disease*** | 3 (11%) | 1 (3%) | 1 (4%) |
| P Value | | 0.23 | 0.28 |
| ***Prior Venous Thromboembolism*** | 0 (0%) | 2 (7%) | 1 (4%) |
| P Value | | 0.28 | 0.5 |
| ***Migraine (with or without aura)*** | 3 (11%) | 2 (7%) | 2 (8%) |
| P Value | | 0.34 | 0.35 |
| ***Family history stroke*** | 9 (32%) | 9 (30%) | 13 (54%) |
| P Value | | 0.24 | 0.21 |
| *Smoking status* | | | |
| Current smoker | 10 (36%) | 7 (23%) | 4 (15.5%) |
| P Value | | 0.15 | 0.06 |
| Ex-smoker | 14 (50%) | 19 (63%) | 16 (61.5%) |
| P Value | | 0.14 | 0.18 |
| Never smoker | 4 (14%) | 4 (13%) | 6 (23%) |
| P Value | | 0.32 | 0.23 |
| ***Statin therapy*** | 21 (75%) | 27 (90%) | 25 (96%) |
| P Value | | 0.11 | 0.051 |

**Legend for Table 1.6**: P Values relate to $\chi^2$ or Fisher exact testing between patients with asymptomatic versus early or late symptomatic carotid stenosis with TCD data. Values are means (±SD) or absolute counts, with percentages in parentheses. Statistically significant P values in bold. MES: Micro-embolic signals; TCD: transcranial Doppler ultrasound

**Table 1.7: Comparison of thrombin generation data in early versus late symptomatic carotid stenosis patients with TCD data.**

| Marker | MES-negative Early Symptomatic (N=14) | MES-negative Late Symptomatic (N=14) | P Value |
|---|---|---|---|
| Lag time (min) | 6.12 (±1.55) | 5.72 (±1.08) | 0.22 |
| Peak thrombin (nM) | 376.8 (±105.4) | 325.4 (±71.2) | **0.029** |
| Time to peak thrombin (min) | 9.59 (±1.93) | 9.45 (±1.31) | 0.76 |
| ETP (nM*min) | 2245.6 (±573.6) | 2031.7 (±434.9) | 0.07 |

Values are means (±SD). Statistically significant p values in bold.

**Figure 1.1: Intrinsic and extrinsic coagulation pathways/cascade.** TFPI: Tissue Factor Pathway Inhibitor (Figure reproduced with permission from Kinsella JA. PhD thesis, *School of Medicine, Trinity College Dublin*, 2012)

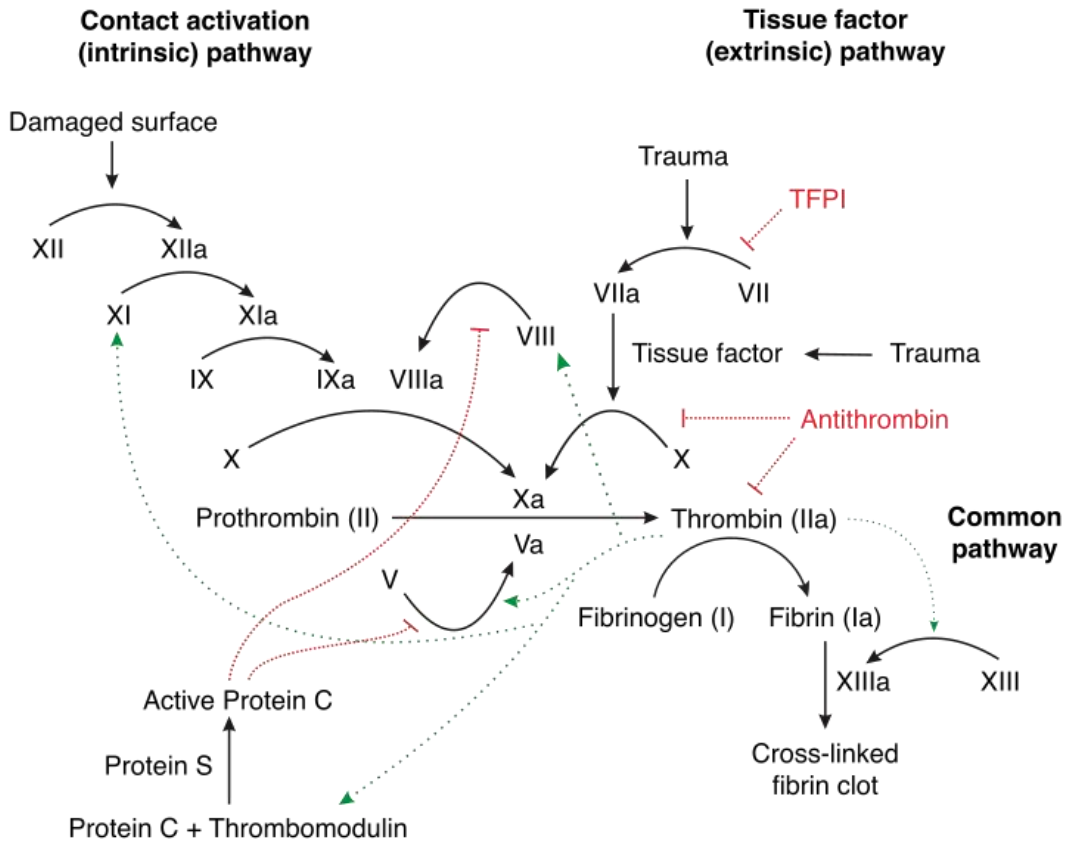


**Figure 1.2:** Example of Thrombin Generation Curve. **A:** Lag-time: Time to initiation of thrombin generation; **B:** Peak thrombin generated; **C:** ttPeak: Time to Peak thrombin generated; **D:** Start-tail: Time to end point of thrombin generation; **E:** ETP: Area under thrombin generation curve. All times measured from t=0 min. (Figure reproduced with permission from Kinsella JA. PhD thesis, *School of Medicine, Trinity College Dublin*, 2012)

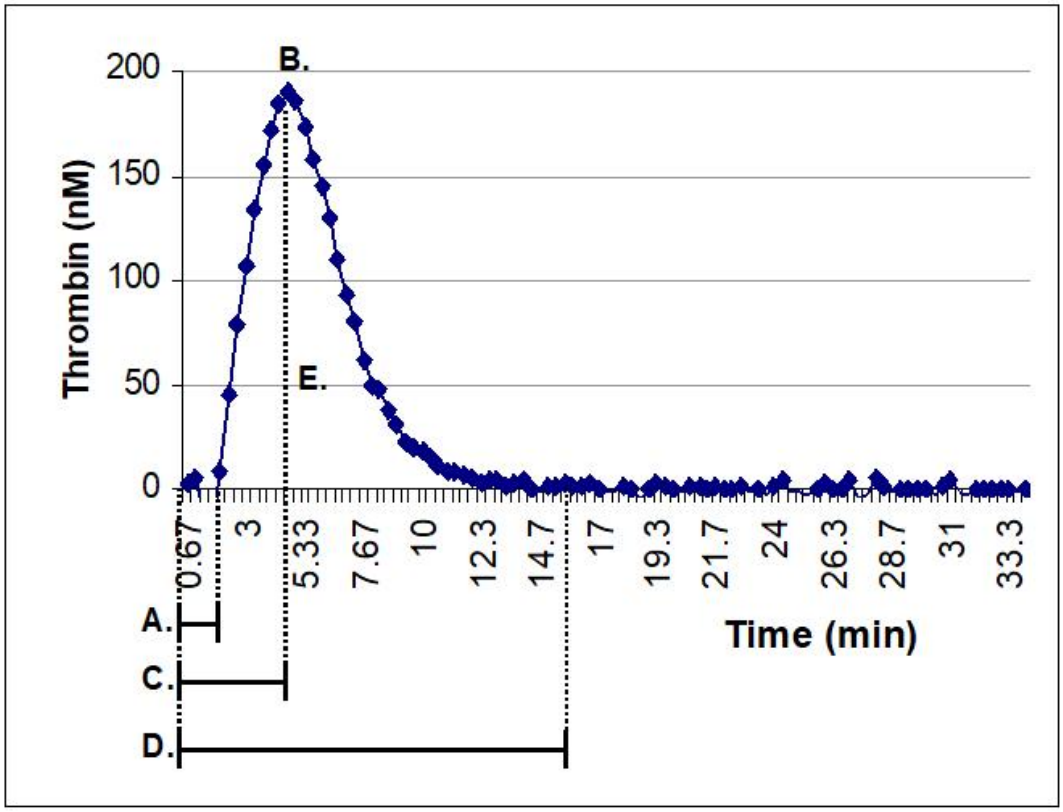


**Figure 1.3:** Example of a HITS within the visual spectral waveform display (large white arrow). The embolus is unidirectional, < 300 ms in duration, and was accompanied by a high-pitched 'chirp' on the simultaneous audio recording. (Figure reproduced with permission from Murphy SJX, MSc thesis, *School of Medicine, Trinity College Dublin*, 2020)

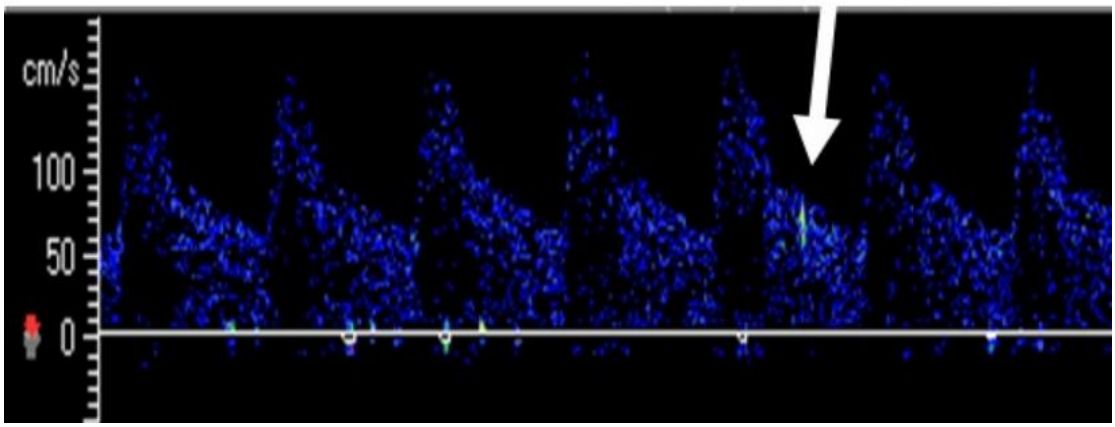
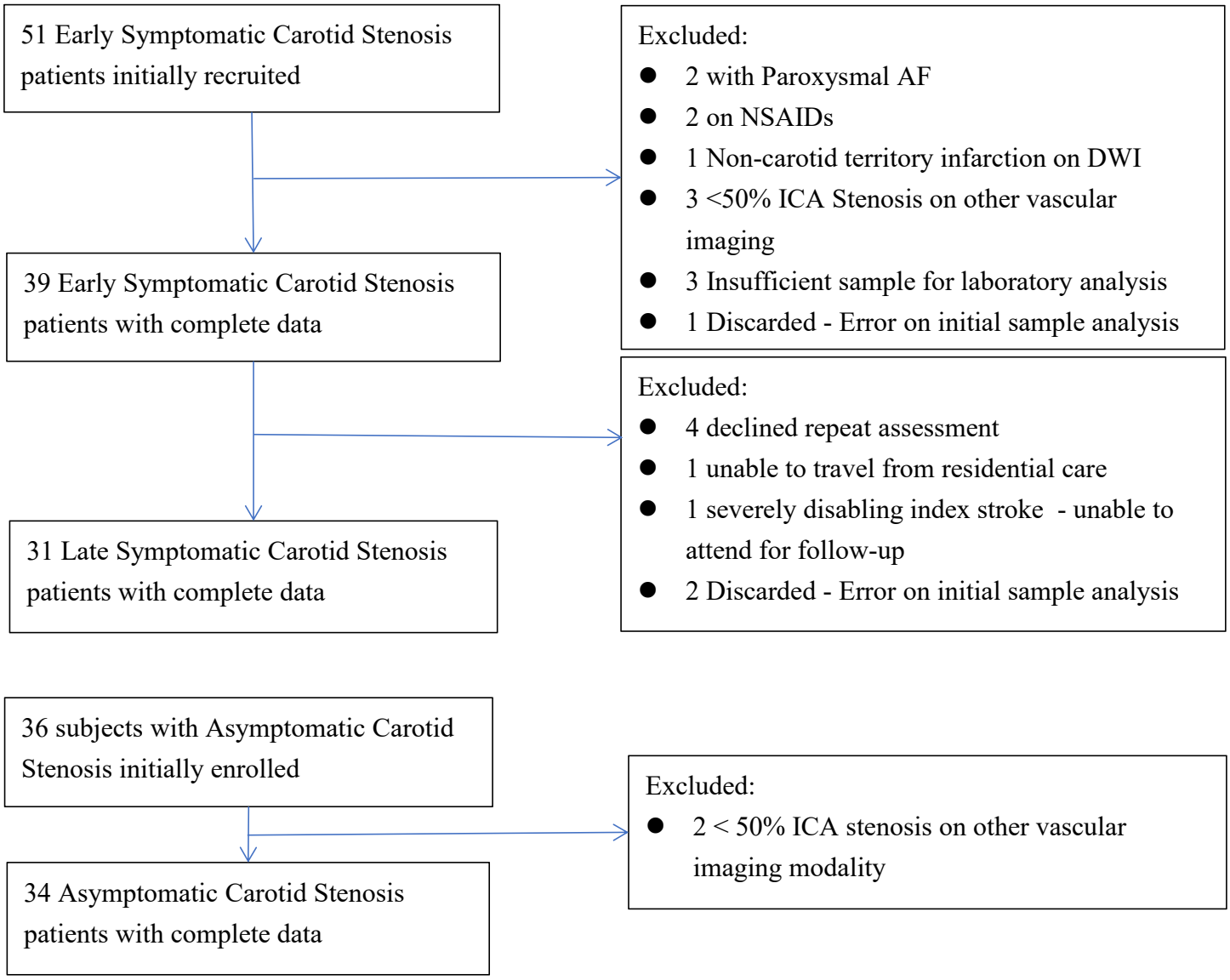


**Figure 1.4**: Flow diagram of Symptomatic and Asymptomatic Carotid Stenosis patients screened and subsequently included in or excluded from the study




AF = Atrial Fibrillation; NSAIDs = Non-steroidal anti-inflammatory drugs;
DWI = Diffusion-weighted imaging; ICA = Internal carotid artery.

# 2. Platelet biomarkers in atherosclerotic extracranial carotid artery stenosis: An updated systematic review of the literature

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## 2.1 Introduction

The annual risk of ipsilateral stroke in hospital-based series varies between 0.7-1.4% in patients with ≥60% to ≥70% asymptomatic carotid stenosis (ACS) without micro-embolic signals (MES) on transcranial Doppler ultrasound (Markus *et al.,* 2010; Spence *et al.,* 2010). The risk of recurrent ipsilateral stroke was approximately 17% in the first year after randomisation in patients with 70-99% symptomatic carotid stenosis (SCS) in the North American Symptomatic Carotid Endarterectomy Trial (NASCET) treated with best medical therapy alone (NASCET, 1991). There are differences in the composition of atherosclerotic plaques (Golledge *et al.,* 1997), endothelial activation status (Kinsella *et al.,* 2014) and coagulation system potential (Kinsella *et al.,* 2015) between patients with SCS and ACS. Differences in platelet biomarker profiles may also partly explain why there is a disparity in the risk of stroke between patients with SCS and ACS (McCabe *et al.,* 2005; Kinsella *et al.,* 2013; Murphy *et al.,* 2019; Kinsella *et al.,* 2017; Murphy *et al.,* 2018), and in patients with carotid stenosis *vs*. controls (McCabe *et al.,* 2005; McCabe *et al.,* 2004; Cha *et al.,* 2003; Stolz *et al.,* 2002; Novo *et al.,* 2005; Yip *et al.,* 2006).

When platelets are exposed to an atherosclerotic plaque, they may go through some or all stages of the platelet activation process by initially adhering to the plaque contents or sub-endothelial matrix, which may then be followed by shape change, secretion of their granule contents and aggregation to one another. One can use sensitive and

specific laboratory techniques to assess various aspects of the platelet activation process, as well as platelet function/reactivity in response to agonist stimulation (Table 2.1). As noted in previous review, this area of translational research has received limited attention in patients with ACS and SCS (Kinsella *et al.,* 2013).

### 2.1.1. Aims

The **aims** of this systematic review were to collate data on platelet biomarkers in blood to improve our understanding of the pathogenesis of TIA and stroke in patients with ACS and SCS, and to determine whether platelet biomarkers might potentially assist with risk-stratification in patients with carotid stenosis. We also aimed to assess the prevalence of antiplatelet-high on-treatment platelet reactivity (HTPR [previously termed 'antiplatelet resistance']) in patients with carotid stenosis and to determine whether there was a clear evidence-base to alter antiplatelet therapy based on antiplatelet-HTPR status in patients with carotid stenosis. We **hypothesised** that these data would enhance understanding of the role of platelet biomarkers in the pathogenesis of TIA and stroke in patients with carotid stenosis, and the potential value of platelet biomarkers in risk-stratification and treatment decision making in this patient cohort.

## 2.2 Methodology

This systematic review was conducted in accordance with the current Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement to update a prior review of this topic which had included all retrieved studies from 1975 up to August 2011 (Kinsella *et al.,* 2013). Medline (Pubmed and Ovid), Embase, Web of Science / Web of Knowledge and Google Scholar were searched for completed human studies on platelet activation or platelet function in ACS or SCS, published in the English language between 1975 and May 2020. This updated review identified other new studies published before August 2011 because we searched an additional database on this occasion (Google Scholar), but the majority of new studies were published after August 2011. The following search terms were used for all databases aside from Embase: 'carotid stenosis', 'platelet activation', 'platelet function', 'platelet reactivity', 'platelet aggregation', 'flow cytometry', 'antiplatelet resistance', 'high on-treatment platelet reactivity', as well as the most-commonly available platelet function/reactivity testing platforms at the time of our review (VerifyNow®, PFA-100®, Multiplate®, PLATELETWORKS®). Additional search terms and their combinations were used for Embase: 'thrombocyte function,' 'thrombocyte activation,' 'blood clotting parameters,' 'carotid artery obstruction,' and 'asymptomatic disease.'

### 2.2.1. Study Selection and Data Extraction:

#### *2.2.1A. Inclusion criteria*

We included peer-reviewed data on platelet activation and/or function in ACS and SCS patients *vs*. a 'control' group, SCS *vs*. ACS, SCS and ACS patients undergoing intervention, and platelet activation or reactivity/function in patients with 'additional neuroimaging evaluation.

### *2.2.1B. Exclusion criteria*

We excluded case reports, small case series (<15 study participants) and review articles (aside from data included in our previous comprehensive review (Kinsella *et al.,* 2013)), studies assessing platelet function *in vitro*, and articles on urinary biomarkers as indirect measures of platelet activation or function. Two independent reviewers (Arun Subramanian [AS] and Siobhan Delaney) screened the title and abstracts of newly retrieved citations not included in our prior review, and hand-searched the reference lists of published articles. These two reviewers extracted the following data on pre-specified forms: e.g. authors; journal; year of publication; study design; inclusion and exclusion criteria; baseline clinical and demographic data or study population; sample size; type of platelet activation or function/reactivity testing platform; platelet and other biomarkers analysed; prescribed antiplatelet regimens and doses; and type of blood sample used in the study. Any discrepancies were resolved by consensus between the 2 reviewers. Each newly-retrieved manuscript since our last review was also critically-appraised by Prof. Dominick McCabe, who also adjudicated on any disagreements between reviewers.

Articles were categorised *a priori* according to the groups outlined in the inclusion criteria. We subsequently categorised evidence within these groups according to the method of assessment of platelet activation and function *ex vivo*. If the terms 'antiplatelet-resistance or 'non-responsiveness' were originally used in source articles, we replaced these with the term 'antiplatelet-HTPR' to maintain consistency throughout the text. At the beginning of each section of the results , the key findings from the original review (Kinsella *et al.,* 2013) were presented, followed by an overview of any new data.

### 2.2.2. Statistical Methodology

Descriptive statistical methods were used to present data, along with published estimates of statistical significance in relevant studies. $P < 0.05$ was considered to represent statistical significance, unless otherwise specified.

### 2.2.3. Quality Assessment and Risk of Bias

The Risk Of Bias In Non-randomised Studies-of Interventions (ROBINS-I) tool was employed by two independent reviewers (AS and Deirdre R Smith) to assess the quality and risk of bias of included studies (Sterne *et al.,* 2016). The overall risk of bias was considered to be equal to the most severe level of bias found in any of the 7 domains tested. Any discrepancies were resolved by reaching consensus between the 2 reviewers. We also critically appraised new data in each section in turn, without proceeding to a meta-analysis.

## 2.3 Results

The search identified 1754 articles. After critical review, 273 peer-reviewed articles relating to the assessment of platelet activation and function in patients with ACS/SCS were reviewed. Forty-three met the inclusion criteria, 17 of which had not been included in the earlier review (Kinsella *et al.,* 2013) (study sample size: 18-329 participants; figure).

### 2.3.1. Asymptomatic Carotid Artery Stenosis versus Controls (Tables 2.1A and 2.1B)

#### *2.3.1A. Platelet Activation (Table 2.2A)*

***Soluble and Surface Markers***

Five small-medium sized case-control studies (sample size range: 11 – 329 participants), showed increased platelet activation in patients with ≥30% ACS despite treatment with antiplatelet therapy in the majority of included patients (Cha *et al.,* 2003; Stolz *et al.,* 2002; Novo *et al.,* 2005; Balla *et al.,* 2006; Enomoto *et al.,* 2010), with one neutral study (Jurk *et al.,* 2010)

#### *2.3.1B. Platelet Function/Reactivity (Table 2.2B)*

The prevalence of aspirin-HTPR in moderate-severe ACS patients on aspirin, alone or in combination with dipyridamole or clopidogrel, has recently been shown to vary between 23% on the 'low shear stress' VerifyNow and Multiplate analysers (Murphy *et al.,* 2020) to 46-57% on the 'moderately high-shear stress' PFA-100 analyser (Kinsella *et al.,* 2017; Murphy *et al.,* 2020), based on cross-sectional/case-control definitions of HTPR (Lim *et al.,* 2015) in patients with ≥ 50-100% ACS. The prevalence of clopidogrel-HTPR in moderate-severe ACS patients who were on clopidogrel, alone or in combination with aspirin, varied between 75-100% on the

'low shear stress' VerifyNow and Multiplate analysers (Murphy *et al.,* 2020) to 25% on the 'moderately high-shear stress' PFA-100 analyser (Murphy *et al.,* 2020).

### *2.3.1C. Asymptomatic Carotid Stenosis and 'Coated Platelets' (Table 2.2A)*

Coated platelets are a subgroup of activated platelets arising from dual agonist-stimulation with collagen and thrombin, which express high levels of surface procoagulant proteins and have increased prothrombinase activity (Kirkpatrick *et al.,* 2014). One large single-centre study indicated that quantification of coated platelets in combination with assessment of stenosis severity may aid risk-stratification and identification of patients with ≥50-99% ACS who may be at higher risk of ipsilateral TIA or stroke (Kirkpatrick *et al.,* 2014). 77% of patients were on some form of antiplatelet therapy, most commonly aspirin monotherapy (57%). However, event rates were 2-3 times higher than in other studies and the study only included US military veterans.

### 2.3.2. Symptomatic Carotid Artery Stenosis versus Controls (Tables 2.2A, 2.2B and 2.2C)

#### *2.3.2A. Platelet Activation*

#### *Soluble and Surface Markers*

One study showed no increase (McCabe *et al.,* 2004), but 6 studies showed elevated soluble markers of platelet activation (Jurk *et al.,* 2010; Balla *et al.,* 2006; Frijns *et al.,* 1997; Hashimoto *et al.,* 2003; Blake *et al.,*2003; Shah *et al.,* 1985) in patients with ≥30% to >70% SCS *vs*. controls (Table 2.3A) (Kinsella *et al.,* 2013). Five studies revealed elevated cell 'surface markers' of platelet activation in patients with ≥50-100% SCS compared with controls (Jurk *et al.,* 2010; Cha *et al.,* 2003; McCabe *et al.,* 2004; Zeller *et al.,* 1999; Yip *et al.,* 2006) (Table 2.3B). Increased CD62P expression, neutrophil-platelet complexes and monocyte-platelet complexes were reported in patients within 1 week of TIA/ischaemic stroke due to 'large artery atherosclerosis' compared with controls (Table 2.3B) (Turgut *et al.,* 2011). However, in addition to the limitations outlined in the Table, the spectrum of severity of extracranial SCS was not specified. Platelet activation status was also assessed at rest and following stimulation with Thrombin Receptor Activating peptide (TRAP) or Adenosine Diphosphate (ADP) in patients approximately 3-6 months after an ischaemic stroke associated with ≥ 50-70% SCS *vs*. vascular disease controls (Lukasik *et al.,* 2013). Percentages of 'resting' platelet-derived microparticles (PMPs) or 'TRAP- or ADP-stimulated' PMPs were significantly higher in stroke patients *vs*. controls (Table 2.3B). These data could be interpreted as suggesting that quantification of platelet-derived microparticles (proposed to have a role in the development of atherosclerosis) may be a more sensitive method of detecting increased platelet activation status than measurement of traditional surface markers of platelet activation (CD62P or PAC-1 binding) in SCS patients. However, only 40% of stroke patients and 34% of controls had 'carotid plaques', so an important proportion

of stroke patients might have had intracranial rather than extracranial stenosis to enable their inclusion in this TOAST subgroup.

### *2.3.2B. Platelet Function/Reactivity (Table 2.3C)*

Two new studies included data on antiplatelet-HTPR status in patients with ≥50-100% (Kinsella *et al.,* 2017); and ≥50-99% (Murphy *et al.,* 2020) SCS. The prevalence of aspirin-HTPR in SCS patients on aspirin, alone or in combination with dipyridamole or clopidogrel, varied between 9.5-13.9% on the 'low shear stress' VerifyNow and Multiplate analysers (Kinsella *et al.,* 2017), and between 11-64% overall on the 'moderately high-shear stress' PFA-100 analyser (Kinsella *et al.,* 2017; Murphy *et al.,* 2020; McCabe *et al.,* 2005). The prevalence of clopidogrel-HTPR in SCS patients on clopidogrel, alone or in combination with aspirin, varied between 0-83.3% on these analysers (Murphy *et al.,* 2020).

### 2.3.3. Symptomatic versus Asymptomatic Carotid Stenosis

#### *2.3.3A. Platelet activation*

***Soluble and Surface Markers***

One study showed elevated sP-selectin but no increase in soluble CD40L levels (Jurk *et al.,* 2010) in patients with >70% SCS *vs*. >50% ACS (Jurk *et al.,* 2010; Kinsella *et al.,* 2013) (Table 2.4A). Five studies showed increased cell surface markers of platelet activation (Jurk *et al.,* 2010; Cha *et al.,* 2003) or leucocyte-platelet complexes (Kinsella *et al.,* 2013; McCabe *et al.,* 2005; Murphy *et al.,* 2019) with flow cytometry in patients with variable degrees of SCS *vs*. ACS (Table 2.4B). The Platelets and Carotid Stenosis (PACS) study also showed that the median % lymphocyte-platelet complexes were higher in patients with ≥ 50-100% recently symptomatic *vs*. ≥ 50-99% asymptomatic MES-negative patients who might traditionally be considered to be a 'lower risk' symptomatic subgroup (Table 2.4B) (Kinsella *et al.,* 2013). The HaEmostasis In carotid STenosis (HEIST) confirmed that the percentage of lymphocyte-platelet complexes was higher in early symptomatic *vs*. asymptomatic MES-ve patients with ≥ 50-99% stenosis (Table 2.4B) (Murphy *et al.,* 2019).

#### *2.3.3B. Platelet Function/Reactivity*

The PACS study showed no significant differences in the prevalence of aspirin-HTPR at high shear stress on the PFA-100 between early symptomatic (55%), late symptomatic (28%) or late symptomatic post-intervention patients (21%) *vs*. asymptomatic patients (46%) on aspirin alone or in combination with dipyridamole or clopidogrel (Table 2.4C) (Kinsella *et al.,* 2017). However, the prevalence of aspirin-HTPR was lower in late symptomatic post-intervention patients *vs*. asymptomatic patients on aspirin monotherapy (10% *vs*. 50%). Furthermore, the prevalence of aspirin-HTPR decreased during follow-up between the early and late phases in symptomatic patients on aspirin, alone or in combination with dipyridamole or clopidogrel (59% *vs*. 26%), and in the subgroup on aspirin monotherapy (62% *vs*.

15%) (Kinsella *et al.,* 2017) (Table 2.4C). The HEIST study subsequently found a lower prevalence of aspirin-HTPR in early symptomatic (28.6%) but not in late symptomatic patients (38.9%) compared with asymptomatic patients (56.7%) on the 'moderately high shear stress' PFA-100 C-EPI assay, but not on the 'low shear stress' VerifyNow® or Multiplate® assays (Table 2.4C) (Murphy *et al.,* 2020). The findings were attributed to a higher median daily dose of aspirin in the early symptomatic *vs*. asymptomatic patient population (225 mg *vs*. 75 mg). Early phase symptomatic patients had a higher prevalence of aspirin-HTPR on the PFA-100 (28.6%) compared with the VerifyNow (9.5%; P = 0.049), but not Multiplate assays (11.9%; P = 0.10). The prevalence of antiplatelet–HTPR was positively influenced by higher shear stress levels, but not MES status (Table 2.4C) (Murphy *et al.,* 2020).

### *2.3.3C. Platelet Count and Reticulated Platelets in Symptomatic versus Asymptomatic Carotid Stenosis*

Platelet counts were higher in early and late symptomatic *vs*. asymptomatic ≥70% carotid stenosis (McCabe *et al.,* 2005), in early symptomatic *vs*. asymptomatic ≥50% carotid stenosis (Kinsella *et al.,* 2013), and in early and late symptomatic *vs*. asymptomatic ≥ 50% carotid stenosis (Murphy *et al.,* 2018) (Table 2.4B). Furthermore, the % reticulated platelet fraction, quantified on an automated Sysmex XE-2100 Haematology Analyser as a marker of platelet production and turnover, was also higher in early (5.78%), late symptomatic (5.11%), and post-intervention (5.28%) than asymptomatic patients (3.48%) in one study (Murphy *et al.,* 2018).

### 2.3.4. Symptomatic and Asymptomatic Carotid Artery Stenosis Patients undergoing Carotid or Pharmacological Intervention

#### *2.3.4A. Platelet activation around the time of carotid intervention*

Two studies showed increased platelet activation (Roblesset *al.,* 2002; Assadian *et al.,* 2008), whereas 2 showed no increase in platelet activation at the time of carotid endarterectomy (CEA) (Vogten *et al.,* 2008), or stenting (Liu *et al.,* 2009) (Table 2.5A)

#### *2.3.4B. Platelet function/reactivity around at the time of endarterectomy*

Platelet reactivity increased in 4 studies (Vogten *et al.,* 2008; Robless *et al.,* 2002; Assadian *et al.,* 2008; Webster *et al.,* 2004), and decreased in one study around the time of CEA (Backovic *et al.,* 2016) (Tables 2.4A, 2.4B and 2.4C). The relationship between the presence of 'unstable carotid plaques' (based on grey-scale median measurements) and platelet reactivity on aspirin on the PFA-100 on day 2 after CEA was assessed in patients with ≥70% carotid stenosis (Lewszuk *et al.,* 2015) (88% symptomatic; Table 2.5C). Aspirin-HTPR was reported in 29% of patients (N=19) (Lewszuk *et al.,* 2015). However, one cannot draw reliable conclusions from this study because of inconsistent definitions of unstable plaques and unreliable definitions of aspirin-HTPR status (Lewszuk *et al.,* 2015). Clopidogrel-HTPR status was assessed with Multiplate whole blood aggregometry in patients with carotid stenosis who had undergone CEA on 75mg aspirin daily, 24 hours, 7 days and 30 days after subsequently commencing 75 mg of clopidogrel daily post-operatively (Table 2.5C) (Backovic *et al.,* 2016). The prevalence of clopidogrel-HTPR decreased from 79.5% at 24 hours to 36.6% at 7 days and 25% at 30 days post-CEA. These findings may have partly reflected evolving inhibition of platelet function with clopidogrel over time without administration of a loading dose, in addition to partial resolution of the acute phase response following surgery. Clopidogrel-HTPR at 30

days was identified in 53.3% of patients who were heterozygous (N=19) or homozygous (N=1) for the CYP2C19*2 genetic polymorphism *vs*. only 14.6% who did not have this genotype (P<0.001) (Backovic *et al.,* 2016). However, the authors arbitrarily defined clopidogrel-HTPR in patients who had received '30 days of clopidogrel' using criteria established in their local laboratory, and clopidogrel-HTPR status based on ADP-induced aggregation alone was not reported, thus limiting the ability to interpret these data in the context of other studies in the literature.

### *2.3.4C. Platelet function/reactivity around the time of carotid artery stenting (CAS) (Tables 2.4B and 2.4C)*

Platelet reactivity increased after CAS in one study (Szapary *et al.,* 2009), was unchanged by the procedure in one study (Liu *et al.,* 2009), and increased in some patients but remain unchanged in other patients in another study (Tsujimoto *et al.,* 2016). One study assessed patients undergoing CAS for >80% ACS or >50% SCS who were on some combination of 100mg aspirin daily and either clopidogrel 75 mg daily and/or cilostazol 200mg for a minimum of 7 days pre-operatively at the discretion of the treating physician (Tsujimoto *et al.,* 2016) (Table 2.5C). Platelet aggregation was assessed before and 4 days after CAS in response to stimulation with various concentrations of ADP and collagen. Increased ADP-induced or collagen-induced platelet aggregation between baseline and 4 days post-CAS was observed in 47%, with no significant change in 53% of patients. Although hyperintense plaques on time-of-flight MRA were associated with a greater risk of increased platelet aggregation over time (OR: 25.2, 95% CI: 2.00-316.2), the incidence of new DWI lesions post-CAS was not associated with platelet reactivity status. This study suggests that CAS may provoke increased platelet aggregation in an important proportion of patients on combination antiplatelet treatment. However, despite the scientific strengths of this study, the more complex aggregometry protocols and unclear rationale for the use of triple antiplatelet therapy in some patients limit

generalisability of the results. In patients with angiographically-confirmed 'peripheral artery disease' on aspirin and clopidogrel who underwent diagnostic angiography (carotid and lower extremity) or surgical or endovascular therapeutic intervention, aspirin-HTPR was observed in 20% and clopidogrel-HTPR in 39% of carotid stenosis patients on the VerifyNow (Yeo *et al.,* 2018) (Table 2.5C). Although 26% of the entire study population had experienced a prior TIA or stroke, it was not clear whether all had prior symptoms in the carotid territory (Yeo *et al.,* 2018).

### *2.3.4D. Platelet function/reactivity/activation in carotid stenosis patients changing antiplatelet therapy*

Four studies assessed platelet aggregometry in SCS patients undergoing modification of their antiplatelet regimen (King *et al.,* 2011; Markus *et al.,* 2005; Nakagawa *et al.,* 2014; Backovic *et al.,* 2016) with variable impacts on platelet reactivity profiles (Tables 2.4B, 2.4C and 2.5), and one study assessed unstimulated and ADP-induced platelet-fibrinogen binding (as a marker of platelet activation status) in response to adding clopidogrel to aspirin therapy around the time of CEA (Payne *et al.,* 2004) (Tables 2.4A and 2.4B). Details are summarised in relevant tables.

### 2.3.5. Assessment of platelet activation or reactivity/function in patients with 'additional neuroimaging evaluation' (Table 2.6)

In addition to the TCD studies outlined above, 5 studies assessed platelet activation status or reactivity/function in patients with additional neuroimaging evaluation.

There were elevated sP-Selectin levels and reduced cell surface CD62P expression in MES +ve *vs*. MES-ve patients within 4 days of acute 'atherothrombotic' ischaemic stroke, with increased sP-Selectin levels in MES+ve *vs*. MES-ve ACS patients (Ritter *et al.,* 2009). The authors suggested that the increased levels of sP-Selectin and reduced platelet surface CD62P expression in MES+vs *vs*. MES-ve acute stroke patients indicated shedding of this antigen from the platelet surface in MES+ve patients. However, cell surface CD62P expression was not significantly lower in asymptomatic MES+ve *vs*. MES -ve patients, the exact proportion of stroke patients with extracranial carotid rather than intracranial or other arterial stenoses was not specified, the precise degree of carotid stenosis was not reported, and comparisons of data between SCS and ACS patients were not performed (Ritter *et al.,* 2009).

Dawson *et al.* analysed TCD data in patients with SCS and ACS (Dawson *et al.,* 2012). At least 95% of symptomatic patients had >50% carotid stenosis, and those with < 50% stenosis had a 'symptomatic ulcerated plaque'. Twenty-one per-cent had aspirin-HTPR on the PFA-100® C-EPI assay, 12.9% on the VerifyNow® Aspirin Rapid Platelet Function Assay (RPFA), and 8.1% on both. The prevalence of aspirin-HTPR on at least one device was higher in MES-positive than MES-negative patients (50% *vs*. 17.4%; P = 0.018). The prevalence of aspirin-HTPR was not significantly different between MES+ve and MES-ve patients on the PFA-100, but was significantly higher on the VerifyNow (Dawson *et al.,* 2012). This study could be interpreted as suggesting that the low shear stress VerifyNow might be more sensitive than the high shear stress PFA-100 at detecting differences in Aspirin-HTPR status

between MES +ve and MES-ve patients overall, regardless of symptomatic status, but the number of subjects involved in some of these subgroup analyses was limited.

The IMPACT trial prospectively randomised 35 patients with >90% 'ACS' on Duplex ultrasound, who had no ipsilateral cerebrovascular events within the preceding 4 months, to receive either a 300mg or 600mg loading dose of clopidogrel 4 days before CAS, followed by 75mg daily thereafter (Van Der Heyden *et al.,* 2013). All received 100mg aspirin daily and had TCD recordings at the time of the procedure as well as platelet function testing pre-CAS after 4 days of clopidogrel. There were no differences in the number of MES between treatment groups, or in the % ADP-induced platelet aggregation (P ≥0.85) or P2Y12 reaction units on the VerifyNow (P=0.77), indicating that there was no benefit of loading with a higher dose of clopidogrel before stenting.

A retrospective study analysed the potential importance of assessing antiplatelet-HTPR status on the VerifyNow, when testing was performed either before CAS **or** within two days of CAS in patients with ≥ 50% stenosis (Song *et al.,* 2013). There was no difference in the prevalence of aspirin-HTPR between patients with and without new ischaemic lesions, but clopidogrel-HTPR was more common in patients with than in those without new DWI lesions (82.2% *vs*. 41.9%). Multivariate logistic regression analysis, after adjustment for age, gender and degree of stenosis, suggested that clopidogrel-HTPR was associated with an increased risk of developing new ischaemic lesions after CAS (OR 6.804, P=0.001). However, this study was retrospective, the precise proportion who had VerifyNow testing pre-or post CAS was not specified, so some patients may only have developed antiplatelet-HTPR in response to stenting. Therefore, one cannot conclude that clopidogrel-HTPR definitely predicts development of new DWI lesions post-CAS from this hypothesis-generating study.

In phase I of a study by Nakagawa *et al*., patients with >80% ACS (N = 16) or >50% SCS (N=12) received aspirin-clopidogrel combination therapy for ≥4 weeks before VerifyNow testing; antiplatelet therapy was not altered based on HTPR status (Nakagawa *et al.,* 2014). In phase II, another group of patients (19 ACS and 17 SCS) received aspirin-clopidogrel for ≥4 weeks, but patients with clopidogrel-HTPR on VerifyNow testing two days before CAS also received 200mg/day of cilostazol at that point (N=13). Fourteen per-cent of patients in phase I and 11% in phase II had aspirin-HTPR; 43% in phase I and 36% in phase II had clopidogrel-HTPR. Amongst patients with clopidogrel-HTPR in phase II who subsequently received adjunctive cilostazol, P2Y12 inhibition was enhanced *vs*. before commencing cilostazol. New ipsilateral ischaemic lesions on MR-DWI performed 1 day after CAS were less common in phase II than in phase I, but there were no differences in the incidence of 'clinically-evident thromboembolic or haemorrhagic events' between the 2 study phases. However, the study was small, time from symptom onset in symptomatic patients was not recorded, so one cannot conclude that the use of triple antiplatelet therapy was safe or effective in this setting.

### 2.3.6. Quality Assessment and Risk of Bias

21 of 43 (48.8%) studies were considered to have a 'low risk' of bias and a further 17/43 (39.5%) studies had some domains categorised as 'sound for a non-randomised study but not comparable to a rigorous randomised trial'. The overall risk of bias was deemed to be low or moderate in 38/43 (88%) included studies (Table 2.7).'

## 2.4 Discussion

This systematic review increases our understanding of the importance of platelets in the pathogenesis of cerebrovascular symptoms in patients with carotid stenosis and of the profile of platelet biomarkers over time in patient subgroups.

There is limited evidence that platelets are excessively activated in patients with ≥ 30% ACS *vs*. controls, with 2 positive studies in patients with > 50% ACS stenosis on treatment with antiplatelet therapy in whom carotid intervention might be considered. One large single-centre study indicated that quantification of coated platelets in combination with assessment of stenosis severity may aid risk-stratification in patients with ≥ 50-99% ACS. To our knowledge, this is the first and only study to suggest that platelet biomarkers have prognostic value in ACS patients. However, due to the observational, single-centre study design, corroborative studies are required.

There is now convincing evidence that platelets are excessively activated in patients with ≥ 30% SCS *vs*. controls despite treatment with commonly-prescribed antiplatelet regimens such as aspirin, aspirin + dipyridamole, clopidogrel or ticlopidine (≥11 positive small-medium sized studies and one neutral study). Although most studies could not determine whether increased platelet activation preceded symptom onset or arose secondary to a TIA/stroke, these data provide a rationale to investigate a strategy of targeting SCS patients with more intensive antithrombotic treatment to reduce the risk of subsequent vascular events.

Platelet activation status does not necessarily correlate with platelet function/reactivity status and new data pertaining to on-treatment platelet function/reactivity and HTPR status have emerged over the last decade. The prevalence range for antiplatelet-HTPR recorded in the literature is between 23-57% for aspirin and 25-100% for clopidogrel in patients with ≥ 50-99% ACS (Table 2.2B)

(Kinsella *et al.,* 2017; Murphy *et al.,* 2020). The prevalence of Aspirin-HTPR varied between 9.5-64% and the prevalence of clopidogrel-HTPR varied between 0-83% in patients with ≥ 50% SCS (Tables 2.2C and 2.4C). One study also showed an increased prevalence of aspirin-HTPR in MES+ve *vs*. MES-ve patients (Dawson *et al.,* 2012). However, prescribed doses of antiplatelet therapy and the timing of assessment of antiplatelet-HTPR status after commencing treatment varied between studies, and prevalence estimates were also dependent on the device used to assess platelet function/reactivity which may be positively influenced by higher shear stress levels (Murphy *et al.,* 2020). Therefore, because of the very wide prevalence ranges observed in different studies, it is not clear which if any of these platelet function testing platforms are likely to inform future treatment decisions in the important proportion of ACS and SCS patients with antiplatelet-HTPR on their prescribed antiplatelet regimen. This might be of clinical relevance due to emerging data that antiplatelet-HTPR status may predict the risks of recurrent vascular events/outcomes in TIA and ischaemic stroke patients overall (Lim *et al.,* 2020; Rutten *et al.,* 2014). Although some studies showed that aspirin + clopidogrel combination therapy decreased ADP-induced platelet activation *vs*. aspirin alone (Payne *et al.,* 2004), and decreased ADP-induced platelet aggregation *vs*. aspirin + dipyridamole combination therapy (King *et al.,* 2011), these data were not linked to clinical ischaemic or haemorrhagic outcomes. Accordingly, routine use of *ex vivo* platelet function/ reactivity testing to tailor antiplatelet therapy is not currently recommended outside of a research setting in patients selected for optimal medical or carotid interventional treatment (deBorst *et al.,* 2015; Leunissen *et al.,* 2015). Adequately-sized, prospective studies are urgently warranted to address this important issue in patients with ACS, SCS and non-cardioembolic TIA/ischaemic stroke overall (Lim *et al.,* 2020). These studies should ideally include more than one type of platform to assess antiplatelet-HTPR status because no single device has been proven to be superior to another at predicting outcomes in patients with carotid stenosis to date.

The combination of data on elevated platelet counts, reticulated platelet fraction and leucocyte-platelet complexes supports the hypothesis that there is an ongoing stimulus to increased platelet production and secretion/turnover, and enhanced platelet activation before or early after TIA/ischaemic stroke in recently symptomatic *vs*. ACS patients (Tables 2.3A and 2.3B). The available prospective data support the hypothesis that these findings are not just reflective of an acute-phase response. These platelet biomarkers may contribute to the pathogenesis of first and subsequent strokes in patients with recently symptomatic and asymptomatic ≥50% carotid stenosis, including those who are MES-ve (Kinsella *et al.,* 2013; Murphy *et al.,* 2019). FBC measurements, including automated measurements of the % reticulated platelet fraction are easy to perform, but their association with outcomes after initial presentation needs to be addressed in future prospective, multi-centre studies.

Seven studies/substudies showed that platelets may become more reactive or activated following CEA or CAS, either as an acute phase response to intervention or peri-procedural treatment, whereas 2 showed no change in platelet activation status following intervention (Tables 2.4A, 2.4B and 2.4C). Some neutral platelet activation studies may also partly reflect the relative insensitivity of the chosen surface markers used to assess platelet activation status (e.g. CD62P expression and PAC-1 binding), and the prescribed peri-procedural antithrombotic regimens. These data inform us that assessment of antiplatelet-HTPR status in the peri-procedural period may be influenced by the procedure, but it is not known whether these findings predict immediate or long-term clinical outcomes.

The Asymptomatic Carotid Emboli Study and other prospective studies have shown that the presence of MES on TCD increases the risk of ipsilateral cerebrovascular events in patients with ACS (Markus *et al.,* 2010; Spence *et al.,* 2010) and SCS (King

*et al.,* 2011; Markus *et al.,* 2005). Pilot studies have revealed increased platelet activation (Ritter *et al.,* 2009) and a higher prevalence of aspirin-HTPR in MES+ve *vs*. MES-ve patients (Dawson *et al.,* 2012). Other studies showed no clear association between platelet reactivity and MES status in ACS patients (Van Der Heyden *et al.,* 2013), or between platelet activation (Kinsella *et al.,* 2013; Murphy *et al.,* 2019) or reactivity (Kinsella *et al.,* 2017; Murphy *et al.,* 2020) and MES status in SCS *vs*. ACS. However, simultaneous assessment of platelet activation and MES status has revealed evidence of increased platelet activation in recently symptomatic *vs*. asymptomatic MES-ve patients, thus improving our insight into the potential pathogenesis of TIA/stroke in patients with recently symptomatic ≥ 50% carotid stenosis (Kinsella *et al.,* 2013; Murphy *et al.,* 2019) who might have been considered to be at lower risk of vascular events based on TCD data alone.

This systematic review has a number of **limitations.** Many studies were of limited size and most were single-centre, so some hypotheses need to be retested in larger or independent, multicentre cohorts. Most small-medium case-control studies have indicated that patients with mild, moderate or severe SCS exhibited increased platelet activation compared with healthy controls. However, it is unclear whether increased platelet activation predisposes to TIA or stroke in carotid stenosis patients, whether these findings are reflective of an acute phase response to cerebral or ocular ischaemia/infarction, or whether there is a combination of both factors at play. Lack of explicit recruitment criteria and precise categorisation of stenosis severity hindered precise interpretation of some studies and applicability of data to patients in whom carotid intervention might be considered. Some studies did not clearly describe the interval between TIA/stroke onset and study inclusion which is now critically important when deciding about the need for best medical therapy alone, CEA or CAS. Furthermore, in studies on antiplatelet-HTPR status, the timing of measurement of HTPR status following index TIA/stroke, and whether or not initial 'loading doses' of

antiplatelet agents were prescribed was not always available. As outlined above, the majority of studies (88%) were deemed to have a low-moderate risk of bias. Although we could not completely exclude positive publication bias, this comprehensive review included a wide range of positive, neutral and negative studies, thus minimising selection bias in our own systematic review process. We cannot comment further on the 'quality of evidence' from the included studies because we did not use e.g. the Grading of Recommendations, Assessment, Development and Evaluation (GRADE) Working Group (www.gradeworkinggroup.org) approach to grade the evidence in relevant sections of our systematic review; this was beyond the scope of this study.

## 2.5 Conclusions

There is increasing evidence that platelets are excessively activated in patients with recently symptomatic carotid stenosis compared with healthy controls and *vs*. those with asymptomatic carotid stenosis, including those who are MES-ve, and may become activated/hyper-reactive following carotid intervention. A clinically important proportion of patients with SCS and ACS have antiplatelet-HTPR on commonly-prescribed antiplatelet therapy, but available data do not currently support routine alteration of treatment based on *ex vivo* platelet function/reactivity tests. Further prospective multicentre studies are required to determine whether models combining clinical, neurovascular-imaging and platelet biomarker data can enhance our ability to predict outcome events, and facilitate optimised antiplatelet therapy in individual patients with carotid stenosis. Data on platelet counts, reticulated platelets and antiplatelet-HTPR status from user-friendly *ex vivo* tests of platelet function/reactivity in whole blood are easily suited to larger, multi-centre studies which could inform such risk-modelling experiments using platelet biomarkers in whole blood in patients with ACS and SCS. Quantification of leucocyte-platelet complexes could also be performed in multiple specialist centres, because standardisation of whole blood flow cytometry protocols is now feasible across sites on newer generation flow cytometers (e.g. the Becton Dickinson flow cytometer which is located in the Meath Foundation Research Lab at TUH-AMNCH), and holds promise for risk-stratification in this patient cohort.

**Figure 2.1: Flow chart summarising search strategy in keeping with the PRISMA statement**

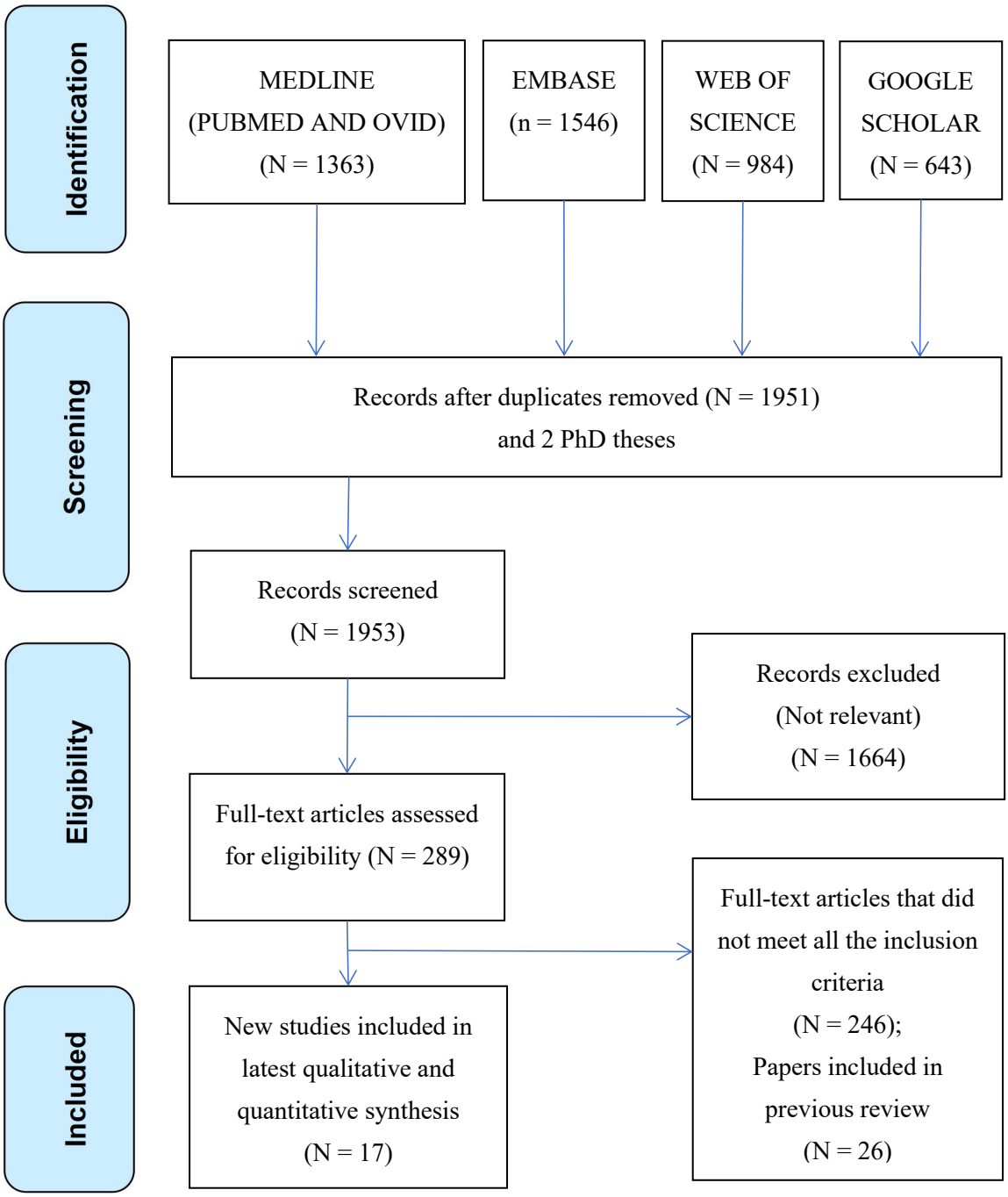

**Table 2.1: Overview of commonly-available techniques used to assess platelet activation status and platelet function technology platforms used to assess platelet function/reactivity in this systematic review (Some data adapted from Subramanian A et al., 2022)**

| Platelet Activation Assay / Platelet Function-Reactivity Test | Principle Employed | Platelet-Specific Biomarker Assay |
|---|---|---|
| **Plasma Soluble P-selectin** | **ELISA:** Marker of platelet activation as sP-selectin is secreted directly into plasma after fusion of platelet α-granules with the platelet surface membrane. Also marker of endothelial cell activation as sP-selectin is also secreted from Weibel-Palade bodies during endothelial activation. As with all assays on platelet poor plasma (PPP), sample preparation involves centrifugation and manipulation, and is subject to artefactual *in vitro* platelet activation, with the potential loss of important platelet subpopulations. Requires trained personnel and is time-consuming. | No |
| **Serum Soluble CD40** | **ELISA:** Assay to assess levels of soluble CD40, the receptor for CD40 Ligand, which has been reported to be present on platelets, B cells, monocytes, macrophages, dendritic cells, and endothelial cells. Requires trained personnel and is time-consuming. | No |
| **Plasma or Serum Soluble CD40 Ligand** | **ELISA:** CD40 Ligand is a transmembrane protein which has been identified on stimulated CD4+ve T cells, stimulated platelets, mast cells, basophils, and vascular smooth muscle cells. Surface-expressed CD40L on platelets is then cleaved from the platelet, subsequently generating a soluble fragment (soluble CD40 ligand) which may have pro-inflammatory and pro-coagulant activity etc. Requires trained personnel and is time-consuming. | No |
| **Serum 11-dehydro-thromboxane $B_2$** | **ELISA:** Thromboxane $A_2$ is a platelet agonist and vasoconstrictor which may be derived from platelets or endothelial cells. Thromboxane $A_2$ is converted into its stable metabolite thromboxane $B_2$. Assays to assess serum thromboxane $B_2$ levels are an indirect measure of inhibition of thromboxane biosynthesis by aspirin. Requires trained personnel and is time-consuming. | No |
| **Plasma Platelet Factor 4 and β-Thromboglobulin** | **ELISA:** Platelet *α-granules* release several substances, including these platelet-specific proteins upon activation which can be quantified in PPP. Requires trained personnel and is time-consuming. | Yes |
| **Optimal Aggregometry** | **Responsiveness to agonists in platelet rich plasma (PRP)** at **'low shear stress',** mimicking conditions which might be seen in veins, large arterioles and normal arteries. Of note, in flowing blood, each lamina of blood exerts some degree of shear stress on its neighbour (liquid shear stress), and the vessel wall also exerts shear stress on the flowing blood (wall shear stress). Shear stress levels rise *in vivo* as blood flows past an arterial stenosis and platelets may be activated by high shear stress rates. Agonists during low shear stress optical aggregometry can be tailored to suit the antiplatelet regimen prescribed in individual patients to assess antiplatelet-HTPR status on e.g. aspirin (Arachidonic Acid) or P2Y12 antagonists (ADP). When an agonist is added to PRP, platelets aggregate and the transmission of light through the sample increases. For example, if aspirin is not inhibiting platelet function adequately, addition of Arachidonic Acid will induce aggregation and the transmission of light through the sample increases. The change in light transmission is typically detected photo-electrically and recorded over time. This technique cannot assess platelet function/reactivity in whole blood, assays are subject to artefactual *in vitro* platelet activation during sample collection and processing, requires trained personnel and is labour-intensive. Individual labs may define criteria for antiplatelet-HTPR status based on prior published data or their own local reference ranges. | Yes |
| **Impedence Aggregometry** | **Responsiveness to agonists in whole blood at low shear stress.** Agonists can be tailored to suit the antiplatelet regimen prescribed in individual patients to assess antiplatelet-HTPR status on e.g. aspirin (Arachidonic Acid) or $P2Y_{12}$ antagonists (ADP). This technique requires a high blood volume, trained personnel, is labour-intensive and semi-quantitative. Individual labs may define criteria for antiplatelet-HTPR status based on prior published data or their own local reference ranges. | Yes |
| **Platelet Function Analyser (PFA-100®)** | **Assessment of platelet adhesion/aggregation and antiplatelet-HTPR status in whole blood in response to moderately 'high shear stress' ($5000s^{-1}$) and biochemical stimulation with agonists** in the test cartridge. Rapid, user-friendly, reproducible, performed in the physiological milieu of whole blood, and no specific expertise required once trained. May detect antiplatelet effects of aspirin (Collagen-Epinephrine cartridge) and $P2Y_{12}$ antagonists (INNOVANCE P2Y™ cartridge) and additional antiplatelet effects of dipyridamole over aspirin (Collagen-ADP cartridge). The antiplatelet agents in question are expected to prolong 'closure times' on the relevant assays beyond the normal ranges for controls who are not on antiplatelet therapy, with 'cutpoints' for defining antiplatelet-HTPR status based on prior published criteria by the manufacturer or their own local reference range. The shear rate and doses of agonists are fixed, and test results are influenced by VWF antigen levels. | Yes |
| **VerifyNow®** | **Assessment of platelet aggregation and antiplatelet-HTPR status in whole blood in response to agonist stimulation at 'low shear stress'.** Rapid, user-friendly, reproducible, and no specific expertise required once trained. May detect antiplatelet effects of aspirin (arachidonic acid in Aspirin cartridge [Aspirin Reaction Units = ARU]) or $P2Y_{12}$ antagonists (ADP, iso-thrombin receptor activating peptide and PAR-4 activating peptide in the P2Y12/PRUTest cartridge [P2Y12 Reaction Units = PRU]). The antiplatelet agents in question are expected to shorten 'reaction units' on the relevant assays below the normal ranges for controls who are not on antiplatelet therapy, with 'cutpoints' for defining antiplatelet-HTPR status based on prior published criteria by the manufacturer. The shear rate and doses of agonists are fixed. | Yes |
| **Multiplate® Impedance Aggregometry** | **Assessment of platelet aggregation and antiplatelet-HTPR status in whole blood at 'low shear stress'.** Rapid, reproducible. May detect antiplatelet effects of aspirin (Arachidonic Acid/Aspirin assay [Units]) and $P2Y_{12}$ antagonists (ADP assay [Units]). The antiplatelet agents in question are expected to shorten the recorded 'units' on the relevant assays below the normal ranges for controls who are not on antiplatelet therapy, with 'cutpoints' for defining antiplatelet-HTPR status typically based on prior published criteria by the manufacturer. The shear rate and recommended doses of agonists are fixed, but capacity to use variable doses of agonists exists. Pipetting steps required during agonist preparation and sample processing. | Yes |
| **Flow Cytometry** | **Assessment of platelet activation status** by measuring platelet surface expression of activation markers (e.g. CD62P [released from platelet alpha and dense granules], CD63 [released from lysosomes or dense granules], PAC1 [IgM murine monoclonal antibody which only binds to activated platelet GpIIb/IIIa receptors], CD40-Ligand [see above]). **Assessment of leucocyte-platelet complexes** in whole blood has been shown to be a more sensitive marker of platelet activation status in carotid stenosis patients (including neutrophil-platelet, monocyte-platelet and lymphocyte-platelet complexes). **Assessment of platelet-derived microparticles** (proposed to have a role in the development of atherosclerosis) also feasible.<br>One can also **assess platelet reactivity** in response to agonist stimulation, tailored to suit the antiplatelet regimen prescribed in individual patients. Individual labs may define criteria for antiplatelet-HTPR status based on their own local reference ranges, unless analysis is done in a 'centralised lab'.<br>Technique is sensitive and specific, flexible, and assays can be performed in whole blood or PRP. Trained personnel required, labour-intensive; expensive equipment and reagents required on site, unless centralised assays are performed. | Yes |

## Table 2.2A: Soluble and Surface Markers of Platelet Activation Status in Asymptomatic Carotid Stenosis *vs.* Controls and Coated Platelets

| 1st Author | Year | Study Design | Patient Population | Control Population | Soluble Platelet Biomarkers | | | | Antiplatelet Regimen | Sample Type |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | sP-selectin | sCD40 | CD40L | 11-dehydro TxB2 | | |
| Stolz | 2002 | Case-control | >70% (N = 19) and < 70% (N = 11) Carotid stenosis | Healthy subjects (N = 13) | ↑ in > 70% Carotid stenosis *vs*. Controls: 452.7 ± 169.7 *vs*. 256.7 ± 74.8 ng/ml, P < 0.01 | | | | Not reported | Serum |
| Novo | 2005 | Case-control | < 50% Carotid stenosis (N = 42) | Subjects without Carotid stenosis (N = 21) | | | ↑ 6.2 (1.4–15.8) *vs*. 1.4 (0.5–4.5) ng/ml; P < 0.0001 | | Aspirin | Plasma |
| Balla | 2006 | Case-control | >30% Carotid stenosis/occlusion (N = 60) | Healthy subjects with no Carotid plaques (N = 30) | | ↔ 85 ± 56.9 *vs*. 79.3 ±18.7 pg/mL; P = 0.34 | ↑ 6.9 ± 5 *vs*. 4.5 ± 3.0 ng/mL; P = 0.038 | | Aspirin, Clopidogrel, Ticlopidine | Serum |
| Enomoto | 2010 | Case-control | >50% Carotid stenosis (N = 10) | Age-matched non-atherosclerotic Controls (N = 8) | | | ↑ P < 0.01 | ↑ P < 0.05 | Aspirin | PRP |
| Jurk | 2010 | Case-control | >50% Carotid stenosis (N = 48) | Age-matched healthy subjects without cardiovascular disease (N = 30) | ↔ P > 0.05 | | ↔ 1.49 ± 0.82 *vs*.1.16 ± 0.55; P > 0.05 | | Aspirin, Ticlopidine | Whole blood |
| | | | | | **Surface Platelet Biomarkers** | | | | | |
| | | | | | **CD62P** | **CD63** | **CD40** | **CD40L** | | |
| Stolz | 2002 | Case-control | >70% (N = 19) and <70% (N = 11) Carotid stenosis | Healthy subjects (N = 13) | ↑ 35.7 ± 9.9% in >70% stenosis, P < 0.001; ↑ 21.2 ±12.6% in <70% stenosis, P < 0.01 *vs*. Controls (7.5 ± 2.2%) | | | | Not reported | Whole blood |
| Cha | 2003 | Longitudinal, Observational Case-control | >50% Carotid stenosis (N = 20) | Healthy subjects (N = 24) | ↑ P < 0.01 | ↑ P < 0.01 | | ↔ | None | Whole blood |
| Jurk | 2010 | Case-control | >50% Carotid stenosis (N = 48) | Age-matched healthy controls without cardiovascular disease (N = 30) | ↔ P > 0.05 | ↔ P > 0.05 | | | Aspirin, Ticlopidine | Whole blood |
| | | | | | **Coated Platelets** | | | | | |
| Kirkpatrick | 2014 | Prospective Cohort | Asymptomatic carotid stenosis: Consecutive eligible patients with moderate (≥ 50%) to severe (80-99%) stenosis (N = 329) | | Incidence of ipsilateral stroke or TIA in patients with ≥50% stenosis and ≥45% coated-platelets was 21.5 per 100 person-years *vs*. 1.27 per 100 person-years in patients with ≥50% stenosis and <45% coated-platelets (P < 0.0001) | | | | Aspirin monotherapy, Aspirin + Clopidogrel, Other antiplatelets; Total N = 253 (77%) | PRP |

**Legend for Table 2.2A**: ↑ = increased, ↔ = not significantly different. Where data are available, absolute values are reported as mean ± standard deviation (SD) or mean ± 2 SD or median (range). TIA, Transient Ischaemic Attack; ICA, Internal Carotid Artery; sP-selectin, Soluble P-selectin; sCD40, Soluble CD40; CD40L, CD40 Ligand; 11-dehydro TxB2, 11 dehydrothromboxane $B_2$; SD, Standard Deviation.

## Table 2.2B: Markers of Platelet Function and Reactivity in Asymptomatic Carotid Stenosis *vs*. Controls

| 1st Author | Year | Study Design | Patient Population | Control Population | PFA-100 C-EPI CT | PFA-100 INNOVANCE P2Y CT | 'Platelet Aggregometry' | Antiplatelet-HTPR (%) | Antiplatelet Regimen | Sample Type |
|---|---|---|---|---|---|---|---|---|---|---|
| Kinsella | 2017 | Longitudinal Observational Case-control | Asymptomatic ≥50-99% carotid stenosis (N = 31) | Healthy controls (N = 18) | Aspirin Monotherapy: 148 sec; Aspirin alone or in combination with Dipyridamole MR or Clopidogrel: 164 sec | N/A | N/A | 50% on Aspirin monotherapy; 46% on Aspirin alone or in combination with Dipyridamole MR or Clopidogrel | Aspirin N = 22 (71%), Aspirin / Dipyridamole N=2 (6%), Clopidogrel N = 5 (16%), Aspirin + Clopidogrel N = 2 (6%) | Whole blood |
| Murphy | 2020 | Longitudinal Observational Case-control | Asymptomatic ≥50-99% carotid stenosis (N = 34) | Healthy controls (N = 19) for some; Manufacturer's normal range for other assays | Aspirin monotherapy or Aspirin in combination with Dipyridamole MR or Clopidogrel: 160.5 Sec | Clopidogrel monotherapy or combination therapy: 301 sec | **VerifyNow** Aspirin ARU: 460.5 P2Y12 PRU: 239.5 **Multiplate** ASP: 22 U ADP: 62.5 U | 23-57% across all devices for Aspirin-HTPR on Aspirin alone or in combination with Dipyridamole MR or Clopidogrel; 25-100% across all devices for Clopidogrel-HTPR on Clopidogrel alone or in combination with Aspirin | Aspirin N = 22 (64.7%), Aspirin / Dipyridamole N = 7 (20.6%) Clopidogrel N = 2 (5.9%), Aspirin + Clopidogrel N = 2 (5.9%) | Whole blood |

**Legend for Table 2.2B**: PFA-100 = Platelet Function Analyser 100; C-EPI CT, Collagen-Epinephrine Closure Time; Platelet aggregometry includes modified aggregometry techniques (VerifyNow and Multiplate); HTPR, High on-Treatment Platelet Reactivity; ARU, Aspirin Reaction Units; PRU, P2Y12 Reaction Units; ASP, Aspirin Test with Arachidonic Acid (Multiplate Assay); ADP, Adenosine Diphosphate Test (Multiplate Assay); U, Units.

## Table 2.3A: Soluble Markers of Platelet Activation Status in Symptomatic Carotid Stenosis vs. Controls

| 1st Author | Year | Study Design | Patient Population | Control Population | Soluble Platelet Biomarkers | | | | | Antiplatelet Regimen | Sample Type |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | sP-selectin | sCD40 | CD40L | β-thromboglobulin | PF4 | | |
| Frijns | 1997 | Case-control | ≥30% carotid stenosis/occlusion (N = 34) | No cardiovascular, malignant, inflammatory or autoimmune diseases (N = 34) | ↑ 200 ±73 *vs*. 135±41 ng/ml; P < 0.0001 | | | | | Aspirin, Dipyridamole Ticlopidine | Plasma |
| Hashimoto | 2003 | Case-case | ≥50% carotid stenosis (N = 27) | <50% carotid stenosis (N = 274) | ↑ 53.2 ± 27.5 *vs*. 38.9 ± 20.5 ng/ml; P < 0.05 | | | | | Aspirin, Ticlopidine | Serum |
| McCabe | 2004 | Longitudinal case-control | Early (N = 19) and late phase (N = 16) post-TIA/stroke with ≥70% ICA stenosis /occlusion | No history of cerebrovascular disease or carotid stenosis on ultrasound (N = 27) | ↔ P ≥ 0.09 (Early and Late phase) | | | | | Aspirin, Aspirin + Dipyridamole, Clopidogrel | PPP |
| Blake | 2003 | Case-case | ≥30% ICA/CCA stenosis (N = 46) | N/A | | | ↑ 2.54 *vs*. 1.58 ng/ml; P < 0.02 in patients with intraplaque lipid on High-Resolution MRI | | | Not specified | Plasma |
| Balla | 2006 | Case-control | >30% carotid stenosis/occlusion (N = 60) | Healthy subjects without carotid plaques (N = 30) | | ↔ 85 ± 56.9 *vs*. 79.3 ± 18.7pg/mL; P = 0.34 | ↑ 6.9 ± 5 *vs*. 4.5 ± 3.0ng/mL; P < 0.038 | | | Aspirin, Clopidogrel, Ticlopidine | Serum |
| Shah | 1985 | Case-control | Prior TIA/Stroke (N = 58) 77% with carotid artery stenosis/occlusion (degree of stenosis not specified) | Young healthy individuals (N = 20) and older age-matched adults (N = 15) | | | | ↑ in thromboembolic (50.2±4.1 ng/ml), cardioembolic (56.3±5.2 ng/ml), uncertain aetiology (56.0±4.8 ng/ml) TIA / stroke *vs*. Controls (31.1 ±2.5 ng/ml; P < 0.001 | ↑ in thromboembolic TIA/stroke (14.9±3.5 ng/ml) *vs*. Controls (6.5±1.4 ng/ml); P < 0.05 | Aspirin | PPP |
| Jurk | 2010 | Case-control | Symptomatic >70% carotid stenosis (N = 25) | Age-matched healthy controls without cardiovascular disease (N = 30) | ↑ P < 0.05 | | ↑ 2.99 ± 1.08 *vs*. 1.16 ± 0.55ng/ml, P < 0.05 | | | Aspirin, Ticlopidine | Plasma |

**Legend for Table 2.3A**: ↑ = increased, ↔ = not significantly different. Where data are available, absolute values are reported as mean ± SD or mean ± 2 SD or median (range). sP-selectin, Soluble P-selectin, sCD40, Soluble CD40; PF4, Platelet Factor 4; TIA, Transient Ischaemic Attack; ICA, Internal Carotid Artery; CCA, Common Carotid Artery; SD, Standard Deviation.

## Table 2.3B: 'Surface Markers' of Platelet Activation Status in Symptomatic Carotid Stenosis *vs.* Controls

| 1st Author | Year | Study Design | Patient Population | Control Population | Surface Platelet Biomarkers | | | | | | | Antiplatelet Regimen | Sample Type |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | CD62P | CD63 | CD40L | PAC1 | NPC | MPC | LPC | | |
| McCabe | 2004 | Longitudinal, Observational Case-control | Early (N = 19) and late phase (N = 16) post TIA/stroke with ≥70% ICA stenosis/occlusion | No history of cerebrovascular disease or carotid stenosis on ultrasound (N = 27) | ↑ in late phase only P = 0.01 | ↔ | | ↔ | ↔ | ↔ | ↔ | Aspirin, Clopidogrel, Aspirin+ Dipyridamole | Whole blood |
| Cha | 2003 | Longitudinal, Observational Case-control | Atherosclerotic Ischaemic Stroke' (on IV heparin or S/C heparinoid; N = 25) | Healthy subjects (N = 24) | ↑ P < 0.01 | ↑ P < 0.01 | ↑ P < 0.01 | | | | | None | Whole blood |
| Zeller | 1999 | Case-control | Acute Symptomatic Carotid occlusion or >50% carotid, vertebral or basilar stenosis (N = 47) | Controls with no history of cerebrovascular disease (N = 72) | ↑ 9.4 ± 5.8 *vs.* 4.9 ± 2.8%; P < 0.001 | ↑ 15.7 ± 8.9 *vs.* 10.8 ± 4.5%; P < 0.001 | | | | | | Aspirin | PRP |
| Yip | 2006 | Longitudinal, Observational Case-control | ≥70% Symptomatic ICA stenosis (N = 35) | Subjects with angina pectoris undergoing cardiac catheterization as 'at-risk controls' (N = 20); Age-and gender-matched healthy volunteers (N = 20) | ↑ 2.93 ± 1.09 % *vs.* 1.74 ± 0.54 % in at-risk controls, and 1.40 ± 0.64% in healthy controls; P < 0.0001 | | | | | | | Aspirin + Clopidogrel | PRP |
| Jurk | 2010 | Case-control | Symptomatic >70 % carotid stenosis (N = 25) | Age-matched healthy controls without cardiovascular disease (N = 30) | ↑ P < 0.05 | ↑ P < 0.05 | | | ↑ 22.42 ± 3.2% *vs.* 10.3 ± 2.7%, P < 0.05 | ↑ 43.3 ± 3.4% *vs.* 17.4 ± 10.8%, P < 0.01 | | Aspirin, Ticlopidine | Whole blood |
| Turgut | 2011 | Case-control | Within 1 week of TIA/Stroke due to 'Large Artery Atherosclerosis' according to TOAST (% with extracranial and intracranial carotid or other stenoses unclear; prescribed antithrombotic regimens before venepuncture not specified; N = 31) | Age-matched healthy controls (N = 37) | ↑ 7.49 ± 5.06 *vs.* 3.89 ± 4.16; P = 0.003 | | | | ↑ 15.7 ± 3.99 *vs.* 11.2 ± 3.88, P < 0.0001 | ↑ 35.1 ± 11.6 *vs.* 24.6 ± 12.0, P = 0.002 | | Not specified at time of venepuncture; excluded patients on antithrombotic therapy before index event | Whole blood |
| Lukasik | 2013 | Observational Case-control | 98-172 days after 'Large-artery ischaemic stroke' (N = 94, 40% with 'carotid plaques') | 'Vascular disease age-, gender- and risk factor-matched controls with no prior TIA/Stroke/MI (N = 76; 34% with 'carotid plaques', so severity of extracranial carotid stenosis in controls unclear) | ↓ 3.1 *vs.* 3.6% unstimulated (P = 0.04); ↓ 79.5 *vs.* 89.5% with TRAP (P < 0.001); ↓ 44.1 *vs.* 52.6 with ADP (P = 0.009) | | | ↔ 2.4 *vs.* 2.2% unstimulated (P = 0.18); ↓ 63.6 *vs.* 74.2 % with TRAP (P = 0.009); ↓ 56.3 *vs.* 68.5% with ADP (P = 0.003) | | | | Aspirin | Whole blood |

**Legend for Table 2.3B**: ↑ = increased, ↓ = decreased, ↔ = not significantly different. Where data are available, absolute values are reported as mean ± SD or mean ± 2 SD or median (range). ICA, Internal Carotid Artery; LPC, Lymphocyte-Platelet Complexes; MPC, Monocyte-Platelet Complexes; NPC, Neutrophil-Platelet Complexes; TIA, Transient Ischaemic Attack; TOAST, Trial of ORG 10172 in Acute Stroke Treatment; SD, Standard Deviation; TRAP, Thrombin Receptor Activating Peptide; ADP, Adenosine Diphosphate

## Table 2.3C: Markers of Platelet Function and Reactivity in Symptomatic Carotid Stenosis *vs*. Controls

| 1st Author | Year | Study Design | Patient Population | Control Population | PFA-100 C-EPI CT | PFA-100 INOVANCE P2Y CT | Platelet aggregometry | Antiplatelet-HTPR (%) | Antiplatelet Regimen | Sample Type |
|---|---|---|---|---|---|---|---|---|---|---|
| McCabe | 2005 | Longitudinal, Observational Case-control | Stroke/ TIA in territory of ≥70% carotid stenosis/occlusion in early (N = 11) and late phases (N = 9) | Healthy subjects with no history of cerebrovascular disease (N = 23) | ↔ Early phase (P = 0.3); ↑ Late phase (P < 0.001) | N/A | No patients defined as Aspirin-HTPR on PFA-100 had Aspirin-HTPR on aggregometry | (7/11) 64% in early phase; (1/9) 11.1% in late phase on PFA-100 C-EPI cartridge | Aspirin | Whole blood for PFA-100; PRP for aggregometry |
| Kinsella | 2017 | Longitudinal Observational Case-control | Symptomatic ≥50-100% Carotid Stenosis in early (N = 46) and late phases (N = 35) | Healthy controls (N = 18) | Early phase: 152 Sec Late phase: 223 Sec (On aspirin alone or in combination with dipyridamole or clopidogrel) | N/A | N/A | Aspirin-HTPR: 55% in early phase; 28% in late phase on aspirin alone or in combination with dipyridamole or clopidogrel | Aspirin, Aspirin + Dipyridamole, Clopidogrel, Aspirin + Clopidogrel | Whole blood |
| Murphy | 2020 | Longitudinal Observational Case-control | Symptomatic ≥50% carotid stenosis in early (N = 43) and late phases (N =37) | Healthy controls (N = 19) for some; manufacturer's normal range for other assays | Early phase: 249 Sec Late phase: 229 Sec (On aspirin alone or in combination with dipyridamole or clopidogrel) | Early phase: 86 Sec, Late phase: 301 Sec (On Clopidogrel monotherapy or combination therapy) | **VerifyNow** ARU: 416.5 in early phase ARU: 435 in late phase; PRU: 245.5 in early phase PRU: 142.5 in late phase **Multiplate** ASP (U): 21 in early phase ASP (U): 21 in late phase ADP (U):76 in early phase ADP (U): 58 in late phase | Aspirin-HTPR across all devices on aspirin alone or in combination with dipyridamole or clopidogrel: 9.5–28.6% early phase, 13.9–38.9% late phase Clopidogrel-HTPR across all devices on Clopidogrel monotherapy or combination therapy: 16.7-83.3% early phase 0-83.3% in late phase | Aspirin, Aspirin + Dipyridamole, Clopidogrel, Aspirin + Clopidogrel | Whole blood |

**Legend for Table 2.3C**: ↑ = increased, ↔ = not significantly different. PFA-100, Platelet Function Analyser 100; C-EPI CT, Collagen Epinephrine Closure Time; Platelet aggregometry includes modified aggregometry techniques (VerifyNow and Multiplate); HTPR, High on-Treatment Platelet Reactivity; ARU, Aspirin Reaction Units; PRU, P2Y12 Reaction Units; ASP, Aspirin Test with Arachidonic Acid (Multiplate Assay); ADP, Adenosine Diphosphate Test (Multiplate Assay).

## Table 2.4A. Soluble Markers of Platelet Activation Status in Symptomatic *vs*. Asymptomatic Carotid Stenosis

| 1st Author | Year | Study Design | Patient Population | sP-selectin | CD40L | Antiplatelet Regimen | Sample Type |
|---|---|---|---|---|---|---|---|
| Jurk | 2010 | Case-control | Symptomatic >70% (N = 25) *vs*. Asymptomatic >50% carotid stenosis (N = 48) | ↑ P < 0.05 | ↔ 2.99 *vs*. 1.49 ng/ml; P > 0.05 | Aspirin, Ticlopidine | Plasma |

**Legend for Table 2.4A**: ↑ = increased, ↔ = not significantly different. Where data are available, absolute values are reported as mean ± SD, mean ± 2 SD or median (range). sP-selectin: Soluble P-selectin; CD40L, Soluble CD40 Ligand.

## Table 2.4B: Surface Platelet Biomarkers in Symptomatic *vs.* Asymptomatic Carotid Stenosis

| 1st Author | Year | Study Design | Patient Population | CD62P | CD63 | CD40 L | PAC1 | NPC | MPC | LPC | Platelet count (x $10^9$/L) | Antiplatelet Regimen | Sample Type |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| McCabe | 2005 | Longitudinal, Observational Case-Case/ Control | Acute Symptomatic (N = 19) and Late Symptomatic (N = 16) *vs.* Asymptomatic ≥70% carotid stenosis (N = 16) | ↔ Acute Symptomatic *vs.* Asymptomatic: 1.3% *vs.* 1.7%; P = 0.9 ↔ Late Symptomatic. *vs.* Asymptomatic 2.1% *vs.* 1.7%; P = 0.09 | ↔ Acute Symptomatic *vs.* Asymptomatic: 8.8% *vs.* 7.5%; P = 0.2 ↔ Late Symptomatic. *vs.* Asymptomatic: 7.8% *vs.* 7.5%; P = 0.3 | | ↔ Acute Symptomatic *vs.* Asymptomatic: 2.9% *vs.* 2.6%; P = 0.7 ↔Late Symptomatic *vs.* Asymptomatic: 3.0% *vs.* 2.6%; P = 0.3 | ↑ Acute Symptomatic *vs.* Asymptomatic: 2.7% *vs.* 2.2%; P = 0.004; ↔ Late Symptomatic *vs.* Asymptomatic: 2.4% *vs.* 2.2%; P = 0.07 | ↑ Acute Symptomatic *vs.* Asymptomatic: 5.2% *vs.* 4.4%; P = 0.046; ↔ Late Symptomatic *vs.* Asymptomatic: 4.5% *vs.* 4.4%; P = 0.3 | ↑ Acute Symptomatic *vs.* Asymptomatic: 2.6% *vs.* 2.1%; P = 0.02. ↔ Late Symptomatic *vs.* Asymptomatic: 2.2% *vs.* 2.1%; P = 0.9 | ↑ Acute Symptomatic *vs.* Asymptomatic: 275 *vs.* 223, P = 0.007 (EDTA); 231 *vs.* 193; P = 0.04 (Citrate) ↑ Late Symptomatic *vs.* Asymptomatic: 266 *vs.* 223, P = 0.05 (EDTA); 239 *vs.* 193, P = 0.02 (Citrate) | Aspirin, Clopidogrel, Aspirin + Dipyridamole | Whole blood |
| Cha | 2003 | Longitudinal, Observational Case-Case/Control | Atherothrombotic stroke (N = 25)§ *vs.* Asymptomatic >50% carotid stenosis (N = 20) | ↔ P > 0.05 | ↔ P > 0.05 | ↑ P < 0.01 | | | | | | None | Whole blood |
| Jurk | 2010 | Case-Case/Control | Symptomatic >70% (N = 25) *vs.* Asymptomatic >50% carotid stenosis (N = 48) | ↑ P < 0.05 | ↑ P < 0.05 | | | | | | | Aspirin, Ticlopidine | Whole blood |
| Kinsella | 2013 | Longitudinal, Observational Case-Case/Control | Early Symptomatic ≥ 50 - 100% (N = 46) and Late Symptomatic (N = 35) *vs.* Asymptomatic ≥50-99% carotid stenosis (N = 31) | ↔ Early Symptomatic 1.88% (P = 0.88); ↔ Late Symptomatic 1.87% (P = 0.46) *vs.* Asymptomatic 1.97% | ↔ Early Symptomatic. 9.78% (P = 0.64); ↔ Late Symptomatic 10.1% (P = 0.1) *vs.* Asymptomatic. 11% | | | ↔ Early Symptomatic 2.9% (P= 0.22); ↔ Late Symptomatic 2.76% (P=0.53) *vs.* Asymptomatic. 2.72% | ↔ Early Symptomatic 5.5% (P = 0.3); ↔ Late Symptomatic 5% (P = 0.69) *vs.* Asymptomatic. 4.6% | ↑ Early Symptomatic 2.8 % (P = 0.001); ↔ Late Symptomatic 2.5% (P = 0.21) *vs.* Asymptomatic 2.4% (↑ LPC also in Early Symptomatic *vs.* Asymptomatic MES-ve subgroup; P = 0.009) | ↑ Early Symptomatic [211] (P = 0.03, Citrate); ↔ Late Symptomatic [219] (P = 0.10) *vs.* Asymptomatic [200] (Citrate) | Aspirin, Clopidogrel, Aspirin + Dipyridamole Aspirin + Clopidogrel | Whole blood |
| Murphy | 2018 | Longitudinal, Observational Case-Case/Control | Early Symptomatic ≥50-99% (N = 43) and Late Symptomatic ≥50-99% (N = 37) *vs.* Asymptomatic ≥50-99% stenosis (N = 34) | | | | | | | | ↑ Early Symptomatic [216] (P = 0.04); ↑ Late Symptomatic [219] (P = 0.044 in Citrate) *vs.* Asymptomatic [194] | Aspirin, Clopidogrel, Aspirin + Dipyridamole Aspirin + Clopidogrel | Whole Blood |
| Murphy | 2019 | Longitudinal, Observational Case-Case/Control | Early Symptomatic ≥50-99% (N = 43) and Late Symptomatic ≥50-99% (N = 37) *vs.* Asymptomatic ≥50-99% stenosis (N = 34) | ↔ Early Symptomatic 4.8% (P = 0.09); ↔ Late Symptomatic 4.3% (P = 0.8) *vs.* Asymptomatic 4.1 % | ↔ Early Symptomatic 13.6 % (P = 0.18); ↔ Late Symptomatic 13.2% (P = 0.32) *vs.* Asymptomatic 11.5% | | | ↔ Early Symptomatic 2.7% (P = 0.06); ↑ Late Symptomatic 3% (P = 0.02) *vs.* Asymptomatic 2.5% | ↔ Early Symptomatic 5.8 % (P = 0.09); ↔ Late Symptomatic 4.4 % (P = 0.9) *vs.* Asymptomatic 4.2 % | ↑ Early Symptomatic *vs.* Asymptomatic (2.8 *vs.* 2.2%; P < 0.001); ↔ Late Symptomatic *vs.* Asymptomatic (2.35 *vs.* 2.2%; P = 0.5)* | | Aspirin, Clopidogrel, Aspirin + Dipyridamole Aspirin + Clopidogrel | Whole Blood |

**Legend for Table 2.4B**: ↑ = increased, ↔ = not significantly different. Where data are available, absolute values are reported as means or medians. PAC1, Procaspase-Activating Compound 1; NPC, Neutrophil-Platelet Complexes; MPC, Monocyte-Platelet Complexes; LPC, Lymphocyte-Platelet Complexes; MES, Microembolic Signals; SD, Standard Deviation. § All atherothrombotic stroke patients were on IV heparin or S/C heparinoid. *↑ LPC also in Early Symptomatic *vs.* Asymptomatic MES-ve subgroup (2.7 *vs.* 2.17%, P = 0.02).

## Table 2.4C: Platelet Function and Reactivity in Symptomatic *vs.* Asymptomatic Carotid Stenosis

| 1st Author | Year | Study Design | Patient Population | PFA-100 (C-EPI CT) | VerifyNow Aspirin (ARU) | Multiplate Aspirin (U) | % Antiplatelet-HTPR | Antiplatelet Regimen | Sample Type |
|---|---|---|---|---|---|---|---|---|---|
| Kinsella | 2017 | Longitudinal Observational Case-Case/Control | Early Symptomatic ≥50-100% (N = 40) & Late Symptomatic (N = 29) *vs.* Asymptomatic ≥50-99% carotid stenosis (N = 26) on aspirin alone or in combination with dipyridamole or clopidogrel. | ↔ Early Symptomatic (152 s, P = 0.55) and Late Symptomatic (223 s, P = 0.27) *vs.* Asymptomatic carotid stenosis (164 s) | | | ↔ Early Symptomatic (55%) and Late Symptomatic (28%) *vs.* Asymptomatic carotid stenosis (46%, P ≤ 0.48) on aspirin alone or in combination with dipyridamole or clopidogrel.<br>↓ Subgroup of Late Symptomatic Post-Intervention *vs.* Asymptomatic carotid stenosis on aspirin monotherapy (10% *vs.* 50%, P = 0.03). | Aspirin, Aspirin + Dipyridamole, Clopidogrel, Aspirin + Clopidogrel | Whole blood |
| | | Nested Longitudinal study in Symptomatic patients | Early Symptomatic ≥50-100% *vs.* Late Symptomatic ≥50-100% on aspirin alone or in combination with dipyridamole or clopidogrel (N = 27) | ↓ Early Symptomatic (149s) *vs.* Late Symptomatic carotid stenosis (205s, P = 0.023) | | | ↓ Late *vs.* Early Symptomatic carotid stenosis on aspirin alone or in combination with dipyridamole or clopidogrel (26% *vs.* 59% P = 0.01), and on aspirin monotherapy (15% *vs.* 62%; P = 0.016).<br>↔ Aspirin-HTPR between MES +ve *vs.* MES-ve subgroups | | |
| Murphy | 2020 | Longitudinal Observational Case-Case/Control | Early Symptomatic ≥50-99% (N = 43) and Late Symptomatic (N = 37) *vs.* Asymptomatic ≥50-99% carotid stenosis (N = 34) on aspirin alone or in combination with dipyridamole or clopidogrel; Early Symptomatic ≥50-99% (N = 6) and Late Symptomatic (N = 6) *vs.* Asymptomatic ≥50-99% carotid stenosis (N = 4) on clopidogrel alone or in combination with aspirin (Note: % clopidogrel-HTPR data included in this table in column 8) | ↑ C-EPI on Aspirin in Early Symptomatic (249s, P = 0.016) *vs.* Asymptomatic (161s)<br>↔ C-EPI on Aspirin in Late Symptomatic (229s, P = 0.11) *vs.* Asymptomatic (161s) | ↓ ARU on Aspirin in Early Symptomatic (417 U, P = 0.01) *vs.* Asymptomatic (461 U)<br>↔ ARU on Aspirin in Late Symptomatic (435, P = 0.13) *vs.* Asymptomatic (461 U) | ↔ U on Aspirin in Early Symptomatic (21 U, P = 0.35) *vs.* Asymptomatic (22 U)<br>↔ U on Aspirin in Late Symptomatic (21 U, P = 0.51) *vs.* Asymptomatic (22 U) | ↓ Aspirin-HTPR in Early Symptomatic *vs.* Asymptomatic only statistically significant on PFA-100 (28.6 *vs.* 56.7%, P = 0.028) Aspirin-HTPR across all devices on aspirin alone or in combination with dipyridamole or clopidogrel:<br>9.5 – 28.6% in Early Symptomatic<br>13.9 – 38.9% in Late Symptomatic<br>23.3 – 56.7% in Asymptomatic<br>% Clopidogrel-HTPR across all devices on clopidogrel alone or in combination with aspirin:<br>16.7 – 83.3% in Early Symptomatic<br>0 – 83.3% in Late Symptomatic<br>25 – 100% in Asymptomatic | Aspirin, Aspirin + Dipyridamole, Clopidogrel, Aspirin + Clopidogrel | Whole blood |
| | | Nested Longitudinal study in Symptomatic patients | Early Symptomatic ≥50-99% *vs.* Late Symptomatic ≥50-99% on aspirin alone or in combination with dipyridamole or clopidogrel (N = 37) | | | | ↔ Aspirin-HTPR prevalence between early and late phase symptomatic patients on any of the assays on either aspirin monotherapy or on aspirin in combination with dipyridamole or clopidogrel (P > 0.05).<br>↔ Aspirin-HTPR between MES +ve *vs.* MES-ve subgroups | | |

**Legend for Table 2.4C**: ↑ = increased, ↔ = not significantly different, ↓ = decreased. PFA-100, Platelet Function Analyser 100; C-EPI, Collagen Epinephrine; CT, Closure Time; MES, Micro-embolic Signals on transcranial Doppler ultrasound; ARU, Aspirin Reaction Units; HTPR, High On Treatment Platelet Reactivity; U, Units on Aspirin Test with Arachidonic Acid (Multiplate Assay).

## Table 2.5A: Platelet activation and platelet count in symptomatic carotid stenosis patients undergoing intervention.

| 1st Author | Year | Study Design | Patient Population | Surface Platelet Biomarkers | | | | Platelet Count | Fibrinogen platelet binding | Antiplatelet Regimen | Sample Type |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | CD62P | CD41 | PAC1 | LPC | | | | |
| Robless | 2002 | Longitudinal observational | Carotid stenosis (degree of stenosis not specified) on Aspirin undergoing CEA (90% symptomatic; N = 40) | ↑ After carotid dissection: P < 0.01; and cross-clamp removal: P <0.05 | | ↑ After carotid dissection P < 0.05 | | | | Aspirin, Dipyridamole | Whole blood |
| Payne | 2004 | Randomised controlled trial | Carotid stenosis before CEA (84% symptomatic; mean stenosis 80%; N = 100). CEA patients on routine aspirin therapy (150 mg daily) were randomised to receive additional treatment with 75 mg clopidogrel (N = 46) or placebo (N = 54) the night before surgery | | | | | | ↔ After addition of Clopidogrel to Aspirin | Aspirin, Aspirin + Clopidogrel | Whole blood |
| Assadian | 2008 | Longitudinal, observational | Symptomatic 'high grade' carotid stenosis undergoing CEA, assigned to intra-operative 5000 units of UFH (N = 10) or 0.5mg/kg enoxaparin (N = 10) | ↑ Serially during operation in overall population (P < 0.001)* | ↑ Serially during operation in overall population (P = 0.002)* | | ↑ Peri-operatively in UFH-treated group *vs*. enoxaparin-treated group (P ≤ 0.034) | ↓ During CEA P = 0.036 ** | | None | Whole blood |
| Vogten | 2008 | Longitudinal, observational | Carotid stenosis (degree of stenosis not specified) peri-CEA on Rx with ASA (100 mg daily; N = 18) or combination therapy with ASA (100 mg daily) + clopidogrel (75 mg daily; N = 9) (Proportion symptomatic unclear; N = 27) | ↔ Unchanged at 7 time points pre-operatively, intra-operatively and post-operatively in each treatment group | | | | | | Aspirin; Aspirin + Clopidogrel | Whole blood |
| Liu [35] | 2009 | Longitudinal, observational | ≥70% symptomatic carotid stenosis undergoing carotid stenting (N = 40) | | | ↔ P > 0.05 at 30mins, 18 hours and 6 days post-op *vs*. baseline | | | | Aspirin, Clopidogrel | Whole blood |

**Legend for Table 2.5A**: ↑ = increased, ↔ = not significantly different. Where data are available, absolute values are reported as mean ± SD or mean ± 2 SD or median (range). CEA, Carotid Endarterectomy; UFH, Unfractionated heparin; ASA, Aspirin; PAC1, Procaspase Activating Compound 1; LPC, Leucocyte-Platelet Complexes; SD, Standard Deviation.

* No significant difference in biomarker expression between UFH- and enoxaparin-treated groups.

** Automated analysis of EDTA-anticoagulated whole blood on a Sysmex XE-2100 haematology analyser showed that reticulated platelets also significantly increased between cross-clamping and re-establishment of blood flow in the operated ICA in the overall patient population (P = 0.001), with no inter-group differences.

## Table 2.5B: Platelet function/reactivity in carotid stenosis patients undergoing intervention (Old Studies)

| 1st Author | Year | Study Design | Patient Population | PFA-100 C-EPI CT | Platelet Aggregometry | Flow Cytometry Reactivity (Inducible activation) | Antiplatelet Regimen | Sample Type |
|---|---|---|---|---|---|---|---|---|
| Robless | 2002 | Longitudinal observational | Carotid stenosis (degree of stenosis not specified) undergoing CEA (90% symptomatic; N = 40) | | ↑ Spontaneous: 21 to 42% (P < 0.001), and ADP-induced Aggregation: 56 to 80% (P < 0.01) during CEA | | Aspirin, Dipyridamole | Whole blood |
| Hayes | 2003 | Longitudinal, observational | Carotid Stenosis (degree of stenosis not specified) undergoing CEA (N = 120; 10% asymptomatic) | | ↑ ADP-induced aggregometry with >25 *vs.* < 25 emboli (P < 0.0012) | ↑ Platelet response to ADP (percentage of cells binding fibrinogen) with >25 *vs.* < 25 micro-emboli on TCD (P < 0.0001) | Aspirin | Whole blood (flow cytometry); PRP (aggregometry) |
| Webster | 2004 | Longitudinal, observational | Carotid stenosis (degree of stenosis not specified) undergoing CEA (? proportion symptomatic; N = 41) | | ↑ Arachidonic acid-induced aggregation post-heparin (P ≤ 0.006) | | Aspirin | PRP |
| Payne | 2004 | Randomised controlled trial | Carotid stenosis before CEA (84% symptomatic; mean stenosis 80%; N = 100). CEA patients on routine aspirin therapy (150 mg daily) were randomised to receive additional treatment with 75 mg clopidogrel (N = 46) or placebo (N = 54) the night before surgery | | ↔ Addition of Clopidogrel to Aspirin did not influence arachidonic acid-induced platelet aggregation | ↓ ADP-induced platelet-fibrinogen binding with Clopidogrel+Aspirin *vs.* Aspirin alone (66.76% *vs.* 75.52%; P = 0.03) | Aspirin, Aspirin + Clopidogrel | Whole blood |
| Markus | 2005 | Randomised, double blind study | Recently (<3 months) symptomatic ≥50% carotid stenosis on Aspirin (N= 40) *vs.* Aspirin and Clopidogrel (N = 31) | | ↑ Mean maximum intensity of collagen-induced platelet aggregation at day 7 to 106.7% of baseline in Aspirin monotherapy group, and ↓ to 70.9% of baseline in Aspirin & Clopidogrel group | | Aspirin, Aspirin + Clopidogrel | PRP |
| Assadian | 2008 | Longitudinal, observational | Symptomatic 'high grade' carotid stenosis undergoing CEA, assigned to intra-operative 5000 units of UFH (N = 10) or 0.5mg/kg enoxaparin (N = 10) | ↔ | N/A | ↑ ADP-induced CD62P (P = 0.03) and CD41 (P < 0.001) expression, and TRAP-6 induced CD41 expression (P = 0.002) during surgery in overall population* | None | Whole blood |
| Vogten | 2008 | Longitudinal, observational | Carotid stenosis (degree of stenosis not specified) peri-CEA on Rx with ASA (100 mg daily; N = 18) or combination therapy with ASA (100 mg daily) + clopidogrel (75 mg daily; N = 9) (Proportion symptomatic unclear; N = 27) | | ↑ Baseline AA-induced platelet aggregation in ASA *vs.* ASA + Clopidogrel group: 14.5% *vs.* 10.3% (P < 0.05); Platelet aggregation (5 minutes post-heparin): 19.7% in ASA group (P < 0.01 *vs.* baseline) and 22.5% in ASA + Clopidogrel group (P < 0.05 *vs.* baseline) | | Aspirin, Aspirin + Clopidogrel | PRP |
| Szapary | 2009 | Longitudinal, observational | ≥70% carotid stenosis (10 symptomatic, 8 asymptomatic; (N = 18) undergoing CAS | | ↑ADP-induced platelet aggregation 5 days post-intervention *vs.* immediately post-procedure (P < 0.0001) | | Aspirin, Clopidogrel | PRP |
| King | 2011 | Longitudinal, observational | ≥50% Symptomatic carotid stenosis starting ASA+Clopidogrel (N = 30) or ASA+Dipyridamole (N = 30) | | ↓ ADP-induced aggregation in the ASA+Clopidogrel *vs.* ASA+Dipyridamole group at 48 hours: 86.5 ± 28.8 *vs.* 101.7 ± 13.0 (P < 0.001) | | Aspirin + Clopidogrel, Aspirin + Dipyridamole | PRP |

**Legend for Table 2.5B**: ↑ = increased, ↔ = not significantly different, ↓ = decreased. PFA-100, Platelet Function Analyser 100; C-EPI, Collagen Epinephrine; CT, Closure Time; CEA, Carotid Endarterectomy; CAS, Carotid Artery Stenting; ADP, Adenosine Diphosphate; TRAP-6, Thrombin Receptor Activating Peptide 6; ASA, Aspirin. * No significant difference in biomarker expression between UFH- and enoxaparin-treated groups.

## Table 2.5C: Platelet function/reactivity in carotid stenosis patients undergoing intervention (New Studies)

| 1st Author | Year | Study Design | Patient Population | VerifyNow Aspirin | VerifyNow P2Y12 | PFA-100 C-EPI CT | Platelet Aggregometry | Antiplatelet Regimen | Sample Type |
|---|---|---|---|---|---|---|---|---|---|
| Lewszuk | 2015 | Longitudinal observational | ≥70% carotid stenosis (N = 58 Symptomatic and N = 8 Asymptomatic) undergoing CEA. Treated with Aspirin 75 mg daily or low molecular weight heparin (LMWH - dose not specified) pre-op; in those on LMWH, aspirin 75 mg daily started 2 hours after surgery | | | 'Aspirin-HTPR' day 2 post-op in 19 patients (29%). However, reliable data interpretation impossible (see main text) | | Aspirin | Whole blood |
| Backovic | 2016 | Longitudinal observational | Carotid stenosis patients who had undergone CEA (N = 112); % Stenosis and % Symptomatic unclear. All on Aspirin 75 mg daily pre-op and post-op. Clopidogrel 75 mg daily started post-op | N/A | N/A | N/A | 'Clopidogrel-HTPR' present if 'ratio of ADP-induced: TRAP-induced platelet aggregation' was in the upper quartile of their local range after patients had received 30 days of Clopidogrel. ↓ Clopidogrel-HTPR over time: 79.5% at 24 hours, 36% at 7 days, and 25% at 30 days post-CEA on Aspirin and Clopidogrel | Aspirin + Clopidogrel | Whole blood |
| Tsujimoto | 2016 | Longitudinal observational | Patients undergoing CAS for >80% Asymptomatic (N = 21) or >50% Symptomatic (N = 17) carotid stenosis. Combination Antiplatelet therapy for 7 days prior to and ≥ 3 months post-CAS: Aspirin (100 mg/day), Clopidogrel (75 mg/day) and/or Cilostazol (200 mg/day) | | | | ↑ ADP-induced or collagen-induced optical platelet aggregation between baseline and 4 days post-CAS in 18 patients (47%) with no significant change noted in 20 patients (53%) | Aspirin (N = 32), Clopidogrel (N=37) Cilostazol (N = 13) | PRP |
| Yeo | 2017 | Longitudinal observational | Angiographically confirmed PAD (N = 154), 49 of whom had 'severe or symptomatic' carotid stenosis requiring carotid angiography and /or stenting. Precise % stenosis was not specified | 20% (10/49) with carotid stenosis had Aspirin-HTPR (ARU ≥ 550) | 39% (19/49) had Clopidogrel-HTPR (PRU ≥ 235) | N/A | N/A | Aspirin + Clopidogrel | Whole blood |

**Legend for Table 2.5C**: ↑ = increased, ↔ = not significantly different, ↓ = decreased. PFA-100, Platelet Function Analyser 100; C-EPI, Collagen Epinephrine; CT, Closure Time; CEA, Carotid Endarterectomy; CAS, Carotid Artery Stenting; ADP, Adenosine Diphosphate; TRAP-6, Thrombin Receptor Activating Peptide 6.

## Table 2.6: Assessment of platelet activation or platelet reactivity/function in patients with 'neuroimaging evaluation'

| 1st Author | Year | Study Design | Patient Population | PFA-100 C-EPI | VerifyNow Aspirin | VerifyNow P2Y12 | TCD: MES +ve *vs*. MES-ve | MRI DWI | Antiplatelet Regimen | Sample Type |
|---|---|---|---|---|---|---|---|---|---|---|
| Ritter | 2009 | Case-Case | Acute 'Atherothrombotic' Stroke patients on intravenous heparin, aiming for APTT ratio of 1.5-2.0 (N = 16); Asymptomatic >50% carotid stenosis on low dose aspirin (N = 30) | N/A | N/A | N/A | ***Acute Stroke***: ↑ sP-selectin (178 *vs*. 121 ng/ml; P = 0.02); ↓ CD62P (4.5 *vs*. 9 AU; P=0.004); ↔ Thrombospondin, % MPCs and NPCs (P ≤ 0.09) in MES +ve *vs*. MES –ve patients. ***Asymptomatic***: ↑ sP-selectin (122 *vs*. 80 ng/ml; P = 0.0007); ↔ CD62P, Thrombospondin, % MPCs and NPCs (P ≥ 0.15) in MES +ve *vs*. MES –ve patients | N/A | Stroke patients on 'low dose' aspirin and IV heparin (Target APTT ratio: 1.5-2.0) Asymptomatic patients on Aspirin | Plasma (sP-selectin); Whole blood (Flow cytometry) |
| Dawson | 2011 | Longitudinal observational Case-Case | Symptomatic >50% carotid stenosis or Symptomatic ulcerated plaque (N = 53, within 48 hours-6 months of TIA/ischaemic stroke onset); or Asymptomatic >50% carotid stenosis (N = 9) (1 patient on aspirin 150 mg daily; all others on aspirin 75 mg daily) | 21% (N = 13) of entire study population had Aspirin-HTPR (CT < 179 sec) | 12.9% (N = 8) of entire study population had Aspirin-HTPR (ARU > 550) | | ↔ Aspirin-HTPR on PFA-100 in MES+ve *vs*. MES-ve patients: 31.3% *vs*. 17.4% (P = 0.29). ↑ Aspirin-HTPR on VerifyNow in MES+ve *vs*. MES-ve patients: 31.3% *vs*. 6.5% (P = 0.02) | N/A | Aspirin | Whole blood |
| IMPACT Trial | 2013 | Randomised single centre trial | Asymptomatic patients >90% carotid stenosis undergoing stenting, randomised to Clopidogrel 300mg (N = 19) or 600mg (N = 16) 4 days before CAS, followed by 75 mg daily. Platelet function assessed at baseline and pre-CAS after 4 days of clopidogrel | | | ↔ PRU between 300mg *vs*. 600mg groups (231 *vs*. 228, P = 0.77). No association between PRU and MES data on TCD | | N/A | Aspirin + Clopidogrel | Whole blood |
| Song | 2013 | Retrospective review | Patients undergoing stenting for ≥50% Symptomatic (N = 57) or ≥70% Asymptomatic carotid stenosis (N = 19) on Aspirin 100 mg/d + Clopidogrel 75 mg/d x ≥7 days | | 12 patients (15.8%) had Aspirin-HTPR (ARU ≥ 550) | 50 patients (65.8%) had Clopidogrel-HTPR (PRU ≥ 240) | | ↔ 'Aspirin HTPR' with *vs*. without new DWI lesions (P = 0.34). ↑ Clopidogrel HTPR with *vs*. without new DWI lesions (82.2% *vs*. 41.9%; P = 0.001) | Aspirin + Clopidogrel | Whole blood |
| Nakagawa | 2014 | Longitudinal, interventional | Patients undergoing CAS for >50% symptomatic or >80% asymptomatic stenosis divided into: Phase I: (Aspirin 100mg/d + Clopidogrel 75 mg/d, N = 28); Phase II (Aspirin + Clopidogrel, N = 36), with Cilostazol 200 mg/d added in those with Clopidogrel-HTPR (N = 13) | | ↔ ARU Phase I *vs*. Phase II: 477 *vs*. 460 (P =NS); ↔ARU in Phase II from before *vs*. after adding Cilostazol in patients with Clopidogrel-HTPR: 493 *vs*. 498 (P = NS) | ↔ PRU Phase I *vs*. Phase II: 235 *vs*. 223 (P = NS); ↓ PRU in Phase II from before *vs*. after adding Cilostazol in patients with Clopidogrel-HTPR: 300 *vs*. 240 (P = 0.006) | | New ipsilateral ischaemic lesions on DWI less common in Phase II (2/36 patients) *vs*. Phase I (7/28 patients; P = 0.034) | Aspirin, Clopidogrel, +/- Cilostazol | Whole blood |

**Legend for Table 2.6**: ↑ = increased, ↔ = not significantly different, ↓ = decreased. PFA-100, Platelet Function Analyser-100; C-ADP, Collagen-Adenosine Diphosphate; C-EPI, Collagen-Epinephrine; CT, Closure Time; TCD, Transcranial Doppler ultrasound; MES, Micro-embolic Signals; MRI, Magnetic Resonance Imaging; DWI, Diffusion Weighted Imaging; CEA, Carotid Endarterectomy; ASA, Aspirin; ARU, Aspirin Reaction Units. (Platelet biomarker and neuroimaging data from studies by Kinsella *et al*. (8, 20) and Murphy *et al*. (21,34) have been summarised in the relevant tables above and have not been duplicated here).

**Table 2.7: Risk of bias assessment with the ROBINS-I tool (Sterne et al., 2016)**

| Studies | Confounding | Selection | Measurement of Intervention | Missing Data | Measurement of Outcomes | Deviation from Intervention | Reported Results | Overall |
|---|---|---|---|---|---|---|---|---|
| ***Older Studies*** | | | | | | | | |
| **Shah 1985** | S | L | L | L | L | L | L | **S** |
| **Frijns 1997** | S | L | L | L | L | L | L | **S** |
| **Zeller 1999** | M | L | L | L | L | L | L | **M** |
| **Robless 2002** | M | L | L | L | L | L | L | **M** |
| **Stolz 2002** | L | L | L | L | L | L | L | **L** |
| **Hashimoto 2003** | L | L | L | L | L | L | L | **L** |
| **Blake 2003** | L | L | L | L | L | L | L | **L** |
| **Hayes 2003** | M | L | L | L | L | L | L | **M** |
| **Cha 2003** | M | L | L | L | L | L | L | **M** |
| **Webster 2004** | L | L | L | L | L | L | L | **L** |
| **McCabe 2004** | L | L | L | L | L | L | L | **L** |
| **Payne 2004** | L | L | L | L | L | L | L | **L** |
| **Assadian 2005** | M | L | L | L | L | L | L | **M** |
| **McCabe 2005 (Platelets)** | M | L | L | L | L | L | L | **M** |
| **Novo 2005** | L | L | L | L | L | L | L | **L** |
| **McCabe 2005 (JNNP)** | L | L | L | L | L | L | L | **L** |
| **Markus 2005** | L | L | L | L | L | L | L | **L** |
| **Yip 2006** | L | L | L | L | L | L | L | **L** |
| **Balla 2006** | M | L | L | L | L | L | L | **M** |
| **Vogten 2008** | M | L | L | L | L | L | L | **M** |
| **Ritter 2008** | M | L | L | L | L | L | L | **M** |
| **Liu 2009** | L | L | L | L | L | L | L | **L** |
| **Szapary 2009** | L | L | L | L | L | L | L | **L** |
| **Enomoto 2010** | S | L | L | L | L | L | M | **S** |
| **Jurk 2010** | S | M | L | L | L | L | M | **S** |
| **King 2011** | L | L | L | L | L | L | L | **L** |
| ***Newer studies*** | | | | | | | | |
| **Turgut 2011** | M | L | L | L | L | L | L | **M** |
| **Dawson 2012** | M | L | L | L | L | L | L | **M** |
| **Kinsella 2013** | L | L | L | L | L | L | L | **L** |
| **Van Der Heyden 2013** | L | L | L | L | L | L | L | **L** |
| **Song 2013** | M | L | L | L | L | L | M | **M** |
| **Lukasik 2013** | L | L | L | L | L | L | L | **L** |
| **Nakagawa 2014** | M | L | L | L | L | L | L | **M** |
| **Kirpatrick 2014** | M | L | L | L | L | L | L | **M** |
| **Lewszuk 2015** | C | L | L | L | C | L | L | **C** |
| **Tsujimoto 2016** | M | L | L | L | L | L | L | **M** |
| **Backovic 2016** | L | L | L | L | L | L | L | **L** |
| **Kinsella 2017** | L | L | L | L | L | L | L | **L** |
| **Yeo 2017** | M | L | M | L | M | L | L | **M** |
| **Murphy 2018** | L | L | L | L | L | L | L | **L** |
| **Murphy 2019** | L | L | L | L | L | L | L | **L** |
| **Murphy 2019 (T&H)** | L | L | L | L | L | L | L | **L** |
| **Murphy 2020** | M | L | L | L | L | L | L | **M** |

**Legend for Supplemental Table 2.7**: Total = 43 studies. L = Low; M = Moderate; S=Serious; C = Critical. **Overall risk of bias** considered to be equal to the most severe level of bias found in any domain. Low risk of bias = 21/43 studies (48.8%); Moderate risk of bias = 17/43 studies (39.5%); Serious risk of bias = 4/43 studies (9.3%); Critical risk of bias = 1/43 studies (2.3%). Low or moderate risk of bias = 38/43 studies (88.4%); Serious or critical risk of bias = 5/43 studies (11.6%).

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