

**Profiles of von Willebrand factor antigen,
von Willebrand factor propeptide and ADAMTS13
activity in patients with ischaemic cerebrovascular
disease after commencing or changing antiplatelet
therapy**

A dissertation submitted for a Master in Science
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Declaration

I designed all of the studies described in this thesis with the advice and supervision of my mentors and colleagues listed in the acknowledgements section below. I performed all the ELISA assays for this study to analyse the VWF:Ag and VWFpp levels. I also performed all of the FRET assays myself with the assistance and supervision of colleagues acknowledged below. I entered all of my data onto a database and performed the statistical analysis of the data with the help and expert supervision of my colleagues, Dr Stephen Murphy and Prof. Dominick McCabe.

This work represents a sub-study of the **Optimal Antiplatelet Therapy in TIA and Ischaemic Stroke (OATS)** Study, which has previously been undertaken by colleagues in our research group, and which formed the basis of Dr Soon Tjin Lim's PhD thesis at TCD. However, this MSc thesis contains new data on von Willebrand factor antigen and VWF propeptide expression in this study patient population, along with novel data on ADAMTS13 activity. I declare that this thesis has not been submitted as an exercise for a degree at this or any other University and 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 Deirdre R. Smith on 12th July 2020

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Summary

Background: There are very limited data on the impact of commonly-prescribed antiplatelet regimens on von Willebrand Factor antigen (VWF:Ag), von Willebrand Factor propeptide (VWFpp) and A Disintegrin And Metalloprotease with ThromboSpondin type 1 motif number 13 (ADAMTS13) activity levels in patients with 'non-TTP related' TIA or ischaemic stroke.

Aims: The aims of this thesis were to simultaneously investigate the profiles of VWF:Ag, VWFpp and ADAMTS13 activity in patients within 4 weeks of TIA or ischaemic stroke onset (baseline) and then at 14 +/- 7 days (14d) and ≥ 90 days (90d) after commencing or altering antiplatelet therapy. We also aimed to assess the relationship between these endothelial +/- platelet biomarkers and platelet function/reactivity on user-friendly tests of platelet function at high and low shear stress, and whether blood group status influenced the expression of these biomarkers in CVD patients'

Methods: We performed a prospective, longitudinal observational analytical study in a clinically well-categorised population of CVD patients (N = 51) who were starting or changing to aspirin monotherapy or clopidogrel/ADP receptor antagonist monotherapy, or changing to aspirin-dipyridamole combination therapy. VWF:Ag and VWFpp levels were quantified with an enzyme-linked immunosorbent assay (ELISA) on thawed, double-spun platelet poor plasma. ADAMTS13 activity was assessed with a Fluorescence Resonance Energy Transfer (FRET) assay on thawed double-spun platelet poor plasma. We also assessed the relationship between these biomarkers and relevant platelet function/reactivity tests using a moderately high shear stress test of platelet adhesion and aggregation (PFA-100[®]) and low shear stress tests of platelet aggregation (VerifyNow[®] and Multiplate[®]).

Results: VWF:Ag levels significantly decreased between baseline and 90d (17.99 vs. 15.62 μ g/mL; P = 0.001) and the VWFpp/VWF:Ag ratio significantly increased at both 14d (0.09; P = 0.025) and 90d (0.10; P = 0.005) vs. baseline (0.08) in our overall CVD patient population. In the subgroup of patients who commenced or changed to clopidogrel monotherapy, VWF:Ag levels significantly decreased during follow-up at both 14d (17.43 μ g/mL; P = 0.007) and 90d (17.17 μ g/mL; P < 0.001) compared with baseline (20.29 μ g/mL). VWFpp levels remained stable over time, but the VWFpp/VWF:Ag ratio also significantly increased at both 14d (0.08; P = 0.014) and 90d (0.08; P = 0.024) vs. baseline (0.07), and ADAMTS13 activity was significantly lower at baseline vs. 90d (134.55% vs 147.2%; P = 0.047) in this subgroup starting clopidogrel. There were no significant changes in the levels of VWF:Ag (P \geq 0.23) or VWFpp (P \geq 0.34), or in the VWFpp/VWF:Ag ratio (P \geq 0.08) in the subgroups who started aspirin or changed to aspirin-dipyridamole combination therapy. There was a positive relationship between VWF:Ag and VWFpp levels at all 3 timepoints in the overall CVD patient population (baseline R² = 13.6%, P = 0.004; 14d R² = 14.2%, P = 0.004; 90d R² = 18.5%, P = 0.001), and in the aspirin-dipyridamole (baseline R² = 20.7%, P = 0.029; 14d R² = 27.7%, P = 0.012; 90d R² = 38.4%, P = 0.003) and clopidogrel monotherapy subgroups (baseline R² = 32.3%, P = 0.005; 14d R² = 33.7%, P = 0.004; 90d R² = 31.6%, P = 0.006). There was also a weak but significant inverse relationship between VWF:Ag levels and ADAMTS13 activity in the overall patient population at 90d only (R² = 8.7%, P = 0.02).

There was no obvious relationship between levels of VWF:Ag, VWFpp or ADAMTS13 activity and platelet function/reactivity or antiplatelet-HTPR status according to either cross-sectional or longitudinal definitions on the relevant assays in the aspirin monotherapy or clopidogrel monotherapy groups in OATS. However, there was a significant inverse relationship between VWF:Ag and PFA-100 C-ADP closure times at baseline ($R^2 = 32.0\%$, $P = 0.008$) and 14d ($R^2 = 46.4\%$, $P = 0.001$), and also a significant inverse relationship between VWFpp and PFA-100 C-ADP closure times at baseline ($R^2 = 18.3\%$, $P = 0.043$) and 14d ($R^2 = 24.3\%$, $P = 0.018$) in the subgroup of patients who changed to aspirin-dipyridamole combination treatment. In addition, there was a significant inverse relationship between VWF:Ag levels and PFA-100 INNOVANCE P2Y closure times ($R^2 = 55.5\%$, $P < 0.001$) and between VWFpp levels and PFA-100 INNOVANCE P2Y closure times ($R^2 = 20.9\%$, $P = 0.028$) only at 14d in this treatment subgroup. There was a positive relationship between ADAMTS13 activity and C-ADP closure times at baseline only in the starting aspirin-dipyridamole subgroup ($R^2 = 19.9\%$, $P = 0.036$), most likely mediated via VWF:Ag based on prior literature on this topic. The proportion of patients with blood group O and non-blood group O was similar in the overall CVD patient population and in all 3 treatment subgroups ($P \geq 0.194$). However, mean VWF:Ag levels were lower in the overall patient population and in the subgroup of patients who were starting aspirin monotherapy who had blood group O vs. those with non-blood group O at baseline ($P \leq 0.043$) and at 90d ($P \leq 0.01$).

Discussion and Conclusions: The prospective pilot studies outlined in this thesis have shown that starting or changing antiplatelet therapy may reduce circulating VWF:Ag levels and increase the VWFpp/VWF:Ag ratio without altering VWFpp expression in an overall CVD patient population, mainly attributed to commencement of clopidogrel monotherapy in this study. Taken together with the data on ADAMTS13 activity, our results support the hypothesis that these findings appear to be attributed to increased clearance of VWF:Ag over time, particularly in the subgroup who commenced clopidogrel. These data improve our understanding of the protean effects of clopidogrel on haemostatic and thrombotic profiles in CVD patients aside from direct platelet P2Y12 receptor blockade. Blood group O is associated with lower circulating VWF:Ag levels, thus adding to the limited literature on this topic in patients with TIA or ischaemic stroke. The significant inverse relationship between both VWF:Ag and VWFpp with PFA-100 C-ADP closure times early after TIA/ischaemic stroke in the subgroup of patients who changed to aspirin-dipyridamole combination treatment also improves our understanding of the endothelial +/- platelet biomarkers which can influence the results of these assays.

Larger studies in a well-phenotyped CVD patient population which simultaneously assess all of these biomarkers, including the VWFpp/VWF:Ag ratio, are warranted to confirm these findings and to understand the precise mechanisms by which certain antiplatelet regimens, including aspirin-clopidogrel combination therapy, might affect VWF:Ag clearance. Furthermore, comprehensive assessment of these biomarkers should improve our understanding of the mechanisms influencing the *ex vivo* response to commonly-prescribed antiplatelet therapy on relevant high shear stress tests of platelet function/reactivity to potentially aid risk-stratification and tailor antiplatelet treatment to suit individual CVD patients in future. We aim to address this issue in local and multicentre studies which have been prospectively planned by our research group.

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Abbreviations

ADAMTS13	A Disintegrin And Metalloprotease with ThromboSpondin type 1 motif number 13
AP-1	Adaptor protein 1
ARU	Aspirin Reaction Unit
C-ADP	Collagen-ADP
CDUS	Carotid Duplex Ultrasound
C-EPI	Collagen-Epinephrine
CT	Computerised Tomography
CTA	Computerised Tomography Angiography
CVD	Cerebrovascular Disease
DVT	Deep Vein Thrombosis
DWI	Diffusion Weighted Imaging
ECG	Electrocardiogram
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme Linked Immunosorbent Assay
ER	Endoplasmic Reticulum
ESO	European Stroke Organisation
FBC	Full Blood Count
FLAIR	Fluid Attenuation Inversion Recovery
GP	Glycoprotein
HTPR	High On-Treatment Platelet Reactivity
IHD	Ischaemic Heart Disease
MES	Micro-embolic signals
MR	Modified Release
MRA	Magnetic resonance angiography
MRI	Magnetic resonance imaging
NSAID	Non-steroidal Anti-inflammatory Drug
PDW	Platelet Distribution Width
PBS	Phosphate Buffered Saline
PDE	Phosphodiesterase
PFA-100[®]	Platelet Function Analyser-100, Dade-Behring, Germany
PG	Prostaglandin
PPP	Platelet Poor Plasma

PRU	P2Y12 Reaction Units
SD	Standard deviation
TIA	Transient Ischaemic Attack
TGN	Trans-Golgi network
TOAST	Trial of ORG 10172 in Acute Stroke Treatment
tPA	tissue-type Plasminogen Activator
TOE	Trans-oesophageal Electrocardiogram
TTE	Trans-thoracic Electrocardiogram
TxA₂	Thromboxane A ₂
UL-VWF	Ultra Large von Willebrand factor
VWF:Ag	von Willebrand factor Antigen
VWFpp	von Willebrand factor propeptide
WPB	Weibel-Palade bodies

1 Introduction

1.1 Cerebrovascular Disease - Transient Ischaemic Attack (TIA) and Stroke

1.1.1 TIA

A transient ischaemic attack (TIA) may be **clinically defined**, using a well-established, ‘time-based definition’, as a transient episode of focal brain, retinal or spinal cord dysfunction that lasts for less than 24 hours in duration, and which, after adequate investigations, is presumed to be of a non-traumatic vascular origin (Bamford, 1992; Easton et al., 2009). This 24 hour ‘cut point’ chosen to distinguish between a TIA and stroke was based on the hypothesis that the ischaemia causing symptoms of a TIA would resolve completely without causing any ‘permanent neuronal damage’. This is in contrast to the ischaemia causing stroke which would be associated with an area of infarction and permanent neuronal injury which should be detectable by microscopy. This time-based diagnosis of TIA was generally accepted prior to the availability of more sensitive, modern neuroimaging techniques which can detect hyperacute cerebral ischaemia or infarction.

Symptoms of TIA will depend on the area of the nervous system affected, but are typically ‘negative phenomena’, including transient monocular blurring or blindness, dysphasia, dysarthria, dysphagia, hemiparesis, hemisensory loss, ataxia, but may also include positive phenomena such as diplopia, vertigo, tingling or pins and needles. TIAs are often referred to by members of the public as ‘mini-strokes’ or ‘warning strokes’. The importance of making an accurate diagnosis is paramount because these patients have a higher risk of having a stroke than the normal population. Twenty years ago, it was reported that approximately 10.5% of patients diagnosed with a first-ever TIA had a stroke within the following 90 days, and of these, 50% occurred within the first 2 days after the index event (Johnston, Gress, Browner, & Sidney, 2000). However, more recent data from the SOS TIA

Registry estimated the risk of stroke to be 1.5% at 2 days, 2.1% within 7 days, 2.8% within 30 days, 3.7% at 90 days, 5.1% at 1 year (Pierre Amarenco et al., 2016) and 9.5% at 5 years after a TIA (P. Amarenco et al., 2018). Establishing a diagnosis of a TIA can often be quite difficult for non-stroke specialists because of the transient nature of the symptoms which have usually resolved before the patient attends for medical assessment. For example, only 43% to 48% of patients referred to a specialised Rapid Access Stroke Prevention service with a 'suspected TIA' had a clinical diagnosis of TIA confirmed following assessment by an expert vascular neurologist or stroke physician (Bradley et al., 2013; Fitzpatrick et al., 2019; Kinsella, Tobin, Cogan, & McCabe, 2011). Common conditions which can mimic a TIA include migraine, seizures, syncope, anxiety, benign paroxysmal positional vertigo (BPPV) and radiculopathy.

Significant advances in neuroradiological assessment over recent decades have identified that 30-50% of patients confirmed to have had a TIA using the classical time-based clinical definition have neuro-radiological evidence of cerebral ischaemia or infarction. In almost half of these patients, acute diffusion-weighted MRI (DW MRI) changes may be fully reversible, although in the remainder, the radiological changes evolve into an established infarct despite only having transient clinical symptoms. The likelihood of having persistent radiological changes of infarction increases with longer symptom duration during the 24 hour period after symptom onset (Kidwell et al., 1999; Rovira et al., 2002). In addition, approximately 5% of clinically diagnosed TIA patients have evidence of abnormal neurological signs which are identified more than 24 hours after symptom onset (Warlow, 1996; C. P. Warlow, 2001).

More recently, new definitions of a TIA have been proposed, based on both the duration of symptoms and findings on neuroimaging. Most TIAs resolve within 60 minutes, and if symptoms last for more than this, the likelihood that they will completely resolve is < 15%

(Albers et al., 2002). This led to a proposal by Albers *et al.* to initially redefine a TIA as ‘a brief episode of neurological dysfunction caused by focal brain or retinal ischemia, with clinical symptoms typically lasting 1 hour, and without neuroimaging evidence of acute infarction’. This initial ‘**tissue-based**’ definition was dependent on the absence of ‘permanent’ end-organ damage on the basis of clinical, neuro-imaging and neuropathological findings rather than on a time-based cut point (Albers et al., 2002). The American Stroke Association subsequently adopted a modified tissue-based definition of a TIA in 2009 to include ‘any transient episode of neurological dysfunction caused by focal brain, retinal or spinal cord ischaemia, without acute infarction on neuroimaging’, with MRI being the most sensitive imaging modality. This is important because a TIA is redefined as a stroke in patients with symptoms lasting < 24 hours, but with evidence of ischaemia or infarction on neuroimaging. However, the European Stroke Association (ESO) did not revise its definition in a similar manner, citing the lack of a ‘perfect biomarker’ for permanent ischaemic brain injury and also because of the large variation in access to different types of neuroimaging, which in turn impacts on diagnostic ability. Conventional neuroimaging, like computerised tomography (CT) and non-DW MRI have lower sensitivity in detecting early acute ischaemia or small infarcts which may take at least hours to evolve following symptom onset (Ay et al., 2005; Boulanger et al., 2005; Coutts et al., 2005; Kidwell et al., 1999; Rovira et al., 2002). For example, 31% of patients will have no acute changes on CT of brain and would be classified as having had a ‘tissue-based diagnosis of TIA’ rather than a ‘tissue-based diagnosis of stroke’ if CT is used instead of DW MRI (DWI) (Förster et al., 2012). Although MRI and DWI is the neuroimaging investigation of choice in patients with suspected TIAs, and may enable the detection of certain patterns of ischaemia to focus appropriate investigations to identify the underlying aetiology of the cerebrovascular event (Förster et al., 2012), CT of brain is still widely used in the initial diagnosis and urgent management of TIAs when MRI is not available, not tolerated or is contraindicated.

Furthermore, DW-MRI may ‘inadvertently categorise’ patients as having had an acute ischaemic stroke if they undergo neuroimaging very early after symptom onset before their ischaemic changes on DW MRI have had time to resolve, so establishing a tissue-based diagnosis of TIA vs. stroke is not always practical.

A number of clinical predictive models have been developed to assist with identification of TIA patients at highest risk of stroke after a TIA to expedite assessment and investigations in these patients. A 6-point, clinically-based, ‘ABCD’ risk score was initially publicised in 2005 to identify patients at highest risk of recurrent stroke early after a TIA. The score was derived from the following clinical features: **A**ge, **B**lood pressure, **C**linical symptoms and **D**uration of symptoms (Rothwell et al., 2005), and was associated with the presence of radiologically-identified acute ischaemia or infarction after a TIA in some studies (Merwick et al., 2010; Redgrave, Coutts, Schulz, Briley, & Rothwell, 2007). DWI abnormalities on MRI are also independently associated with an increased risk of subsequent vascular events following TIA (Purroy et al., 2004). The addition of diabetes as a predictor to the original ABCD scoring system (ABCD²), and the subsequent inclusion of neuroimaging findings and the presence or absence of >50% internal carotid artery stenosis (ABCD³-I) has helped to further predict future stroke risk after a TIA following assessment by a stroke specialist (Johnston et al., 2007; Merwick et al., 2010; Wolf, Held, & Hennerici, 2014). However, it is important to note that ABCD² scoring by referring doctors is also frequently inaccurate, and non-stroke specialists may underestimate stroke risk compared with experts in TIA and stroke care (Bradley et al., 2013).

1.1.2 Stroke

Symptoms of stroke have been recognised for over 4000 years, as evidenced by descriptions of stroke presentations and treatment documented on 2 clay tablets from ancient

Babylonians, one of which is in display in the Louvre Museum in Paris and the other in the British Museum in London (Karim & Amin, 2018). An inscription by ancient Egyptians, dated approximately 2455 BCE, on the tomb of the vizier Weshptah, seems to describe stroke (York & Steinberg, 2010) and Hippocrates described an episode of ‘sudden paralysis’ which is generally attributed to stroke. The term ‘stroke’ is derived from the Greek word ‘apoplexia’ which can be translated as ‘to strike with violence’ or ‘to cripple by a stroke’. The first use of the term ‘stroke’ in a medical context was recorded in 1599, when a sudden onset of symptoms was attributed to a ‘stroke of God’s handle’ (Barnhart, 1995).

A stroke may be **clinically defined** as ‘a sudden onset of focal rather than global neurological dysfunction, with symptoms lasting for more than 24 hours or resulting in death before 24 hours, and in which, after adequate investigations, is presumed to be of a non-traumatic vascular origin’(Bamford, 1992; "Cerebrovascular diseases: prevention, treatment, and rehabilitation. Report of a WHO meeting," 1971). Some clinicians have adopted a ‘tissue-based’ diagnosis of stroke, as outlined above, and one must accept that there are some limitations to the clinical definition. For example, cervical artery dissection accounts for up to 25% of younger TIAs or strokes and 2% of strokes overall and may occur after neck manipulation or after head and neck trauma (Biller et al., 2014). Furthermore, major subarachnoid haemorrhage or major brain stem haemorrhage may present with global rather than focal neurological dysfunction in some cases. In 2013, the American Heart Association/American Stroke Association updated their definition of stroke to one which also includes ‘silent infarction’ of cerebral, spinal or retinal tissue, and also silent haemorrhages. By doing so, they have removed the association with clearly defined clinical symptoms. Therefore, their updated tissue-based definition defines ischaemic stroke as ‘an episode of neurological dysfunction caused by focal cerebral, spinal, or retinal infarction’. Infarction in this context was defined as (a) ‘brain, spinal cord, or retinal cell death attributable to ischaemia, based on imaging, pathological or other objective evidence of

cerebral, spinal cord, or focal retinal ischaemic injury in a defined vascular distribution; or (b) clinical evidence of cerebral, spinal cord, or retinal focal ischaemic injury based on symptoms persisting ≥ 24 hours or until death, when other aetiologies have been excluded' (Sacco et al., 2013). This decision will impact on individual health and life insurance premiums in the USA because silent brain infarction (SBI) confers a two-fold increase in the risk of future stroke, and has been identified in at least 20% of 'clinically stroke-free' older adults over the age of 70 years (Gupta et al., 2016). However, these patients still have a lower risk of future stroke than those with a 'clinical ischaemic stroke syndrome' (Mohan et al., 2011). Such extensive expansion of this definition of stroke is fraught with difficulty because subcortical white matter changes have been identified in 87% of patients between 60-70 years in a population-based study in Rotterdam (de Leeuw et al., 2001). This issue also has very important implications for clinical and epidemiological studies estimating the prevalence of TIA or stroke and survival after stroke if one uses different definitions in different centres worldwide. The World Health Organization, European Stroke Organisation and the World Stroke Organisation have not included 'silent pathology' within their definition of stroke, so there is no universal agreement on this issue at present.

Therefore, for practical purposes during the conduct of the study which led to this MSc thesis, clinical time-based definitions of TIA and stroke were used, unless otherwise specified.

1.1.3 Stroke Epidemiology

Stroke is a leading cause of acquired physical disability in adults in middle-high income countries and is associated with substantial care costs (Rajsic et al., 2019). Stroke is also the second commonest cause of death worldwide and was responsible for 5.5 million deaths in 2016 (2.9 million deaths in men and 2.6 million deaths in women) (GBD 2016 Stroke

Collaborators, 2019). Worldwide, deaths from haemorrhagic stroke are slightly more frequent than deaths from ischaemic stroke (2,838,062 vs 2,690,171) (GBD 2016 Stroke Collaborators, 2019) even though approximately 80% of strokes are due to cerebral infarction and 20% are attributed to haemorrhage (Krishnamurthi et al., 2014). Stroke is also the second most frequent contributor to disability-adjusted life years (DALYs) (Gorelick, 2019). A DALY is a measure of overall disease burden, expressed as the number of years lost due to ill-health, disability or early death. The concept was developed in the 1990s to compare overall health and life expectancy between different countries. One DALY can be regarded as ‘one lost year of healthy life’. The sum of these DALYs across the population, or the burden of disease, can be thought of as a measure of the gap between current health status and an ideal health situation where the entire population lives to an advanced age, free of disease and disability (WHO Health statistics and informationsystems:https://www.who.int/healthinfo/global_burden_disease/metrics_daly/en/) Recently, the regional and global lifetime risk of stroke was assessed in the Global Burden of Disease Study (Feigin et al., 2018). In 2016, the global lifetime risk of stroke was estimated to be 24.9% from the age of 25 years onwards, with an 18.3% risk of ischaemic stroke and an 8.2% risk of haemorrhagic stroke (The sum of the lifetime risks of ischaemic stroke and haemorrhagic stroke, as determined in separate analyses, was greater than the overall risk of stroke because it is possible to have both subtypes of stroke over a lifetime.) (Feigin et al., 2018). The mean global lifetime risk of stroke increased from 22.8% in 1990, a relative increase of 8.9% (Feigin et al., 2018); the risk of stroke also doubles for each successive decade after the age of 55 years (Asplund et al., 2009). The age of stroke occurrence appears to have fallen, with an increase in the incidence at younger ages (Avan et al., 2019). According to the World Stroke Organisation, almost 60% of all strokes each year are in people under the age of 70 years, and in 8%, they are younger than 44 years (GBD 2016 Stroke Collaborators, 2019). The estimated lifetime risk of stroke was 23.5% in high

socio-demographic index (SDI) countries, 31.1% in high-middle SDI countries (highest risk), and 13.2% in low SDI countries (lowest risk) (Feigin et al., 2018). The lower risk in low SDI countries is attributed to other competing causes of early mortality. The highest estimated lifetime risks of stroke were in East Asia (38.8%), Central Europe (31.7%) and Eastern Europe (31.6%), with the lowest risk in eastern sub-Saharan Africa (11.8%) (Feigin et al., 2018). The prevalence of stroke is expected to rise over time due to our expanding and ageing population, in association with improved survival rates from stroke arising from recent major advances in hyperacute ischaemic stroke care and targeted stroke prevention treatment regimens (Ovbiagele et al., 2013). Because patients with haemorrhagic stroke were not included in this study, this aspect of stroke has not been dealt with in further detail as it is beyond the scope of this introduction.

1.2 Von Willebrand Factor Antigen

1.2.1 Biosynthesis, structure and function

von Willebrand factor (VWF) is a multimeric plasma glycoprotein which undergoes an interesting sequence of changes between production and storage within vascular endothelial cells and platelets (Gralnick et al., 1985). It is named after Erik von Willebrand, who first described a new type of inherited bleeding predisposition distinct from haemophilia A in 1926, now known as von Willebrand disease (VWD) (von Willebrand, 1931). The relevant protein, von Willebrand factor (VWF), was not identified for a further 30 years. VWF has a dual primary role in platelet haemostasis: it mediates platelet adhesion and binds and stabilises factor VIII. However, VWF also has a potential role in a variety of ‘non-haemostatic’ pathways, including intimal thickening, tumour cell apoptosis, various inflammatory processes and transport of various other proteins (the ‘molecular bus’) (Rauch et al., 2013).

VWF multimers are formed exclusively in endothelial cells and megakaryocytes which are the precursor cells of platelets. There appear to be subtle differences in VWF synthesis within the endothelial cells of various vascular beds e.g. endothelial cells of small vessels in the lung and brain produce higher levels of VWF than vessels of a similar size in the liver and kidney (Yamamoto, de Waard, Fearn, & Loskutoff, 1998).

VWF is transcribed within the nucleus of endothelial cells and megakaryocytes, and these single, identical VWF molecules, which at this stage are termed ‘pre-pro-VWF’, each contain 2,813 amino acids (figure 1). Using electron microscopy, the description of this long string of amino acids has recently been revised to take account of the different structural domains which have various functions relating to haemostasis and multimer formation: SP-D1–D2–D’–D3–A1–A2–A3–D4–C1–C2–C3–C4–C5–C6–CK (Zhou et al., 2012).

The D1-D2 domains are composed of 741 amino acids which constitute VWF propeptide (VWFpp), and the remaining domains contain 2050 amino acids and constitute VWF:Ag.

Twenty two amino acids make up a signal peptide (SP) segment proximal to the VWFpp section. A furin cleavage site is located between the D2 domain of VWFpp and the D' domain of VWF:Ag. A binding site for factor VIII has been identified on the D'/D3 domains. The A1 domain mediates both the binding of VWF:Ag to GPIIb α on platelets and also to types I, III, IV and VI collagen. VWF also binds to type I and type III collagen via the A3 domain. ADAMTS13 cleaves VWF within the A2 domain, specifically between tyrosine 1605 and methionine 1 (Bierings & Voorberg, 2016). The C4 domain of VWF:Ag can bind to integrin $\alpha_2\beta_3$ (glycoprotein IIb/IIIa [GPIIb/IIIa]) on platelets. (Haberichter, 2015).

The 'pre-pro-VWF' molecules are translocated to the endoplasmic reticulum (ER) where the SP segment is removed; two single VWF molecules bind to one another by disulphide bonds which are formed via covalent binding of cysteine pairs located on the C-dimer, thus leading to the formation of a single stable dimer (Katsumi, Tuley, Bodo, & Sadler, 2000). The dimers then proceed into the acidic environment of the trans-Golgi network where the presence of both the D1-D2 domain (VWFpp) and D' domain (VWF:Ag) are required for correct orientation of the dimers to enable inter-dimeric cross-linking (Haberichter, 2015). *O*-linked glycans are added, carbohydrates are further processed, and sulfation occurs, forming amino-terminal (*N*-terminal) multimers with variable molecular weight, ranging from 500 to 20 000 kDa. (Haberichter, 2015) and containing up to 60 subunits of VWF dimers (D. D. Wagner, 1990). This is the so-called 'zipper model' of a multimerization process which is catalysed by VWFpp (Lenting, Christophe, & Denis, 2015).

Interestingly, the VWF sequence of amino acids contains a four-fold higher percentage of cysteine residues than that seen in average human proteins (8.3% versus 2.3%) (Miseta & Csutora, 2000). These free cysteine residues are essential for the disulphide bonding which enables formation of the dimers, their collection into multimers and subsequent accurate folding of VWF into Weibel-Palade bodies (Shapiro et al., 2014).

Weibel-Palade bodies (WPBs) were first described by Ewald Weibel and George Palade in 1964 using a transmission electron microscope. They are elongated secretory organelles which are specific to vascular endothelial cells and are secreted in response to physiological stimuli, such as injury or inflammation. They are rod shaped, 1–5 μm long and have a diameter of 0.1-0.3 μm (Valentijn, Valentijn, Jansen, & Koster, 2008). WPBs are predominantly comprised of VWF (Ewenstein, Warhol, Handin, & Pober, 1987), although they do contain several other proteins which contribute to inflammation, angiogenesis and tissue repair. These include tissue-type plasminogen activator (tPA), P-selectin, interleukin-8, eotaxin-3, angiopoietin-2, osteoprotegerin, endothelin-1, endothelin-converting enzyme and calcitonin gene-related peptide (Valentijn, Sadler, Valentijn, Voorberg, & Eikenboom, 2011). The biogenesis of the WPBs arises in the Golgi apparatus where VWF tubules stimulate the trans-Golgi network (TGN) to form membranous protrusions, leading to budding of vesicles and formation of immature WPBs (Valentijn et al., 2008). Recent studies have indicated that VWF finds itself at a crossroads at this point because a proportion of VWF will become stored in WPBs and the remainder will become encompassed within short-lived TGN-derived vesicles and proceed directly down the ‘constitutively’ secreted pathway (Bierings & Voorberg, 2016). The gate-keeper for this process is the adaptor protein 1 (AP-1) (Lopes da Silva & Cutler, 2016) which also plays an essential structural role in the formation of WPBs (Lui-Roberts, Collinson, Hewlett, Michaux, & Cutler 2005).

For those VWF multimers being stored within the WPB, the slightly acidic environment of the WPB (pH approx. 5.5) leads to 100-fold compaction of the VWF multimers into long, self-assembled, right-handed helical tubules. The centre of these tubules contain the D1-D2 (VWFpp) domain and the D’-D3 domain of the main VWF structure. The remaining A1-CK domains protrude from the helical tubule and lead to consistent spacing between the different multimers. The six small C domains (C1-C6) between the D4 and CK domains form a stem. The A2, A3, and D4 domains form a raceme (that is, a flower cluster, with separate flowers

attached by short equal stalks at equal distances along a central stem), with three pairs of large, opposed flower-like domains (Zhou et al., 2011). This structure also allows the carriage of many of the other proteins found in WPBs, some of which as yet have an uncertain role) (IL-8 and tissue-type plasminogen activator), whereas others interact directly with VWF (e.g. P-selectin, osteoprotegerin) (Valentijn et al., 2011). In addition, the tubular conformation and compaction of VWF multimers has been shown to be crucial for the rapid unfurling of the 100 μm long, 'platelet-catching VWF filaments' following exposure to neutral pH after exocytosis in cell culture and in living blood vessels. If tubules are disassembled prior to exocytosis, then short or tangled filaments are released and platelet recruitment is reduced (Michaux et al., 2006).

Secretion of VWF from endothelial cells occurs via 3 different pathways: regulated, basal and constitutive, and each pathway releases VWF in different multimeric states (Lopes da Silva & Cutler, 2016). Regulated secretion of VWF from WPBs occurs in response to agonist-induced endothelial stimulation (e.g. thrombin, histamine) when multiple WPBs coalesce into a single, super-sized 'secretory pod' which fuses with the plasma membrane (Valentijn et al., 2010) in a process known as multigranular exocytosis. Ultra-large von Willebrand factor (UL-VWF) multimers are then released in large quantities, mainly from the apical (lumen-facing) surface of the endothelial cells into the circulating plasma, with some release to the basolateral side. This form of VWF can be regarded as the 'emergency supply', released locally at the site of vascular injury and having the highest platelet-binding activity (Lopes da Silva & Cutler, 2016). These 'regulated release' UL-VWF multimers have been shown to form extremely lengthy string-like structures which can be up to several millimetres in length and range in size from 500 to 20,000 kDa (J. F. Dong et al., 2002). The variable molecular weight of VWF is due to differences in the number of multimers present. Basally-secreted VWF has been identified as the main source of circulating plasma VWF and occurs when single WPBs sequentially tether, dock, prime and fuse with the endothelial

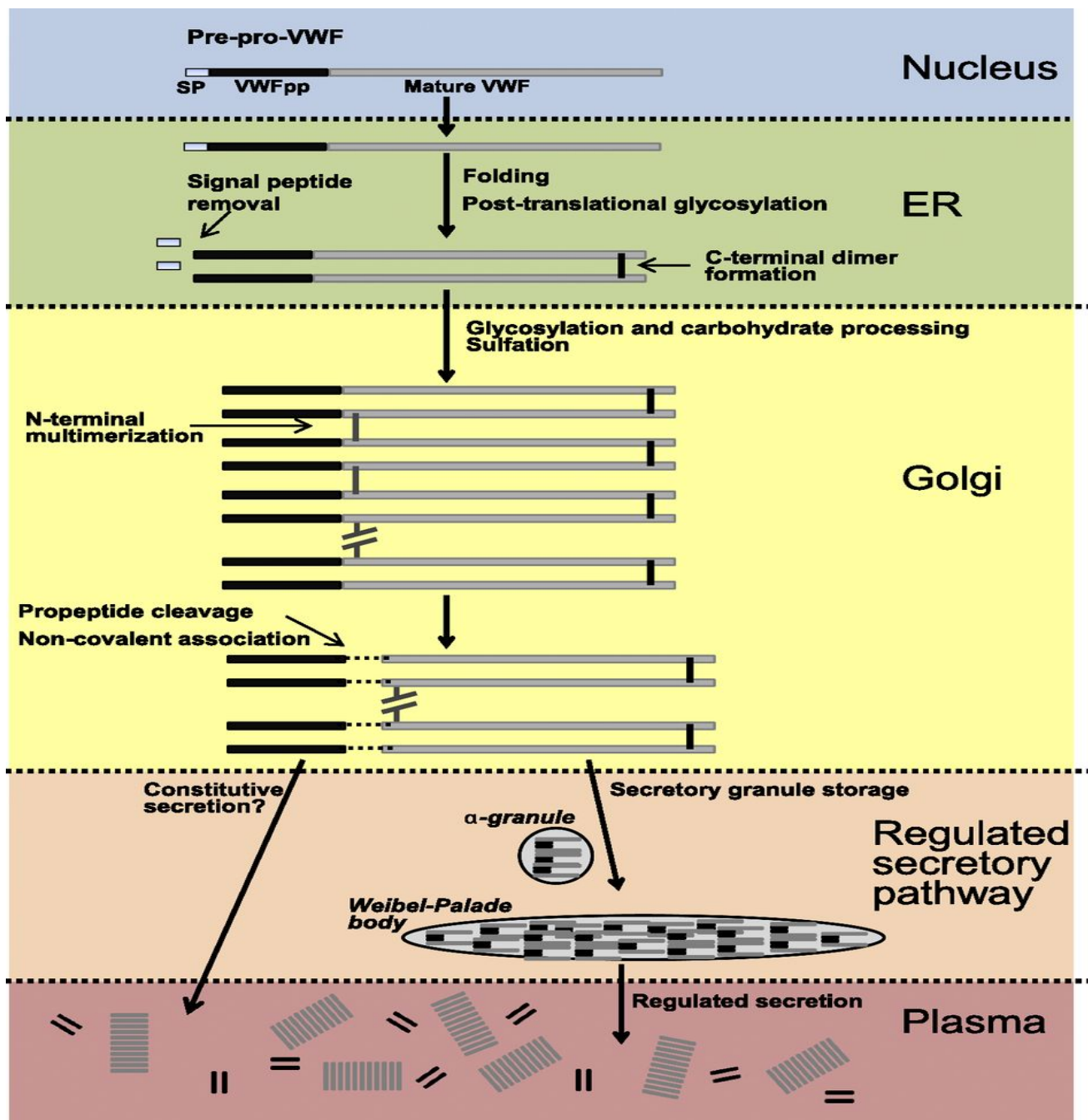
cell membrane, with each step involving specific protein complexes (Lenting et al., 2015; Mourik & Eikenboom, 2017). The single WPB ultimately releases both VWF and the other WPB proteins into the circulation via a fusion pore. In a small but significant proportion of single fusion events, the WPB attaches to the plasma membrane via small fusion pores of approximately 12 nm in diameter (Babich et al., 2008) but because of the small size of these fusion pores, larger proteins such as P-selectin and VWF are retained within the collapsed WPB following fusion, whereas smaller membrane-bound protein such as CD63 and soluble IL-8 are released through the open pore. This process has been referred to as ‘lingering kiss exocytosis’ (Babich et al., 2008). During weak stimulation of the WPBs, up to 25% of single fusion events were of this nature. This process could enable endothelial cells to adjust their response to different secretagogues, thus facilitating release of contents with a lower ratio of VWF to cytokines which are less thrombotic and more pro-inflammatory (Nightingale & Cutler, 2013). This basal secretion of VWF from WPBs occurs continuously from the apical surface of the endothelial cells in the absence of any stimuli. The VWF multimers released by this pathway are also predominantly UL-VWF multimers, but they are structurally slightly different to the UL-VWF multimers of ‘regulated’ VWF and do not produce VWF strings which can initiate thrombus formation. Instead, they are immediately cleaved on secretion by ADAMTS-13 and subsequently circulate in plasma as intermediate VWF multimers (Lopes da Silva & Cutler, 2016). The ‘constitutively-released’ VWF is composed of a proportion of VWF which leaves the trans-Golgi network, but instead of being packaged into WPBs, is directed solely towards the basolateral aspect of endothelial cells by means of a gatekeeper protein, adaptor protein 1 (AP-1). This VWF pool is released towards the subendothelial matrix in the absence of stimuli and is composed of low molecular weight VWF multimers which fuse with the plasma membrane of the endothelial cells to constitute the majority of collagen-bound subendothelial VWF. Endothelial cells combine basal,

regulated and constitutional secretion of their WPB contents, whereas alpha granules of platelets only release VWF upon platelet activation (Lenting et al., 2015).

VWF:Ag and VWFpp remain non-covalently bound within WPBs following proteolytic cleavage in the trans-Golgi network by the enzyme furin (Haberichter, Jozwiak, Rosenberg, Christopherson, & Montgomery, 2002). Immediately upon secretion from WPBs, VWFpp dissociates from VWF:Ag and circulates independently (Giblin, Hewlett, & Hannah, 2008). Circulating plasma VWF:Ag is predominantly derived from endothelial cells with only a small proportion secreted from platelets (Kanaji, Fahs, Shi, Haberichter, & Montgomery, 2012). Therefore, measurement of VWF:Ag levels can clearly be used as a marker of endothelial activation, but it is not a 'specific biomarker of endothelial activation' because these levels are also influenced by other factors, including blood group, ADAMTS13 activity, platelet activation status and systemic inflammation (Bongers et al., 2006; McCabe et al., 2015). Of note, circulating VWF:Ag has a half-life of 8 to 12 hours in healthy individuals (J. Eikenboom et al., 2013; Haberichter, 2015).

Figure 1 outlines the progress of VWF through the endothelial cell and ultimate secretion although this diagram does not include basal secretion from the WPBs.

Figure 1: VWF:ag And VWFpp: biology and clinical utility (Diagram reproduced with kind permission from Professor Sandra L. Haberichter) (Haberichter, 2015)



Legend for Figure 1: VWF intracellular processing. VWF is transcribed as pre-pro-VWF in the nucleus (blue) and is translocated to the Endoplasmic Reticulum (ER) (green), where it undergoes signal peptide removal, glycosylation, and dimer formation. In the Golgi apparatus (yellow), O-linked glycans are added, carbohydrates are processed, VWF is sulphated and multimerized, and VWF propeptide is cleaved from mature VWF but remains non-covalently associated. VWF is trafficked to the regulated secretory pathway (peach) and stored in WPBs or α -granules before release into plasma (red), where VWFpp and VWF dissociate and circulate independently of one another.

1.2.2 Role of Shear Stress

Under conditions of low shear stress, the UL-VWF strings circulate in a loosely-folded globular conformation (Schneider et al., 2007) and although UL-VWF can bind to coagulation factor VIII, it does not readily bind to endothelium or platelets (Jackson, 2007). It appears that the size of VWF multimers under normal flow conditions is precisely controlled by ADAMTS13 cleavage (Furlan, Robles, & Lammle, 1996).

A wide range of haemodynamic conditions exist *in vivo*, with exposure of flowing blood to low shear stress rates ($< 500 \text{ s}^{-1}$) in venules and veins, and moderately high shear rates of 5000 s^{-1} in small arterioles. However, thrombus formation also alters the haemodynamic environment due to a reduction in the diameter of the vessel lumen through which the blood flows. Therefore, to maintain the same volume of blood passing through a vessel, velocity of flow must increase, thus resulting in higher shear stress rates. Immediately distal to a severe coronary atherosclerotic stenosis, shear stress rates may be as high as $40,000 \text{ s}^{-1}$ (Bluestein, Niu, Schoepfoerster, & Dewanjee, 1997), and blood flow and different shear stress rates influence different aspects of the platelet activation and aggregation process (Ruggeri, Dent, & Saldivar, 1999) (Schneider et al., 2007).

At higher shear stress rates above 5000 s^{-1} , UL-VWF rapidly changes shape from a globular to an elongated conformation with the subsequent exposure of A3 collagen binding sites. This facilitates the attachment of UL-VWF to the surface of endothelial cells, thereafter changing it from an inactive structure into a highly-reactive one (Sorrentino, Studt, Medalia, & Tanuj Sapra, 2015). Once tethered to the sub-endothelium and immobilised, UL-VWF will experience further tensile force, leading to the disruption of bonds between the A1 domain and its neighbouring D'D3 region, with consequential exposure of the region of the A1 domain which binds to the GP1b α receptor on platelets (Mourik & Eikenboom, 2017; Sugimoto, Dent, McClintock, Ware, & Ruggeri, 1993) (Lof, Muller, & Brehm, 2018). It has

been postulated that the A1 and A2 domains may have an auto-inhibitory role to play, either by restraining the A1-GP1b α interaction or by protecting the A2 domain from cleavage by ADAMTS13, or via both mechanisms (Lof et al., 2018).

The role of VWF in platelet activation and aggregation is paramount under conditions of high-shear stress, including in arterioles, in the microcirculation and in the presence of critical arterial stenoses. These areas correspond to the segments of the vascular tree where efficient primary haemostasis is essential to arrest bleeding. Conversely, although VWF-mediated platelet aggregation is necessary at sites of vascular injury, if VWF was activated under normal flow conditions, it would rapidly induce thrombotic occlusions (Lof et al., 2018).

As shear rates increase above 1000 s⁻¹, platelet aggregate formation becomes more dependent on VWF; above 10,000 s⁻¹, development of a thrombus is almost entirely dependent on VWF:Ag (Jackson, 2007). Savage *et al.* proposed that above a shear rate of 500 to 800 s⁻¹, platelet adhesion could only be initiated by the interaction between an immobilized VWF A1 domain and the platelet membrane glycoprotein Ib α (GPIb α) (Savage, Saldivar, & Ruggeri, 1996). Of note, larger multimers are more responsive to shear stress than smaller VWF multimers resulting in higher binding ability for collagen and platelet receptors GP1b α and GPIIb/IIIa compared with smaller multimers (Stockschlaeder, Schneppenheim, & Budde, 2014).

The PFA-100, one of the platelet function/reactivity devices used during the OATS study, exposes platelets within a whole blood sample to a moderately high shear rate of 5,000 to 6,000 s⁻¹ which is comparable to the shear rate to which platelets are exposed in a moderately stenosed artery (Kroll, Hellums, McIntire, Schafer, & Moake, 1996). Conversely, the VerifyNow and Multiplate devices operate under low shear stress conditions.

1.2.3 VWF and plasma cholesterol

Plasma membrane cholesterol has been shown to play an important role in regulating the secretion of VWF from endothelial cells. Depleting cholesterol from endothelial cells may slow WPB exocytosis, whereas supplementing cells with cholesterol may increase VWF secretion (Cookson, Conte, Dempster, Hannah, & Carter, 2013). This relationship between cholesterol and WPB exocytosis may therefore contribute to the pathways which influence the risk of vascular events because elevated plasma VWF levels have been observed in association with chronically elevated plasma cholesterol levels (Cookson et al., 2013). Conversely, long-chain omega III polyunsaturated fatty acids appear to reduce WPB degranulation, and this mechanism may contribute to some of their anti-inflammatory and proposed 'vasoprotective effects' (Burgin-Maunders, Brooks, & Russell, 2013).

1.2.4 Platelet-derived VWF:Ag

There is increasing interest in the role of platelet-derived VWF, in particular in light of reports that there are significant differences in its structure compared with endothelium-derived VWF. Most notably, there is an absence of ABO blood group carbohydrate determinants on platelet-derived VWF (Brown, Collins, & Bowen, 2002), with a marked reduction (>50%) in its total sialic acid and galactose expression compared with endothelium-derived VWF (McGrath, McRae, Smith, & O'Donnell, 2010). The latter finding is particularly relevant as this appears to confer specific resistance to ADAMTS13 proteolysis, thus facilitating platelet-plug formation (McGrath et al., 2013). Platelet-derived VWF represents approximately 20% of total VWF protein and contains larger sized multimers, with a higher ratio of VWF activity to antigen (Gralnick et al., 1985). It has been reported that whilst platelet-derived VWF is not essential for normal thrombosis and haemostasis, it does play a role in ischaemic stroke in a mouse model (Verhenne et al., 2015).

However, more recent studies demonstrated that platelet-derived VWF was not sufficient on its own to promote atherosclerosis, even in the absence of ADAMTS13.

1.2.5 VWF:Ag in ischaemic stroke and TIA overall and in aetiological subgroups

The association between VWF:Ag and coronary artery disease has been widely studied, with relatively fewer studies on VWF:Ag in ischaemic cerebrovascular disease. Elevated levels of VWF:Ag have been observed in the early phase (Bongers et al., 2006; Catto et al., 1997; McCabe et al., 2004; McCabe et al., 2015; Tobin et al., 2017) and late phase after TIA or ischaemic stroke compared with controls (Hanson et al., 2011; McCabe et al., 2004; Tobin et al., 2017). One pilot study revealed that VWF:Ag levels were elevated in the early and late phases after TIA or ischaemic stroke overall, and in CVD patient subgroups with large artery atherosclerosis, small vessel disease, cardioembolism and TIA/stroke of indeterminate aetiology compared with controls without a history of cerebrovascular disease (McCabe et al., 2004). VWF levels were also found to be significantly elevated in 120 patients within 24 hours of cardio-embolic ischaemic stroke onset compared with controls, with no significant differences between in VWF expression between stroke subtypes (Licata et al., 2009). Hanson *et al.* showed that VWF:Ag levels were increased in the early phase after large artery atherosclerotic (LAA) stroke, cardioembolic (CE) stroke, cryptogenic stroke, and stroke secondary to small vessel disease (SVD) compared with controls (Hanson et al., 2011). At 3 months, VWF:Ag levels remained elevated in LAA stroke, CE stroke and cryptogenic stroke, but not in stroke due to SVD compared with controls (Hanson et al., 2011). A subsequent systematic review and meta-analysis of various biomarkers in lacunar stroke versus ‘non-lacunar stroke’ and ‘non-stroke controls’ by Wiseman in 2014 revealed that VWF and other markers of endothelial dysfunction (homocysteine, E-selectin, intercellular adhesion molecule-1 (ICAM-I) were higher in acute lacunar stroke versus non-stroke controls, with conflicting data in the late phase after stroke (Wiseman, Marlborough, Doubal,

Webb, & Wardlaw, 2014). VWF levels were also significantly lower in acute lacunar stroke compared with other ‘non-lacunar’ ischaemic stroke subtypes (Wiseman et al., 2014). A recent prospective, observational, case-control study by members of our research group measured plasma VWF:Ag levels in CVD patients at baseline (within 4 weeks of TIA or ischaemic stroke onset), and at 14 days and at ≥ 90 days later compared with healthy controls (Tobin et al., 2017). That study revealed that VWF:Ag levels were increased in an overall TIA and ischaemic stroke population at baseline, and in patients with recently symptomatic carotid stenosis at all timepoints compared with controls (Tobin et al., 2017).

1.2.6 VWF:Ag in patients with carotid stenosis

A number of studies have assessed VWF:Ag levels in patients with carotid stenosis and many of these studies have been summarised recently (S. Murphy, 2016, 2020). A cross-sectional study which assessed plasma VWF levels in 53 patients with TIA and ipsilateral or contralateral carotid artery stenosis on ultrasound of the extracranial neck arteries did not find an association between VWF antigen levels and the degree of carotid stenosis (Blann, Farrell, Picton, & McCollum, 2000). Another prospective cohort study which investigated ‘progression of atherosclerosis’ in 258 participants with carotid plaques reported a positive association between plaque area on carotid ultrasound and VWF levels (Nilsson et al., 2002). A cross-sectional, population-based study by With Noto *et al* investigated the relationship between carotid plaque morphology and echogenicity on carotid ultrasound and endothelial dysfunction (With Noto, Bogeberg Mathiesen, Amiral, Vissac, & Hansen, 2006). The authors found significantly higher plasma VWF antigen levels in 133 ‘mainly asymptomatic carotid stenosis’ patients (17% had a history of stroke in any vascular territory) compared with 138 ‘cardiovascular disease controls’ who did not have carotid stenosis (6% of patients had a history of ‘any stroke’) (With Noto et al., 2006). However, this study was limited by the fact that it did not focus on patients with either truly asymptomatic carotid stenosis or

with stroke in the vascular territory supplied by an extracranial internal carotid artery stenosis. One study in 67 patients undergoing carotid artery stenting found increased plasma VWF:Ag levels in the period immediately following stenting compared with just pre-stenting (Xia, Yang, Qu, Cheng, & Wang, 2011). Those patients who subsequently developed carotid restenosis had significantly higher plasma VWF:Ag levels 24 hours after stenting than those who did not have restenosis at the 1 year follow-up timepoint. Yang *et al.* measured VWF:Ag and Endothelin-1 levels in a small group of patients who underwent carotid artery stenting (CAS) for >50% symptomatic carotid stenosis or >70% asymptomatic carotid stenosis (Yang et al., 2012). Fourteen of the 61 patients subsequently developed carotid restenosis. Bloods were taken at 'baseline', and then at 1 hour, 2 weeks, 1 month and 6 months after CAS. VWF:Ag and endothelin-1 levels were significantly higher at 1 hour and at 6 months after CAS than at baseline, but not at 12 months compared with baseline. There were no significant differences in the baseline levels of VWF:Ag or endothelin-1 between the subgroups of patients who did or did not develop restenosis over time. Other data from our research group in patients with recently symptomatic and asymptomatic carotid stenosis from the Platelets and Carotid Stenosis study will be summarised in the section on VWFpp below (Kinsella et al., 2014).

1.2.7 Potential value of VWF in predicting the risk of first or recurrent vascular events

The Progetto Lombardo Atero-Trombosi (PLAT) Study was a prospective, observational, multicentre study of clinical outcomes (documented recurrent atherothrombotic events up to 2 years after inclusion) in patients with vascular disease i.e. previous TIA, symptomatic coronary artery disease or peripheral arterial disease (Cortellaro et al., 1992). There was no significant association between plasma VWF:Ag levels and the risk of recurrent vascular events in patients with previous TIA. One large study which investigated the association between several markers of 'haemostatic function' and the risk of future ischaemic stroke in

more than 14,700 middle-aged participants who were free from cardiovascular disease and who had no history of prior stroke revealed a strong positive association between VWF levels and the incidence of ischaemic stroke (Folsom et al., 1999). The Rotterdam study is a prospective, population-based cohort study which was established in 1990 to investigate factors influencing the occurrence of neurological, cardiovascular, ophthalmological, endocrinological or psychiatric diseases in an ageing population. The population of a suburb of Rotterdam, Ommoord, were invited to participate at regular intervals. The association between VWF:Ag levels, ADAMTS13 activity and ischaemic stroke was assessed in this population. The authors concluded that high VWF levels were associated with an increased stroke risk in the general population (Wieberdink et al., 2010). In atrial fibrillation patients on anticoagulation, raised VWF levels doubled the risk of stroke, cardiovascular death or major bleeding during follow-up; however, although the addition of VWF levels to certain prediction scoring systems statistically improved the prediction of certain endpoints, the impact on practical clinical decision making was minimal in that study (Garcia-Fernandez et al., 2017).

1.3 Von Willebrand Factor Propeptide (VWFpp)

1.3.1 Biosynthesis, structure and function

The production and structure of VWFpp has been alluded to in section 1.2.1 above. In short, the string of amino acids which constitutes VWF has five different structural domains: D1–D2–D'–D3–A1–A2–A3–D4–C1–C2–C3–C4–C5–C6–CK (Zhou et al., 2012). The D1-D2 domains contain 741 amino acids and constitute VWFpp; the remaining 2050 amino acids constitute VWF:Ag. Proteolytic cleavage of VWFpp from VWF:Ag occurs in the trans-Golgi network via the action of the enzyme furin (Haberichter et al., 2002), but VWFpp and VWF:Ag remain non-covalently bound to each other during their time in the WPBs. Immediately upon secretion from WPBs, VWFpp dissociates from VWF:Ag and circulates independently (Giblin et al., 2008).

As discussed in section 1.2.1, VWFpp is essential for intracellular processing and correct storage of VWF:Ag, and it also works as a catalyst for the multimerization process. Without the input of VWFpp, the accurate and precise functioning of VWF:Ag would be compromised.

By definition, there should initially be 1 unit of VWF:Ag for every 1 unit of VWFpp in 1 mL of plasma in healthy individuals, with a steady-state VWFpp/VWF:Ag ratio of approximately 1.0 (Haberichter, 2015). Individuals with blood group O have lower VWF:Ag levels due to slightly faster clearance of VWF:Ag than that seen in non-blood group O individuals (Haberichter et al., 2006). However, VWFpp level is not influenced by blood group (J. Eikenboom et al., 2013; Nossent et al., 2006). Therefore, although VWFpp has a shorter half-life than VWF in blood, individuals with blood group O have slightly elevated VWFpp/VWF:Ag ratios than those who are non-blood group O (Haberichter, 2015) (Nossent et al., 2006).

Although both VWF:Ag and VWFpp are secreted from damaged endothelial cells, it has been suggested that VWFpp levels are a more sensitive marker of acute vascular endothelial cell dysfunction than VWF:Ag levels (Kinsella et al., 2014). Because VWFpp is stored and then secreted, with a half-life of 2 to 3 hours, it must have a specific function in plasma (J. Eikenboom et al., 2013). One proposal is that VWFpp can bind to mature VWF in the circulation and regulate the haemostatic abilities of VWF by moderating VWF binding to platelet GpIb α (Madabhushi et al., 2012). However, it is likely that VWFpp has other, as yet unidentified, functional effects and roles.

1.3.2 Von Willebrand Factor Propeptide (VWFpp) in ischaemic stroke and TIA

Due to its rapid turn-over and maximum half-life of 3 hours, as stated above, it has been postulated that VWFpp could prove to be a more sensitive plasma marker of acute endothelial activation than VWF:Ag (Habe et al., 2012; Kinsella et al., 2014; van Mourik et al., 1999; Vischer et al., 1997). In a pilot longitudinal, observational analytical study by our research group, VWF:Ag and VWFpp levels were simultaneously quantified in 91 patients within 4 weeks of TIA or ischaemic stroke (baseline), and then 14 days and > 90 days after starting or altering antiplatelet therapy (Tobin et al., 2014). In the overall study population, VWF:Ag, VWFpp levels and VWF:Ag/VWFpp ratio were unchanged at 14 days and 90 days compared with baseline. Pre-planned subgroup analysis revealed that the addition of dipyridamole MR to aspirin led to a significant reduction in VWF:Ag levels at both 14 days and 90 days, without a significant change in VWFpp levels. However, both VWF:Ag and VWFpp levels remained stable at 14 days and 90 days after starting aspirin and after changing from aspirin to clopidogrel. A further prospective, observational, case-control study by Tobin *et al.*, measured plasma VWFpp levels at baseline (within 4 weeks of TIA or ischaemic stroke), 14 days and 90 days later and compared these data with those in healthy controls (Tobin et al., 2017). Unadjusted VWFpp levels were significantly higher in patients

at baseline, 14d and 90d than in controls. However, differences in VWFpp between groups were no longer statistically significant after correction for age, hypertension, hyperlipidaemia or smoking status between groups. The VWF:Ag/VWFpp ratio was similar in patients and controls and it was concluded that VWFpp was not superior to VWF:Ag at detecting acute endothelial activation in this overall CVD patient cohort (Tobin et al., 2017). An additional study by our research group compared plasma VWF:Ag and VWFpp levels in patients with $\geq 50\%$ asymptomatic carotid stenosis with levels in patients with $\geq 50\%$ recently symptomatic carotid stenosis in the 'early' (≤ 4 weeks) and 'late' (≥ 3 months) phases after TIA or ischaemic stroke (Kinsella et al., 2014). VWFpp levels were significantly higher in early, late and late post-intervention symptomatic patients than asymptomatic patients. VWF:Ag levels decreased significantly in symptomatic patients followed up from the early to late phase after symptom onset. Early symptomatic micro-embolic signal negative (MES-negative) patients had significantly higher VWFpp levels than asymptomatic MES-negative patients (Kinsella et al., 2014).

Two independent case-control studies (COCOS and ATTAC) also studied VWF:Ag and VWFpp in CVD patients (van Schie et al., 2010). The 'COCOS' study compared data from 101 patients within 7-14 days of first-ever TIA or ischaemic stroke with 106 healthy, age-matched controls. Subgroup analysis of the 'ATTAC' study compared data from 171 young patients (men aged 18–45, and women aged 18–55) who had experienced a first-ever TIA or ischaemic stroke 3 months earlier with 171 age-matched healthy controls. In the COCOS study, VWF:Ag levels were significantly higher, with a non-significant trend towards increased VWFpp levels and a lower VWFpp:VWF:Ag ratio in patients vs. controls. In a subgroup of patients from the COCOS study who also had blood samples taken 3 months after TIA or stroke onset, there were no significant differences in VWF:Ag or VWFpp levels

between the early and late phases after symptom onset. In the ATTAC study, there were also significantly higher VWF:Ag levels in patients *vs.* controls, but the differences in VWFpp levels and VWFpp/VWF:Ag ratio did not reach statistical significance (van Schie et al., 2010).

1.4 ADAMTS13

1.4.1 Biosynthesis, structure and function

In 1982, patients with chronic relapsing thrombotic thrombocytopenic purpura (TTP) were noted to have ‘ultra-large VWF’ multimers which in turn led to an increase in microvascular thrombosis, platelet consumption and haemolysis (Moake et al., 1982). A relationship was recognised between VWF proteolysis and TTP, and subsequent to this, a deficiency of a plasma ‘von Willebrand factor cleaving protease’ (VWFCP) was recognised in these patients. This VWFCP was identified as a plasma metalloprotease which cleaves the Tyr¹⁶⁰⁵–Met¹⁶⁰⁶ bond in the central A2 domain of the VWF subunit as it circulates in plasma in large multimers which range in size from 500 to 20,000 kDa (Furlan et al., 1996; Tsai, 1996). The activity of this metalloprotease results in downregulation of the potent thrombogenic potential of these ultra-large VWF multimers during the process of platelet aggregation, thus limiting the formation of platelet-rich thrombi in arterioles. VWFCP was shown to be a member of the ADAMTS family of metalloproteases because it possesses the characteristic combination of A Disintegrin And Metalloprotease (reprolysin-type) with a Thrombospondin type 1 repeat (Hurskainen, Hirohata, Seldin, & Apte, 1999; Kaushal & Shah, 2000; Tang & Hong, 1999). It was the 13th member of this group of enzymes to be identified and hence was named ‘ADAMTS13’. Although it is similar in structure to other ADAMTS proteases, ADAMTS13 has distinctive features with diverse modes of ligand binding.

The ADAMTS13 gene has been localised to human chromosome 9q34 and contains 29 exons. ADAMTS13 enzyme is primarily synthesised in hepatic stellate cells which are located in the interstitial spaces between hepatocytes (Uemura et al., 2005). However, it has also been shown to be synthesised in vascular endothelial cells (Shang, Zheng, Niiya, & Zheng, 2006), platelets (Suzuki et al., 2004), in glomerular podocytes, endothelium and

basement membrane (Manea et al., 2007) and glial cells (Tauchi et al., 2012). Over 200 different mutations of the ADAMTS13 gene have been identified (van Dorland et al., 2019). The mature ADAMTS13 transcript has a size of 4.7 kb and gives rise to a precursor protein of 1427 amino acid residues which contains multiple sites or potential sites for glycosylation. It has been postulated that ADAMTS13 activity may vary between different tissues, in particular between the liver and skeletal muscle (Tsai, 2005). Acute liver disease may be associated with decreased circulating levels of ADAMTS13 (Hugenholtz et al., 2013). Three independent gene polymorphisms of the ADAMTS13 gene and smoking were identified as major predictors of plasma ADAMTS13 antigen levels, with smoking exposure being associated with a 3.6% decrease in antigen levels (Ma et al., 2017). This finding confirms an earlier study which noted lower ADAMTS13 levels in male Arab smokers (Al-Awadhi, Jadaon, Alsayegh, & Al-Sharrah, 2012). ADAMTS13 activity has not been shown to be influenced by ABO blood groups to date (Chion, Doggen, Crawley, Lane, & Rosendaal, 2007; Kokame, Sakata, Kokubo, & Miyata, 2011). However, the latter of these 2 studies, which included 3616 Japanese participants, also demonstrated that plasma ADAMTS13 activity decreases with age, especially after the age of 60 years, and appeared lower in men than in women (Kokame et al., 2011).

In contrast to other ADAMTS proteases, ADAMTS13 is secreted into the circulation as an active enzyme with a plasma half-life of approximately 2-3 days (Furlan, Robles, Morselli, Sandoz, & Lammler, 1999). The estimated normal plasma concentration of ADAMTS13 is approximately 1 µg/ml (0.7–1.4 µg/ml = 3.5–7.0 nM) (Rieger et al., 2006). The mechanisms by which ADAMTS13 is cleared or metabolized *in vivo* are unknown. The sugar chains of ADAMTS13 are capped by sialic acids, suggesting that the hepatic asialoglycoprotein receptor could be involved in ADAMTS13 clearance (Hiura et al., 2010).

1.4.2 ADAMTS13 in ischaemic stroke and TIA

A small number of studies have assessed ADAMTS13 activity or antigen levels in patients with TIA or ischaemic stroke (Andersson et al., 2012; Bongers et al., 2006; Bustamante et al., 2018; McCabe et al., 2015; Sonneveld et al., 2015). A prospective, pilot observational analytical case-control study compared ADAMTS13 activity and VWF:Ag levels in 53 patients in the early phase (≤ 4 weeks) and in 34 of these patients in the late phase (≥ 3 months) after TIA or ischaemic stroke on aspirin with 22 aspirin-naïve controls (McCabe et al., 2015). In addition, the relationship between ADAMTS13 activity and platelet function/reactivity was assessed by measuring Collagen-ADP (C-ADP) and Collagen-Epinephrine closure times on a platelet function analyser called the PFA-100[®]. There was a significant reduction in ADAMTS13 in the early phase ($P < 0.01$) but not in the late phase after TIA or stroke compared with controls ($P = 0.19$) (McCabe et al., 2015). There was also a significant inverse relationship between ADAMTS13 activity and VWF:Ag levels in the early phase ($r = -0.31$; $P = 0.024$), but not in the late phase after TIA or stroke ($P = 0.74$). There was a positive correlation between ADAMTS13 activity and C-ADP closure times in early phase patients only, likely mediated via VWF:Ag levels. (McCabe et al., 2015). The Rotterdam study found that low ADAMTS13 activity was significantly associated with the risk of ischaemic stroke, independent of age, sex and other established cardiovascular risk factors (Sonneveld et al., 2015). Furthermore, the addition of ADAMTS13 activity to risk prediction models for ischaemic stroke improved the accuracy of these models beyond those which included standard risk factors (age, sex, systolic blood pressure, treatment of hypertension, total and HDL cholesterol, smoking, and diabetes). In fact, the contribution of ADAMTS13 to stroke risk prediction was comparable with the effects of systolic blood pressure, cholesterol, and smoking (Sonneveld et al., 2015). This prospective, population-based study also reported that individuals with both high VWF:Ag levels and low ADAMTS13 activity had at least a 1.73-fold increased risk of all-cause and cardiovascular

mortality compared with individuals with both low VWF levels and high ADAMTS13 activity, although the number of individuals in these subgroups was small (Sonneveld et al., 2016). This study suggests that VWF:Ag levels and ADAMTS13 activity are independent risk factors for subsequent vascular events, and might also have additive effects.

A further study involving women aged 18-49 years found that high levels of VWF:Ag and low levels of ADAMTS13 antigen were both associated with an increased risk of stroke and myocardial infarction (MI) in a 'dose-dependent manner' (Andersson et al., 2012). The combined risk of having both high levels of VWF:Ag and low ADAMTS13 antigen levels was higher than that associated with either of these individual risk factors. These risks were further increased by the use of an oral contraceptive pill (Andersson et al., 2012). Of those recruited, 167 women had a history of ischaemic stroke, 202 had a history of a myocardial infarction and there were 626 healthy controls. It is important to note that the blood samples were collected at a median of 95 months post-ischaemic stroke, and at 69 months post-MI.

A more recent study found that reduced ADAMTS13 antigen levels were associated with a poorer response to recanalization therapies following acute ischaemic stroke with either IV tPA or mechanical thrombectomy (Bustamante et al., 2018). Of interest, one single-nucleotide polymorphisms (SNP) in the ADAMTS13 gene (rs4962153) was found to be associated with an increased risk of ischaemic stroke overall (Odds ratio (OR): 1.25; 95% CI: 1.01–1.54) and with the risk of cryptogenic ischaemic stroke (OR: 1.50; 95% CI: 1.09–2.07) in a population of patients < 70 years old in western Sweden (Hanson, Jood, Nilsson, Blomstrand, & Jern, 2009). Larger studies in diverse patient populations are warranted to confirm these findings.

1.5 Aspirin, Dipyridamole and Clopidogrel

1.5.1 Aspirin

Leaves and bark from the willow tree have been used for their beneficial effects on health for over 2,400 years and have been cited in ancient texts from Assyria, Sumer and Egypt as a treatment for fever and arthralgia. Hippocrates (440-377 B.C.) wrote about the medical uses of the bark and leaves of salix, the willow tree (Pearce, 2014). The first “clinical trial” was a report outlining five years of experiments by Reverend Edward Stone, a vicar in Chipping Norton in the UK, using dried, powdered willow bark to successfully treat fevers. These findings were published by the Royal Society in 1763 (Stone, 1763). In the 1820s, a number of chemists in Germany, France and Italy worked on isolating the active component of the willow tree bark, but it was not until 1838 that Raffaele Piria, an Italian chemist, succeeded in producing salicylic acid in its pure state. However, 100% salicylic acid is toxic if absorbed by ingestion, inhalation or via the skin, and so, it was unusable in this form. In 1853, a French chemist called Charles Frederic Gerhardt created acetylsalicylic acid by buffering it with sodium and acetyl chloride, thus making it tolerable after oral intake. Historically, it has been believed that a German chemist called Felix Hoffmann rediscovered Gerhardt’s formula in 1897, and prepared the first pure acetylsalicylic acid which was devoid of the unpalatable side effects of sodium salicylate, thus successfully treating his father who suffered from arthritis. Hoffmann worked for the German company, Bayer, at this time and is said to have convinced the company to market the drug in 1899 because of the improvement in his father’s symptoms. However, it now appears that Hoffmann’s former colleague, Arthur Eichengrun, played a much more significant supervisory role in the synthesis of aspirin than that for which he was previously given credit (Pearce, 2014). There are 2 versions of where the name ‘Aspirin’ came from. The first is that ‘Aspirin’ is derived from the ‘A’ in acetyl chloride, the ‘spir’ in *spiraea ulmaria* (meadowsweet flower; the plant from which the salicylic acid is derived) and the “in” which was a familiar ending for

medicines at that time (Connelly, 2014). A more emotive account is that it was named after an early Italian bishop who was the patron saint of headaches, Saint Aspirinius (Farre, Tamargo, Mateos-Caceres, Azcona, & Macaya, 2010). In any case, aspirin was finally patented by Bayer on February 27th 1900.

Acetylsalicylic acid is an acetylating agent whose acetyl group irreversibly covalently binds to and acetylates a serine residue (Ser 530) in the active catalytic pocket of the platelet COX-1 enzyme (Toth, Muszbek, & Komaromi, 2013). It can also inhibit COX-2 by acetylation of another serine residue (Ser 516), although this reaction is approximately 170-fold times slower than the reaction with COX-1 (Vane, Bakhle, & Botting, 1998). As a result of these bonds, neither COX isoform is capable of converting arachidonic acid into prostaglandin H₂ (PGH₂), thus leading to a reduction in the production of thromboxane A₂ (TXA₂) and other prostaglandins. Of note, platelet production of TXA₂ in response to various stimuli (collagen, thrombin and ADP) results in an increased platelet aggregation response and in vasoconstriction (Cheng et al., 2002). On the other hand, vascular endothelial cell production of prostacyclin results in inhibition of platelet aggregation and vasodilation (Majed & Khalil, 2012). However, in response to low dose aspirin, the antithrombotic effects of TXA₂ inhibition dominate over the prothrombotic effects of prostacyclin inhibition (Patrono et al., 2001). Aspirin also results in the acetylation of other proteins in the blood coagulation pathway, including fibrinogen, which in turn enables more effective fibrinolysis (Undas, Brummel-Ziedins, & Mann, 2014).

1.5.2 Dipyridamole

Dipyridamole was originally launched in 1959 as an anti-anginal medication due to its coronary vasodilatory properties. Subsequent to this, its ability to impede the formation of

clots was noted in animals and humans, which in turn led to its development as an anti-thrombotic agent.

Dipyridamole is a pyrimidi-pyrimidine compound which exercises its antithrombotic actions in a number of ways, as reviewed recently by my collaborator, Dr Soon Tjin Lim. Dipyridamole inhibits adenosine uptake by erythrocytes, which may in turn facilitate vasodilation and platelet inhibition (Kim & Liao, 2008; Lim, Submitted 2020). This leads to an increase in extracellular concentrations of adenosine, in particular at the platelet-vascular interface, which is a potent inhibitor of platelet aggregation (Arch & Newsholme, 1978). Furthermore, dipyridamole inhibits phosphodiesterase 3 (PDE3) and phosphodiesterase 5 (PDE5) in platelets, thus increasing intra-platelet levels of cAMP, a potent inhibitor of 'platelet activation'. Dipyridamole can also exert a vasodilatory effect on vascular smooth muscle by inhibiting PDE3- and PDE5-mediated cAMP/cGMP degradation. It may also promote prostaglandin I₂ (PGI₂) production by vascular smooth muscle cells which, in turn, also stimulates cAMP production, resulting in further vasodilatory and anti-platelet effects (Gresele et al., 1983; P. Gresele, Momi, & Falcinelli, 2011; Lim, Submitted 2020). It has been demonstrated that dipyridamole may inhibit 'platelet aggregation' by inhibition of PDE5 (Aktas, Utz, Hoenig-Liedl, Walter, & Geiger, 2003). Furthermore, it has been proposed that dipyridamole mediates the antiplatelet effects of a combination of NO and PGI₂ by increasing intracellular levels of cAMP/cGMP (Bult, Fret, Jordaens, & Herman, 1991a, 1991b; Lim, Submitted 2020).

Dipyridamole may exert 'anti-inflammatory effects' by inhibiting secretion of monocyte chemoattractant protein-1 (MCP-1) and matrix metalloproteinase-9 (MMP-9) from monocytes, resulting in reduced nuclear translocation of NF-κB and inhibition of transcription and generation of further MCP-1 (Lim, Submitted 2020; Weyrich et al., 2005). Additionally, dipyridamole may downregulate Macrophage-1 Antigen (MAC-1), leading to inhibition of neutrophil adhesion to cultured endothelial cells obtained from ischaemic stroke patients

(Hallevi, Hazan-Halevy, & Paran, 2007; Lim, Submitted 2020). Other anti-inflammatory effects of dipyridamole include reduction of lymphocyte recruitment, and reduced activation and secretion of other pro-inflammatory mediators (H. Dong, Osmanova, Epstein, & Brocke, 2006). Monocytes/macrophages and microglial cells play a pivotal role in driving inflammation in evolving ischaemic stroke lesions (Chiba & Umegaki, 2013). Dipyridamole has been shown to exert anti-inflammatory effects on human monocytes, which may contribute to its secondary preventive effects following ischaemic stroke (Lim, Submitted 2020; Massaro et al., 2013). Dipyridamole may also inhibit ICAM-1 and MMP-9 expression in human brain endothelial cells exposed to TNF α , and may reduce levels of MMP-9 and apoptosis in ‘oxygen-glucose starved’ cerebral endothelial cells (Guo, Stins, Ning, & Lo, 2010; Lim, Submitted 2020). More recently, dipyridamole has also been shown to reduce circulating VWF:Ag levels (Tobin et al., 2014; Zhao et al., 2005) and may have indirect ‘anticoagulant effects’ by reducing peak and total thrombin generation *ex-vivo* following TIA or ischaemic stroke (Tobin et al., 2013).

1.5.3 Clopidogrel

Clopidogrel is one of the thienopyridine family of antiplatelet agents whose ability to inhibit ADP-induced platelet activation was first reported in 1987 (Feliste et al., 1987). It is a pro-drug of which at least 50% of orally administered clopidogrel is systemically absorbed, but 85-90% of this absorbed clopidogrel is hydrolysed to an inactive carboxylic acid metabolite, SR26334 (Jiang, Samant, Lesko, & Schmidt, 2015). Similar to the other thienopyridines, the inactive pro-drug is initially activated by a number of isoforms of the hepatic cytochrome P450 (CYP) superfamily, including CYP2C19, CYP1A2 and CYP2B6, and subsequently CYP2C19, CYP2C9, CYP2B6 and CYP3A4 in a two-step process (Cattaneo, 2012; Jiang et al., 2015). The contribution of CYP1A2, CYP2B6, and CYP2C19 to the formation of 2-oxo-clopidogrel was 35.8, 19.4, and 44.9%, respectively. The contribution of CYP2B6, CYP2C9,

CYP2C19, and CYP3A4 to the formation of the active metabolite was 32.9, 6.76, 20.6, and 39.8%, respectively (Kazui et al., 2010).

The active metabolite of clopidogrel has an elimination half-life of 30-60 minutes with peak plasma levels of the active metabolite (about 3 mg/l) observed approximately one hour after administration of the dose. The pharmacokinetics of the main circulating metabolite increase in a linear fashion proportional to dose. The resultant, highly-unstable active metabolite (R-130964) forms a disulphide bond with the P2Y₁₂ ADP receptor on the platelet surface, thereby leading to irreversible inhibition of the receptor which persists for the life span of the platelet (Bonello et al., 2010; Savi, Laplace, Maffrand, & Herbert, 1994). The influence of certain loss-of-function CYP single nucleotide polymorphisms on the metabolism of clopidogrel, and the potential impact of same in the clinical setting is now very topical. However, this is beyond the scope of the introduction to this MSc thesis, so will not be discussed in further detail at this stage.

Three antiplatelet regimens, namely aspirin, aspirin-dipyridamole combination therapy and clopidogrel monotherapy, were the main evidence-based treatments used to treat CVD patients during the conduct of the OATS study. The *ex vivo* response to these regimens was assessed by my colleague, Dr Soon Tjin Lim, prior to the publication of the POINT trial outside of China (Johnston et al., 2018) and prior to the subsequent meta-analyses of the data from the FASTER (Kennedy et al., 2007), CHANCE (Y. Wang et al., 2013) and POINT trials (Hao et al., 2018; Johnston et al., 2018; Pan et al., 2019). Meta-analyses of these trials have informed recent clinical guideline documents on the benefits and risks of short-term aspirin-clopidogrel combination therapy (Hao et al., 2018; Pan et al., 2019; Powers et al., 2019; Prasad et al., 2018) but this regimen was not studied for this component of the OATS study.

1.5.4 High on-Treatment Platelet Reactivity (HTPR)

High on-Treatment Platelet Reactivity (HTPR), previously known as ‘antiplatelet non-responsiveness’, has been well documented in patients with CVD with substantial variability in the *ex vivo* response to commonly prescribed antiplatelet agents (Fiolaki et al., 2017; Lim et al., 2015). The majority of definitions of antiplatelet-HTPR in the literature are ‘cross-sectional/case-control definitions’ where the results of platelet function/reactivity testing in a patient at a single timepoint are compared with those obtained from the manufacturer’s normal range or from a group of healthy controls (Bonello et al., 2010). A novel ‘longitudinal definition of antiplatelet-HTPR’, which was initially proposed by our research group, refers to ‘failure to inhibit platelet function/reactivity compared with the patient’s own baseline value before undergoing a change in antiplatelet therapy by more than twice the coefficient of variation of the assay’ (Tobin et al., 2011; Tobin et al., 2013). Few studies have assessed longitudinal antiplatelet-HTPR status on aspirin, clopidogrel (Tobin et al., 2013) or dipyridamole (Tobin et al., 2011), but this approach enabled identification of additional inhibition of platelet function after adding dipyridamole to aspirin which could not have been identified using traditional case-control definitions of HTPR status (Tobin et al., 2011).

The prevalence of *ex vivo* antiplatelet-HTPR in patients with TIA or ischaemic stroke on these standard antiplatelet therapies varies according to the definitions and platelet function devices employed, but most are derived from case-control definitions of HTPR. With aspirin monotherapy, the prevalence of aspirin-HTPR varies between 3-62% (Helgason et al., 1994; Lim et al., 2015; von Lewinski, Riggert, & Paulus, 2009). The prevalence of antiplatelet-HTPR with clopidogrel monotherapy is 8-61% (Fukuoka et al., 2011; Jover et al., 2014; Lim et al., 2015), and the prevalence of dipyridamole-HTPR is 56–59% when dipyridamole is added to aspirin in the early, subacute or late phases after TIA/stroke onset (Lim et al., 2015;

Tobin et al., 2011). The TRinity AntiPlatelet responsiveness (TRAP) study assessed 'longitudinal aspirin-HTPR status' in patients who were studied within 4 weeks of TIA or ischaemic stroke, and then again at ≥ 14 days and ≥ 90 days after starting aspirin (75-300 mg daily; N = 26 at baseline) (Tobin et al., 2013). Using a novel longitudinal definition of antiplatelet-HTPR, 24% of patients at 14 days and 18% at 90 days demonstrated aspirin-HTPR with the C-EPI cartridge (Tobin et al., 2013). Using this longitudinal definition, the prevalence of aspirin-HTPR was much lower than that anticipated from a study employing a cross-sectional/case-control definition of aspirin-HTPR in the early phase after TIA/stroke (24% vs. 60%, P = 0.003), and there was a trend towards a lower prevalence of aspirin-HTPR in the late phase after symptom onset also (18% vs. 43%, P = 0.3) (McCabe et al., 2005). A direct, prospective comparison of the prevalence of antiplatelet-HTPR using case-control/cross-sectional and longitudinal definitions of antiplatelet-HTPR is being performed as part of the ongoing OATS study (McCabe DJH, personal communication).

The whole blood platelet function analysers which were used to assess HTPR status in the OATS study, from where platelet function/reactivity data were derived for this MSc thesis, were the PFA-100[®], VerifyNow[®] and Multiplate[®] testing platforms (see section 2.3.)

1.6 Aims

The aims of this pilot, prospective, longitudinal, observational component of the OATS study were to assess:

1. Whether there were any changes in plasma VWF:Ag and VWFpp levels, VWFpp/VWF:Ag ratio or ADAMTS13 activity *ex vivo* in patients with a recent TIA or ischaemic stroke following the commencement or alteration of commonly-prescribed antiplatelet therapy;
2. The relationship between VWF:Ag levels, VWFpp levels and ADAMTS13 activity at different stages after a recent TIA or ischaemic stroke;
3. The relationship between these biomarkers and *ex vivo* platelet function/reactivity on user-friendly tests of platelet function in whole blood at both moderately high shear stress and low shear stress;
4. Whether blood group status influenced the expression of these biomarkers in CVD patients.

1.7 Hypotheses

We hypothesised that:

1. VWF:Ag levels would decrease over time after altering antiplatelet therapy, especially on changing to aspirin-dipyridamole combination therapy (Tobin et al., 2014; Zhao et al., 2006) and that ADAMTS13 activity might be reduced in the early phase but would increase during follow-up at the late phase after TIA/ischaemic stroke (McCabe et al., 2015);

2. There would be a positive relationship between VWF:Ag and VWFpp levels, but there might not be a significant inverse relationship between VWF:Ag levels and ADAMTS13 activity in our CVD patient population (S. Murphy, 2020) as had been shown previously with different methodology (McCabe et al., 2015).
3. VWF:Ag or VWFpp levels would be associated with the results of a moderately high shear stress test, but not those of low shear stress tests of platelet function/reactivity;
4. Patients with blood group O would have lower circulating VWF:Ag levels than non-blood group O CVD patients.

2. Methods

2.1 Study Participants

2.1.1 Ethical Approval

This study was initially fully approved by the St. James's Hospital/Adelaide and Meath Hospital Research Ethics Committee (REC Ref: 2011/35/03). The latest, minor amendments to the OATS study protocol were fully approved by the SJH / AMNCH Research Ethics Committee in 2018 [REF: 2018-06; List 21 (5)] and by the St. James's Hospital / Tallaght University Hospital Joint Research Ethics Committee (JREC) in 2019 [REF: 2019-07; List 27 (14)]. Written consent was obtained from all patients (or proxy consent from their relatives/next of kin, where appropriate) prior to venesection. Detailed information regarding the purpose and nature of the study, as well as the procedures involved, was provided to all participants both verbally and via written information sheets.

2.1.2 Inclusion Criteria

Patients were eligible for inclusion in this aspect of the OATS study if they:

- Were within 4 weeks of onset of a TIA or ischaemic stroke and their treating physician decided to commence or change antiplatelet treatment;
- Had undergone detailed clinical and laboratory assessment during the OATS study before (**baseline**), 14 +/- 7 days after (**14d**), and at least 90 days (**90d**) after starting or changing antiplatelet treatment with complete longitudinal data at each timepoint;
- Were ≥ 18 years of age.

2.1.3 Exclusion Criteria

Patients were excluded from this aspect of the OATS study if they:

- Myocardial infarction, DVT, PE or surgery within the preceding three months;
- Ongoing unstable angina or unstable symptomatic peripheral vascular disease;
- Platelet count $< 100 \times 10^9/L$;
- Known bleeding or clotting diathesis, including known platelet-related bleeding disorders;
- Active proven vasculitis;
- Active neoplasia;
- Non-steroidal anti-inflammatory drug (NSAID) intake, other than aspirin, in the preceding 11 days;
- Patients unlikely to be able to attend for clinical follow-up and repeat testing at 14 +/- 7 days.
- Incomplete longitudinal data with data at only one or two of the 3 follow-up timepoints in the OATS study.

2.1.4 Patient Recruitment

Recruited patients were assigned to one of three treatment subgroups for this aspect of the OATS study:

1. ‘Starting Aspirin monotherapy subgroup’: Patients who were ‘antiplatelet naive’ and whose treating physician decided to commence them on aspirin monotherapy.
2. ‘Starting Aspirin-Dipyridamole subgroup’: Patients who were already on aspirin monotherapy or another antiplatelet regimen and a decision was made by their treating physician to change them to aspirin-dipyridamole MR combination therapy.
3. ‘Starting Clopidogrel monotherapy subgroup’: Patients who were already on either aspirin monotherapy or aspirin-dipyridamole combination therapy and their treating physician decided to change them to clopidogrel monotherapy or monotherapy with other emerging irreversible ADP receptor antagonists (e.g. prasugrel).

2.1.5 Initial Clinical Assessment, Timing and Follow-up

All patients included in this pre-planned component of the OATS study had been given a clinical diagnosis of TIA or ischaemic stroke by their Consultant Neurologist or Stroke Physician after comprehensive neurovascular work-up, which was coordinated by the patients' treating physician according to ESO guidelines (European Stroke Organisation Executive & Committee, 2008). All participants also underwent a specific research study assessment between 26th October 2011 and 13th January 2016 by one of two clinically-experienced Research Registrars in Vascular Neurology (Dr Soon-Tjin Lim [STL] or Dr Stephen JX Murphy (SM]) using a standardised study proforma. The diagnosis of TIA or ischaemic stroke was also confirmed in all cases by Dr Lim and/or by Prof McCabe before study recruitment (Lim ST. Platelet activation status, on-treatment platelet reactivity and reticulated platelets in the early and late phases after TIA/ischaemic stroke. PhD in Clinical Medicine, School of Medicine, Trinity College Dublin; submitted 2020).

All patients had appropriate, detailed neurovascular blood tests performed, including FBC, ESR, CRP, coagulation profile, renal and liver profiles, fasting lipids, fasting glucose, HbA1C, TFTs, vitamin B12 and plasma folate. Homocysteine levels were checked during routine work-up in the laboratory in most patients during the latter part of the recruitment period. CT of brain and/or MRI of brain (with FLAIR, T2-weighted, T1-weighted, DWI, and T2* sequences) unless MRI was contra- indicated or not tolerated, colour Doppler ultrasound of carotid and vertebral arteries, and extracranial CTA or magnetic resonance angiography (MRA), ECG, 24 hour Holter monitoring, transthoracic echocardiography (TTE) or trans-oesophageal echocardiography (TOE) with bubble and Valsalva were performed during routine clinical work-up, as deemed appropriate by the treating physician.

The underlying mechanism responsible for TIA or Stroke was categorised by Dr Lim according to the Trial of Org 10172 in Acute Stroke Treatment (TOAST) classification (Adams et al., 1993) and ASCOD classification systems (P. Amarenco et al., 2013) (Lim ST. Platelet activation status, on-treatment platelet reactivity and reticulated platelets in the early and late phases after TIA/ischaemic stroke. PhD in Clinical Medicine, School of Medicine, Trinity College Dublin; submitted 2020). In this MSc thesis, patients were categorised according to the TOAST classification system alone. The TOAST classification system was developed to improve categorisation of ischaemic strokes on the basis of aetiology because this affects prognosis, outcome and management. The five main categories are large artery atherosclerosis, lacunar (small vessel occlusion), cardioembolic, stroke of other determined aetiology and stroke of undetermined aetiology. The ‘other determined’ category includes patients with non-atherosclerotic vasculopathies, dissection, hypercoagulable states or haematological disorders. Stroke of ‘undetermined aetiology’ includes patients in whom no cause is found despite an extensive evaluation, where the evaluation was incomplete or in patients with 2 or more potential competing causes of stroke. This rating system has good inter-rater agreement and intra-rater reproducibility (Adams et al., 1993). It is still the most widely-used system for establishing ischaemic stroke aetiology (Radu, Terecoasă, Băjenaru, & Tiu, 2017).

As stated above, all patients studied for this MSc thesis underwent detailed clinical and laboratory assessment before (**baseline**), 14 +/- 7 days after (**14d**), and at least 90 days (**90d**) after starting or changing antiplatelet treatment. Some patients who were recruited to the main OATS study did not have complete data on their specific antiplatelet regimen at each timepoint e.g. if they needed to change to an alternative antithrombotic regimen because of adverse effects, recurrent vascular events or the need for anticoagulation due to a subsequent diagnosis of paroxysmal atrial fibrillation. Some patients who only had data at two

timepoints were included in Dr Lim's PhD thesis and other aspects of the OATS study, but were excluded from analysis in this MSc thesis.

2.1.6 Venepuncture and sample collection

After resting for at least 20 minutes to minimise platelet activation *in vivo*, careful atraumatic venepuncture was performed in all patients by one of my PhD colleagues (STL or SM) using a standardised protocol, as described previously (McCabe et al., 2004; S. J. X. Murphy et al., 2018) (Lim ST. Platelet activation status, on-treatment platelet reactivity and reticulated platelets in the early and late phases after TIA/ischaemic stroke. PhD in Clinical Medicine, School of Medicine, Trinity College Dublin; submitted 2020). A tourniquet was initially applied to the arm to aid with identification of the vein and insertion of the needle. 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.). The tourniquet was subsequently released during collection of the first 3 ml of blood that was drawn into a 3 ml sterile Vacutainer[®] tube containing 0.105M (3.2%) buffered sodium citrate and subsequently discarded. 2 ml sterile 3.2% buffered sodium citrate-anticoagulated samples were taken for analysis of platelet function with the VerifyNow[®] system (Accriva Diagnostics). Six further samples were drawn into sterile 3 ml Vacutainers[®] containing 3.2% buffered sodium citrate. The first and second of these citrate-anticoagulated samples were used for whole blood flow cytometric analysis and for measurement of platelet function with the platelet function analyser (PFA-100[®], Dade-Behring, Germany). The next three 3.2% citrate-anticoagulated samples were used for preparation of platelet poor plasma (PPP), as outlined below. The final 3.2% citrate-anticoagulated sample was used for measurement of the platelet count, MPV, PDW and 'automated reticulated platelet count and fractions' in citrate-anticoagulated blood between

2 and 4 hours after venepuncture. Subsequently, one 3 ml double-walled Vacutainer[®] tube containing 'recombinant hirudin anticoagulant' was taken for analysis of platelet function on the Multiplate[®] system. Thereafter, three 3ml sterile Vacutainer[®] tubes containing K₂ EDTA were obtained. This first sample was used to measure the full blood count (FBC), including measurement of the mean platelet volume (MPV) and platelet distribution width (PDW). This FBC was performed between 2 hours and 4 hours after venepuncture to standardise the effects of EDTA-induced platelet swelling which is very minimal between 2 and 4 hours after blood sampling (Trowbridge, Slater, Kishk, Woodcock, & Martin, 1984). The two remaining EDTA tubes containing whole blood were stored at -70 to -80°C for future, planned pharmacogenetic studies. A 3.5ml tube containing lithium-heparin was then taken for storage of serum, and two 6ml tubes containing K₂-EDTA were drawn to prepare serum for storage and for ABO blood grouping in our clinical haematology laboratory. Finally, two additional 6ml serum tubes were taken and stored for later analysis of serum thromboxane B₂ levels, and other serum markers which might affect the results of our other assays (Lim ST. Platelet activation status, on-treatment platelet reactivity and reticulated platelets in the early and late phases after TIA/ischaemic stroke. PhD in Clinical Medicine, School of Medicine, Trinity College Dublin; submitted 2020).

This MSc thesis specifically focused on the prospectively-planned analysis of levels of VWF:Ag, VWFpp and ADAMTS13 activity in PPP derived from the 3.2% citrate-anticoagulated samples (see below), and on the relationship between these biomarkers and the results of ABO blood grouping and platelet function/reactivity assays from the OATS study. In addition, we also categorised the on-treatment platelet reactivity status of patients within the 3 treatment subgroups to determine whether there were any differences in levels of VWF:Ag, VWFpp and ADAMTS13 activity in those with *vs.* those without antiplatelet-HTPR.

2.2 Laboratory Methods

2.2.1 Sample separation and freezing- Platelet Poor Plasma (PPP)

As outlined previously, PPP was prepared by STL or SM from the three 3.2% buffered sodium citrate-anticoagulated blood samples within one hour of venepuncture. (Murphy SJX. 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. MSc in Clinical Medicine, School of Medicine, Trinity College Dublin; awarded 2020); (Lim ST. Platelet activation status, on-treatment platelet reactivity and reticulated platelets in the early and late phases after TIA/ischaemic stroke. PhD in Clinical Medicine, School of Medicine, Trinity College Dublin; submitted 2020). The samples were centrifuged at 2250 x 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 x 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 -70 to -80°C for the pre-planned VWF:Ag and VWFpp ELISA analysis. The bottom third of each of these centrifuged samples, which was considered to be 'non-double spun PPP', was also immediately frozen and stored at -70 to -80°C for subsequent assessment of the pre-planned ADAMTS13 activity with a FRET assay. Samples were subsequently thawed for re-aliquoting, then refrozen and later thawed once more at 37°C for 20 minutes before analysis in the VWF:Ag and VWFpp ELISA assays.

2.2.2 Enzyme Linked Immunosorbant Assay (ELISA)

Reagents

- Polyclonal rabbit Anti-Human VWF antibody (DAKO, Denmark)
- Polyclonal rabbit Anti-Human VWF/HRP antibody (DAKO, Denmark)
- Anti-human VWF propeptide (MW1939 Clone CLB-Pro 35 coating antibody, and clone CLB-Pro 14.3 detection antibody) [Sanquin Reagents, Amsterdam, The Netherlands].
- Phosphate buffered saline with 0.1% Tween[®] (PBS-T): Phosphate Buffered Saline tablets Tween-20 (Polyoxyethylenesorbitan Monolaurate *Tween) containing 10 mM phosphate buffer, 2.7 mM potassium chloride and 137 mM sodium chloride, pH 7.4 + 0.1% Tween-20 [Sigma-Aldrich Ireland Ltd (Merck), Arklow, Co Wicklow]
- 3,3',5,5'-tetramethylbenzidine (TMB): 1-Step Ultra TMB-ELISA Substrate [Fisher Scientific, Ballycoolin, Dublin 15]
- Coagulation Reference [LanganBach Services, Bray, Co Wicklow]
- 3% Bovine Serum Albumin (BSA) [Sigma-Aldrich Ireland Ltd (Merck), Arklow, Co Wicklow]

Methods

The concentration of VWF:Ag and VWFpp in each PPP sample was quantified, as previously described (Kinsella et al., 2014; O'Donnell, McKinnon, Crawley, Lane, & Laffan, 2005; Preston et al., 2009). This component of the methodology section was written in close collaboration with my colleague, Dr Stephen Murphy for his own MSc thesis in our Department (S. Murphy, 2020).

For the VWF:Ag ELISA, polyclonal rabbit anti-human VWF antibody (DAKO) was diluted 1:1000 in a carbonate buffer (50mM sodium carbonate diluted in distilled water) at pH 9.6, and 100µl was pipetted into each well of a 96-well flat bottomed MaxiSorb plate (BioSciences, Dun Laoghaire, Co Dublin) to act as a capture antibody. The plate was covered

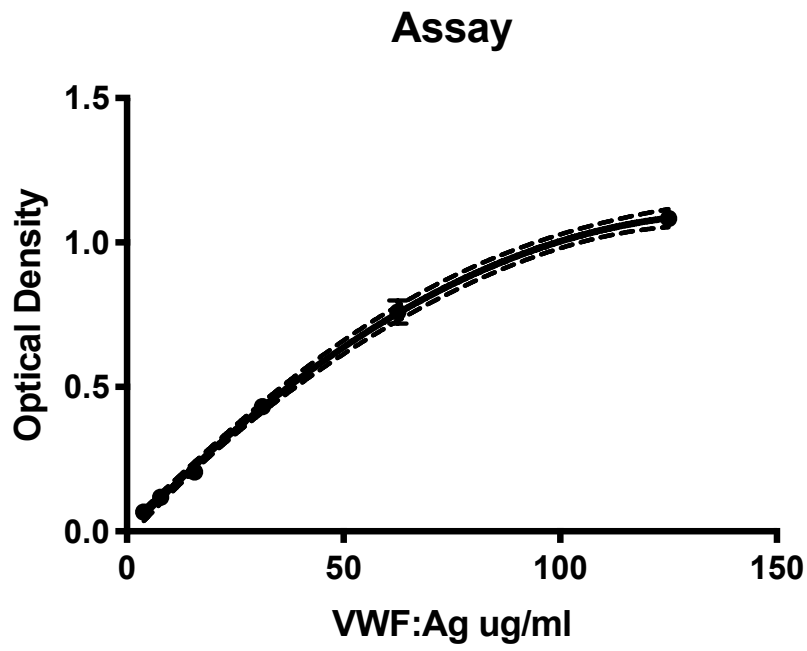
with an air-tight plastic film and incubated overnight at 4°C. The following morning, the plate was washed three times with 200µl of PBS-T per well. The reaction was blocked by the addition of 150µl of 3% (w/v) bovine serum albumin in PBS-T to each well and incubated for 1 hour at 37°C. The plate was then washed again three times with 200µl of PBS-T per well. Coagulation reference plasma (VWF:Ag concentration 10µg/ml) was serially diluted with buffer and added to the plate in duplicate. Double-spun PPP samples, which had been collected and prepared as outlined above, were warmed to 37°C and diluted 1:400 and 1:800 with PBS-T. 100µl of each of these diluted samples was added to the plate in duplicate and incubated at 37°C for 2 hours. The plate was washed again three times with 200µl of PBS-T per well. 100µl of polyclonal rabbit anti-human VWF:Ag/HRP antibody (DAKO), diluted 1:1000 in a 50mM carbonate buffer at pH 9.6, was pipetted into each well and incubated for 1 hour at 37°C to act as a detection antibody for the VWF:Ag ELISA. The plate was again washed with 200µl of PBS-T per well three times. Finally, 100µl of TMB was added to each well and the reaction was subsequently stopped by the addition of 50 µl of 2N H₂SO₄ to each well at the same pipetting rate as the previous step. The VWF:Ag ELISA data were quantified by spectrophotometry at 450nm using a VERSA Max Tuneable Microplate Reader.

The VWFpp ELISA was performed in a similar manner, but anti-human VWF propeptide MW1939 Clone CLB-Pro 35 was used as the capture antibody (Plesmanlaan 125) after being diluted 1:100 in 50mM carbonate buffer at pH 9.6. 100µl of this 1:100 diluted capture antibody was added to each well, covered and incubated overnight at 4°C. The following morning, the plate was washed three times with 200µl of PBS-T per well, and the reaction was blocked by the addition of 150µl of 3% (w/v) bovine serum albumin in PBS-T to each well. The plate was covered and incubated at 37°C for 2 hours and was then washed three times with 200µl of PBS-T per well. Coagulation reference plasma (VWFpp concentration 1µg/ml) was serially diluted with PBS-T and added to the plate in duplicate. Double-spun

PPP samples from patients were also warmed to 37°C and diluted 1:20 and 1:40 with PBS-T; 100µl of each of these diluted samples was added to the plate in duplicate and the plate was covered and incubated for 2 hours at 37°C. The plate was then washed with 200µl of PBS-T per well three times. 100µl of Clone CLB-Pro 14.3 detection antibody (Plesmanlaan 125) was diluted 1:100 in PBS-T, added to each well, covered and incubated at 37°C for 2 hours for VWFpp quantification. The plate was then washed again with 200µl of PBS-T per well three times. Finally, 100µl of TMB was added to each well and the reaction was subsequently stopped by the addition of 50 µl of 2N H₂SO₄ per well at the same pipetting rate as the previous step. The VWFpp ELISA data were also quantified by spectrophotometry at 450nm using a VERSA Max Tuneable Microplate Reader.

VWF:Ag and VWFpp levels were recorded as µg/ml. All results were interpreted via linear regression of fluorescence with a second order quadratic polynomial curve to enable precise quantification of the data with PRISM® 2018 software.

Figure 2.1: Example of a reference curve from a VWF:Ag assay (S. Murphy, 2020).



2.2.3 Fluorescence Resonance Energy Transfer (FRET) assay to quantify ADAMTS13 activity

Reagents

- FRETs-VWF73 probe [Peptides International, Louisville, USA]
- Pooled normal reference plasma
- Dimethyl Sulfoxide (DMSO) [Sigma-Aldrich Ireland Ltd (Merck), Arklow, Co Wicklow]

Reaction Buffer at pH 6.0

- 5 mM Bis-Tris (Trizma: 2-Amino-2-(hydroxymethyl)-1,3-propanediol) [Sigma-Aldrich Ireland Ltd (Merck), Arklow, Co Wicklow]
- 25 mM CaCl₂ (Calcium Chloride Anhydrous) [Sigma-Aldrich Ireland Ltd (Merck), Arklow, Co Wicklow]
- 0.005% Tween-20[®] (Polyoxyethylenesorbitan Monolaurate *Tween) [Sigma-Aldrich Ireland Ltd (Merck), Arklow, Co Wicklow]

Method

ADAMTS13 activity in our patient samples was quantified with a Fluorescence Resonance Energy Transfer (FRET) assay (Kokame, Nobe, Kokubo, Okayama, & Miyata, 2005) (S. Murphy, 2020). Non double-spun platelet poor plasma, which had been collected and prepared as outlined above, was warmed to 37°C. Pooled normal reference plasma was added to the first 4 wells of a 96-well plate at volumes of 1 µl, 2 µl, 4 µl and 6 µl. Each patient sample was tested in duplicate at 2 µl and 4 µl volumes only. Reaction buffer was added to each well to reach a total volume of 100 µl in each well. 35 µl of DMSO was added to the lyophilised FRETs-VWF73 probe to give a stock concentration of 100 µl, and then diluted 1:25 with reaction buffer to reach a final concentration of 4 µM. Finally, 100 µl of diluted FRETs-VWF73 substrate was added to each well to give a total volume of 200 µl in each well and a final substrate concentration of 2 µM. The plate was analysed on a Varioskan Lux Plate Reader with a $\lambda_{ex} = 340\text{nm}$ and $\lambda_{em} = 450\text{nm}$, with measurements performed every 5 minutes between 0 and 60 minutes. The reaction rate was calculated by linear regression of fluorescence and expressed as a percentage ADAMTS13 activity relative to pooled normal plasma (Figures 2.2A, 2.2B and 2.2C).

Figure 2.2A: Example of a graph of fluorescence over time obtained from different concentrations of reference plasma which were used to produce a standard curve for the ADAMTS13 assay (S. Murphy, 2020).

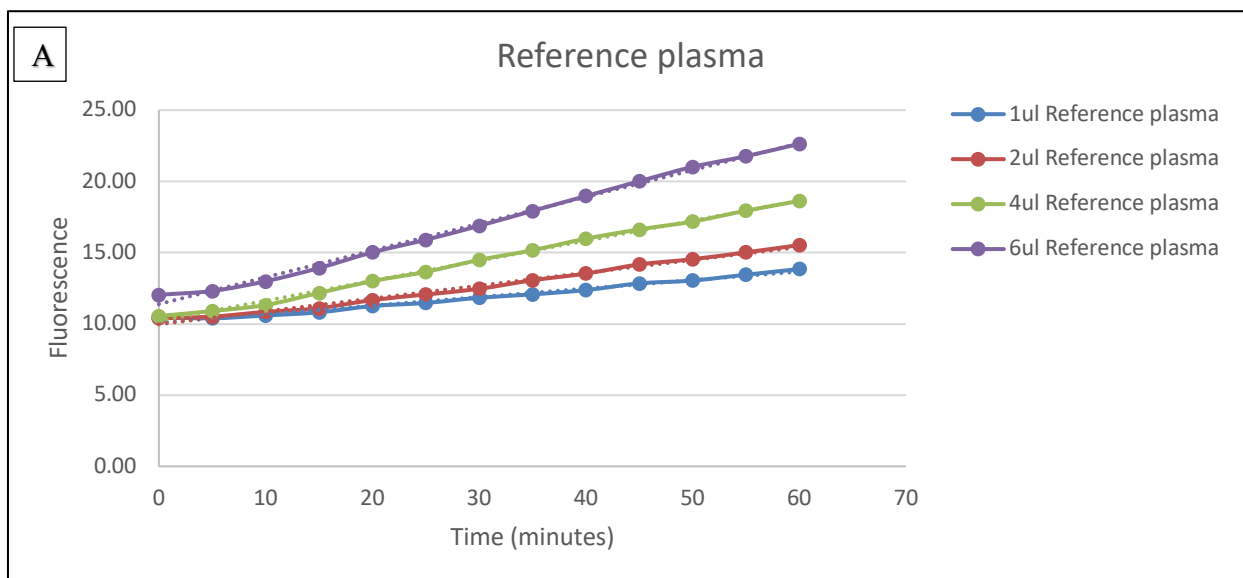


Figure 2.2B: The standard curve was created by combining the slopes of the reference curves produced in figure 2.2A. The high R^2 value indicates that this line is very close to being perfectly linear; any curves with R^2 values < 0.95 were rejected (S. Murphy, 2020).

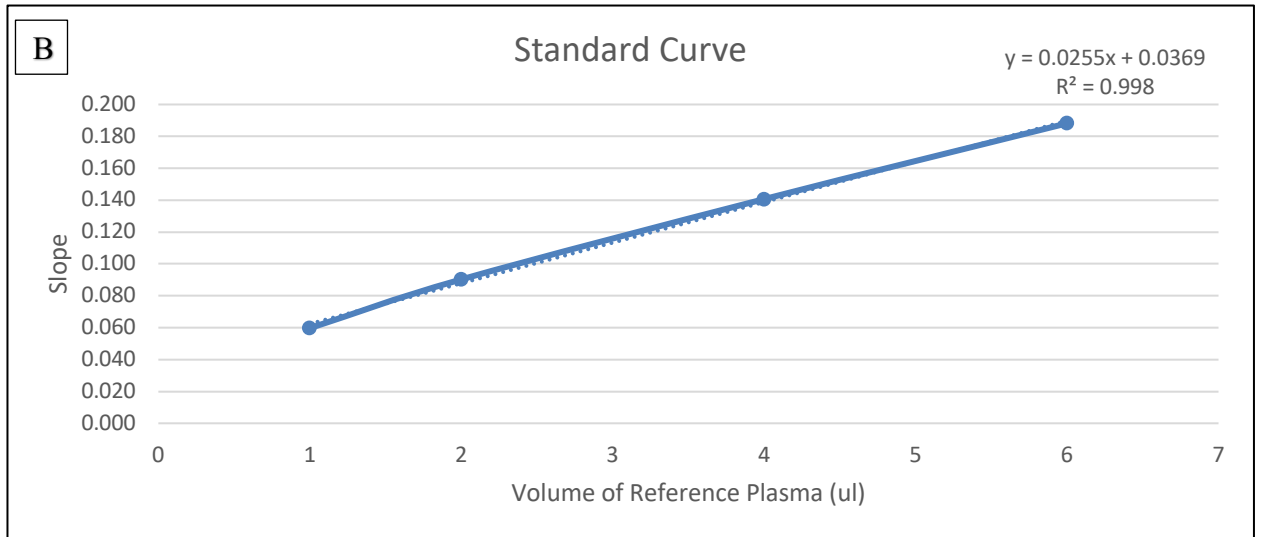
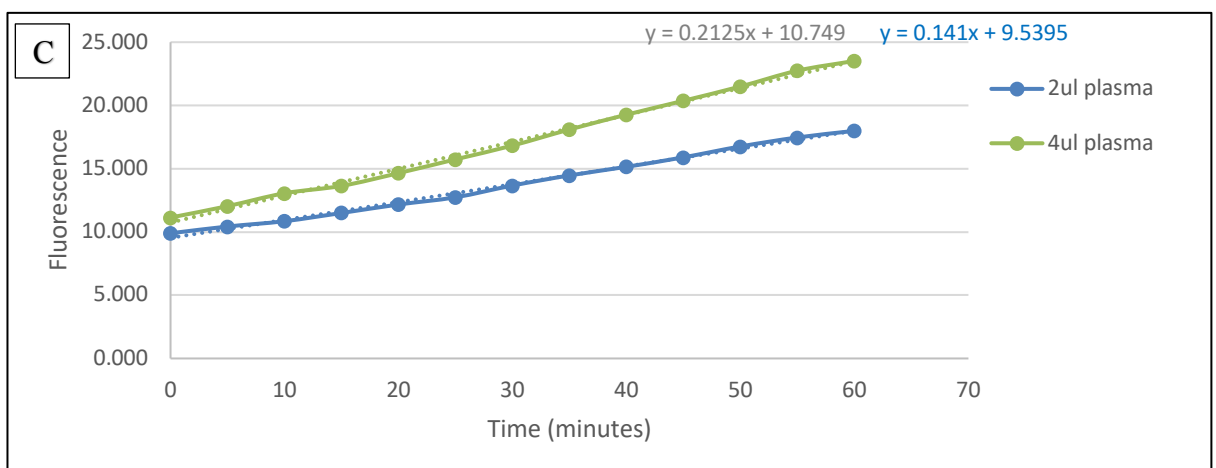


Figure 2.2C: Similar to figure 2.2A, a graph of fluorescence versus time was plotted for both volumes ($2\mu\text{l}$ and $4\mu\text{l}$) of patient plasma. The slope of each line was interpolated from the standard curve (figure 2.2B) in order to quantify ADAMTS13 activity as a percentage of $1\mu\text{l}$ of pooled normal reference plasma (S. Murphy, 2020).



2.3 Devices and definitions used for HTPR

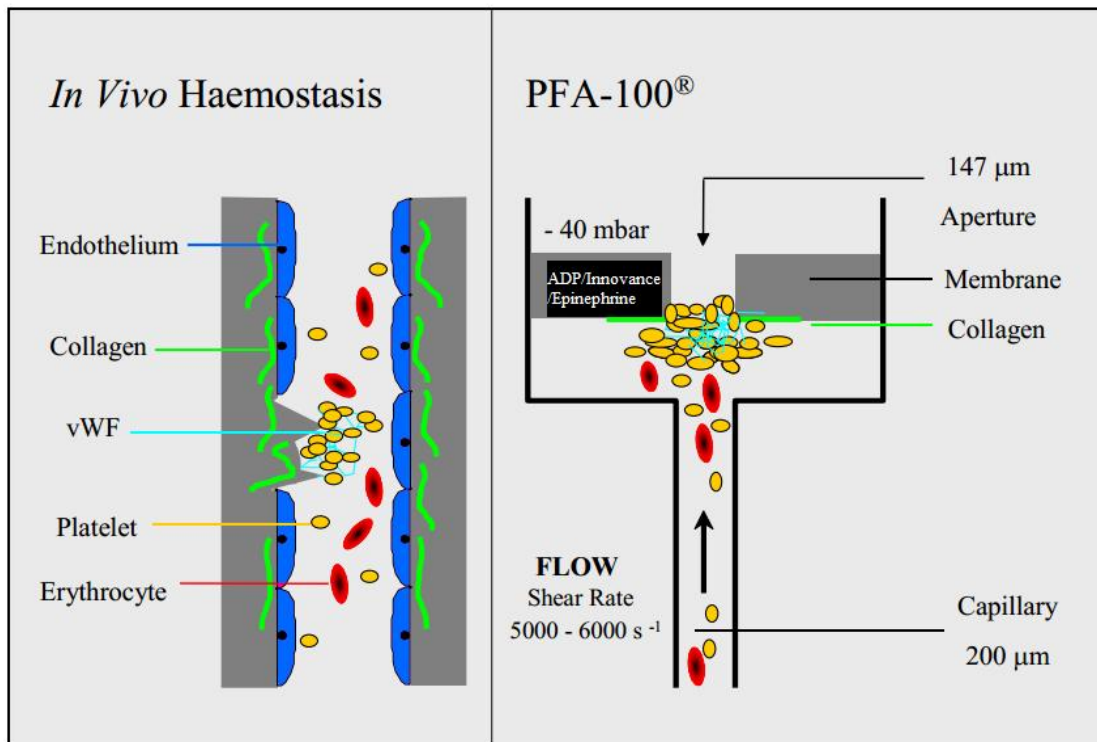
2.3.1 PFA-100

The PFA-100[®] (Siemens, Germany) was developed to assess platelet function in whole blood by simulating the *in vivo* haemostatic process at **moderately high sheer stress rates** (Kundu et al., 1995). A whole blood sample is aspirated at a moderately high shear rate of 5,000 to 6,000 s⁻¹ through a 200 µm capillary, the distal end of which has a nitrocellulose membrane with a central 147 µm aperture. The shear rate is comparable to the shear rate to which platelets are exposed in a moderately stenosed artery (Kroll et al., 1996). The nitrocellulose membrane is coated with 2 µg collagen in combination with one of the following:

- 50 µg ADP (C-ADP cartridge)
- 10 µg epinephrine bitartrate (C-EPI cartridge)
- 20 µg ADP / 5 ng Prostaglandin E₁ / 459µg CaCl (INNOVANCE[®] PFA P2Y cartridge)

Saline ‘trigger solution’ is dispensed onto the membrane during the test and this serves to solubilise the relevant agonist(s). The combination of biochemical stimulation and high shear stress activates the platelets which adhere to the membrane and aggregate to one another to form a platelet plug which will eventually occlude the aperture. The time taken from application of the saline trigger solution to complete aperture occlusion is called the ‘closure time’; the maximum time recorded by the device is 300 seconds. Closure times > 300 seconds are recorded as ‘test time exceeded’ and we arbitrarily recorded these as 301s for statistical analyses.

Figure 2.3: Diagrammatic representation of the *in vivo* haemostatic process and the PFA-100[®] cup-capillary system within a test cartridge showing a platelet plug occluding the central aperture of the biologically active membrane (Figure adapted from Murphy SJX, PhD Thesis, TCD 2017).



Legend for Figure 2.3: *In vivo* haemostasis: A defect in the lining of the endothelial surface of a blood vessel, such as that caused by the rupture of an atherosclerotic plaque, will lead to turbulent blood flow distal to the plaque rupture, thus increasing the shear stress platelets to which platelets are exposed. This in turn activates the platelets, and if sub-endothelial collagen is exposed, platelets will adhere to the plaque and then aggregate to each other. Ultimately, a platelet-rich thrombus forms that helps stabilise the plaque, but leads to a risk of distal platelet thromboembolism. (Figure redrawn from slide kindly donated by Dade-Behring, Germany, and adapted by Prof. D McCabe).

The C-EPI cartridge is much more sensitive than the C-ADP cartridge at detecting aspirin-induced platelet dysfunction, because ADP can stimulate platelet aggregation independent of the arachidonic acid pathway. Furthermore, the C-ADP cartridge is not sensitive at detecting the antiplatelet effects of clopidogrel *ex vivo* using cross-sectional/case-control

definitions of clopidogrel-HTPR (Kinsella et al., 2013), so the INNOVANCE P2Y cartridge has been designed for this purpose (Edwards, Jakubowski, Rechner, Sugidachi, & Harrison, 2012; Tsantes et al., 2012).

All PFA-100 INNOVANCE P2Y assays were performed first on the device by my colleague, Dr Soon Tjin Lim, for his PhD thesis at TCD between 100-120 minutes after venepuncture. To standardise our test methodology with previous studies (McCabe, Harrison et al. 2005; Tobin, Kinsella et al. 2011; Kinsella, Tobin et al. 2013), PFA-100 C-ADP and C-EPI testing was performed in all cases between 120 and 150 minutes after venepuncture (Lim ST. Platelet activation status, on-treatment platelet reactivity and reticulated platelets in the early and late phases after TIA/ischaemic stroke. PhD in Clinical Medicine, School of Medicine, Trinity College Dublin; submitted 2020).

As described previously (Lim ST. Platelet activation status, on-treatment platelet reactivity and reticulated platelets in the early and late phases after TIA/ischaemic stroke. PhD in Clinical Medicine, School of Medicine, Trinity College Dublin; submitted 2020), the following cross-sectional/case-control and longitudinal definitions of antiplatelet-HTPR on the PFA were employed in our department:

Cross-sectional definition of Aspirin-HTPR on the PFA-100: Aspirin-HTPR was defined as failure to prolong C-EPI closure times beyond the mean + 2 standard deviations of our control range (176 seconds) in patients on aspirin monotherapy (Tobin, Kinsella et al. 2011; Kinsella, Tobin et al. 2013).

Novel longitudinal definition of Aspirin-HTPR on PFA-100: The intra-assay CV for C-EPI cartridge assay in our lab was 7.5%. Based on recent pilot data from our lab, ‘aspirin-HTPR’ was also defined as failure to prolong C-EPI closure times compared with the patient’s baseline value on no antiplatelet treatment by more than twice the intra-assay CV

of the assay i.e. failure to prolong C-EPI closure times by >15% of the patient's baseline C-EPI closure time (Kinsella, Tobin et al. 2013; Tobin, Kinsella et al. 2013).

Cross-sectional definition of Clopidogrel-HTPR on the PFA-100: Clopidogrel-HTPR was defined as failure to prolong INNOVANCE[®] PFA P2Y closure times beyond the normal control range, as per the established manufacturer's definition i.e. failure to prolong INNOVANCE[®] PFA P2Y closure times > 106s in patients on clopidogrel [Siemens Inc. INNOVANCE P2Y assay, package insert, Erlangen, Germany, 2015].

Novel longitudinal definition of Clopidogrel-HTPR on the PFA-100: In our laboratory, the intra-assay co-efficient of variation (CV) for the INNOVANCE[®] PFA P2Y cartridge was 7.8% (Lim ST. Platelet activation status, on-treatment platelet reactivity and reticulated platelets in the early and late phases after TIA/ischaemic stroke. PhD in Clinical Medicine, School of Medicine, Trinity College Dublin; submitted 2020). Clopidogrel-HTPR was defined as failure to prolong INNOVANCE[®] PFA P2Y closure times on clopidogrel compared with baseline values before starting clopidogrel by more than twice the CV of the assay i.e. failure to prolong INNOVANCE[®] PFA P2Y closure times by > 15.6% of the patient's baseline INNOVANCE[®] PFA P2Y closure time.

Recently-defined novel longitudinal definition of Dipyridamole-HTPR on the PFA- 100: In our laboratory, the intra-assay CV for the C-ADP assay was 7%. 'Dipyridamole HTPR' was defined as failure to prolong C-ADP closure times compared with the patient's baseline on aspirin monotherapy by more than twice the CV of the assay when dipyridamole was added to aspirin therapy i.e. failure to prolong C-ADP closure times by >14% of the patient's baseline C-ADP closure time (Tobin, Kinsella et al. 2011) (Lim ST. Platelet activation status, on-treatment platelet reactivity and reticulated platelets in the early and late phases

after TIA/ischaemic stroke. PhD in Clinical Medicine, School of Medicine, Trinity College Dublin; submitted 2020).

2.3.2 VerifyNow[®]

The VerifyNow platform uses a modified light transmission aggregometry paradigm to assess inhibition of platelet function/reactivity **under low shear stress conditions** in response to stimulation with fixed doses of different platelet agonists in single-use cartridges containing fibrinogen-coated beads (van Werkum et al., 2008) (figure 2.4 and figure 2.5). The reagents bound to the fibrinogen beads are arachidonic acid in the Aspirin cartridge [Accriva Diagnostics VerifyNow-ASA (aspirin) assay package insert. San Diego, CA. 2012]; and adenosine diphosphate (ADP), iso- thrombin receptor activating peptide (iso-TRAP), and PAR-4 activating peptide in the P2Y12 cartridge [Accriva Diagnostics VerifyNow-P2Y12 (P2Y12) assay package insert; San Diego, CA. 2012] (Lim ST. Platelet activation status, on-treatment platelet reactivity and reticulated platelets in the early and late phases after TIA/ischaemic stroke. PhD in Clinical Medicine, School of Medicine, Trinity College Dublin; submitted 2020).

During the test, a 2ml 3.2% sodium citrate-anticoagulated whole blood sample tube is inserted into the selected cartridge where it is mixed with the relevant agonist(s) and fibrinogen-coated beads (figure 2.5). Activated platelets will bind to these beads, causing clumping and subsequent precipitation of the complexes of platelet-fibrinogen coated beads out of solution. A beam of light is passed through the mixed sample and the light absorbance through the solution is measured 16 times per second. The amount of platelet-fibrinogen bead complexes falling out of solution directly influences the amount of light subsequently detected. The rate and magnitude of aggregation are measured over a predetermined period

of time. Using an algorithm, these results are then described in Aspirin Reaction Units (ARU) for the Aspirin cartridge, and P2Y₁₂ Reaction Units (PRU) for the P2Y₁₂ cartridge.

All VerifyNow 'Aspirin assays' were performed were performed by my colleague, Dr Soon Tjin Lim, for his PhD thesis at TCD between 60-100 minutes, and the P2Y₁₂ assays were performed between 80-100 minutes after venepuncture.

Figure 2.4: Photograph and diagrammatic representation of VerifyNow light absorbance system (figure adapted from the Accriva product demonstration manual which had in turn been adapted from Murphy SJX, PhD Thesis, TCD 2017).

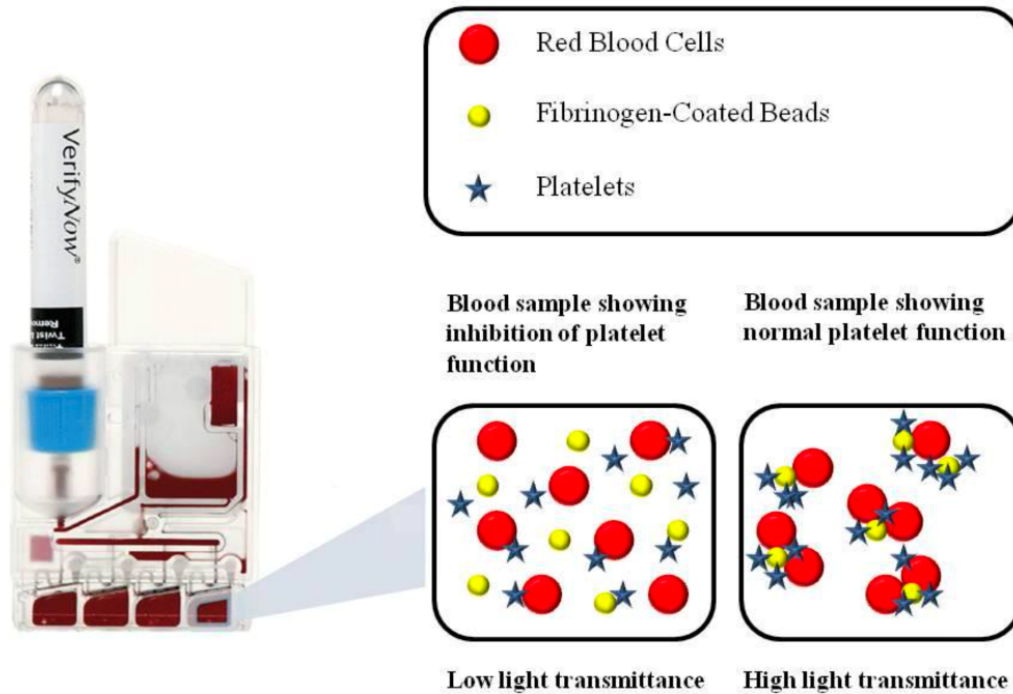
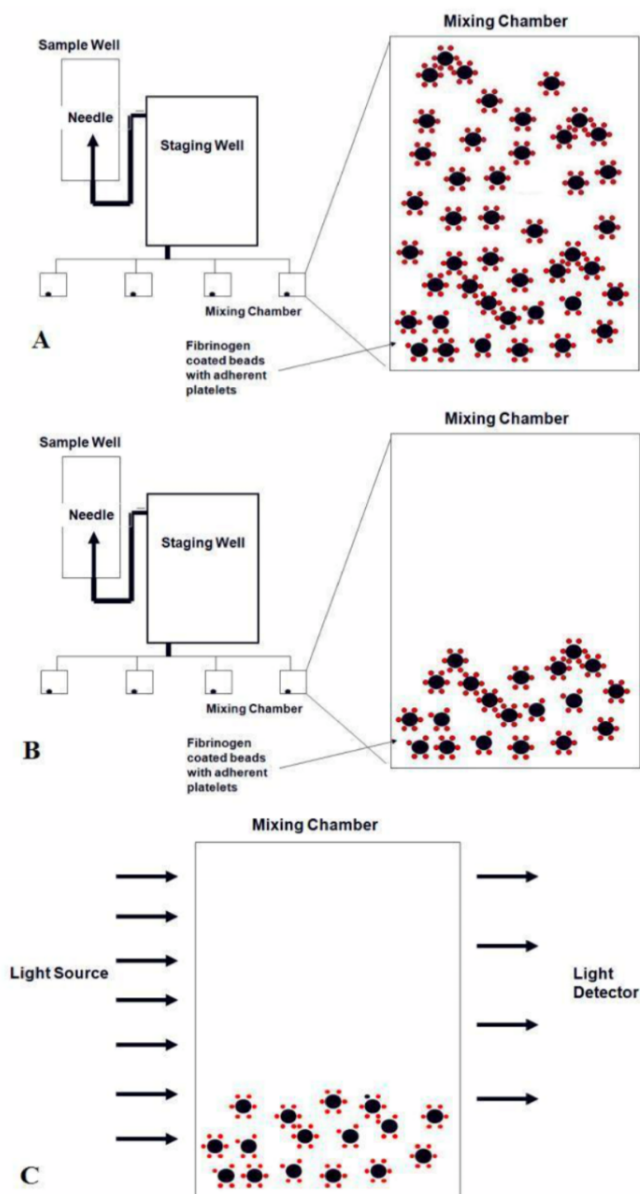


Figure 2.5: Illustration of the VerifyNow Test principle (Figure adapted from Murphy SJX, PhD Thesis, TCD 2017).



Legend for Figure 2.5:

A: A 2 ml citrate-anticoagulated blood sample is loaded into a sample well at the start of the test. Blood is aspirated into a staging well and distributed to the mixing chambers containing fibrinogen coated beads and a metal ball to mix the sample.

B: During the test, the platelets adhere to the fibrinogen-coated beads depending on the degree of inhibition of platelet function and fall out of solution.

C: Light from a source is passed through the sample, and the amount of light detected is dependent on the amount of platelet-fibrinogen bead complexes falling out of solution. These data are converted to Aspirin Reaction Units (ARU) in the Aspirin cartridge, and P2Y12 Reaction Units (PRU) in the P2Y12 cartridge.

Current cross-sectional definition of Aspirin-HTPR on the VerifyNow: Aspirin-HTPR in patients on aspirin monotherapy was defined according to the manufacturer's definition as Aspirin Reaction Units (ARU) ≥ 550 on the Aspirin cartridge (Kinsella, Tobin et al. 2013) (Lim ST. Platelet activation status, on-treatment platelet reactivity and reticulated platelets in the early and late phases after TIA/ischaemic stroke. PhD in Clinical Medicine, School of Medicine, Trinity College Dublin; submitted 2020).

Novel longitudinal definition of Aspirin-HTPR on VerifyNow[®]: The intra-assay CV for the Aspirin cartridge was 0.1% in our laboratory. Aspirin-HTPR was defined as failure to shorten the ARU on aspirin compared with the patient's baseline on no antiplatelet treatment by more than twice the CV of the Aspirin assay i.e. failure to shorten the ARU by $\geq 0.2\%$ of patient's baseline ARU (Lim ST. Platelet activation status, on-treatment platelet reactivity and reticulated platelets in the early and late phases after TIA/ischaemic stroke. PhD in Clinical Medicine, School of Medicine, Trinity College Dublin; submitted 2020).

Cross-sectional definition of Clopidogrel-HTPR on the VerifyNow: Clopidogrel-HTPR in patients on clopidogrel has been defined as P2Y12 reaction units (PRU) ≥ 194 on the P2Y12 cartridge [Accumetrics Inc. VerifyNow-P2Y12 (P2Y12 assay) package insert. San Diego, CA. 2006] (Lim ST. Platelet activation status, on-treatment platelet reactivity and reticulated platelets in the early and late phases after TIA/ischaemic stroke. PhD in Clinical Medicine, School of Medicine, Trinity College Dublin; submitted 2020).

Novel longitudinal definition of Clopidogrel-HTPR on VerifyNow: The intra-assay CV for the P2Y12 cartridge in our laboratory was 5.5%. Clopidogrel-HTPR was defined as failure to shorten PRUs on clopidogrel compared with the patient's own baseline value by more than twice the CV of the P2Y12 assay i.e. failure to shorten the PRU by $\geq 11\%$ of patient's baseline PRU (Lim ST. Platelet activation status, on-treatment platelet reactivity and

reticulated platelets in the early and late phases after TIA/ischaemic stroke. PhD in Clinical Medicine, School of Medicine, Trinity College Dublin; submitted 2020).

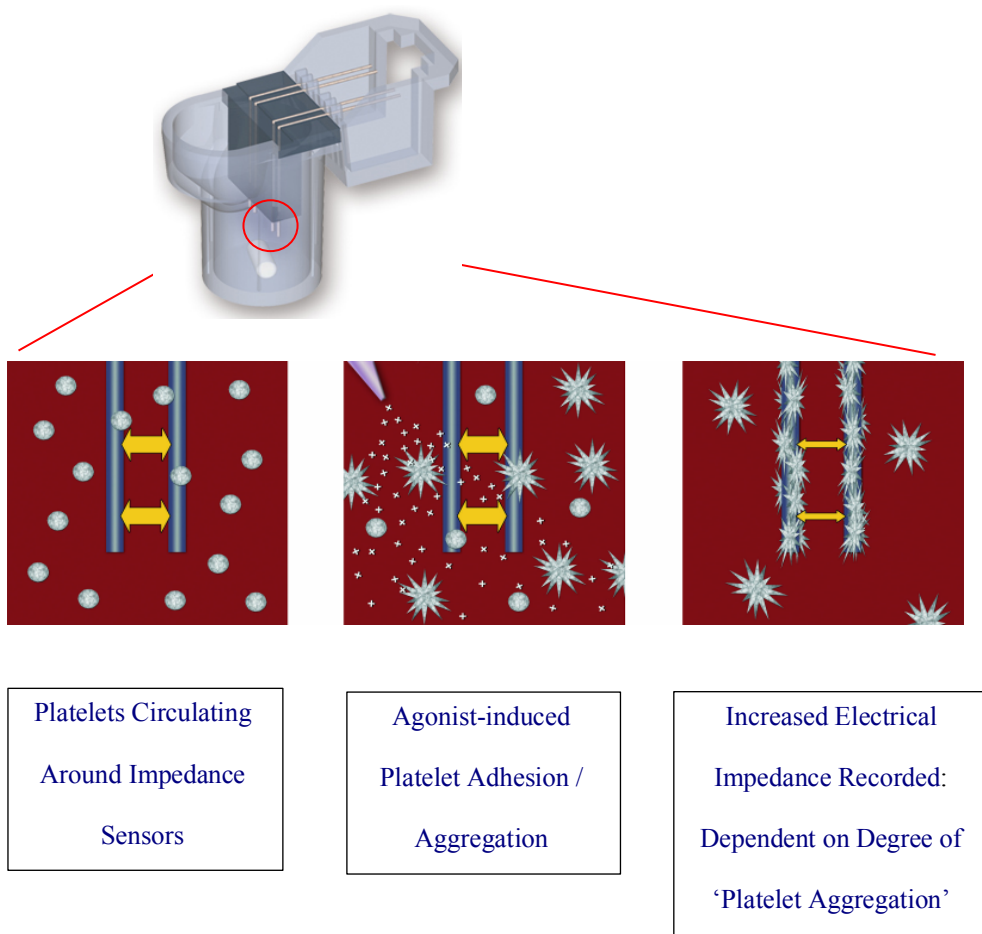
2.3.3 Multiplate[®] Assay

The Multiplate whole blood platelet aggregation assay is performed on a multichannel, whole blood aggregometer which measures impedance to conduction of an electrical current at **low shear stress** as platelets adhere to 2 adjacent electrodes and aggregate to one another within a cuvette following exposure to platelet agonists (figure 2.6) (Lim ST. Platelet activation status, on-treatment platelet reactivity and reticulated platelets in the early and late phases after TIA/ischaemic stroke. PhD in Clinical Medicine, School of Medicine, Trinity College Dublin; submitted 2020).

The disposable test cuvettes contain two sets of independent impedance sensors. 300µl of hirudin-anticoagulated whole blood is added to 2 test cuvettes, diluted 1:2 with 300µl of 0.9% NaCl, heated to 37°C, and mixed with a magnetic stirrer for 3 minutes (Siller-Matula, Gouya, Wolzt, & Jilma, 2009). Platelets are then stimulated by the addition of either 20µL of 15mM arachidonic acid (Aspirin test; final concentration 0.5mM; Roche Multiplate ASP assay package insert 2014) or 20µL of 0.2mM ADP (ADP test; final concentration 6.5µM; Roche Multiplate ADP assay package insert 2014) to the individual cuvettes *in vitro* to measure the antiplatelet effects of aspirin or clopidogrel, respectively. Stimulated platelets adhere to the 2 adjacent electrodes and aggregate to one another within the cuvettes, thus increasing impedance at a rate proportional to the platelet reactivity in the sample (Lim ST. Platelet activation status, on-treatment platelet reactivity and reticulated platelets in the early and late phases after TIA/ischaemic stroke. PhD in Clinical Medicine, School of Medicine, Trinity College Dublin; submitted 2020). The increase in electrical impedance is recorded continuously for 6 minutes after the addition of the agonists, and the mean values of the two

independent determinations are expressed in units (U = one tenth of the Area Under the Curve), indicative of the extent of platelet adhesion and aggregation in the sample in patients on aspirin or clopidogrel, respectively. All Multiplate assays were performed by my colleague, Dr Soon Tjin Lim, for his PhD thesis at TCD between 100 to 120 minutes after venepuncture (Lim ST. Platelet activation status, on-treatment platelet reactivity and reticulated platelets in the early and late phases after TIA/ischaemic stroke. PhD in Clinical Medicine, School of Medicine, Trinity College Dublin; submitted 2020).

Figure 2.6: Multiplate® test principle within the Multiplate cuvette (figure courtesy of Verum Diagnostica GmbH [owned by Roche]; supplied to Prof. McCabe and adapted for this MSc thesis).



Cross-sectional definition of HTPR on Multiplate: The manufacturer's recommended cross-sectional definition of Aspirin-HTPR is $> 40U$ for the Aspirin test [Roche Inc, Multiplate ADP assay package insert, Berlin; 2015] (Lim ST. Platelet activation status, on-treatment platelet reactivity and reticulated platelets in the early and late phases after TIA/ischaemic stroke. PhD in Clinical Medicine, School of Medicine, Trinity College Dublin; submitted 2020).

Novel longitudinal definition of Aspirin-HTPR on Multiplate: In our laboratory, the intra-assay CV for the Aspirin test was 7.3% (N = 8 assays). Aspirin-HTPR was defined as failure to reduce aggregation on the Multiplate Aspirin test compared with the patient's baseline value on no antiplatelet treatment by more than twice the intra-assay CV of the assay i.e. failure to decrease the AUC by $> 14.6\%$ of the patient's baseline AUC (Lim ST. Platelet activation status, on-treatment platelet reactivity and reticulated platelets in the early and late phases after TIA/ischaemic stroke. PhD in Clinical Medicine, School of Medicine, Trinity College Dublin; submitted 2020).

Cross-sectional definition of Clopidogrel-HTPR on Multiplate: The manufacturer's recommended cross-sectional definition of clopidogrel-HTPR is $> 46U$ on the ADP test [Roche Inc, Multiplate ADP assay package insert, Berlin; 2015] (Lim ST. Platelet activation status, on-treatment platelet reactivity and reticulated platelets in the early and late phases after TIA/ischaemic stroke. PhD in Clinical Medicine, School of Medicine, Trinity College Dublin; submitted 2020).

Novel longitudinal definition of Clopidogrel-HTPR on Multiplate: In our laboratory, the intra-assay CV for the ADP test was 7.8% (N = 8 assays). Clopidogrel-HTPR was defined as failure to reduce aggregation on the ADP test with clopidogrel compared with the patient's own baseline value by more than twice the CV of the assay, i.e. failure to decrease the AUC by $> 15.6\%$ of the patient's baseline AUC (Lim ST. Platelet activation status, on-treatment

platelet reactivity and reticulated platelets in the early and late phases after TIA/ischaemic stroke. PhD in Clinical Medicine, School of Medicine, Trinity College Dublin; submitted 2020).

As with the other 2 devices, there is no established cross-sectional definition of dipyridamole-HTPR on the Multiplate platform. Therefore, an exploratory longitudinal definition of dipyridamole-HTPR was devised during the design of the OATS study, employed during Dr Soon Tjin Lim's thesis and used for my MSc thesis.

Exploratory novel longitudinal definition of Dipyridamole-HTPR on the Multiplate ADP assay: 'Dipyridamole HTPR' was provisionally defined as failure to decrease the AUC of the Multiplate ADP test compared with the patient's baseline AUC on aspirin monotherapy by more than twice the CV of the assay when dipyridamole was added to aspirin therapy i.e. failure to decrease the AUC by > 15.6% of the patient's AUC at baseline.

2.4 Statistics

2.4.1 Statistical Methodology

Paired or unpaired t-tests were used for comparison of matched and unmatched parametric variables, and the Wilcoxon signed rank and Wilcoxon rank sum tests were used for comparison of paired and unpaired non-parametric variables, respectively. Chi-squared or Fisher exact tests were used to compare proportions between groups. Linear regression analysis examined the relationship between various biomarkers and also the relationship between individual biomarkers and relevant platelet function/reactivity assays. Most calculations were performed with Minitab version 17[®], but chi-squared and Fisher exact tests were performed on GraphPad Prism Version 8.3.0 (328).

3. RESULTS

3.1 Demographic and Vascular Risk Profiles of Study Participants

In total, 51 patients with a mean age of 59 years who had complete clinical and laboratory data at baseline and also at the 14d and 90d follow-up timepoints were included in this aspect of the OATS study. The detailed demographic and vascular risk factor profiles of the overall patient population and of the individual treatment subgroups at enrolment are outlined in Table 1. Descriptive statistical analysis revealed that the mean age of patients was significantly higher in the clopidogrel monotherapy subgroup than in the aspirin monotherapy (66.4 vs. 54.6 years, $P = 0.003$) and aspirin-dipyridamole subgroups (66.4 vs. 56.1 years, $P = 0.02$). The proportion of patients who had a history of ischaemic heart disease was also higher in the clopidogrel monotherapy subgroup than in either the aspirin monotherapy ($P = 0.012$) or the aspirin-dipyridamole subgroups ($P = 0.001$). Hypertension was more prevalent in the clopidogrel monotherapy subgroup compared with the aspirin monotherapy subgroup ($P = 0.027$), and diabetes mellitus was more prevalent in the clopidogrel monotherapy subgroup compared with the aspirin-dipyridamole subgroup ($P = 0.02$). The proportion of patients in whom their index TIA/ischaemic stroke occurred whilst on antiplatelet treatment was significantly higher in the clopidogrel monotherapy subgroup compared with the aspirin monotherapy subgroup (60% vs. 0%; $P = 0.0006$), but not in comparison with the aspirin-dipyridamole group (21%; $p = 0.054$). The frequency of statin use at recruitment was significantly lower in those starting aspirin compared with those starting aspirin-dipyridamole combination therapy ($P = 0.0023$) or clopidogrel monotherapy ($P = 0.0028$). There were no other significant differences in demographic or vascular risk factors between the treatment subgroups.

Table 3.1: Demographic and Vascular Risk Profiles of Study Participants at enrolment. Values are means [\pm SD] or absolute values with percentages in parentheses (%), where appropriate. IHD* = Prior history of ischaemic heart disease, including myocardial infarction or angina; DVT/PE** = Deep venous thrombosis or pulmonary embolism; Hyperlipidaemia*** = Patient on statin therapy for a prior diagnosis of hyperlipidaemia or total cholesterol > 5.0 mmol/L or LDL > 3.5 mmol/L at the time of baseline assessment when the OATS study was originally designed; Blood Group O****: %s calculated based on the number of patients with available results of blood grouping.

Parameter	All Patients (N = 51)	Starting Aspirin Monotherapy (N = 12)	Starting Aspirin- Dipyridamole (N = 19)	Starting Clopidogrel Monotherapy (N = 20)
Mean Age in Years [\pm SD]	59 [\pm 12.6]	55 [\pm 10.3]	56 [\pm 14.49]	66 [\pm 8.95]
Sex (M/F) [% M]	30/21 [58.8%]	5/7 [41.7%]	10/9 [52.6%]	15/5 [75%]
Index Stroke/TIA (% Stroke)	3/48 (5.9%)	1/11 (8.3%)	1/18 (5.3%)	1/19 (5%)
Prior Stroke/TIA	7 (13.7%)	0	2 (11%)	5 (25%)
IHD*	9 (17.6%)	0	0	9 (45%)
Hypertension	24 (47.1%)	3 (25%)	7 (37%)	14 (70%)
Diabetes Mellitus	8 (15.7%)	2 (17%)	0	6 (30%)
Atrial Fibrillation/Flutter at Recruitment	1 (2%)	1 (8%)	0	0
Prior DVT/PE**	0	0	0	0
Peripheral Vascular Disease	1 (2%)	0	0	1 (5%)
Migraine (With or Without Aura)	12 (23.5%)	4 (33%)	4 (21%)	4 (20%)
Never Smoker	28 (54.9%)	7 (58%)	10 (53%)	11 (55%)
Ex-Smoker	7 (13.7%)	1 (8%)	4 (21%)	2 (10%)
Smoking at Recruitment	16 (31.4%)	4 (33%)	5 (26%)	7 (35%)
Hyperlipidaemia***	36 (70.6%)	6 (50%)	14 (73.7%)	16 (80%)
Statin Therapy at Recruitment	27 (52.9%)	1 (8%)	13 (68%)	13 (65%)
Family History of Stroke	14 (27.5%)	4 (33%)	7 (37%)	3 (15%)
Mean NIHSS / NIHSS Score (N = Stroke Patients Only)	2 (N = 5)	1 (N = 3)	0 (N = 1)	1 (N = 1)
Blood Group O****	18/42 (42.9%)	4/8 (50%)	7/15 (46.7%)	7/19 (36.8%)
Unknown Blood Group	9/51 (17.6%)	4/12 (33%)	4/19 (21.1%)	1/20 (5%)
Index Event on Antiplatelets	16 (33.3%)	0	4 (21%)	12 (60%)
Index Event on Aspirin	14 (27.5%)	0	3 (15.8%)	11 (55%)
Index Event on Aspirin-Dipyridamole	1 (2%)	0	0	1 (5%)
Index event on Clopidogrel	1 (2%)	0	1 (5.3%)	0

The TIA/stroke aetiological subtypes in patients according to the TOAST Classification system are outlined in Table 3.2.

Table 3.2: Aetiological subtyping by TOAST classification in all patients and in each treatment subgroup. Values represent absolute numbers (%).

Stroke/TIA subtype	All Patients (N = 51)	Starting Aspirin Monotherapy (N = 12)	Starting Aspirin - Dipyridamole (N = 19)	Starting Clopidogrel (N = 20)
Large Artery Atherosclerotic	1 (2%)	1 (8%)	0	0
Lacunar	8 (16%)	2 (16%)	4 (21%)	2 (10%)
Cardioembolic	1 (2%)	1 (8%)	0	0
Undetermined Aetiology	41 (80%)	8 (67%)	15 (79%)	18 (90%)
Other Determined	0	0	0	0

3.2 Results of Laboratory Assays

3.2.1. VWF:Ag levels, VWFpp levels and ADAMTS13 activity in the overall CVD patient population:

In the overall patient population, the reduction in VWF:Ag expression between baseline and 14 days did not reach statistical significance ($P = 0.07$), but VWF:Ag levels did significantly decrease between baseline and 90d ($P = 0.0001$) (Table 3.3A). However, the ratio of VWFpp/VWF:Ag significantly increased between baseline and both 14d ($P = 0.0025$) and 90d ($P = 0.005$) (Table 3.3A). There was no significant difference in the levels of VWFpp or ADAMTS13 activity between baseline and either 14d or 90d in the overall study population.

Table 3.3A: Plasma biomarkers before (baseline) and 14d and 90d after starting or changing antiplatelet therapy in the overall CVD patient population. P values refer to comparisons between baseline and follow-up data at 14d and 90d, respectively. Values are means (\pm SD). Significant P values highlighted in bold.

	Baseline (N = 51)	14d (N = 51)	90d (N = 51)
VWF:Ag ($\mu\text{g/ml}$)	17.99 (+/- 8.43)	16.36 (+/- 7.02)	15.62 (+/- 6.46)
P value		0.07	0.001
VWFpp ($\mu\text{g/ml}$)	1.31 (+/- 0.47)	1.31 (+/- 0.49)	1.30 (+/- 0.40)
P value		0.95	0.83
VWFpp/VWF:Ag Ratio	0.08 (+/- 0.04)	0.09 (+/- 0.05)	0.10 (+/- 0.05)
P value		0.025	0.005
ADAMTS13 Activity (%)	150.95 (+/- 34.87)	155.75 (+/- 42.51)	149.43 (+/- 37.20)
P value		0.34	0.76

3.2.2. VWF:Ag levels, VWFpp levels and ADAMTS13 activity in patients starting aspirin monotherapy:

The median daily dose of aspirin taken by this group of patients was 75mg at 14d (range: 75-300mg) and all patients were on 75mg daily at 90d.

There was no significant difference in VWF:Ag or VWFpp levels, the VWFpp/VWF:Ag ratio or ADAMTS13 activity between baseline and 14d or 90d in patients commencing aspirin monotherapy (Table 3.3B).

Table 3.3B: Plasma biomarkers before (baseline) and 14d and 90d after commencing aspirin monotherapy. P values refer to comparisons between baseline and follow-up data at 14d and 90d, respectively. Values are means (\pm SD).

	Baseline (N = 12)	14d (N = 12)	90d (N = 12)
VWF:Ag (μg/ml)	16.51 (+/-7.73)	16.09 (+/- 5.92)	14.98 (+/- 6.58)
P value		0.8	0.23
VWFpp (μg/ml)	1.39 (+/- 0.47)	1.35 (+/- 0.53)	1.31 (+/- 0.42)
P value		0.6	0.34
VWFpp/VWF:Ag Ratio	0.10 (+/- 0.06)	0.10 (+/- 0.09)	0.11 (+/- 0.08)
P value		0.81	0.76
ADAMTS13 Activity (%)	149.08 (+/- 30.19)	161.75 (+/- 37.03)	140.25 (+/- 41.84)
P value		0.12	0.46

3.2.3 VWF:Ag levels, VWFpp levels and ADAMTS13 activity in patients changing to aspirin-dipyridamole combination therapy:

The median daily dose of aspirin taken by this subgroup of patients was 75mg at baseline (range: 0-300mg), 14d (range: 75-300mg) and 90d (range: 75-300mg). One patient was on no antiplatelet therapy and one was on 75mg of clopidogrel daily at baseline, thus accounting for the lowest end of the dose range of 0mg of aspirin at baseline in this subgroup. All 19 patients were taking 200mg of dipyridamole MR twice daily at both 14d and 90d.

In this pilot study, levels of VWF:Ag and VWFpp, the VWFpp/VWF:Ag ratio and ADAMTS13 activity did not significantly change between baseline and 14d or 90d in patients changing to aspirin-dipyridamole combination therapy (Table 3.3C).

Table 3.3C: Plasma biomarkers at baseline and 14d and 90d in patients changing to aspirin-dipyridamole combination therapy. P values refer to comparisons between baseline and follow-up data at 14d and 90d, respectively. Values are means (\pm SD).

	Baseline (N = 19)	14d (N = 19)	90d (N = 19)
VWF:Ag (μg/ml)	15.83 (+/- 8.83)	15.39 (+/- 8.31)	14.38 (+/- 7.20)
P value		0.80	0.29
VWFpp (μg/ml)	1.26 (+/- 0.57)	1.31 (+/- 0.58)	1.31 (+/- 0.49)
P value		0.56	0.48
VWFpp/VWF:Ag Ratio	0.09 (+/- 0.04)	0.10 (+/- 0.04)	0.11 (+/- 0.04)
P value		0.43	0.08
ADAMTS13 Activity (%)	169.39 (+/- 38.59)	159.21 (+/- 37.58)	157.57 (+/- 37.05)
P value		0.27	0.17

3.2.4 VWF:Ag levels, VWFpp levels and ADAMTS13 activity in patients starting clopidogrel monotherapy:

The median daily dose of aspirin taken by this subgroup of patients at baseline was 75mg (range: 0-300mg), but none were on aspirin at 14d or 90d. One patient was on combination therapy with 75mg of aspirin daily and 200mg of dipyridamole MR twice daily at baseline before changing directly to clopidogrel. The median daily dose of clopidogrel was 75mg at both 14d and 90d in 19 of 20 patients in this treatment subgroup. One patient was on prasugrel 10mg daily at 14d and 90d, as per the choice of the treating physician, but we included her in this ‘starting clopidogrel monotherapy subgroup’ as she was on an irreversible ADP receptor antagonist.

In the subgroup of patients starting clopidogrel monotherapy, there was a significant reduction in VWF:Ag levels between baseline and both 14d (P = 0.007) and 90d (P < 0.001) (Table 3.3D). There were no significant changes in VWFpp expression over time, but the ratio of VWFpp/VWF:Ag increased between baseline and 14d (P = 0.0014) and 90d (P = 0.024) (Table 3.3D). Although the differences in ADAMTS13 activity between baseline and 14 days did not reach statistical significance (P = 0.08), ADAMTS13 activity was significantly lower at baseline than at 90 days in patients starting clopidogrel (P = 0.047) (Table 3.3D).

Table 3.3D: Plasma biomarkers at baseline and 14d and 90d after changing to clopidogrel monotherapy. P values refer to comparisons between baseline and follow-up data at 14d and 90d, respectively. Values are means (±SD). Significant P values highlighted in bold.

	Baseline (N= 20)	14d (N=20)	90d (N=20)
VWF:Ag (µg/ml)	20.92 (+/- 7.97)	17.43 (+/- 6.46)	17.17 (+/-5.59)
P value		0.007	< 0.001
VWFpp (µg/ml)	1.29 (+/- 0.38)	1.28 (+/- 0.38)	1.28 (+/- 0.30)
P value		0.83	0.85
VWFpp/VWF:Ag Ratio	0.07 (+/- 0.02)	0.08 (+/- 0.03)	0.08 (+/- 0.02)
P value		0.014	0.024
ADAMTS13 Activity (%)	134.55 (+/- 25.21)	148.86 (+/- 50.38)	147.20 (+/-34.7)
P value		0.08	0.047

3.3 Regression analysis between VWF:Ag levels, VWFpp levels and ADAMTS13 activity.

There was a significant positive relationship between VWF:Ag and VWFpp levels in the overall patient population at baseline ($R^2 = 13.6\%$, $P = 0.004$), 14d ($R^2 = 14.2\%$, $P = 0.004$) and 90d ($R^2 = 18.5\%$, $P = 0.001$). There was also a significant inverse relationship between VWF:Ag and ADAMTS13 in the overall patient population at 90d ($R^2 = 8.7\%$, $P = 0.020$). There was no other significant relationship between VWF:Ag levels and ADAMTS13 activity, or between VWFpp levels and ADAMTS13 activity in the overall patient population at any other timepoint ($P \geq 0.093$).

There was no significant relationship between any of these biomarkers at any timepoint in the subgroup commencing aspirin monotherapy, but the number of subjects included was small ($N = 12$; $P \geq 0.069$) (Tables 3.4A, 3.4B and 3.4C). There was a significant positive relationship between VWF:Ag and VWFpp levels at baseline ($R^2 = 20.7\%$, $P = 0.029$), 14d ($R^2 = 27.7\%$, $P = 0.012$) and 90d ($R^2 = 38.4\%$, $P = 0.003$) in patients changing from aspirin monotherapy to aspirin-dipyridamole (Table 3.5A). Furthermore, in patients starting clopidogrel monotherapy, there was also a significant positive relationship between VWF:Ag and VWFpp levels at baseline ($R^2 = 32.3\%$, $P = 0.005$), 14d ($R^2 = 33.7\%$, $P = 0.004$) and 90d ($R^2 = 31.6\%$, $P = 0.006$) (Tables 3.6A, 3.6B and 3.6C). However, there was no significant relationship between levels of VWF:Ag and ADAMTS13 activity, or between VWFpp levels and ADAMTS13 activity in patients changing from either aspirin monotherapy to aspirin-dipyridamole or in patients starting clopidogrel monotherapy at any timepoint (Tables 3.5A, 3.5B, 3.5C and 3.6A, 3.6B, 3.6C).

3.4 Regression or correlation analysis between VWF:Ag levels, VWFpp levels, ADAMTS13 activity and relevant platelet function/reactivity assays

Regression analysis or correlation analysis, where appropriate, was also performed to assess the potential relationship between these biomarkers and the results of relevant platelet function/reactivity studies which had previously been performed in the three treatment subgroups during the OATS study. Because the data were not all normally distributed, Minitab regression analysis was used for analysis of relationships between parametric data and Minitab Pearson correlation coefficient analysis for assessment of relationships between non-parametric datasets.

3.4.1 Aspirin monotherapy subgroup

In the *patient subgroup starting aspirin monotherapy*, the relevant platelet function/reactivity data which were analysed were derived from the PFA-100 C-EPI, VerifyNow Aspirin and Multiplate Aspirin assays. There was a significant positive correlation between VWF:Ag levels and the Multiplate Aspirin assay results at 90d (Pearson Coefficient = 0.728, P = 0.017). However, there were no other significant relationships between VWF:Ag levels, VWFpp levels or ADAMTS13 activity and the results of any other assays at baseline, 14d or 90d in this treatment subgroup ($P \geq 0.052$) (Tables 3.4A, 3.4B and 3.4C).

Table 3.4A: Regression analysis (or correlation analysis, where specified) between VWF:Ag levels, VWFpp levels and ADAMTS13 activity at baseline, and relationship between these biomarkers and relevant platelet function/reactivity assays at baseline in patients prior to commencing aspirin monotherapy. *Pearson Correlation Coefficient.

	VWF:Ag	VWFpp	ADAMTS13	PFA-100 C-EPI	VerifyNow Aspirin	Multiplate Aspirin
VWF:Ag	N/A	No relationship R ² = 0% P = 0.93	No relationship R ² = 0% P = 0.91	No relationship R ² = 25.9% P = 0.052	No relationship *PC = -0.037 P = 0.91	No relationship R ² = 21% P = 0.08
VWFpp	No relationship R ² = 0% P = 0.93	N/A	No relationship R ² = 0% P = 0.64	No relationship R ² = 4% P = 0.26	No relationship *PC = 0.075 P = 0.82	No relationship R ² = 0% P = 0.59
ADAMTS13 Activity	No relationship R ² = 0% P = 0.91	No relationship R ² = 0% P = 0.64	N/A	No relationship R ² = 0.6% P = 0.33	No relationship *PC = -0.198 P = 0.54	No relationship R ² = 0% P = 0.72

Table 3.4B: Regression analysis (or correlation analysis, where specified) between VWF:Ag levels, VWFpp levels and ADAMTS13 activity at 14d, and relationship between these biomarkers and relevant platelet function/reactivity assays at 14d in patients on aspirin monotherapy. *Pearson Correlation Coefficient.

	VWF:Ag	VWFpp	ADAMTS 13	PFA-100 C-EPI	VerifyNow Aspirin	Multiplate Aspirin
VWF:Ag	N/A	No relationship R ² = 0% P = 0.45	No relationship R ² = 0% P = 0.35	No relationship *PC = -0.198 P = 0.5	No relationship R ² = 0% P = 0.64	No relationship R ² = 2.1% P = 0.298
VWFpp	No relationship R ² = 0% P = 0.45	N/A	No relationship R ² = 0% P = 0.66	No relationship *PC = 0.292 P = 0.36	No relationship R ² = 0% P = 0.47	No relationship R ² = 3.6% P = 0.27
ADAMTS13 Activity	No relationship R ² = 0% P = 0.35	No relationship R ² = 0% P = 0.66	N/A	No relationship *PC = 0.221 P = 0.49	No relationship R ² = 0% P = 0.67	No relationship R ² = 0.2% P = 0.34

Table 3.4C: Regression analysis (or correlation analysis, where specified) between VWF:Ag levels, VWFpp levels and ADAMTS13 activity at 90d, and relationship between these biomarkers and relevant platelet function/reactivity assays at 90d in patients on aspirin monotherapy. *Pearson Correlation Coefficient.

	VWF:Ag	VWFpp	ADAMTS13	PFA-100 C-EPI	VerifyNow Aspirin	Multiplate Aspirin
VWF:Ag	N/A	No relationship R ² = 0% P = 0.88	No relationship R ² = 22.2% P = 0.07	No relationship *PC = -0.376 P = 0.28	No relationship R ² = 2.8%, P = 0.29	Positive relationship *PC = 0.728 P = 0.017
VWFpp	No relationship R ² = 0% P = 0.88	N/A	No relationship R ² = 10.9% P = 0.16	No relationship *PC = -0.173 P = 0.63	No relationship R ² = 0%, P = 0.89	No relationship *PC = 0.584 P = 0.08
ADAMTS13 Activity	No relationship R ² = 22.2% P = 0.07	No relationship R ² = 10.9% P = 0.16	N/A	No relationship *PC = 0.491 P = 0.29	No relationship R ² = 9.8%, P = 0.197	No relationship *PC = 0.024 P = 0.95

3.4.2 Aspirin-dipyridamole treatment subgroup

In the *aspirin-dipyridamole treatment subgroup*, regression analysis was performed to assess the relationship between VWF:Ag levels, VWFpp levels and ADAMTS13 activity with data from the PFA-100 C-ADP and INNOVANCE P2Y, VerifyNow P2Y12 and Multiplate ADP assays (Tables 3.5A, 3.5B and 3.5C).

(a) At baseline, there was a significant inverse relationship between VWF:Ag levels and C-ADP closure times ($R^2 = 32.0\%$, $P = 0.008$) (Figure 3.1) (Table 3.5A). In addition, there was a significant inverse relationship between VWFpp levels and C-ADP closure times at baseline ($R^2 = 18.3\%$, $P = 0.043$), and a significant positive relationship between ADAMTS13 activity and C-ADP closure times at baseline ($R^2 = 19.9\%$, $P = 0.036$) (Figure 3.2). There was no significant relationship between VWF:Ag levels, VWFpp levels or ADAMTS13 activity at baseline with baseline data from the low shear stress VerifyNow P2Y12 or Multiplate ADP assays ($P \geq 0.412$ for the VerifyNow P2Y12 and $P \geq 0.084$ for the Multiplate ADP assays).

(b) At 14d, there was a significant inverse relationship between VWF:Ag levels and closure times on both the PFA-100 C-ADP ($R^2 = 46.4\%$, $P = 0.001$) and PFA-100 INNOVANCE P2Y assays ($R^2 = 55.5\%$, $P < 0.001$) (Table 3.5B). There was also a significant inverse relationship between VWFpp levels and C-ADP closure times ($R^2 = 24.3\%$, $P = 0.018$) and INNOVANCE P2Y closure times ($R^2 = 20.9\%$, $P = 0.028$) (Table 3.5B). There was no significant relationship between ADAMTS13 activity and C-ADP or INNOVANCE P2Y closure times at 14d ($P \geq 0.27$) (Table 3.5B).

(c) At 90d, there was a weak but significant positive relationship between ADAMTS13 activity and the Multiplate ADP units ($R^2 = 18.1\%$, $P = 0.04$), but none of the other relationships reached statistical significance ($P \geq 0.051$) (Table 3.5C).

Figure 3.1: Regression analysis and fitted line plot showing a significant inverse relationship between baseline VWF:Ag levels and baseline C-ADP closure times in patients before changing to aspirin-dipyridamole combination therapy (P = 0.008).

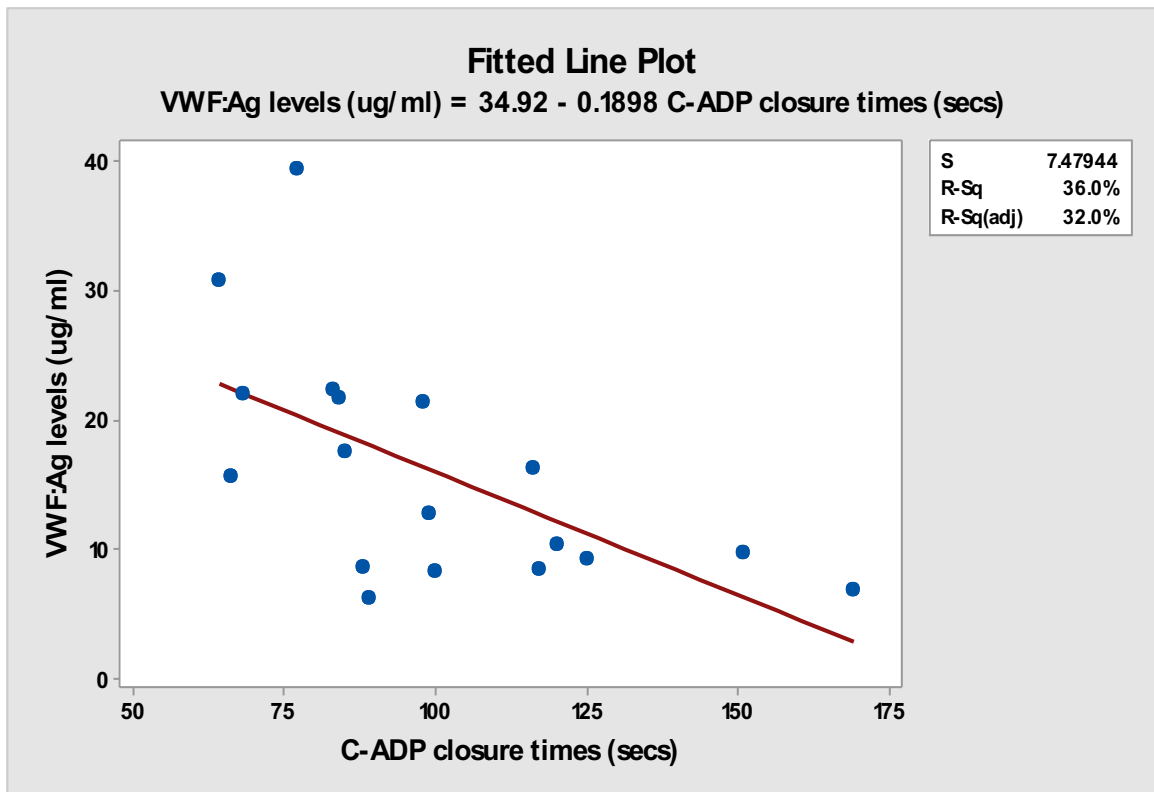


Figure 3.2: Regression analysis and fitted line plot showing a significant positive relationship between baseline ADAMTS13 activity and baseline C-ADP closure times in patients before changing to aspirin-dipyridamole combination therapy (P = 0.036)

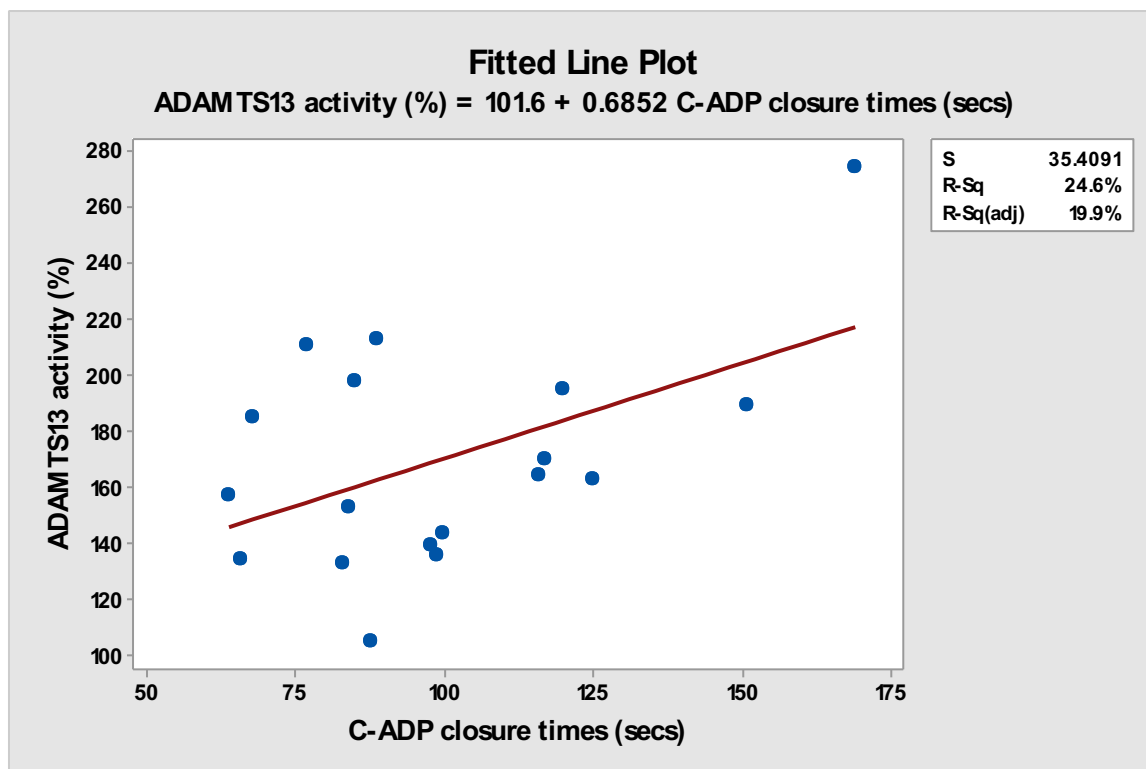


Table 3.5A: Regression analysis (or correlation analysis, where specified) between VWF:Ag levels, VWFpp levels and ADAMTS13 activity at baseline, and relationship between these biomarkers and relevant platelet function/reactivity assays at baseline in patients changing to aspirin-dipyridamole combination therapy. *Pearson Correlation Coefficient. Significant P values highlighted in bold.

	VWF:Ag	VWFpp	ADAMTS13	PFA-100 C-ADP	PFA-100 INNOVANCE P2Y	VerifyNow P2Y12	Multiplate ADP
VWF:Ag	N/A	Positive relationship R ² =20.7%, P = 0.029	No relationship R ² = 0% P = 0.82	Negative relationship R ² = 32% P =0.008	No relationship *PC = -0.306 P = 0.23	No relationship R ² = 0% P = 0.41	No relationship R ² = 0% P = 0.898
VWFpp	Positive relationship R ² =20.7% P = 0.029	N/A	No relationship R ² = 0% P = 0.47	Negative relationship R ² =18.3% P =0.043	No relationship *PC = -0.351 P = 0.17	No relationship R ² = 0% P = 0.45	No relationship R ² = 4.7% P = 0.19
ADAMTS13 Activity	No relationship R ² = 0% P = 0.82	No relationship R ² = 0% P = 0.41	N/A	Positive relationship R ² = 19.9% P = 0.036	No relationship *PC = -0.049 P = 0.85	No relationship R ² = 0% P = 0.61	No relationship R ² = 12.4% P = 0.08

Table 3.5B: Regression analysis between VWF:Ag levels, VWFpp levels and ADAMTS13 activity at 14d, and relationship between these biomarkers and relevant platelet function/reactivity assays at 14d in patients changing to aspirin-dipyridamole combination therapy. Significant P values highlighted in bold.

	VWF:Ag	VWFpp	ADAMTS13	PFA-100 C-ADP	PFA-100 INNOVANCE P2Y	VerifyNow P2Y12	Multiplate ADP
VWF:Ag	N/A	Positive relationship R ² = 27.7% P = 0.012	No relationship R ² = 0%, P = 0.37	Negative relationship R ² = 46.4%, P = 0.001	Negative relationship R ² = 55.5%, P < 0.001	No relationship R ² = 0% P = 0.65	No relationship R ² = 0% P = 0.53
VWFpp	Positive relationship R ² = 27.7% P = 0.012	N/A	No relationship R ² = 0%, P = 0.97	Negative relationship R ² = 24.3%, P = 0.018	Negative relationship R ² = 20.9%, P = 0.028	No relationship R ² = 0% P = 0.41	No relationship R ² = 0% P = 0.75
ADAMTS13 Activity	No relationship R ² = 0%, P = 0.37	No relationship R ² = 0%, P = 0.97	N/A	No relationship R ² = 1.6% P = 0.27	No relationship R ² = 0% P = 0.41	No relationship R ² = 12.7% P = 0.075	No relationship R ² = 0% P = 0.64

Table 3.5C: Regression analysis (or correlation analysis, where specified) between VWF:Ag levels, VWFpp levels and ADAMTS13 activity at 90d, and relationship between these biomarkers and relevant platelet function/reactivity assays at 90d in patients changing to aspirin-dipyridamole combination therapy. *Pearson Correlation Coefficient. Significant P values highlighted in bold.

	VWF:Ag	VWFpp	ADAMTS13	PFA-100 C-ADP	PFA-100 INNOVANCE P2Y	VerifyNow P2Y12	Multiplate ADP
VWF:Ag	N/A	Positive relationship R ² = 38.4% P = 0.003	No relationship R ² = 2.6% P = 0.24	No relationship R ² = 0% P = 0.078	No relationship *PC = -0.309 P = 0.25	No relationship R ² = 0% P = 0.35	No relationship R ² = 0% P = 0.91
VWFpp	Positive relationship R ² = 38.4% P = 0.003	N/A	No relationship R ² = 0.8% P = 0.298	No relationship R ² = 0.2% P = 0.33	No relationship *PC = 0.089 P = 0.74	No relationship R ² = 0% P = 0.69	No relationship R ² = 0% P = 0.97
ADAMTS13	No relationship R ² = 2.6% P = 0.24	No relationship R ² = 0.8% P = 0.298	N/A	No relationship R ² = 5% P = 0.19	No relationship *PC = -0.301 P = 0.26	No relationship R ² = .3% P = 0.32	Positive relationship R ² = 18.1% P = 0.044

3.4.3 Clopidogrel monotherapy subgroup

In the *starting clopidogrel monotherapy subgroup*, regression or correlation analysis was performed to assess the relationship between VWF:Ag levels, VWFpp levels and ADAMTS13 activity with data from the PFA-100 INNOVANCE P2Y, VerifyNow P2Y12 and Multiplate ADP assays. There was no statistically significant relationship, or correlation where appropriate, between VWF:Ag levels, VWFpp levels or ADAMTS13 activity and the results of any of these platelet function/reactivity assays at baseline, 14d or 90d ($P \geq 0.130$) (Tables 3.6A, 3.6B and 3.6C).

Table 3.6A: Regression analysis (or correlation analysis, where specified) between VWF:Ag levels, VWFpp levels and ADAMTS13 activity at baseline, and relationship between these biomarkers and relevant platelet function/reactivity assays at baseline in patients prior to commencing clopidogrel monotherapy. *Pearson Correlation Coefficient. Significant P values highlighted in bold.

	VWF:Ag	VWFpp	ADAMTS13	PFA-100 INNOVANCE P2Y	VerifyNow P2Y12	Multiplate ADP
VWF:Ag	N/A	Positive relationship R ² = 32.3% P = 0.005	No relationship R ² = 0% P = 0.74	No relationship *PC = -0.350 P = 0.13	No relationship R ² = 0% P = 0.56	No relationship R ² = 0% P = 0.98
VWFpp	Positive relationship R ² = 32.3% P = 0.005	N/A	No relationship R ² = 0% P = 0.87	No relationship *PC = -0.016 P = 0.95	No relationship R ² = 0% P = 0.84	No relationship R ² = 0% P = 0.68
ADAMTS13	No relationship R ² = 0% P = 0.74	No relationship R ² = 0% P = 0.87	N/A	No relationship *PC = 0.257 P = 0.28	No relationship R ² = 0% P = 0.96	No relationship R ² = 0% P = 0.88

Table 3.6B: Regression analysis (or correlation analysis, where specified) between VWF:Ag levels, VWFpp levels and ADAMTS13 activity at 14d, and relationship between these biomarkers and relevant platelet function/reactivity assays at 14d in patients on clopidogrel monotherapy. *Pearson Correlation Coefficient. Significant P values highlighted in bold.

	VWF:Ag	VWFpp	ADAMTS 13	PFA-100 INNOVANCE P2Y	VerifyNow P2Y12	Multiplate ADP
VWF:Ag	N/A	Positive relationship R ² = 33.7% P = 0.004	No relationship R ² = 2.1% P = 0.25	No relationship *PC = 0.100 P = 0.68	No relationship R ² = 0% P = 0.58	No relationship R ² = 0% P = 0.88
VWFpp	Positive relationship R ² = 33.7% P = 0.004	N/A	No relationship R ² = 0% P = 0.57	No relationship *PC = 0.186 P = 0.45	No relationship R ² = 0% P = 0.73	No relationship R ² = 0% P = 0.81
ADAMTS13	No relationship R ² = 2.1% P = 0.25	No relationship R ² = 0% P = 0.57	N/A	No relationship *PC = 0.169 P = 0.49	No relationship R ² = 0% P = 0.496	No relationship R ² = 3.1% P = 0.22

Table 3.6C: Regression analysis (or correlation analysis, where specified) between VWF:Ag levels, VWFpp levels and ADAMTS13 activity at 90d, and relationship between these biomarkers and relevant platelet function/reactivity assays at 90d in patients on clopidogrel monotherapy. *Pearson Correlation Coefficient. Significant P values highlighted in bold.

	VWF:Ag	VWFpp	ADAMTS13	PFA-100 INNOVANCE P2Y	VerifyNow P2Y12	Multiplate ADP
VWF:Ag	N/A	Positive relationship R ² = 31.6% P = 0.006	No relationship R ² = 0% P = 0.41	No relationship *PC = -0.029 P = 0.91	No relationship R ² = 0% P = 0.88	No relationship R ² = 0% P = 0.82
VWFpp	Positive relationship R ² = 31.6% P = 0.006	N/A	No relationship R ² = 0% P = 0.81	No relationship *PC = 0.035 P = 0.89	No relationship R ² = 0% P = 0.67	No relationship R ² = 0% P = 0.87
ADAMTS13	No relationship R ² = 0% P = 0.41	No relationship R ² = 0% P = 0.81	N/A	No relationship *PC = 0.163 P = 0.51	No relationship R ² = 0% P = 0.46	No relationship R ² = 0% P = 0.92

3.5 Relationship between biomarkers and High on-Treatment Platelet Reactivity (HTPR) status

High on Treatment Platelet Reactivity (HTPR) status was determined according to both cross-sectional and longitudinal definitions of antiplatelet-HTPR in the aspirin and clopidogrel monotherapy subgroups, but could only be dichotomised according to the longitudinal definition of dipyridamole-HTPR in the aspirin-dipyridamole subgroup (see section 2.3. for definitions).

In the aspirin monotherapy subgroup, it was not possible to perform any reliable comparisons of levels of VWF:Ag, VWFpp or ADAMTS13 activity between patients with *vs.* those without aspirin-HTPR due to the small numbers in one group at both 14d and 90d.

In the aspirin-dipyridamole treatment subgroup, although we have shown that VWF:Ag and VWFpp levels may significantly shorten PFA-100 C-ADP and INNOVANCE P2Y closure times at 14d (see section 3.4.2. above), levels of VWF:Ag, VWFpp and ADAMTS13 activity were not significantly different between those with *vs.* those without dipyridamole-HTPR at 14d or 90d on the PFA-100 C-ADP assay ($P \geq 0.065$).

In the clopidogrel monotherapy subgroup, there were no significant differences in VWF:Ag levels, VWF:pp levels or ADAMTS13 activity between patients with *vs.* those without clopidogrel-HTPR according to either cross-sectional or longitudinal definitions of clopidogrel-HTPR at 14d on the PFA-100 INNOVANCE P2Y, VerifyNow P2Y12 or Multiplate ADP assay ($P \geq 0.099$). There were no significant differences in these biomarkers between patients with *vs.* those without clopidogrel-HTPR according to either cross-sectional or longitudinal definitions at 90d on the Multiplate ADP assay ($P \geq 0.43$), or according to cross-sectional definitions of clopidogrel-HTPR on the PFA-100

INNOVANCE P2Y ($P \geq 0.49$) and VerifyNow P2Y12 assays ($P \geq 0.34$). However, it was not possible to perform any reliable comparisons of biomarkers between patients with *vs.* those without longitudinal definitions of clopidogrel-HTPR on the PFA-100 INNOVANCE P2Y or VerifyNow P2Y12 assays at 90d due to very small numbers in one group.

Of note, there were no significant differences in VWF:Ag levels between any of the 3 treatment subgroups at baseline ($P \geq 0.07$), 14d ($P \geq 0.4$) or 90d ($P \geq 0.18$).

3.6. Analysis of the potential impact of blood group on VWF:Ag levels in CVD patients

Forty-two of the 51 CVD patients included in this component of the OATS study also had blood group data available for analysis. These 42 patients were subdivided into ‘blood group O’ and ‘non-blood group O’ (blood group A, B or AB) CVD patients. Patients with unknown blood groups were excluded from this analysis.

Mean VWF:Ag levels were significantly lower in the overall CVD patient population with blood group O than in those with non-blood group O at baseline (14.57 μ g/ml *vs.* 19.77 μ g/ml; $P = 0.043$) and at 90d (12.45 μ g/ml *vs.* 17.17 μ g/ml; $P = 0.01$), but not at 14d (14.37 μ g/ml *vs.* 16.87 μ g/ml; $P = 0.21$). There were similar findings in the subgroup of patients who were starting aspirin monotherapy in which mean VWF:Ag levels were lower in those with blood group O than in those with non-blood group O at baseline (10.75 μ g/ml *vs.* 17.45 μ g/ml; $P = 0.013$) and at 90d (9.47 μ g/ml *vs.* 18.16 μ g/ml; $P = 0.003$), but not at 14d (12.95 μ g/ml *vs.* 18.97 μ g/ml; $P = 0.118$). There was no significant difference in VWF:Ag levels between blood group O and non-blood group O patients in the aspirin-dipyridamole subgroup or in the clopidogrel monotherapy subgroup, respectively at baseline, 14d or 90d.

There were no significant differences in VWFpp levels ($P \geq 0.13$) or ADAMTS13 activity ($P \geq 0.06$) between blood group O and non-blood group O patients in the overall CVD patient population, or in any of the 3 treatment subgroups at baseline, 14d or 90d.

Regression analysis was also performed to assess the relationship between age and VWF:Ag levels in the total population and in the 3 treatment subgroups. There was a positive relationship between age and VWF:Ag levels in the aspirin-dipyridamole subgroup at 90d only ($P = 0.029$), with no significant relationship noted otherwise in the overall CVD patient population or in any treatment subgroup ($P \geq 0.06$).

Of note, there were no significant differences in VWF:Ag levels between any of the 3 treatment subgroups at baseline, 14d or 90d,

4. Discussion

This prospective longitudinal observational study in a well clinically-categorized population of CVD patients who were starting or changing antiplatelet therapy overall showed that VWF:Ag levels significantly decreased between baseline and 90d, although the differences between baseline and 14d did not reach statistical significance. This finding could reflect a resolution of the acute phase response at 90d compared with baseline, with reduced VWF:Ag secretion or increased clearance, but because all of the patients in this study underwent a change in antiplatelet therapy, these findings could also reflect the impact of treatment change in our patient population over time (see below). There was no significant change in VWFpp levels, but the VWFpp/VWF:Ag ratio did significantly increase between baseline and both 14d and 90d.

There was no significant change in the levels of VWF:Ag or VWFpp or in the VWFpp/VWF:Ag ratio in the subgroups of patients who were starting aspirin or changing to aspirin-dipyridamole combination therapy. These data are in keeping with prior findings indicating that commencement of aspirin does not significantly influence the profile of any of these biomarkers in CVD patients, allowing for the small numbers of subjects recruited to this and our prior pilot study (Tobin et al., 2014). However, we did not confirm the findings by Tobin *et al.* that the addition of dipyridamole to aspirin led to a significant reduction in VWF:Ag levels at 14d and 90d after TIA or ischaemic stroke. This might well reflect a type II error due to the smaller number of subjects treated with this particular treatment regimen in this component of the OATS study compared with the prior TRAP study (N = 19 vs. N = 52) (Tobin et al., 2014), because there was a non-significant trend towards a reduction in VWF:Ag levels in our patient subgroup after commencing aspirin-dipyridamole combination therapy (table 3.3C). Furthermore, the percentage of patients changing to aspirin-dipyridamole combination therapy who were treated with statins at

baseline was higher in the OATS study than in the prior TRAP study (68 vs. 35%; $P = 0.015$) (Tobin et al., 2014). This might be of relevance because statins have been reported to decrease plasma VWF:Ag levels (Sahebkar et al., 2016), so it is also possible that the higher use of statins at baseline in this OATS study subgroup might have led to an underpowering of our study to detect differences in VWF:Ag levels over time. Zhao *et al.* also previously reported that dipyridamole decreased circulating VWF:Ag levels in 11 healthy volunteers and in 11 patients with a prior history of ischaemic stroke within the past 5 years. Aspirin, dipyridamole and clopidogrel were administered to all subjects singly and in combination (including triple therapy) for a 2 week period each and blood samples were taken at the end of each 2 week treatment phase (Zhao et al., 2006). However, in contrast to our OATS study design, they did not specifically assess these biomarkers after the addition of dipyridamole to aspirin, and fewer patients were included. Therefore, reassessment of the longitudinal profile of these biomarkers in a larger patient population would be needed to definitively investigate the impact of adding dipyridamole to aspirin on VWF:Ag expression in CVD patients on other modern secondary prevention regimens.

There were no significant differences in VWF:Ag levels between the 3 treatment groups at baseline, 14d or 90d. However, VWF:Ag levels significantly decreased during follow-up at 14d and 90d compared with baseline in CVD patients who commenced or changed to clopidogrel monotherapy. VWFpp levels remained constant over time, but the VWFpp/VWF:Ag ratio also significantly increased at both 14d and 90d vs. baseline. These results suggest that the data from the subgroup of patients who started clopidogrel were predominantly responsible for the reduction in VWF:Ag levels and the increase in the VWFpp/VWF:Ag ratio during follow-up in our overall OATS patient population. Given that the VWFpp/VWF:Ag ratio should remain unchanged if there is reduced synthesis and secretion of both antigens (Sadler, 1998; D D Wagner et al., 1987), and because VWFpp

levels remained stable during follow-up to the subacute and late phases after TIA or ischaemic stroke, these findings most likely reflect increased clearance of VWF:Ag over time in the subgroup who commenced clopidogrel. The potential mechanisms by which clopidogrel might promote VWF:Ag clearance are not fully understood. Although ADAMTS13 activity did not change in the overall patient population or in the subgroups commencing aspirin or dipyridamole, the hypothesis that the reduction in VWF:Ag levels in patients changing to clopidogrel is due to increased clearance over time is supported by finding of significantly higher levels of ADAMTS13 activity at 90d compared with baseline in this clopidogrel treatment subgroup alone. This profile could have arisen due to consumption or inactivation of ADAMTS13 as it degrades ultra-large VWF multimers in the acute phase after TIA or ischaemic stroke (Mannucci, Capoferri, & Canciani, 2004; McCabe et al., 2015; Reiter, Knobl, Varadi, & Turecek, 2003), with more ADAMTS13 available to clear VWF:Ag in the late phase after symptom onset. However, one must accept that our VWF:Ag assay did not differentiate between levels of small, intermediate, large or very large VWF multimers as our VWF assay measured only total VWF:Ag levels and not ‘individual fractions’ of circulating VWF multimers (McCabe et al., 2015). Furthermore, we did not do e.g. gel electrophoresis to assess the relative contribution of different sized multimers to our overall VWF:Ag pool (Ledford-Kraemer, 2010; Varadi et al., 2009).

Although we must acknowledge that these subgroup findings in patients commencing clopidogrel may also partly reflect resolution of an acute phase response, the consistent and highly statistically significant findings at both 14d and 90d raise the possibility that this is a ‘true treatment effect’, thus enhancing our understanding of the potential direct or indirect influence of clopidogrel on haemostatic and thrombotic pathways aside from direct platelet P2Y₁₂ receptor blockade. These subgroup findings are in contrast to prior pilot data which did not reveal any change in VWF:Ag levels or in the VWFpp/VWF:Ag ratio after starting

clopidogrel (Tobin et al., 2014). The different findings in the 2 studies are not explained by any differences in age, other demographic or vascular risk factor profiles between the subgroups of patients commencing clopidogrel monotherapy in the OATS vs. the TRAP study ($P \geq 0.17$), but it is important to note that ADAMTS13 activity was not simultaneously quantified during the TRAP study (Tobin et al., 2014). Smadja *et al.* did not note any change in VWF:Ag levels compared with baseline after 7 days of clopidogrel treatment in 28 healthy white male volunteers aged 18-35 years old, but levels of VWFpp and ADAMTS13 activity were not assessed (Smadja et al., 2012). Gianetti *et al.* assessed 116 patients with a history of ST elevation myocardial infarction who were undergoing primary percutaneous coronary intervention (PCI) and who had antiplatelet-HTPR identified 5 days after PCI. All patients received a loading dose of 300mg of aspirin and 600mg of clopidogrel, followed by 5 days of 100 mg of aspirin daily and 75mg of clopidogrel daily prior to the randomisation process and initial quantification of levels of VWF:Ag and ADAMTS13 activity. Patients were subsequently randomised to receive a combination of standard dose (SD) aspirin (100mg daily) and clopidogrel 75mg daily or double dose (DD) aspirin (200mg daily) and clopidogrel (150mg daily) for a 6 month period. VWF:Ag levels were significantly lower and ADAMTS13 activity and C-EPI CTs were significantly higher in patients taking DD treatment after 30 days and 180 days compared with the group taking SD treatment (Gianetti et al., 2013). These data suggest that higher doses of either aspirin or clopidogrel may influence expression of these biomarkers, but the same subjects were not assessed after SD or DD treatment, VWFpp levels were not quantified, and the study design was different to OATS.

Of note, the age profile of CVD patients in the clopidogrel monotherapy subgroup in the OATS study was significantly higher than in the aspirin monotherapy subgroup ($P = 0.003$) and in the aspirin-dipyridamole subgroup ($P = 0.017$). It has been reported that VWF:Ag

levels may increase with increasing age (Conlan et al., 1993), so the power to detect these profile changes over time compared with baseline might potentially have been enhanced in the clopidogrel subgroup in OATS. However, we did not find any relationship between age and VWF:Ag in the clopidogrel monotherapy subgroup to suggest that age influenced our results in this treatment subgroup at 14d or 90d (Pearson correlation coefficient 0.326 [P = 0.16] at 14 days, and Pearson correlation coefficient 0.392 [P = 0.087] at 90 days).

As noted in the results section, the clopidogrel subgroup had a significantly higher proportion of patients with hypertension, diabetes or ischaemic heart disease, all of which have previously been associated with higher circulating levels of VWF:Ag (Atiq et al., 2018; Ogbenna, Okpalanze, Adewoyin, & Enifeni, 2018; Spiel, Gilbert, & Jilma, 2008). It is possible that some patients may have had other medications altered by their treating physicians during the course of their study follow-up in each treatment subgroup, e.g. to optimise control of diabetes or hypertension, which might, in turn, have potentially influenced the levels of these biomarkers following TIA or stroke. However, *post hoc* analyses revealed that there were no statistically significant differences in VWF:Ag levels at baseline between patients with and without hypertension (P = 0.61), between those with and without diabetes (P = 0.9), or in those with or without ischaemic heart disease (P = 0.88) who were commencing clopidogrel. Therefore, we have no evidence that these vascular risk factors influenced the results in the starting clopidogrel treatment subgroup. Nevertheless, this should be explored in future larger studies on this topic, with multiple linear regression analysis, as appropriate.

Regression analysis revealed a positive relationship between VWF:Ag and VWFpp levels in the overall patient population and in the aspirin-dipyridamole combination and clopidogrel monotherapy subgroups at all 3 timepoints. This is consistent with prior studies

done on CVD patients (S. Murphy, 2020), healthy volunteers (Mannucci et al., 2004; Marianor, Zaidah, & Maraina, 2015), patients with VWD (Mannucci et al., 2004) and in patients with non-CVD vascular disease (Marianor et al., 2015). We did not find a statistically significant inverse relationship between VWF:Ag and ADAMTS13 activity at baseline or 14d, or between VWFpp levels and ADAMTS13 activity at any stage. This is in keeping with prior data from our group on the relationship between VWF:Ag levels and ADAMTS13 activity in patients with asymptomatic or recently symptomatic carotid stenosis using identical laboratory methodology (S. Murphy, 2020). However, we did find a weak but statistically significant inverse relationship between VWF:Ag and ADAMTS13 activity at 90d only at which stage VWF levels had decreased. This is in contrast to a prior study by McCabe *et al.* which showed a significant inverse relationship between ADAMTS13 activity [quantified with a collagen binding assay] and VWF:Ag levels [quantified with a latex agglutination assay] in the early phase ($r = -0.31$; $P = 0.024$), but not in the late phase after TIA or stroke on aspirin monotherapy ($P = 0.74$) (McCabe et al., 2015). This emphasises the importance of paying careful attention to the precise laboratory techniques employed when comparing results between studies.

There was no obvious relationship between levels of VWF:Ag, VWFpp or ADAMTS13 activity and platelet function/reactivity or antiplatelet-HTPR status on the relevant assays in the aspirin monotherapy or clopidogrel monotherapy subgroups in OATS. However, there was a significant inverse relationship between both VWF:Ag and VWFpp with PFA-100 C-ADP closure times at baseline and 14d, with a positive relationship between ADAMTS13 activity and C-ADP closure times at baseline only in the subgroup of patients who changed to aspirin-dipyridamole combination therapy. These findings are in keeping with the hypothesis that higher VWF:Ag levels may shorten C-ADP closure times on the high-shear stress PFA-100 device, as previously observed in patients with VWD (Castaman et al., 2010;

Cattaneo et al., 1999), and also in a larger group of CVD patients treated with aspirin monotherapy (McCabe DJH. PhD in Neurological Studies entitled: 'Assessment of platelet activation and function in ischaemic stroke, transient ischaemic attack and asymptomatic carotid stenosis'. University College London; 2004: 1-402)(McCabe et al., 2015). However a study of 62 patients with aortic stenosis did not show any significant correlation between PFA-100 closure times and VWF:Ag level, VWFpp level or ADAMTS13 activity (Hollestelle et al., 2011). Our novel finding that VWFpp levels may also shorten PFA-100 C-ADP closure times at baseline and at 14d in patients who changed to aspirin-dipyridamole combination therapy improves our understanding of the influence of this 'endothelial biomarker' on this moderately high-shear stress test of platelet adhesion/aggregation (Kundu et al., 1995) in this CVD treatment subgroup. Furthermore, the inverse relationship between PFA-100 INNOVANCE P2Y closure times and (i) VWF:Ag levels at both baseline and 14d and (ii) VWFpp levels at 14d in CVD patients commencing dipyridamole has not been reported previously to our knowledge. PFA-100 INNOVANCE P2Y testing was not available to our research group at the time of the TRAP study so this warrants further evaluation in a larger CVD patient population undergoing longitudinal testing on this antiplatelet regimen.

It is likely that the observed positive relationship between ADAMTS13 activity and C-ADP closure times at baseline in this component of the OATS study is also predominantly mediated via elevated VWF:Ag levels early after TIA or stroke, as suggested previously (McCabe et al., 2015). The significant positive relationship between ADAMTS13 activity and the Multiplate ADP assay units at 90d after commencing aspirin-dipyridamole combination therapy only warrants further assessment if reagents for this device become routinely available again to determine whether this is a potentially clinically-important finding or whether this result reflects a type I error. However, none of the other relationships between any of the biomarkers and the results of the low shear stress tests of platelet

aggregation reached statistical significance, confirming the hypothesis that VWF:Ag and VWFpp play a more important role in high shear stress tests of platelet function/reactivity.

The lack of any clear differences in levels of VWF:Ag, VWFpp or ADAMTS13 activity between ‘dichotomised subgroups’ of patients with vs. those without antiplatelet-HTPR, according to either cross-sectional or longitudinal definitions, was limited by the small numbers of patients in certain treatment subgroups at 14d and 90d. However, these findings are in keeping with data on VWF:Ag and VWFpp levels in patients on aspirin monotherapy (Tobin et al., 2014), VWF:Ag levels in patients with peripheral artery disease on clopidogrel monotherapy (Linnemann, Schwonberg, Rechner, Mani, & Lindhoff-Last, 2010) and VWF:Ag and VWFpp levels in patients changing to aspirin-dipyridamole combination therapy (Tobin et al., 2014). Taken together with the other findings above, this study also shows that one is more likely to identify an association between levels of VWF:Ag, VWFpp or ADAMTS13 activity and platelet function/reactivity data if one uses continuous rather than categorical data in future studies on this topic investigating biological factors that may influence platelet function/reactivity or HTPR status in CVD patients on commonly-prescribed antiplatelet regimens.

The proportion of patients with blood group O and non-blood group O was similar in the overall CVD patient population and in all 3 treatment subgroups ($P \geq 0.68$) (Table 3.1) Our results in the overall patient population and also in the aspirin monotherapy subgroup at baseline and 90d confirm previous findings that blood group O is associated with lower circulating VWF:Ag levels. Numerically, this was also observed in the aspirin-dipyridamole and clopidogrel subgroups, but the differences did not reach statistical significance ($P \geq 0.273$). There were no significant differences in VWFpp levels ($P \geq 0.132$) or ADAMTS13 activity ($P \geq 0.061$) between blood group O and non-blood group O patients in the overall

CVD population or in any of the treatment subgroups at baseline, 14d or 90d, as noted previously in studies assessing the impact of blood groups on VWF:Ag levels, VWFpp levels or ADAMTS13 activity (Jeroen Eikenboom et al., 2013; Gill, Endres-Brooks, Bauer, Marks, & Montgomery, 1987; Haberichter et al., 2006; Marianor et al., 2015; McGrath et al., 2010; Z. Wang et al., 2017).

This study had some **limitations**. Due to the relatively limited sample size, statistical calculations in some of our subgroups may have been subject to a type II error, as clearly acknowledged in our detailed discussion above. It is possible that some patients may have had other medications altered by their treating physicians during the course of their study follow-up in each treatment subgroup e.g. to optimise control of diabetes or hypertension, which might, in turn, have potentially influenced the levels of these biomarkers following TIA or stroke. However, post-hoc analyses revealed that there were no statistically significant differences in VWF:Ag levels between patients with and without hypertension ($P = 0.6$), between those with and without diabetes ($P = 0.9$), or in those with or without ischaemic heart disease ($P = 0.88$) who were commencing clopidogrel. Therefore, we have no evidence that these vascular risk factors influenced the results in the starting clopidogrel treatment subgroup. Nevertheless, this should be explored in future larger studies on this topic.

Despite our robust clinical follow up of all patients at 14d and 90d, this pilot study was not powered to comment on the value of these biomarkers in predicting the risk of recurrent vascular events in this CVD study population, as has previously been studied by others (De Meyer, Stoll, Wagner, & Kleinschnitz, 2012; Sonneveld et al., 2015; Sonneveld et al., 2016). The OATS study only included a small proportion of patients with TIA or stroke secondary to large artery atherosclerosis (2 % LAA by TOAST) whose platelets would have been exposed to high shear stress *in vivo* because patients with ≥ 50 -99% recently symptomatic carotid stenosis were excluded from the study for at least 3 months after carotid intervention until the acute phase response from surgery or intervention had settled. Therefore, our data predominantly pertain to the other important TIA and stroke patient TOAST subgroups. Because VWF:Ag may be endothelium- or platelet-derived we cannot comment on the relative contribution of these cell types to circulating levels of VWF:Ag in our study population, but prior data indicate that VWF:Ag is predominantly a marker of endothelial

activation (Hollestelle et al., 2006). This study was designed before the results of the POINT trial (Johnston et al., 2018) or the meta-analyses of data from the FASTER, CHANCE and POINT trials were published (Hao et al., 2018; Prasad et al., 2018), so the OATS study did not include a subgroup of patients on aspirin-clopidogrel combination therapy. There was a significant issue with the availability of dipyridamole MR in the community in Ireland in 2018 and 2019 after completion of recruitment to this component of the OATS study, but this issue has now been addressed by another independent supplier in our Country. Therefore, our original OATS study subgroups still represent the **long-term** secondary prevention regimens which are prescribed for the majority of patients following TIA or ischaemic stroke, so the data in our current treatment subgroups are still clinically very relevant.

5. Conclusions and overview of future work

In conclusion, the prospective, innovative pilot studies outlined in this thesis have shown that starting or changing antiplatelet therapy may reduce circulating VWF:Ag levels and increase the VWFpp/VWF:Ag ratio without altering VWFpp expression in an overall CVD patient population, mainly attributed to commencement of clopidogrel monotherapy in this study. Taken together with the data on ADAMTS13 activity, our results support the hypothesis that these findings appear to be attributed to increased clearance of VWF:Ag over time, particularly in the subgroup who commenced clopidogrel. These data improve our understanding of the protean effects of clopidogrel on haemostatic and thrombotic profiles in CVD patients aside from direct platelet P2Y₁₂ receptor blockade. Blood group O is associated with lower circulating VWF:Ag levels, thus adding to the limited literature on this topic in patients with TIA or ischaemic stroke. The significant inverse relationship between both VWF:Ag and VWFpp with PFA-100 C-ADP closure times early after TIA/ischaemic stroke in the subgroup of patients who changed to aspirin-dipyridamole

combination therapy also improves our understanding of the endothelial +/- platelet biomarkers which influence the results of these assays. However, larger studies in a well-phenotyped CVD patient population which simultaneously assess all of these biomarkers, including the VWFpp/VWF:Ag ratio, are warranted to confirm these findings and to understand the precise mechanisms by which certain antiplatelet regimens, including aspirin-clopidogrel combination therapy, might affect VWF:Ag clearance. Furthermore, comprehensive assessment of these biomarkers could improve our understanding of the mechanisms influencing the *ex vivo* response to commonly-prescribed antiplatelet therapy on relevant high shear stress tests of platelet function/reactivity to potentially aid risk-stratification and tailor antiplatelet treatment to suit individual CVD patients in future. We aim to address this issue in local and multicentre studies which have been prospectively planned by our research group.

6. References

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