The VWF-ADAMTS 13 axis and related haematological aspects of acute ischaemic brain injury

Table of Contents

1 PREFACE 7

1.1 Statement of Originality 7

1.2 Abstract 8

1.3 Impact statement 9

1.4 Acknowledgements and declaration 11

1.5 Dedication 13

1.6 Details of Publications/ Presentations 14

1.7 List of Abbreviations 15

1.8 List of Figures 18

1.9 List of Tables 19

2 INTRODUCTION: THE VWF-ADAMTS13 AXIS IN STROKE AND TIA: INTRODUCTION THE VWF-ADAMTS13 AXIS IN STROKE AND TIA 21

2.1 Stroke 21
2.1.1 Incidence and prevalence 21
2.1.2 Mortality and morbidity 21
2.1.3 Risk factors 22
2.1.4 Pathogenesis 22

2.2 Arterial thrombus formation in cardiovascular disease 23
2.2.1 Atherosclerotic plaque development 23
2.2.2 Plaque rupture and thrombus formation 23
2.2.3 Tissue factor pathway 24
2.2.4 Collagen pathway 24
2.2.5 Thrombus development 25
2.2.6 Thrombus propagation 26
2.2.7 Inflammation, atherosclerosis and thrombosis 26
2.2.8 Leucocyte recruitment 28

2.3 Treatment of stroke 29
2.3.1 Pharmacological treatment: thrombolysis 29
2.3.2 Mechanical treatment: thrombectomy 30
2.3.3 Antiplatelet treatment 30
2.3.4 Monitoring antiplatelet effect 31
2.3.5 Aspirin resistance 32

2.3.6 Inflammation, VWF and aspirin resistance 33
2.3.7 Future anti-platelet targets 33
2.3.8 Anticoagulation and ischaemic stroke 34

2.4 Thrombotic thrombocytopenic purpura: introduction to the VWF and ADAMTS13 axis 35

2.5 VWF- ADAMTS13 axis in stroke 35
2.5.1 Von Willebrand Factor 36
2.5.2 VWF and shear stress 37
2.5.3 VWF and ADAMTS13 38
2.5.4 Experimental evidence for role of VWF- ADAMTS13 axis in stroke 38
2.5.5 Stroke, VWF-ADAMTS13 and inflammation 40
2.5.6 Clinical evidence for role of VWF- ADAMTS13 axis in arterial thrombosis 42
2.5.7 VWF and ADAMTS13 in ischaemic stroke: at presentation 42
2.5.8 VWF and ADAMTS13 in ischaemic stroke: at follow up 43
2.5.9 VWF-ADAMTS13 in ischaemic stroke: prospective data and a temporal relationship 43
2.5.10 VWF and ADAMTS13 in ischaemic stroke: ratios and reciprocity 45
2.5.11 ADAMTS13 in thrombolysis 45
2.5.12 Unanswered questions 46

2.6 Thrombin generation 47
2.6.1 Thrombin generation overview 47
2.6.2 Basis for laboratory thrombin generation testing 48
2.6.3 Is thrombin generation increased in acute stroke? 49

2.7 Genetics and ADAMTS13 activity 51

2.8 Genetics and secondary stroke prevention 52
2.8.1 Genetic variation and drug resistance 52
2.8.2 CYP genes: clopidogrel resistance & *CYP2Y19C*2* 52
2.8.3 Clopidogrel resistance in stroke 53
2.8.4 Evidence for thrombin generation in stroke population 53
2.8.5 Current practice 54

3 VWF AND ADAMTS13 IN ACUTE ISCHAEMIC BRAIN INJURY: METHODS 55

3.1 Inclusion and exclusion criteria 55

3.2 Ethical approval 55

3.3 Clinical procedures and data collection 56
3.3.1 Informed consent procedure 56
3.3.2 Screening Period 56
3.3.3 Clinical data collection and assessment points 57

3.4 Research samples 59

3.5 Sample collection and preparation 59
3.5.1 Blood collection protocol: 59
3.5.2 Blood processing protocol: centrifugation & aliquotting 59
3.5.3 Sample storage 59

3.6 VWF analysis- VWF antigen (VWF:Ag) 60
3.6.1 Principle: 60
3.6.2 Equipment 60
3.6.3 Reagents 60

3.6.4 Method 60

3.7 VWF analysis- activity 61
3.7.1 Principle 61
3.7.2 Equipment 61
3.7.3 Reagents 61
3.7.4 Method 61

3.8 ADAMTS13 activity analysis 62
3.8.1 Principle 62
3.8.2 Equipment 62
3.8.3 Reagents 63
3.8.4 Method 63

3.9 ADAMTS13 antigen analysis 64

3.10 Thrombin generation 65
3.10.1 Principle: 65
3.10.2 Equipment 66
3.10.3 Reagents 66
3.10.4 Sample preparation 66
3.10.5 Working reagents 67
3.10.6 Assay protocol 67
3.10.7 Precision 68
3.10.8 Calculations, reference plasma and normalisation 68
3.10.9 Normalisation of the results 69
3.10.10 Normal range 70
3.10.11 Potential errors 71

3.11 Genetic testing: PCR 71
3.11.1 ADAMTS13 genetic variants 71
3.11.2 CYP2C19 genetic variants 72
3.11.3 Polymerase chain reaction (PCR) 73
3.11.4 Gel electrophoresis 73
3.11.5 Restriction digest 74

3.12 Statistics 74

4 VWF AND ADAMTS13: RESULTS AT PRESENTATION 75

4.1 Results at presentation

4.2 VWF, ADAMTS13 and ischaemic stroke 79

4.3 Haemorrhagic stroke 80

4.5 Blood group O vs non-O: association with VWF:Ag, VWF:Act, FVIII and ADAMTS13:Ac 85
4.5.1 Blood group O vs non-O: association with VWF:Ag, VWF:Act, FVIII and ADAMT13 85

4.6 Clinical correlation 87

4.7 Influence of anticoagulant and antiplatelet therapy 87
4.7.1 Anticoagulant therapy 87
4.7.2 Anti-platelet therapy 88

4.8 Laboratory markers and VWF-ADAMTS13: multiple linear regression 88

4.9 Entire cohort: multiple linear regression of clinical influences on the VWF:Ag-ADAMTS13 ratio 90

4.10 Regression: does diagnosis of ischaemic stroke or TIA (combined) influence the VWF:Ag-ADAMTS13Ac ratio adjusted for age and functional score? 93

4.11 Regression: does diagnosis of ischaemic stroke or TIA (separate diagnoses) influence the VWF:Ag-ADAMTS13Ac ratio adjusted for age and functional score? 94

4.12 Regression: In ischaemic brain injury, does age and functional score influence the VWF:Ag-ADAMTS13 ratio? 95

4.13 Regression and the VWF:Ag-ADAMTS13 axis in ischaemic brain injury 96

5 VWF AND ADAMTS13: LONGITUDINAL RESULTS 97

5.1 Longitudinal changes: ischaemic stroke 97

5.2 Longitudinal trends in ischaemic stroke: laboratory variables at every time point 100

5.3 Longitudinal changes in ischaemic stroke: analysis 101

5.4 Longitudinal patterns in TIA 103

5.5 Longitudinal changes in controls 105

5.6 Longitudinal analysis: ischaemic brain injury and final follow up clinical scores 107

5.7 ADAMTS13 quartiles and functional scores 109

5.9 VWF:Ag-ADAMTS13Ac is associated with functional scores 109

5.8 Control group: longitudinal trends functional scores 110

5.9 Recurrence and mortality 110

5.10 Presentation VWF:Ag-ADAMTS13Ac axis and subsequent mortality in acute ischaemic brain injury (IS and TIA combined) 112

5.11 Presenting VWF:Ag-ADAMTS13 and mortality: chi square testing 113

5.12 Presenting VWF:Ag-ADAMTS13 quartile analysis 114

5.13 Role of thrombin generation in stroke and TIAs 116

5.14 Longitudinal changes in IS: impact of thrombolysis 119

5.15 Thrombolysis and VWF:Ag-ADAMTS13Ac in ischaemic stroke 122

5.16 Thrombolysis and thrombin generation 125

5.17 Impact of genetic variation and ischaemic stroke: results and comment 130

5.17.1 ADAMTS13 mutations 130

5.17.2 CYP 2Y19C*2, clopidogrel resistance and secondary stroke prevention 132

6. VWF AND ADAMTS13: DISCUSSION 134

6.1 The VWF:Ag-ADAMTS13Ac axis in acute ischaemic brain injury 134

6.2 Presentation **134**

6.3 Longitudinal **136**

6.4 Morbidity and mortality **137**

6.5 Thrombolysis **139**

6.6 Genetics **140**
6.6.1 ADAMTS13 mutations: discussion 140
6.6.2 Clopidogrel and CYP2C19 genotyping: discussion 140

6.7 Limitations and future improvements **141**

6.8 Conclusion: VWF and ADAMTS13 in ischaemic brain injury **143**

7 CONGENITAL TTP AND PHARMACOKINETICS OF PLASMA INFUSION **144**

7.1 Introduction **144**

7.2 Congenital TTP and pharmacokinetics of plasma infusion: methods **145**
7.2.1 Plasma samples 146
7.2.2 Pharmacokinetic data analysis 146

7.3 Congenital TTP and pharmacokinetics of plasma infusion: results **148**

7.4 Pharmacokinetics of ADAMTS13 **156**

7.5 Discussion: congenital TTP and pharmacokinetics of ADAMTS13 **163**

8 HAEMATOLOGY IN YOUNG STROKE: RELEVANCE OF THE FULL BLOOD COUNT **167**

8.1 Introduction **167**

8.2 Haematology in young stroke: thrombophilia screening for antiphospholipid antibody status **168**
8.2.1 Antiphospholipid syndrome 168
8.2.2 Clinical: 169
8.2.3 Laboratory: 169
8.2.4 Antiphospholipid syndrome- evidence linking to young stroke: 169

8.3 Young stroke full blood count and thrombophilia: methods **171**
i) The full blood count and II) Thrombophilia screening in young stroke 171

8.4 Lab methodology **171**
8.4.1 Lupus anticoagulant 171
Equipment: 172
8.4.2 Anti-cardiolipin antibody IgG/ IgM: 174
8.4.3 Anti-beta-2-glycoprotein 1 IgG/ IgM: 176

8.5 Results: haematological investigation of young stroke **177**
8.5.1 The full blood count in young stroke 177

8.5.2 Results: thrombophilia screening **179**
8.5.3 Lupus anticoagulant testing 180
8.5.4 Anticardiolipin antibody testing 180
8.5.5 Anti-beta-2 glycoprotein 1 antibody testing 181

8.6 Discussion: haematological investigation of young stroke **183**

9 FINAL CONCLUSIONS **187**

10 REFERENCES **188**

1 Preface

1.1 Statement of Originality

I can verify that this thesis is a genuinely original piece of work, consisting of my own account of the investigations and interpretations undertaken throughout the period of registration under supervision for my MD (Res) degree. In collaboration with the Stroke Research Team, I screened and recruited patients. Together with my supervisor Professor Marie Scully, I designed the research. I perfomed the data collection, laboratory analysis and data analysis aside from the pharmacokinetic modelling to which I am indebted to our clinical pharmacology colleagues Professor Oscar della Pasqua and Sean Oosterholt. Please see my full accreditation to all colleagues involved in the Acknowledgements section. I wrote the thesis completely independently.

1.2 Abstract

The main focus of this thesis is examination of the role of the VWF-ADAMTS13 axis in acute ischaemic brain injury. Acute ischaemic stroke and transient ischaemic attack (TIA) are associated with raised von Willebrand factor (VWF) and decreased ADAMTS13 activity but association with outcome has not been studied. We prospectively explored VWF antigen (VWF:Ag) and activity (VWF:Act) levels and ADAMTS13 in 308 adults with acute ischaemic stroke (n=103), TIA (n=80), haemorrhagic stroke (n=16) or controls (n=109), with serial samples taken from presentation until after 6 weeks (median follow up 188 days). Severity was assessed at presentation and at follow up using the National Institutes of Health Stroke Score (NIHSS) and modified Rankin scale (mRS).

We demonstrate resolution of VWF:Ag-ADAMTS13Ac ratio in convalescence in ischaemic stroke (IS, 2.42 to 1.66; p=0.008; TIA, 1.89 to 0.65; p=<0.0001). A raised VWFAg/ ADAMTS13 ratio at presentation of acute IS or TIA was associated with increased mortality, including when adjusted for age. Thrombolysis resulted in prompt reduction of the VWF:Ag- ADAMTS13Ac ratio. Therapeutic targets to VWF and ADAMTS13 activity merit further investigation.

In conjunction with this, we studied the pharmacokinetics of ADAMTS13 activity in plasma of 6 patients with congenital thrombotic thrombocytopenic purpura (TTP) receiving regular infusions. There was variability in ADAMTS13 half-life, suggesting analysis of inter-individual clearance is necessary for future optimisation of treatment, particularly in the era of recombinant ADAMTS13.

Haematological laboratory investigations in patients under 60 years of age presenting to the hyperacute stroke unit (HASU) were retrospectively reviewed, examining 609 stroke and TIA patients over a 31-month period. We examined the haematocrit and platelet count, and potential for primary haematological cause such as myeloproliferative disease (MPD) or TTP. One quarter of the cohort merited further follow up. Thrombophilia testing has now altered so that triple antiphospholipid antibody testing is performed rather than heritable risk factors for venous thrombosis.

The work overlaps between clinical haematology, stroke medicine and laboratory haemostasis; with promise for future investigation and therapy in diseases with pathogenesis common to all.

1.3 Impact statement

The work covered in this thesis opens avenues for future exploration. We have explored the critical balance of VWF and ADAMTS13 in acute ischaemic brain injury. VWF is integral for platelet adhesion to collagen fibres, and platelet aggregation under high shear conditions. ADAMTS13 is responsible for the breakdown and ultimate control of the precursor ultra-large VWF multimers. The balance of these haemostatic markers is pivotal to the haematological disease thrombotic thrombocytopenia purpura (TTP), characterised by a deficiency of ADAMTS13 and consequent disordered haemostasis leading to life-threatening arterial thrombosis. Realization of the importance of ADAMTS13 has revolutionised the treatment of TTP in the past decades, and now promises exciting therapeutic potential in acute ischaemic brain injury.

Acute ischaemic stroke may also be associated with deranged haemostasis. Examining the ratio of VWF antigen (VWF:Ag) to ADAMTS13 activity (Ac) showed a heightened ratio in the acute phase of ischaemic stroke, with resolution in convalescence. Normalisation of the VWF:Ag-ADAMTS13Ac axis appeared more effectively achieved with thrombolysis. Thrombolysis was also associated with the expected resolution in clinical scores, with a marked difference seen in the thrombolysed group mRS from presentation to follow up (3.5 to 0, p=0.002) compared to the non-thrombolysed group (3 to 2, p=0.062). We also demonstrated that a raised VWF:Ag –ADAMTS13 ratio at presentation of acute ischaemic brain injury to be associated with increased mortality, including when adjusted for age. In the era of recombinant ADAMTS13 being used in clinical trials for treatment of TTP, it may be possible to consider a therapeutic role in the manipulation of the baseline VWF:Ag-ADAMTS13 balance in acute ischaemic brain injury. Normalisation of ADAMTS13 may be integral in limiting arterial thrombosis; preventing deterioration of cerebral perfusion in patients presenting with cerebrovascular occlusion, whether acute stroke or TIA. There could be implications for recurrence risk and clinical outcome. This is the largest cohort to prospectively investigate the VWF-ADAMTS13 balance over the hyperacute and convalescent phases of ischaemic stroke, correlating the haemostatic markers with clinical outcome

As a parallel, we have studied the pharmacokinetics of ADAMTS13 replacement in 6 patients with congenital TTP treated with solvent-detergent fresh frozen plasma (SD-FFP), the current therapy utilised to replace the missing ADAMTS13 and prevent clinical relapses. The required volume of plasma limits the ADAMTS13 dose that can be delivered. Clinical trials of a recombinant ADAMTS13, capable of achieving a higher peak ADAMTS13 level, are underway. Our pharmacokinetic analysis of 6 patients with congenital TTP demonstrates great variability in ADAMTS13 half-life, suggesting that investigation of inter-individual clearance of ADAMTS13 is necessary for future optimisation of treatment. Again, since ADAMTS13 acts to control the VWF cascade in acute ischaemia, such insight may suggest therapeutic potential of recombinant ADAMTS13 in hyperacute stroke management.

We also investigated haematological protocols in place on the hyperacute stroke unit via a retrospective review of young stroke and TIA patients, in whom cryptogenic causes are more likely. We examined the full blood count at presentation, specifically whether discrepancies in the haematocrit and/ or platelet count were considered and

followed up, potentially unveiling myeloproliferative disease or TTP; as well as the yield of thrombophilia testing. As a result of this study, we have adapted the laboratory investigation panel to improve clinical utility.

The themes of work focus on the same pathophysiology of disordered haemostasis, and yet overlap between aspects of haematological, cardiovascular and neurological medicine. This work represents multi-specialty and multi-disciplinary collaboration. Working together may mean developments in one medical field heralding transformation in another.

1.4 Acknowledgements and declaration

This thesis describes work that was carried out over a four-year period whilst completing my training as a haematology specialist registrar at University College London Hospital. The initial work was undertaken during my laboratory post as an academic clinical fellow in the Haemostasis Research Unit, before taking up the MD (Res) placement. The clinical fellow post was funded by a grant donated by Baxter (now Takeda).

The research was predominantly focused on the VWF:Ag-ADAMTS13Ac axis in acute ischaemic brain injury, and registered with both the NHS national health authority (East of England research ethics committee) and University College London Hospital Joint Research Office.

In my acknowledgements, I would firstly like to thank all the patients and their families who kindly consented to being involved in this study- from the beginning, and in particular to those who returned to the hospital specifically for follow up visits or who allowed us to visit them at home.

I was personally involved with and responsible for patient recruitment, sample collection and initial sample processing in collaboration with the Stroke Research Team (listed below) on the hyperacute stroke unit. I would like to acknowledge the support from all members of the team, in particular Scheherazade Feerick, Renuka Erande, Caroline Watchurst, Caroline Hogan, Maria Brezitski, Emma Elliott, Krishna Patel and Azra Banaras. Enormous thanks are extended to Shez for her efforts in helping maximum follow up of all patients originally recruited.

In the TIA clinic, I am very grateful to Arvind Chandratheva, Vafa Alakbarzade and Agnieszka Dados for their assistance with patient recruitment, data collection and compiling databases for young stroke management. I am delighted that this enabled collaboration on the young stroke work that has also contributed to this thesis (relevance of the full blood count in young stroke, and thrombophilia screening in young stroke).

I was responsible for managing sample processing and storage in both the Clinical Research Facility and Haemostasis Research Unit. In the laboratory, I am particularly grateful to Chiara Vendramin, whom provided assistance with all laboratory analysis in the HRU: ADAMTS13 activity via FRETS, ADAMTS13 antigen assay development, thrombin generation, PCR, and overall sample and data collection. Chiara was a tower of strength, and I shall always remember this. I appreciate the support of all other laboratory staff as follows:

Clinical Research Facility: Shuhana Uddin (senior biomedical scientist) for teaching local laboratory procedure.

Haemostasis Research Unit staff: Katy Langley for teaching basic laboratory technique, Maria Efthymiou for teaching thrombin generation technique, Debra Ellis for helping with research ethics committee liaison and data organisation and Ferras Alwan for patient help with statistics. Many thanks also to Dr Mari Thomas and Dr JP Westwood for their help, suggestions and review throughout the entire research support, and in particular their very kind support.

Whitfield Street: Deepak Singh for teaching laboratory techniques including use of Sysmex automated analysers, Ian Longair for teaching laboratory technique in ABO blood grouping.

Cancer Institute: Rosemary Gale for teaching PCR technique and interpretation.

With regard to work focused on the pharmacokinetics of ADAMTS13 in congenital TTP post plasma infusion, I would like to acknowledge the significant contributions of Professor Oscar Della Pasqua and Sean Oosterholt in the UCL Clinical Pharmacology and Therapeutics Group for pharmacokinetic analysis. Their invaluable work enabled us to make the most meaningful analysis of our data.

I am extremely grateful for the supervision of Professor Martin Brown, who assisted with the design of the main study question- the role of the VWF:Ag-ADAMTS13Ac axis in acute ischaemic brain injury- as well as ongoing guidance and review of writing.

My final and most profound thanks are extended with the deepest appreciation to my primary supervisor, Professor Marie Scully for her academic guidance, and enormous kindness and understanding.

1.5 Dedication

For my Dad

1.6 Details of Publications/ Presentations

i) Presentation at 3rd European Stroke Organisation Conference; Prague, Czech Republic (2017, given by V Alakbarzade)
"Utility of current thrombophilia screening in young stroke and TIA patients"
Alakbarzade V, Taylor A, Scully M, Chandratheva A.

ii) Presentation (e-poster) at 22nd European Association of Haematology Annual Congress; Madrid, Spain (June 22nd- 25th, 2017)
"The importance of the full blood count, JAK II and ADAMTS13 testing in stroke evaluation: a review of 619 consecutive young stroke and TIA patients"
Taylor A, Alakbarzade V, Dados A, Simister R, Chandratheva A, Scully M.

iii) Presentation (poster) at 59th Annual Meeting and Exposition of American Society of Haematology (December 9-12, 2017) in Atlanta, USA
"The VWF-ADAMTS13 Axis in Acute Ischaemic Brain Injury: a Prospective Study"
Taylor A, Vendramin C, Feerick S, Erande R, Watchurst C, Hogan C, Brown M and Scully M

iv) Publication: "The importance of the full blood count in cerebral ischaemia: a review of 609 consecutive young stroke and TIA patients".
Taylor A, Alakbarzade V, Simister R, Chandratheva A, Scully M.
Journal of Stroke and Cerebrovascular Diseases
September 2018, Volume 27 (9), 2500- 2504

v) Publication: "Utility of current thrombophilia screening in young patients with stroke and TIA"
Alakbarzade V, Taylor A, Scully M, Simister R, Chandratheva A.
Stroke and Vascular Neurology 2018
svn-2018-000169. doi: 10.1136/svn-2018-000169

vi) Publication: "Pharmacokinetics of plasma infusion in congenital thrombotic thrombocytopenic purpura"
Taylor A, Vendramin C, Oosterholt S, Della Pasqua O, Scully M.
J Thromb Haemost 2019, 17 (1): 88-93; https://doi.org/10.1111/jth.14345.

vii) Publication: "Von Willebrand factor-ADAMTS13 ratio at presentation of acute ischaemic brain injury is predictive of outcome".
Taylor A, Vendramin C, Singh D, Brown MM & Scully M
Blood Advances 2020; 4 (2): 398- 407

1.7 List of Abbreviations

Aβ2 GPi	Anti-beta-2-glycoprotein antibody
aCL	Anticardiolipin antibody
ADAMTS13	A disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13
ADP	Adenosine diphosphate
ALT	Alanine aminotransferase
APA	Antiphospholipid antibody
APLS	Antiphospholipid syndrome
BSA	Bovine serum albumin
CADASIL	Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy
CAT	Calibrated automated thrombography
CIS	Consultee information sheet
CRN	Clinical Research Network
CRP	C-reactive protein
CT	Computed tomography scan
ELISA	Enzyme-linked immunosorbent assay
ET	Essential thrombocythaemia
ETP	Endogeneous thrombin potential
FVIIa	Activated factor VII
FVIII	Factor VIII
FIX	Factor IX
FXa	Activated factor X
FDA	Food and Drugs Agency
FRETS	Fluorescent resonance energy transfer
GCS	Glasgow Coma Scale
GP	Platelet glycoprotein

HASU	Hyperacute Stroke Unit
HS	Haemorrhagic stroke
ICAM	Intercellular adhesion molecule
IR	Incremental recovery
IS	Ischaemic stroke
IST-3	The Third International Stroke Trial
LA	Lupus anticoagulant
LDL	Low-density lipoprotein
LT	Lag time
MCAO	Middle cerebral artery occlusion
MHRA	Medicines and Healthcare Regulatory Agency
MPD	Myeloproliferative disease
MPO	Myeloperoxidase
MRI	Magenetic resonance imaging scan
NICE	National Institute for Health and Care Exellence
NIHSS	National Institutes of Health Stroke Scale
OR	Odds ratio
PAI-1	Plasminogen activator inhibitor-1
PAR-4	Protease activated receptor 4
PCR	Polymerase chain reaction
PFA-100	Platelet function analyser
PF4	Platelet factor 4
PI	Plasma infusion
PIS	Patient information sheet
PMF	Primary myelofibrosis
PNP	Pooled normal plasma
PPP	Platelet poor plasma
PV	Polycythaemia vera
rADAMTS13	Recombinant ADAMTS13

RCP	Royal College of Physicians
SAH	Subarachnoid haemorrhage
SD-FFP	Solvent detergent fresh frozen plasma
SNP	Single nucleotide polymorphism
TAFI	Thrombin- activatable fibrinolysis inhibitor
TAT	Thrombin anti-thrombin
TIA	Transient ischaemic attack
tPA	Tissue-type plasminogen activator
TNF-α	Tumour necrosis factor- alpha
TOAST	Trial of Org 10172 in Acute Stroke Treatment classification system
TTP	Thrombotic thrombocytopenic purpura
ULVWF	Ultra-large Von Willebrand factor
VWF	Von Willebrand factor

1.8 List of Figures

Figure 1: Example of thrombin generation curve....65

Figure 2: Example of patient thrombin generation curves compared to reference....69

Figure 3: Comparison of haemostatic markers between groups at presentation....81

Figure 4: Comparison of haemostatic markers between groups at presentation: Mann Whitney testing....83

Figure 5: Longitudinal changes in haemostatic markers in ischaemic stroke....98

Figure 6: VWFAg/ ADAMTS13Ac ratio from presentation to convalescence in ischaemic stroke....99

Figure 7: Paired testing in ischaemic stroke: presentation to final follow up....102

Figure 8: Longitudinal changes in haemostatic markers in TIA....104

Figure 9: Longitudinal changes in haemostatic markers in controls....106

Figure 10: Presentation VWF:Ag, VWF:Act, ADAMTS13AC and ratio VWF:Ag-ADAMTS13Ac in ischaemic brain injury according to subsequent mortality....112

Figure 11: ROC curve analysis: VWFAg-ADAMTS13Ac ratio as predictive for mortality....115

Figure 12: Comparison of thrombin generation between subgroups at presentation....116

Figure 13: Longitudinal trends of thrombin generation: peak thrombin and ETP....117

Figure 14: ADAMTS13 and ratio VWF:Ag- ADAMTS13 in thrombolysed ischaemic stroke versus non-thrombolysed ischaemic stroke....123

Figure 15: Longitudinal changes in thrombin generation in thrombolysed compared to non-thrombolysed ischaemic stroke....127

Figure 16: Change in von Willebrand factor activity over time.....157

Figure 17: Visual predictive plot of ADAMTS-13 activity over time.....160

Figure 18: Individualised simulations of maintaining target trough ADAMTS13 >10IU/dL with total dose amount (fixed frequency) or dosing interval (fixed dose amount)....161

Figure 19: Individualised simulations of maintaining target trough ADAMTS13 >50IU/dL with total dose amount (fixed frequency) or dosing interval (fixed dose amount)....162

Figure 20: Outcomes of raised haematocrit (>0.45) and/or thrombocytosis (platelet count >400 x 10^9/L)....178

Figure 21: Outcomes of thrombocytopenia (platelet count <150 x 10^9/L)....179

Figure 22: Lupus anticoagulant antibody testing....180

Figure 23: Anticardiolipin antibody testing....181

Figure 24: Anti beta-2 glycoprotein 1 antibody testing....182

1.9 List of Tables

Table 1: Modified Rankin score (mRS) 58

Table 2: NIHSS score (National Institutes of Health Stroke Scale) 0-42 58

Table 3: Thrombin generation inter-assay variation 68

Table 4: Example of results on day of evaluation (without normalisation) 69

Table 5: Example of results on day of evaluation (post normalisation) 70

Table 6: Demographic characteristics at presentation 75

Table 7: TOAST classification 76

Table 8: Anti-platelet or anticoagulant medications on admission 76

Table 9: Known medical co-morbidities 77

Table 10: Functional and disability status scores of patients in each subgroup 78

Table 11: Presentation laboratory results 79

Table 12: TIA cohort at presentation 84

Table 13: Comparison of blood group O/ non-O according to presentation haemostatic markers 85

Table 14: Ischaemic stroke cohort blood group comparison 85

Table 15: TIA cohort blood group comparison 86

Table 16: Haemorrhagic stroke cohort blood group comparison 86

Table 17: Control cohort blood group comparison 86

Table 18: Correlation of presentation functional scores and laboratory parameters (Spearman rank non-parametric correlation) 87

Table 19: Regression of presentation laboratory markers (independent variables) against VWF:Ag- ADAMTS13 ratio (dependent variable) 89

Table 21: Regression of diagnosis of ischaemic stroke or TIA at presentation, modified Rankin score and age (independent variables) against VWF:Ag- ADAMTS13 ratio (dependent variable) 93

Table 22: Regression of diagnosis of ischaemic stroke or TIA (split) at presentation, modified Rankin score and age (independent variables) against VWF:Ag- ADAMTS13 ratio (dependent variable) 94

Table 23: Regression of diagnosis of ischaemic stroke or TIA (split) at presentation, modified Rankin score and age (independent variables) against VWF:Ag- ADAMTS13 ratio (dependent variable) 95

Table 24: Haemostatic markers at each time point 100

Table 25: Longitudinal changes in ischaemic stroke: comparison 101

Table 26: Regression of modified Rankin score at final follow up as dependent variable, with presenting VWF:Ag-ADAMTS13Ac ratio as independent variable in ischaemic brain injury 107

Table 27: Regression of modified Rankin score at final follow up as dependent variable, with presenting VWF:Ag-ADAMTS13Ac ratio and presenting modified Rankin score as independent variables in ischaemic brain injury 108

Table 28: ADAMTS13 activity quartiles- is there a correlate with functional scores? 109

Table 29: Mortality: comparison of baseline haemostatic markers..... 111

Table 30: Chi squared testing of survival in ischaemic stroke according to presentation VWFAg-ADAMTS13Ac. 113

Table 31: Mortality according to VWF:Ag-ADAMTS13Ac ratio quartiles in ischaemic stroke and TIA 114

Table 32: Thrombolysis comparison..... 119

Table 33: Thrombolysis in ischaemic stroke: comparison of thrombolysed and non-thrombolysed groups at presentation: clinical scores and haemostatic markers at presentation 120

Table 34: Thrombolysis in ischaemic stroke: comparison of thrombolysed and non-thrombolysed groups over longitudinal follow up..... 121

Table 35: Ischaemic stroke mRS 0-3 at presentation: comparison of VWF:Ag-ADAMTS13Ac ratio at each time point according to whether thrombolysed or not. 124

Table 36: Ischaemic stroke mRS 4-5 at presentation: comparison of VWF:Ag-ADAMTS13Ac ratio at each time point according to whether thrombolysed or not. 125

Table 37: Thrombin generation in ischaemic stroke: comparison according to whether thrombolysed or not 126

Table 38: Ischaemic stroke mRS 0-3 at presentation: comparison of thrombin generation at presentation and final follow up according to whether thrombolysed or not. 129

Table 39: Ischaemic stroke mRS 4-5 at presentation: comparison of thrombin generation at presentation and final follow up according to whether thrombolysed or not. 130

Table 40: Patients with *ADAMTS13* mutations 131

Table 41: CYP2C19 (*2) polymorphism and relationship with haemostatic markers 133

Table 42: Congenital TTP: raw patient data. 150

Table 43: Estimated model parameter values for a two-compartment model with linear elimination (ADAMTS13 activity) 158

Table 44: Median, 5^{th} and 95^{th} percentiles of secondary pharmacokinetic parameters (ADAMTS13 activity) 158

Table 45: Individual secondary pharmacokinetic parameters 159

Table 46: Precision and reproducibility of anticardiolipin antibody assay adjustment 176

2 Introduction: The VWF-ADAMTS13 axis in stroke and TIA: Introduction The VWF-ADAMTS13 axis in stroke and TIA

2.1 Stroke

2.1.1 Incidence and prevalence

Stroke is a major cause of mortality and morbidity in the UK, and worldwide is the second leading cause of death (Lozano et al. 2012). NICE guidelines outline that stroke accounted for over 56,000 deaths in England and Wales in 1999, representing 11% of all deaths (Mant, Wade, and Winner 2004). State of the Nation Stroke statistics (published by the Stroke Association) quote that 1 in every 8 patients will die within 30 days of a stroke (Bray et al. 2016), although the mortality trend has fallen by almost half in the period 1990 to 2010 (Feigin et al. 2003).

2.1.2 Mortality and morbidity

Mortality alone notwithstanding, the significant morbidity among survivors is highlighted by NICE guidelines. Every year in England alone, there are approximately 110,000 people with a first or recurrent stroke and a further 20,000 people with a TIA. The aftermath of stroke affects more than 900,000 people in England, with dependence on others for help with activities of daily living in half of these patients.(Department of Health National Audit Office 2005)

NICE guidelines also illuminate the cost implications. Stroke is estimated to cost the economy around £7 billion per year in England alone: comprised of direct costs to the NHS of £2.8 billion, costs of informal care of £2.4 billion and costs because of lost productivity and disability of £1.8 billion (Mant, Wade, and Winner 2004). The National Stroke Strategy was published by the Department of Health in 2007. This aimed to outline a quality framework and management guidelines- from emergency response to supporting life after stroke. The development of hyperacute stroke units and cohesive multidisciplinary teams demonstrate that care for stroke patients now has a far greater profile in NHS targets for care (Department of Health 2007). In London alone, there are 8 hyperactue stroke units commissioned. Each services a population of approximately 1 million, with streamlining of services allowing for emergency brain scans and imporved delivery of thrombolysis. In line with this, our institution has a daily TIA clinic for assessment of symptoms suggestive of TIA in patients well enough for outpatient scans and investigations.

The Intercollegiate Stroke Working Party has developed the National Clinical Guideline for Stroke, with regular updates listing specific recommendations for almost every aspect of stroke care (Royal College of Physicians 2016). Strong national and international multi-centre collaboration has pushed stroke care forward in terms of comprehensive strategy, from being a condition for which there was little intervention, to a number of evidence-based treatments that can both improve the outcome and decrease risk of recurrence (Brown 2012).

2.1.3 Risk factors

Non-modifiable risk factors for stroke include age, gender and ethnicity. Potentially modifiable risk factors include hypertension, valvular disease, atrial fibrillation, diabetes, hyperlipidaemia, smoking, high alcohol intake, illicit drug use, obesity, lack of exercise, carotid artery disease and transient ischaemic attacks (TIAs, Furie et al. 2011). Primary and secondary prevention measures include lifestyle modification and pharmacotherapy- for example, anticoagulation for all patients in atrial fibrillation with a high stroke risk and no contraindications, and using statins in diabetic patients considered to be at high risk of stroke (Meschia et al. 2014).

In the era of pharmacogenomics, there is increasing interest in the role of genetic factors in stroke risk. There are known monogenic causes of stroke, which tend to result in specific stroke subtypes. The most well-known is cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL), resulting in hereditary small-vessel disease and vascular dementia (Joutel et al. 1996). Monogenic causes of stroke are rare with little contribution to overall population risk (Markus 2010). Stroke is a clinical syndrome of focal neurological loss of function rather than a single disease (Markus 2012). The heterogeneity of pathological mechanisms leading to stroke, both haemorrhagic and ischaemic; is further added to by different genetic aetiologies. With the advent of techniques such as genome-wide association studies and whole genome sequencing, it may be possible to tease out novel stroke genes. If stroke risk variants are identified that explain the majority of genetic risk, incorporated with rarer variants, it may become possible in future to personalise stroke prediction in the individual (Markus 2012).The healthcare economics and feasibility of such a goal may be more controversial.

2.1.4 Pathogenesis

Ischaemic stroke arises from sudden interruption of cerebral blood flow and consequent ischaemia. This usually arises from thrombotic or embolic occlusion of a cerebral artery, while other mechanisms include dissection causing direct occlusion and lacunar stroke potentially from cerebral arteriolar abnormalities. This causes a corresponding neurological deficit, potentially irreversible and possibly devastating in its clinical effects. Acute treatment aims to restore cerebral flow and minimise neuronal damage, via thrombolysis or mechanical thrombectomy. Secondary prevention includes surgical means such as carotid endarterectomy, anticoagulation, antiplatelets, neuroprotective agents and treating associated risk factors such as hyperlipidaemia and hypertension (Deb, Sharma, and Hassan 2010).

Approximately 80% of strokes are caused by focal cerebral ischaemia due to arterial occlusion, with 6.5- 19.6% caused by intracerebral haemorrhage (Feigin et al. 2003; Hughes, Lip, and Guideline Development Group, National Institute for Health and Clinical Excellence 2008). Atherosclerotic plaque rupture of extracranial artery stenoses leads to cerebral thromboembolism (Stoll and Bendszus 2006). Thromboembolism from the heart to brain is the cause of approximately 20% of stroke, such as with atrial fibrillation, known to increase risk of stroke five-fold (Hughes, Lip, and Guideline Development Group National Institute for Health and Clinical Excellence 2008). Within the cerebral microvasculature, ischaemia leads to oxygen and glucose deprivation, neuronal depolarisation and calcium-mediated excitotoxicity causing necrotic and apoptotic cellular death (G Stoll, Kleinschnitz, and Nieswandt

2008). Surrounding the infarct core, there is the well-described penumbra. This tissue is still salvageable depending on whether there is restoration of blood-flow, the principle behind thrombolysis and thrombectomy treatment (Dirnagl, Iadecola, and Moskowitz 1999).

2.2 Arterial thrombus formation in cardiovascular disease

2.2.1 Atherosclerotic plaque development

Atherosclerosis is defined as a chronic disease characterised by lipid retention and arterial intimal chronic inflammation (Lundberg and Hansson 2010 & J. Andersson, Libby, and Hansson 2010). The atherosclerotic process is initiated by cholesterol-laden low-density lipoprotein (LDL) accumulating in the intima, triggering endothelial cell activation (Lippi, Franchini, and Targher 2011). This causes expression of chemokines and leucocyte adhesion molecules, which attract monocytes and T cells. Monocytes differentiate into macrophages, and upregulate receptors, with increased lipoprotein uptake resulting in foam cell formation. Within the atherosclerotic plaque, T-lymphocytes interact to produce proinflammatory cytokines, with sustained inflammation contributing to proteolysis and plaque disruption.

Plaque composition is directly linked to likelihood of erosion and rupture: increased with leucocytosis, high tissue factor expression, proteinases, decreased collagen and oxidative-stress-mediated cellular apoptosis (B. Furie and Furie 2008). Rupture exposes the subendothelial matrix and the plaque-containing thrombogenic material to the arterial circulation. Other platelet-activating material in the plaque includes von Willebrand factor (VWF), fibrin, fibrinogen, oxidised LDL, thrombospondin and various collagen types. These will actively stimulate platelet adhesion, aggregation and secretion of dense granules. As part of the cascade effect, this will elicit platelet-monocyte aggregation and platelet-dependent activation of blood coagulation proteins (B. Furie and Furie 2008 & 'S. Penz et al. 2005).

2.2.2 Plaque rupture and thrombus formation

The mechanism of thrombus formation is highly intricate. The blood vessel endothelium acts to separate subendothelial vessel components from blood cells and soluble plasma proteins involved in blood coagulation and fibrinolysis. Just as with plaque rupture, haemostasis becomes activated after vessel wall injury (Lippi, Franchini, and Targher 2011).

With endothelial activation, adhesion receptors are expressed on the endothelial cell membrane, and Weibel Palade bodies are released into plasma. A prothrombotic cascade ensues- the endothelial lipid bilayer is altered to expose negatively charged phospholipids capable of binding coagulation factors and other haemostatic molecules such as tissue factor. Endothelial damage exposes both collagen and tissue factor (Murray et al. 2010). It is illustrative to understand the mechanism of thrombus formation via 2 pathways: tissue factor and collagen.

2.2.3 Tissue factor pathway

In the *tissue factor pathway*, the source of tissue factor is either from constitutive expression from the vessel wall, or active expression within the vessel wall. Following activation by protein disulphide isomerase, tissue factor complexes with VIIa, leading to thrombin generation as the coagulation cascade is stimulated. There is sequential conversion of inactive blood coagulation proteins to the corresponding active enzymes, also known as the 'thrombin burst'. Factors XI, X and IX are essential for the thrombin burst, to sustain and amplify thrombin generation. This eventually results in the enzymatic action of FXa on prothrombin, with consequent thrombin action on fibrinogen, to generate the definitive fibrin clot. Plasmin- mediated fibrin degradation is regulated by TAFI (thrombin-activatable fibrinolysis inhibitor).

The rapid production of thrombin in turn will activate platelets via cleavage of protease-activated receptor (PAR)-4. PAR-4 is the main thrombin receptor on platelets (Ofosu 2003). Mice lacking PAR-4 are protected from arterial thrombosis in an experimental setting, suggesting that thrombin activation of platelets is essential for the initiation of the haemostatic process (Sambrano et al. 2001). With thrombin cleavage of PAR-4, platelet activation leads to release of contents including adenosine diphosphate (ADP), serotonin and thromboxane A2. These platelet contents are agonists and will activate other platelets, further amplifying signals for thrombus formation. In summary, the tissue factor pathway culminates with platelets being captured on the vessel wall, with ongoing platelet activation and interaction.

2.2.4 Collagen pathway

The *collagen pathway* is activated by endothelial disruption exposing collagen, rapidly leading to platelet deposition from the bloodstream. As above, platelets are captured on the vessel wall but via platelet glycoprotein (Gp) VI interacting with collagen, and glycoprotein (Gp)Ib-V-IX with VWF respectively. Consequent platelet- platelet interaction is mediated via GpIIb/IIIa (also known as αIIbβ3), a platelet integrin serving as fibrinogen receptor, binding to its ligands fibrinogen and VWF (B. Furie and Furie 2008).

GpIIb/IIIa binding to VWF and fibrinogen, mediates a firm adhesion, perpetuating coagulant activity and platelet aggregation (Reininger et al. 2010). GpIa/IIa mediates collagen-platelet binding under the high-shear conditions expected near an atherosclerotic plaque, also promoting the association between collagen and GpVI. Further addition to this haemostatic 'plug' is secured with ADP promoting platelet shape change, granule content release and subsequent aggregation; and thromboxane-2 exerting further activation and recruitment of additional platelets to those already aggregated.

2.2.5 Thrombus development

Inflammatory states such as atherosclerotic plaque rupture will result in endothelial secretion of VWF and subsequent platelet recruitment. VWF and platelet glycoprotein Ib interaction allows platelets to roll on endothelial cells. VWF mediates platelet adhesion to collagen fibres in the atherosclerotic plaque, and will support subsequent platelet aggregation onto such matrices even under high-shear conditions (Reininger et al. 2010). *VWF-/-* mice have demonstrated smaller atherosclerotic lesions than those seen in wild-type mice demonstrating its role in tethering the constitutive components of arterial thrombus formation (Methia et al. 2001 & Davi and Patrono 2007). As above, GpVI adheres platelets to collagen. It also acts as the major agonist for initial platelet activation and granule release. The independence of the collagen pathway in thrombus formation, and the role of GpVI was further demonstrated by the exposure of human atheromatous plaques to flowing blood at arterial wall shear rate (S. Penz et al. 2005). Plaque-induced platelet thrombus formation was observed in fully anticoagulated blood- thereby excluding tissue factor-mediated coagulation. Mice platelets lacking GpVI were unable to adhere to atheromatous plaque or form thrombi. Human platelet thrombus formation onto plaques in flowing blood was halted by antibody blockade of GpVI.

The two distinct pathways –collagen and tissue factor- of thrombus formation are supported by the mouse model work of Reininger et al regarding the molecular and cellular mechanisms behind arterial thrombus formation (Reininger et al. 2010). Atheromatous human plaques were exposed to blood and blood components, with kinetics of thrombus formation followed via thrombin generation and microscopy. Collagenous plaque induced platelet adhesion and aggregation via platelet collagen receptor glycoprotein VI (GpVI). This was proven with two techniques: i) inhibition of GPVI with an anti –GpVI antibody, and ii) pre-treating plaques with a collagenase. Both techniques inhibited plaque-induced thrombus formation. With normal GpVI function, platelet adhesion and aggregation on collagenous plaque components (including fibronectin, collagen and VWF) was shown to occur within 1 minute after atherosclerotic plaque rupture (GpIb-IX-V platelet surface receptor binding VWF and GpVI binding collagen respectively).

Secondly, tissue factor was shown to induce blood coagulation by using a polyclonal anti-tissue factor antibody to knock off 'blood borne' tissue factor. With blood borne tissue factor inhibited, the initiation of coagulation was delayed by 3 minutes, with the formation of thrombin and fibrin triggered by plaque-based tissue factor (held in the lipid-rich core and fibrous caps of the plaque). Plaque tissue factor could stimulate fibrin formation in flow niches provided by the developing platelet aggregates. Coagulation would only trigger in such niches, with no evidence of a blood-borne tissue factor or FXIIa contributing. It was surmised that platelets provide the surface on which the thrombin necessary for clot formation is generated. Plaque tissue factor then exposed to activated platelets prompts coagulation to continue.

Reininger et al's work demonstrated the sequential development of thrombi in arterial thrombus pathogenesis, with GpVI-dependent platelet activation followed by plaque tissue factor-mediated fibrin formation. It is less clear how the collagen and tissue factor pathways interact. It is suggested that the synergism of these pathways

illustrates why one therapeutic target may be ineffectual- such as anticoagulation only limiting the tissue factor pathway, and GpVI blockade only limiting the collagen pathway (Reininger et al. 2010). Ongoing clinical trials regarding GpVI blockade, limiting the early stages of platelet activation, should clarify whether infarct progression can be limited without increasing risk of intracranial bleeding (Denorme and Rondina 2019).

2.2.6 Thrombus propagation

Platelet recruitment continues as the thrombus develops. Some platelets will remain unactivated and may eventually separate from the developing thrombus. Numerous influences include shear, flow, turbulence and the number of platelets available in the circulation (B. Furie and Furie 2008).

Platelet activation via receptor GpIIb/IIIa (integrin αIIbβ3) will induce a conformational change in the receptor, increasing its affinity for ligands fibrinogen and VWF. Fibrinogen is the important ligand at low shear, whereas VWF acts at higher shear. Release of the platelet granule contents after activation is crucial for ongoing thrombus formation. ADP and calcium are released from dense granules, going on to stimulate further platelet activation. ADP has 2 specific receptors on the platelet surface, P2Y1 and P2Y2. Later signalling events, such as involving CD40 ligand and lymphocyte activation molecules, will heighten platelet-platelet affinity and stabilise the thrombus.

It has been argued that pathological arterial thrombosis underlies most causes of death worldwide (Lippi, Franchini, and Targher 2011). It is not doubted that an intricate pathological process is initiated when an atherosclerotic plaque becomes injured. Atherosclerotic plaques consist of lipid, foam cells, necrotic cell debris, matrix components and tissue factor- but also contain platelet-activating molecules including VWF, fibrin, fibrinogen and thrombospondin. Upon plaque rupture, these molecules stimulate platelet adhesion, aggregation and secretion of dense granules- and consequent platelet-monocyte aggregation and platelet-dependent activation of blood coagulation proteins (B. Furie and Furie 2008). It therefore seems logical that VWF plays a primary role in stroke.

2.2.7 Inflammation, atherosclerosis and thrombosis

Beyond the pathogenesis of atherosclerosis, chronic inflammation contributes to thrombosis. As already discussed, activated platelets release a variety of pro-thrombotic and pro-inflammatory mediators including VWF. The C-reactive protein (CRP) is the acute-phase reactant most commonly relied on in clinical practice as a biomarker of inflammation. It has been shown to have an association with increased risk of death after ischaemic stroke, independent of the NIHSS prognostic scoring for stroke outcome as well as a predictive role in stroke recurrence and neurological deterioration (Ghabaee et al. 2014; Kuwashiro et al. 2013; Seo et al. 2012). Inflammatory markers associated with the acute-phase response (IL-6, CRP, fibrinogen, TNF-alpha) were dependent on each other in multivariate analysis, with the generalised inflammatory state strongly associated with secondary ischaemic stroke rather than a particular marker; based on a nested case-control study of 591 strokes (Welsh et al. 2008).

This has been countered with suggestion that CRP itself may not just reflect inflammation but be a direct contributor- causing cerebral vascular endothelial injury and up-regulation of cell adhesion molecule expression in mouse brain endothelial cells (J. Zhang et al. 2006). The correlation between CRP and prognosis appears to be more robust than outcome related to other acute-phase reactants such as TNF-alpha, IL-6, IL-8, IL-10, fibrinogen and leucocytes (Sahan et al. 2013).

There is some evidence to suggest the CRP may have an independent thrombogenic effect, potentiating arterial thrombus formation via tissue factor expression. Matsuda et al examined arterial thrombus formation under the influence of CRP with balloon injury of rabbit neointima, showing increase in thrombus size and tissue factor in transgenic rabbits overexpressing human CRP (Matsuda et al. 2011). A further pathway could be the inhibition of release of intrinsic t-PA and stimulated release of plasminogen activator inhibitor-1 from vascular endothelial cells (Devaraj, Xu, and Jialal 2003′ & U. Singh, Devaraj, and Jialal 2005) . This would suggest that high plasma levels of CRP decrease fibrinolytic activity of endothelial cells, thus contributing to thrombus development and potentially limiting the effect of t-PA therapy with early recanalization failure (Koga et al. 2013). P-selectin may also be pivotal in arterial inflammation and thrombosis. As platelets are activated, P-selectin (CD62P) is released from the alpha-granules, then expressed on the platelet surface (Lukasik et al. 2011). P-selectin interacts with both monocytes and granulocyte, thereby involving the leucocytes with thrombosis (Davi and Patrono 2007 & Hu et al. 2010).

Biomarkers of inflammation also include the D-dimer- a marker of coagulation activation, and certainly relevant for ongoing thrombosis propagation. One study of biomarkers related to early recurrent ischaemic lesions on imaging after acute ischaemic stroke, found that D-dimer might predict such radiological change, independent of other clinical, imaging and laboratory variables (Kang et al. 2009). D-dimer concentration, reflecting circulatory fibrin turnover, could reflect cerebral thrombosis formation, mark systemic hypercoagulability and indeed stimulate pro-inflammatory cytokines release such as IL-6 (Barber et al. 2004 &′Robson et al 1994)**.**

Stabilisation of the thrombus via platelet-platelet recruitment has been discussed above- including CD40, and its ligand CD154. There is evidence that this membrane glycoprotein is integral in inflammation, inducing cellular adhesion molecules and enhancing pro-inflammatory cytokines (Omari and Dorovini-Zis 2003 & Henn et al. 1998). Ishikawa et al found that CD40-deficient and CD40-ligand deficient mice showed decreased blood cell recruitment and vascular permeability of the cerebral microcirculation following ischaemia and reperfusion, with overall reduction of infarct volume compared with wild type mice (Ishikawa et al. 2005). Intravital fluorescent microscopy showed that the CD40 or CD40L deficient mice exhibited a significant reduction in rolling and adhering leucocytes compared with wild type mice at 4-hour reperfusion post stroke. Clinical work with patients with acute cerebral ischaemia showed upregulation of the CD40 system: heightened levels of CD154 on platelets, CD40 on monocytes, increased soluble CD154 in plasma and increased numbers of prothrombotic platelet-monocyte aggregates - even 3 months after previous acute cerebral ischaemia (Garlichs et al. 2003). This was thought to have therapeutic potential, since aspirin does not inhibit CD154 expression, while clopidogrel completely abolishes CD154 upregulation (Hermann et al. 2001).

2.2.8 Leucocyte recruitment

Ischaemia and resultant inflammation largely reflects neutrophil infiltration, adhering to the endothelial cells within a few hours of ischaemia. As leukocytes roll and adhere, there is additional recruitment of adherent platelets, mediated by specific adhesion glycoproteins and signalling pathways such as CD40/40L and cytokine modulation. Leukocyte rolling, adhesion, and transmigration at the site of infection or injury are hallmarks of inflammation. There is increasing interest in lymphocyte-endothelial-platelet interactions in the post-ischaemic cerebral microcirculation (Yilmaz and Granger 2010). Ischaemia means compromised perfusion with consequent upregulation of adhesion molecules on both endothelial and circulating cells, with resultant rolling of neutrophils and platelets. Neutrophil activation causes protease and superoxide release, thereby causing blood-brain barrier disruption and exposing brain antigens to blood. Brain antigens are processed in lymphoid tissue by antigen-presenting cells, and presented to T-lymphocytes via MHC class II. T cells thereby differentiate into proinflammatory Th1, Th17 or cytotoxic T cells (CTL) and immunomodulatory Th2 and T regulatory cells. The lymphocyte effect on neuronal tissue is thereby via the release of pro-and/or anti-inflammatory cytokines. Cytotoxic T cells are activated by MHC I presenting antigen-presenting cells, and may then contribute to the brain injury process via release of membrane attacking and pro- apoptotic substances. Overall T cell activation is time-dependent, contributing to the later phases of reperfusion injury. Protection against brain injury and inflammation has consequently been demonstrated following MCA occlusion/ reperfusion in mice genetically deficient in either CD4+ and/or CD8+ T-lymphocytes (Yilmaz et al. 2006)**.** Whether platelet-lymphocyte interaction contributes enough to modify ischaemic brain injury is unknown. Activated platelets may modulate T-cell responses by releasing mediators such as platelet-release platelet factor 4 (PF4) or via cell surface receptor (eg CD40L) signalling, and altering lymphocyte contribution may be a future therapeutic target for limiting ischaemic stroke (Li 2008).

2.3 Treatment of stroke

Ischaemic brain injury has 3 main modalities of treatment, all aiming to achieve reperfusion of the occluded vessel and thereby reverse ischaemia and neuronal damage. The main modalities are pharmacological- ie thrombolysis and mechanical- ie endovascular thrombus extraction.

2.3.1 Pharmacological treatment: thrombolysis

Thrombolysis and fibrinolysis are terms generally used synonymously in the context of treatment of acute ischaemic stroke. Fibrinolysis sees activation of plasminogen to plasmin, in turn splitting fibrinogen and fibrin into degradation products, causing clot lysis as the fibrin meshwork is dissolved. Control of fibrinolysis is manifest via plasminogen activator inhibition (mainly plasminogen activator inhibitor-1, PAI-1) and plasmin inhibition (mainly alpha-2 antiplasmin).

Thrombolysis aims to dissolve the fibrin clot comprising the occlusive thrombus and thereby allow vessel recanalization. Early intravenous or intra-arterial thrombolysis are the only established therapeutic options at present, aiming to salvage those patients with a remaining penumbra. Later application offers little benefit, and too high a risk of severe intracerebral haemorrhage (NINDS Study Group 1995).

Streptokinase was the original thrombolytic agent, stimulating systemic plasmin generation. It is now less favoured due to its high rate of antigenicity and untoward side effects including fever and hypotension (Murray et al. 2010). Tissue-type plasminogen activator (t-PA) activates plasminogen optimally at the fibrin surface and less so in the circulation, therefore being more fibrin-specific. As plasmin is generated at the fibrin surface, it is protected from inhibition by alpha-2 antiplasmin since the lysine-binding sites are not available. Alteplase is a recombinant tissue plasminogen activator, and the only thrombolytic agent that is MHRA- and FDA-licensed for acute ischaemic stroke (Murray et al. 2010). Haemorrhagic sequelae remain the most noteworthy adverse reactions, but systemic reviews essentially suggest a significant net benefit with an increased number of patients alive and independent (Hacke et al. 2008; Sandercock et al. 2012).

US data previously showed that 22% of ischaemic stroke patients present within 3 hours, but only 8% meet all eligibility criteria for tPA with subsequent thrombolysis (Kleindorfer et al. 2004). The main contraindication for thrombolysis is presenting outwith the time window, with consequent focus on management pathways such as the hyperacute stroke unit (HASU) model, to improve patient eligibility. In the UK and Europe, the eligible time window for thrombolysis is 4-5 hours from the onset of symptoms. The National Stroke Strategy stipulates that thrombolysis should be available to all appropriate patients at the point of need, corroborated by the Royal College of Physicians (RCP) and NICE guidelines (Demaerschalk et al. 2016).

A Cochrane systemic review of all 11 completed thrombolysis trials (2009) had established that there is a net benefit in using thrombolysis therapy in acute ischaemic stroke (Wardlaw JM et al 2009)**.** The benefit is deemed greatest if given within 90 minutes of onset and the risk may outweigh the benefits after 4.5 hours. (Hacke et al. 2008 & 'Lees et al for the ECASS, ATLANTIS trials, 2010). IST-3 (the third international stroke trial) subsequently

showed that overall benefit also applied to patients aged more than 80 years. A sub-group analysis of those thrombolysed at 4.5 to 6 hours post onset of symptoms suggested improved functional recovery, though was not significant (Sandercock et al. 2012).

Although timely application of thrombolysis is now clearly supported by the evidence, it should be noted that thrombolysis does not work in all cases. Even if successful recanalization is achieved, there can be secondary arterial reocclusion (Wardlaw et al. 2012 & Heo et al. 2003). Reperfusion injury is also a possibility, with progressive stroke despite early reperfusion. Although the ischemia may be thwarted, the infarct is not halted and recovery is still limited (Stoll and Bendszus 2006). Poorer clinical outcomes are seen with large artery occlusion, due to failure of early recanalization; particularly in proximal anterior circulation or basilar arterial occlusion (Bhatia et al. 2010; Fischer et al. 2005). Arterial thrombus length and location are now recognised as predictive of poorer outcome, with a consequent drive to investigate intra-arterial mechanical means of clot removal (Evans et al. 2017; Kamalian et al. 2013; Riedel et al. 2011).

2.3.2 Mechanical treatment: thrombectomy

Mechanical thrombectomy, or endovascular thrombus extraction, is an evolving technology. There is growing international recognition of its utility, but caveats are resource availability- such as round-the-clock availability of interventional neuroradiology and significant risk of haemorrhage (Brown 2012). A number of randomised controlled trials have been published comparing this hyperacute stroke treatment in addition to intravascular thrombolysis, with standard treatment (thrombolysis alone). Overall the Intercollegiate Working Stroke Party concludes that the treatment is effective for selected patients with a proximal large artery occlusion as an adjunct to thrombolysis, and for those whom thrombolysis is contraindicated (Royal College of Physicians 2016). The practicalities of delivering such a specialised service, with all the resource required, will be a considerable challenge which developing evidence may further bolster (Brown 2012).

2.3.3 Antiplatelet treatment

The role of secondary prevention is also paramount to prevention of recurrent events, both immediate and long-term; and consists primarily of antiplatelet agents or anticoagulation. Risk of recurrent ischaemic brain injury is significant: 26% within 5 years of a first stroke and 39% by 10 years (Mohan et al. 2011) .This is particularly so in the immediate aftermath of the event, estimated to be as high as 5% within the first week and 20% within the first month by the RCP (Royal College of Physicians 2012).

Beyond thrombolysis, the RCP guideline mandates that every patient should have anti-platelet therapy once significant haemorrhage has been ruled out. Thrombolysed patients should start on antiplatelet therapy after 24 hours once bleeding has been excluded. All acute stroke patients with primary intracerebral haemorrhage excluded

should be treated with an antiplatelet within 24 hours. It is recommended that aspirin 300mg od is used until 2 weeks after the onset of stroke, “at which time definitive long-term antithrombotic treatment should be initiated”.

The antiplatelet effect of aspirin is mediated via the selective and irreversible inhibition of cyclooxygenase conversion of arachidonic acid to thromboxane A2, the latter a potent platelet aggregator and vasoconstrictor. Route of administration is typically oral, although an intravenous preparation is available. Jimenez et al demonstrated that chewing the enteric-coated aspirin formulation markedly inhibited platelet aggregation and thromboxane A2 production within 15 minutes - arguing that chewing essentially converted the aspirin into soluble form (Jimenez et al. 1992).

Aspirin is used in both acute management of ischaemic stroke and long-term secondary prevention. In terms of secondary prevention; aspirin, clopidogrel and dipyridamole are established in stroke prevention (CAPRIE Steering committee 1996 & ʼVerro, Gorelick, and Nguyen 2008). Clopidogrel is a P2Y12 antagonist, irreversibly inhibiting binding of ADP to the P2Y12 receptor on the platelet, so that platelet aggregation is not stabilised (Labarthe et al. 2005). Dipyridamole inhibits adenosine uptake into the platelet, stimulating adenylate cyclase and thereby inhibiting platelet aggregation. Dual therapy with aspirin and dipyridamole is more efficacious than aspirin alone in secondary prevention, supported by the ESPRIT trial (The ESPRIT study group 2006). Clopidogrel monotherapy is licensed by NICE for first-line secondary prevention in ischaemic stroke, with supportive data from comparative trials including CAPRIE and PRoFESS. (CAPRIE steering committee 1996; Diener et al 2008). Aspirin and clopidogrel combined were not shown to be more efficacious than monotherapy, with increased bleeding risks (Halkes et al. 2008)**.** NICE advocates the combination of aspirin and dipyridamole an appropriate alternative to clopidogrel if the latter cannot be tolerated. Aspirin and clopidogrel combined were not shown to be more efficacious than monotherapy, with increased bleeding risks (Halkes et al. 2008)**.**

2.3.4 Monitoring antiplatelet effect

Whether in vitro measurement of antiplatelet agent effect correlates with clinical efficacy is controversial. Laboratory methods include measurement of thromboxane B2 (a thromboxane A2 metabolite, measuring aspirin pharmacological effect), optical and impedance platelet aggregometry, PFA-100 platelet function screening, thromboelastography, activation-dependent changes on the platelet surface and vasodilator-stimulated phosphoprotein phosphorylation (measuring activation-dependent platelet signalling, dependent on the P2Y12 receptor).

In vitro studies have shown aspirin and P2Y12 antagonists do not effectively inhibit plaque-induced platelet activation under arterial flow conditions (S. M. Penz et al. 2007). In clinical terms, measuring platelet function is notoriously difficult with numerous confounding influences and a lack of homogeneity between platelet function analysers (Sternberg et al. 2013). One systemic review of evidence pertaining to laboratory aspirin/clopidogrel resistance found association with poorer clinical outcomes- even with no standardised definition of exactly what

constitutes resistance. It was argued that there are no specific treatment recommendations for patients exhibiting high platelet reactivity or poor platelet inhibition (Feher et al. 2010).

Reproducibility of platelet testing over time is thought to be poor, and so it is difficult to attest as to how long platelets remain activated after an acute ischaemic event (Harrison et al. 2005 & Harrison et al. 2008). There is no single test that can be used to definitively predict a clinical response to aspirin in terms of recurrent ischaemic events (Cattaneo 2007). Individualising pharmacotherapy is therefore difficult.

Platelet function testing may suggest that the effect of aspirin is less than optimal in ischaemic stroke, though its role in secondary prevention is critical. The effect of aspirin in acute stroke, within 48 hours of onset, was assessed by 2 large trials. A moderate but statistically significant benefit on stroke outcome was observed (Chen 1997 & International Stroke Trial Group 1997). The primary effect of aspirin may be due to the prevention of early stroke recurrence rather than limiting the neurological consequences of the acute event (Adams Jr. et al. 2007). Overall the effect of aspirin therapy in acute ischaemic stroke, regarding short term mortality and non-fatal outcome, is less than the benefit witnessed in acute myocardial infarction (Albers et al 2004 & ISIS study group 1988) This may be explained by the variability of pathogenic mechanisms in acute ischaemic stroke- since large artery thrombosis is only identified in a proportion of ischaemic strokes (Davi and Patrono 2007 & ISIS study group 1988).

2.3.5 Aspirin resistance

Before aspirin resistance is diagnosed, compliance with therapy must be considered in the first place. One case-controlled study compared patients within 24 hours of ischaemic stroke and controls taking aspirin whom had no vascular history according to PFA-100 and rapid platelet function analyser results (Dawson et al. 2011). Poor compliance was deemed to account for nearly half of all cases of apparent aspirin 'failure'. However, even in those with objective evidence of recent aspirin ingestion according to urinary salicycluric acid measurement, resistance rates remained similar between the groups. Demonstrable aspirin resistance was supported by a further cohort study of patients admitted with suspected ischaemic stroke and already prescribed daily aspirin (Halawani et al. 2011). Incomplete platelet inhibition by aspirin was present in 43% of patients (n=22), measured by arachidonic acid (AA)- stimulated platelet aggregation. In-patient aspirin administration resulted in a significant decrease in AA aggregation ($p=0.001$), suggesting poor adherence pre-admission. Both studies support incomplete adherence accounting for approximately 50% of incomplete platelet inhibition, but alternative causes of inadequately suppressed platelets may include aspirin-independent mechanisms of recurrent ischaemia.

2.3.6 Inflammation, VWF and aspirin resistance

It is proposed that inflammatory markers in the acute phase, including VWF, may contribute to mitigating the desired aspirin effect (McCabe et al. 2005). Heightened VWF levels were linked with aspirin resistance as reflected by PFA-100 in one case-control study in 50 patients with acute ischaemic stroke all treated with aspirin 300mg/day for at least 10 days (Arslan, Yoldas, and Zorlu 2013). This was corroborated clinically with raised VWF as an explanation for aspirin resistance in recurrent stroke patients (Conway et al. 2003). The relationship of causality and/or effect of high VWF levels in cerebrovascular disease is unclear (van Schie et al. 2011).

Furthermore in the inflammatory state, platelet activation results in P-selectin (CD62P) release and expression on the platelet surface, and interaction with granulocytes and monocytes (Davi and Patrono 2007). In addition, activated platelets also express surface CD40 ligand- interacting with its counterpart on other platelets and endothelial cells. Lukasik et al used flow cytometry and biochemical evaluation of platelet activation markers to investigate the impact of aspirin on acute ischaemic stroke patients and healthy volunteers, measuring soluble CD62P, CD40, thromboxane B2 serum level and whole blood impedance platelet aggregation under arachidonic acid stimulation (Lukasik et al. 2011). Assays were performed at baseline and after 10-day treatment with aspirin (150mg od) and demonstrated no difference in the values of studied platelet-related inflammatory biomarkers between healthy individuals and acute stroke patients despite effective inhibition of COX-1 dependent platelet aggregation. It was argued that this could potentially reflect platelet hyperactivation in the course of the ischaemic event- ie stroke patients on aspirin may have limited platelet COX-1 dependent platelet aggregation, but remain exposed to pro-inflammatory mediators produced in excess by platelets at the time of the ischaemic insult.

In vitro aspirin resistance, determined by PFA-100 closure time, was correlated with increased plasma levels of VWF in a prospective study of patients treated with prophylactic doses of aspirin for secondary prevention of cardiovascular disease. Again, this suggests that a there may be a potential role for elevated VWF levels in explaining recurrent ischaemic events in patients on antiplatelet therapy and other prophylactic measures (Chakroun et al. 2004). The interaction of inflammation with the VWF-ADAMTS13 axis will be examined furthermore.

2.3.7 Future anti-platelet targets

The final common pathway of platelet activation results in the exposure of the GPIIb/IIIa binding site for a variety of ligands: including fibrinogen, VWF and fibronectin, to accord firm adhesion to the extracellular matrix and aggregation. Inhibition of GPIIb/IIIa includes antibodies (abciximab) and peptides from a snake venom disintegrin (eptifibatide); directly inhibiting ligand binding. Intravenous GPIIb/IIIa inhibitor use in acute coronary syndromes is supported by NICE.(National Institute for Health and Clinical Excellence (NICE, 2002 & ' Bhatt et al, 2000)**.** However, the role of GPIIb/IIIa inhibitors in ischaemic stroke was curtailed due to a phase 3 trial showing increased intracranial haemorrhage and mortality, as well as lack of efficacy (Adams et al. 2008).

There is gathering interest in potential therapeutic options for ischaemic stroke that extend beyond platelet aggregation inhibition or GPIIb/IIIa inhibition. Gp1b inhibition in a mouse stroke model demonstrated improved cerebral blood flow on reperfusion, identified to have exact correlation with infarct prevention on MRI compared to controls (Pham et al. 2011). Further to this, an anti GPIb-effect was demonstrated in a guinea pig model using ALX-0081, a divalent humanized nanobody directed against the GPIb-binding site on VWF A1 domain (Momi et al. 2013). Blocking this site allowed dissolution of 'fresh' intracranial thrombi, allowing reperfusion and decreased infarct size. The ALX-0081 treatment was as efficacious as tPA in achieving reperfusion if given early after vessel occlusion. It was argued that targeting the GPIb site rather than GPIIb/IIIa could incorporate limiting the inflammatory component of ischaemic brain injury: ie the GPIb interactions with immune cell recruitment. Interestingly, a combination of ALX-0081 with low-dose tPA appeared to improve thrombolysis without consequent intracranial haemorrhage, the latter perhaps the most devastating complication of the GPIIb/IIIa inhibitors. It is postulated that this confirms that GPIb-VWF interactions are not required for maintaining vascular integrity in the ischemic brain, whereas GPIIb/IIIa appears to be absolutely critical (Nieswandt et al. 2013)**.**

2.3.8 Anticoagulation and ischaemic stroke

Approximately one-quarter of patients presenting with stroke are in atrial fibrillation. It is probable that strokes occurring in patients with arrhythmia have originated from embolus from cardiac thrombus (Royal College of Physicians 2016). There is a risk of haemorrhagic transformation in all ischaemic stroke patients, and up to 6% of ischaemic stroke patients will sustain a symptomatic intracranial haemorrhage. Risk of bleeding is increased by immediate anticoagulation, particularly if the infarct is large. National guidelines have therefore mandated that anticoagulation should indeed be the standard treatment for adult patients with ischaemic stroke or TIA in atrial fibrillation, but only once imaging has ruled out haemorrhage. Anticoagulation is not recommended in uncontrolled hypertension, and delayed for at least 14 days in patients with disabling ischaemic stroke.

Aspirin 300mg od is the typical treatment recommended during this interval for adult stroke patients. Following TIA, it is recommended that anticoagulation is commenced as soon as brain imaging has excluded a bleed, using a low molecular weight heparin, oral direct thrombin or factor Xa inhibitor. The direct oral anticoagulants are now increasingly used as an alternative to warfarin for patients with atrial fibrillation in primary and secondary stroke prevention (Andersen and Olsen, 2007 &' López-López et al. 2017).

The role of anticoagulation in ischaemic stroke patients in sinus rhythm has been extensively investigated. NICE guidelines and the American Stroke Association do not recommend anticoagulants after routine acute ischaemic stroke or to prevent early recurrent stroke unless there is a specific indication such as cerebral venous thrombosis (Adams Jr. et al. 2007 &'National Institute for Health and Clinical Excellence [NICE] 2008). Meta-analysis has shown that anticoagulation does not improve functional outcome in the acute phase of ischaemic stroke, with the reduced rate of recurrence in the anticoagulated arm offset by the increased number of intracranial haemorrhages (Gubitz et al, 2008). Direct comparison of oral anticoagulants versus antiplatelet therapy showed that

anticoagulants reduced risk of stroke and other major vascular events by one third compared to the antiplatelets, but with a higher rate of intracranial haemorrhage (Aguilar et al; 2007). The British Society of Haematology acknowledged that with an ageing population, increasing anticoagulation use in atrial fibrillation and dual anti-platelet use following acute coronary syndrome; clinical situations evolve when both anticoagulation and anti-platelets may be warranted (Keeling et al. 2011). Review of evidence supports that patients with peripheral arterial disease or ischaemic stroke derive equivalent secondary antithrombotic efficacy from warfarin alone, compared to aspirin alone, or to aspirin and dipyridamole in ischaemic stroke (Anand et al. 2007; Halkes et al. 2008; WAVE Investigators 2006). More recently, rivaroxaban has not been shown superior to aspirin alone in prevention of cardiovascular death, myocardial infarction or ischaemic stroke in patients with stable atherosclerotic disease; with combined aspirin and rivaroxaban (2.5mg twice daily) demonstrating better cardiovascular outcomes yet more major bleeding than those on aspirin alone in the COMPASS trial (Eikelboom et al. 2017).The NAVIGATE study specifically examined rivaroxaban versus aspirin in prevention of recurrent stroke after a primary embolic stroke of undefined origin. Rivaroxaban was not superior to aspirin, and had a higher rate of bleeding.(Hart et al. 2018) Trials are underway examining the use of dabigatran and apixaban, while acknowledging that more tailored patient phenotyping may be the key step in determining whether antiplatelets or anticoagulants better for stroke prevention (Menon and Putaala 2018).

2.4 Thrombotic thrombocytopenic purpura: introduction to the VWF and ADAMTS13 axis

Thrombotic thrombocytopenic purpura (TTP) is a thrombotic microangiopathy, constituting a haematological emergency in the acute setting. Disseminated microvascular ischaemia quickly culminates in multiorgan dysfunction and a high mortality rate without prompt management. The hallmark feature of TTP is a deficiency of the serine metalloproteinase ADAMTS13 (a disintegrin-like and metalloprotease with thrombospondin type 1 repeats 13), whether congenital or acquired. Ultra-large von Willebrand factor (ULVWF) is secreted from the endothelium, and would normally by cleaved by ADAMTS13. In ADAMTS13 deficiency, there is no breakdown of ULVWF with subsequent uncontrolled platelet adherence and aggregation (Scully et al. 2012). Widespread microthrombi can propagate with potentially devastating sequelae in the microvasculature of the brain or heart (Scully and Goodship 2014). A profound imbalance of the VWF-ADAMTS13 axis typifies TTP.

2.5 VWF- ADAMTS13 axis in stroke

The exact pathogenesis of ischaemia, inflammation and thrombus in acute brain injury is clearly intricate and involved, encompassing many pathways of activation and co-stimulation. The specific contribution of VWF and ADAMTS13, and the balance between them, in thrombus formation and propagation, arterial occlusion and resultant ischaemic stroke is difficult to disentangle. Understanding the pathogenesis may indicate a potential role for novel therapies focusing on the VWF-ADAMTS13 axis, whether in reducing the infarct size in the acute presentation, or preventing a deterioration of cerebral perfusion in those patients that present with TIA or a more ‘stuttering’ type stroke phenotype.

2.5.1 Von Willebrand Factor

VWF, a large multimeric glycoprotein, has already been discussed as a key player in platelet recruitment and propagation of thrombosis. It enables platelet adhesion and aggregation, even in conditions of high shear stress (De Meyer et al. 2012). VWF is pivotal in the coagulation cascade since it acts as a carrier protein for factor VIII, protecting the latter from degradation, cellular uptake, or binding to activated endothelial cells or platelets (the half-life of FVIII reduced from 12 to 1-2 hours when not bound to VWF). Other roles include action as a vascular damage sensor, attracting platelets to sites of endothelial insult; as well as leucocyte adhesion and inflammatory cell recruitment. Therefore as VWF plays a key role in haemostasis by securing platelet adhesion, this is also is the hallmark of arterial thrombosis (Bernardo et al. 2005).

VWF is synthesized in endothelial cells as a monomer, subsequently made into multimers that are secreted. It is stored in a mixture of multimers in the alpha-granules of platelets and as ultra-large multimers in the Weibel-Palade bodies of endothelial cells (Shim et al. 2008). The multimers are released in response to injury or inflammation.

VWF is releated from endothelial cells in both a constitutive and regulated fashion. There is both regulated, secretagogue-stimulated release of VWF and basal, secretagogue-independent release of VWF from Weibel Palade bodies (Nightingale and Cutler 2013). In vivo release of VWF from storage pools is stimulated by adrenaline, vasopressin and nicotinic acid, and in experimental situations with fibrin, thrombin, histamine and complement proteins (Lip and Blann 1997).

Mediation of initial platelet adhesion occurs through VWF binding to GP1b-X-V receptors on platelets at sites of endothelial denudation, thereby promoting platelet adhesion, aggregation and subsequent thrombus formation (Dong et al. 2002). Under high shear stress conditions, VWF is able to initiate platelet activation through a shear-induced interaction with GpIb-IX-V, and also acting as the bond for the haemostatic plug by binding the activated form of GPIIb/IIIa. Flow microscopic techniques have visualised formation and cleavage of VWF strings following endothelial cell secretion of ULVWF multimers, and found them to be extraordinarily long, some extending to some millimetres in length (Dong et al. 2002).

Ligands for GpIb other than VWF include thrombin, high-molecular-weight kininogen, factor XII, Mac-1 (a β2 integrin expressed in neutrophils and monocytes) and P-selectin (Berndt et al. 2001). It may be the platelet adhesion receptor that is more critical than VWF since VWF-deficient mice have demonstrated delayed rather than absent arterial thrombus, whereas mice lacking the GpIb extracellular domain showed virtually absent platelet adhesion (Bergmeier et al. 2006). While it is not clear which GpIb interactions are critical for stroke thrombus formation, allelic variants of platelet GpIb, with consequent enhanced VWF/ GpIb interactions have been shown to have an increased risk of ischaemic stroke (Maguire et al. 2008). Overall this would be in keeping with clinical data recognising increased VWF serum levels as an independent risk factor for stroke (Bongers et al. 2006).

ADAMTS13 is the specific enzyme able to cleave ultra-large VWF into smaller, less adhesive multimers that circulate in plasma. The same flow microscopy work demonstrating the length of VWF strings showed rapid cleavage in the presence of plasma ADAMTS13, but with no cleavage in the presence of plasma from patients with TTP (Dong et al. 2002). ADAMTS13 thereby prevents VWF–platelet aggregation and thrombosis, which explains the pathophysiology noted in TTP. The ULVWF multimer strings with adhered platelets may occlude small blood vessels in situ. Following embolization, the now well-described clinical scenario of uncontrolled micro- and macrovascular thrombosis results, including potentially devastating cerebral and coronary infarction with simultaneous haemorrhage (Tsai 2010).

2.5.2 VWF and shear stress

With the exposure of VWF and platelets to shear stress, such as found in the normal arteriolar and capillary circulation, there is platelet aggregation. This is essential for effective haemostasis at sites of arteriolar or capillary injury, but suggests there is a microcirculatory baseline prothrombotic state that needs to be held in check (Tsai 2010). Atomic force microscopy has also demonstrated the structural change in VWF with different shear stress regimes (Siedlecki et al. 1996). VWF maintains a compact conformation while converted to a series of multimers with progressively smaller sizes. As shear stress is applied, there is a conformational transition from a globular state to an extended chain conformation with exposure of intra-molecular globular domains at a critical shear stress level. With exposure of more platelet-binding sites, there can be propogation of a prothrombotic platelet plug. This overall suggests a close structural-functional relationship of the VWF molecule (X. Long Zheng 2013).Where there is arteriolar or capillary injury, the conformationally flexible VWF is readily stretched out to an extended form for mediating platelet adhesion and aggregation essential for haemostasis (Tsai 2010).

VWF is cleaved as shear stress causes structural alteration at the domain level (I. Singh et al. 2006, 2009; X. Zhang et al. 2009). As there is structural shift, the A2 domain is exposed, containing the ADAMTS13- specific scissile bond (Wu et al. 2010). The physiological role of ADAMTS13 may therefore be considered to be a counter to intravascular platelet thrombosis, by cleaving VWF before it is activated by shear stress to cause platelet aggregation (Montaner 2015). The unfolded VWF may be protected by thrombospondin-1 from ADAMTS13 cleavage. Inactivation of ADAMTS13 by thrombin and plasmin generated at sites of vessel injury may also help minimize the cleavage of the unfolded VWF. With deficiency of ADAMTS13, VWF- platelet aggregation in the arterioles and capillaries is uncontrolled. VWF, after repetitive exposure to high levels of shear stress, is unfolded to an elongated active form that causes platelet aggregation in the circulation. Intra-vascular platelet thrombosis further increases the shear stress in the microcirculation, begetting more VWF unfolding and platelet aggregation.

2.5.3 VWF and ADAMTS13

The specificity of ADAMTS 13 for the VWF plasma protein is crucial for haemostatic control. There are several mechanisms. As already discussed, the scissile bond (Tyr- 1605=Met-1606) is 'hidden' deep in the VWF molecule and only exposed at a critical degree of shear stress (Shim et al. 2008). Platelets adhering to VWF strings offer tensile stretching for exposure of the A2 domain to cleavage. Secondly, cofactors that bind VWF facilitate recognition by ADAMTS13 (Nishio et al. 2004). Thirdly, ADAMTS13 exosites removed from the active site interact with VWF to enhance protease activity. Kinetic analysis of recombinant ADAMTS13 constructs demonstrate that the ADAMTS13 binding sites are flexible with no fixed spatial distance, and hence able to cater for variable VWF conformational shifts (Gao, Anderson, and Sadler 2008).

2.5.4 Experimental evidence for role of VWF- ADAMTS13 axis in stroke

With its pivotal role in platelet adhesion and thrombus formation, it is logical to consider that VWF may play a significant role in the pathogenesis of ischaemic stroke. Experimental evidence supports this, firstly demonstrating ischaemia to be a potent inducer of VWF secretion (Pinsky et al. 1996 &ʼVischer 2006). Zhao et al used a seminal mouse experimental model to demonstrate the role of VWF in stroke injury (Zhao et al. 2009). Three types of genetically manipulated mice (wild type, heterozygous for VWF deficiency and homozygous for VWF deficiency) were subjected to 2 hours of transient focal cerebral ischaemia by occluding the right middle cerebral artery with a 7.0-siliconized filament, simulating a MCAO (middle cerebral arterial occlusion) stroke model. Laser Doppler flowmetry was used in all mice to confirm induction of ischaemia and reperfusion. Mice were killed after 22 hours of MCAO, with coronal brain sections stained with tetrazolium chloride and brain infarct volumes measured with imaging software. In the mice with homozygous or heterozygous VWF deficiency, there was a significant decrease in infarct volume compared with the wild type mice: 2-fold reduction in *VWF-/-* mice and 40% reduction in *VWF+/-* mice. Reduction in VWF appeared sufficient to dramatically reduce stroke impact.

Secondly, the investigators evaluated the role of ADAMTS13 in ischaemic stroke, hypothesizing it would have a protective role. The same model of induced focal cerebral ischaemia was used in different mice strains, but this time with ADAMTS13 manipulated in addition to VWF: mice were either wild type, *ADAMTS13−/−* or *ADAMTS13−/−/VWF−/−*. Increased infarct volume was demonstrated in ADAMTS13−/− mice compared with wild type. This difference depended on the presence of VWF, since mice deficient in both ADAMTS13 and VWF had infarct volume similar to mice deficient in VWF alone. If deficient in ADAMTS13 alone, there was a larger infarct volume post stroke than in wild type mice.

The final step in this experimental model followed the same procedure, but with recombinant ADAMTS13 (rADAMTS13) added, derived from both human and hamster cell lines to ensure that altered protein glycosylation played no role. Additional rADAMTS13 was infused into wild type mice using the same model, and the reduced

infarct volume was also correlated with functional outcome using a tape removal test- a technique that assesses sensory and motor impairments in mice forepaw function. Mice treated with the rADAMTS13 demonstrated a significantly shortened time to remove adhesive tapes from paws, suggesting a profound improvement in sensorimotor performance. It was surmised that the rADAMTS13 endowed a protective effect when infused after cerebral ischaemia, without causing cerebral haemorrhage.

Overall, it is seen that *VWF-/-* mice are protected from cerebral ischaemia post middle cerebral artery occlusion, with *VWF+/-* mice showing a 40% reduction in infarct volume- suggesting that halving VWF expression was sufficient to dramatically reduce stroke impact (Fujioka et al. 2010; Kleinschnitz et al. 2007; L. Wang et al. 2013). Both Kleinschnitz et al and Fujioka et al showed that infusion of rADAMTS13 prior to reperfusion reduced infarct volume by 30% with improved sensorimotor function at 24 hours, without producing haemorrhage (Fujioka et al. 2010; Kleinschnitz et al. 2009). In *ADAMTS 13-/-* mice, ADAMTS13 infusion reduces thrombosis, platelet adhesion, aggregation and inflammation. Other factors associated with reduction in ADAMTS13 include thrombin, plasmin and interleukins such as IL-6 (L. Wang et al. 2013). Chauhan et al used intravital microscopy of mice mesenteric venules, genetically manipulated for ADAMTS13, to visualise thrombus formation (Chauhan et al. 2006). Venules measuring 25–30-µm in diameter were visualized in the mesentery of live mice one minute after topical superfusion of a calcium ionophore (A23187) acting to increase ionic membrane permeability. This caused endothelial activation without actually disrupting the surface. Thrombus formation was observed in *ADAMTS13−/−* mice (n = 5), whereas no microthrombi formed in *ADAMTS13+/+* mice. It was hypothesized that stimulation of Weibel-Palade bodies to secrete ULVWF in the absence of ADAMTS13 causes spontaneous thrombosis formation even without vascular injury. This was also recreated using histamine as another endothelial stimulant and secretagogue of Weibel-Palade bodies. Microscopy demonstrated that histamine promoted platelet string formation in the venules of *ADAMTS13−/−* mice, inhibited by rADAMTS13. The investigators then assessed the rate of thrombus formation in injured arterioles of *ADAMTS 13-/-* mice compared to *ADAMTS 13 +/+* mice, and found a significant difference- ie cleavage of VWF multimers by ADAMTS13 delays thrombus formation.

A rADAMTS13 concentrate was then used to demonstrate reversal of the prothrombotic phenotype of *ADAMTS 13-/-* mice: the platelet aggregate was destabilised after the infusion, and thrombus growth inhibited. The same effect was observed in wild type mice: the infused protein causing significant delay in occlusion time, suggesting a still robust antithrombotic effect even in wild-type animals. This work provided a visual demonstration of ADAMTS13 behaviour in vivo, able to act at low venous and high arterial shear stress to cleave platelet strings, control interaction with the activated vessel wall, prevent thrombi formation and modulate the thrombotic response in injured arterioles.

Moving on from ischaemic stroke, a recent publication has alluded to the potential use of rADAMTS13 in limiting microthrombi and improving neurological performance after experimental subarachnoid haemorrhage (SAH) in mice. The addition of rADAMTS13 reduced the amount of apoptotic and degenerative neurons, and appeared to decrease neuronal inflammation without altering vasospasm or systemic inflammation. Conversely, rADAMTS13 neither increased the amount of subarachnoid blood nor prolonged the bleeding time. It was postulated by the

authors that this could suggest a role for ADAMTS13 for mitigating the severity of neuronal injury after SAH, implying a role for ADAMTS-13 in limiting the clinical sequelae of haemorrhagic as well as ischaemic stroke (Muroi et al. 2014).

This was supported by further mouse work by Cai et al, demonstrating how rADAMTS13 attenuates brain injury after induced intracerebral haemorrhage in mice (Cai et al. 2015). Inflammation was reduced by rADAMTS13, reflected by levels of chemokines and cytokines, myeloperoxidase activity and neutrophil recruitment at 24 hours. Haemorrhagic lesion volume and cerebral oedema were lessened and functional outcome improved. Conversely, it was found that the anti-inflammatory effect of rADAMTS13 on cultured endothelial cells was reversed by recombinant VWF, again supporting the directly reciprocal relationship. ICAM-1 (intercellular adhesion molecule) was of particular note, known to be associated with inflammation-mediated endothelial damage, and potentiating blood-brain barrier leakage after brain injury (Zhang et al. 2005). ICAM-1 expression was significantly reduced by rADAMTS13 treatment, with reduction of pericyte loss and protection of blood-brain barrier integrity. This was not the case in neutrophil-depleted mice, suggesting that a neutrophil-mediated inflammatory process is involved. Overall it was concluded that rADAMTS13 exerts a protective effect in haemorrhagic brain injury via the reduction in inflammation-mediated blood-brain barrier dysfunction. This holds intriguing promise for future treatment of intracerebral haemorrhage.

There are few data available suggesting that ADAMTS13 levels provide a continuous variable correlating with ischaemic damage versus a threshold effect. Mouse models in the literature have focused on the manipulation of *ADAMTS13* with either homozygous or compound heterozygote mutations resulting in absolute or severe deficiency of ADAMTS13. Whether ADAMTS13 may have direct effects via VWF-independent mechanisms is unclear. A recent review suggests that ADAMTS13 actions may include down-regulation of inflammation, regulation of angiogenesis and degradation of the extracellular matrix (Feng et al. 2016). However, there is no evidence that ADAMTS13 exhibits proteolytic activity on any other substrate than VWF; and examination of clinical trends in the literature largely reflects the VWF-ADAMTS13 synergism seen in the experimental models.

2.5.5 Stroke, VWF-ADAMTS13 and inflammation

The same model of intravital microscopy was then used in a separate publication to investigate a potential link between thrombosis and inflammation (Chauhan et al. 2008). Previous in vitro evidence showed that VWF promoted leucocyte adhesion, and furthermore that leucocyte tethering and rolling under high shear stress was supported by platelets bound to ULVWF (Bernardo et al. 2005; Pendu et al. 2006). It was therefore hypothesized that ADAMTS13, via cleaving action on hyperactive ULVWF, would be able to down-regulate not only thrombosis, but also inflammation. Leukocyte rolling was visualized as a measure of endothelial activation on unstimulated mesenteric veins. ADAMTS13 deficient mice showed increased leukocyte rolling per minute in unstimulated mesenteric veins. The absence of ADAMTS13 activity on a VWF-deficient background did not increase leukocyte

rolling, suggesting that the increased leukocyte rolling observed in *ADAMTS13 -/-* mice was dependent on the presence of VWF.

Further study by the same group showed that ADAMTS13 deficiency resulted in increased Weibel-Palade body release- depicted by measuring endothelial P-selectin expression as a marker for Weibel-Palade body secretion, suggesting this could correlate with increased leukocyte rolling. Histamine was then used to stimulate the endothelium, as a secretagogue of Weibel-Palade bodies, to release ULVWF multimers with resultant VWF- platelet strings anchored to the endothelium. The in vitro phenomenon of decreased leukocyte rolling was repeated. Manipulation of P-selectin activity, an adhesion molecule thought to mediate leukocyte and platelet rolling, via a blocking antibody showed that leukocyte rolling was abolished. Other markers of inflammation were also investigated: mice were challenged with TNF- alpha and mesenteric microvenules visualised using intravital microscopy. Adherent leukocytes were approximately doubled in activated microvenules of *ADAMTS13 -/-* mice compared to *+/+* mice.

Overall, it was surmised that ULVWF multimers, released together with P- selectin from Weibel-Palade bodies by stimuli including hypoxia, inflammatory cytokines or changes in shear stress, could accelerate inflammatory responses in diseases such as atherosclerosis by slowing down leukocytes. This would support a therapeutic potential for a recombinant ADAMTS13 in limiting ULVWF multimers in ischaemic stroke. Potential mechanisms could be neutrophil extravasation across inflamed endothelium via interaction between endothelial-bound VWF and platelets mediated via platelet GP1b, and neutrophil rolling and adhering to activated platelets bound to VWF on the surface of endothelial cells (Bernardo et al. 2005). Since increased baseline leukocyte rolling in *ADAMTS13 -/-* mice has been shown to be platelet and VWF- dependent, it was hypothesized that the increased cerebral inflammation observed in ADAMTS13-deficient mice is platelet GPIb-dependent (Chauhan et al. 2008 & Khan et al. 2012).This latter study by Khan et al used an induced ischaemia mouse model to demonstrate that as well as ADAMTS13-deficient mice exhibiting a significantly enlarged infarct size, there was a relationship with markers of inflammation: increased myeloperoxidase (MPO) activity, neutrophil infiltration, and expression of the pro-inflammatory cytokines interleukin-6 (IL-6) and tumour-necrosis factor- alpha (TNF-α) (Khan et al. 2012). In contrast, VWF-deficient mice exhibited significantly reduced MPO activity, neutrophil infiltration, and inflammatory cytokine induction, suggesting a VWF- mediated role. Mice deficient for both ADAMTS13 and VWF exhibited an identical reduction of the same inflammatory parameters, which was surmised to indicate that the increased inflammation observed in ADAMTS13-deficient mice is VWF-dependent. A final interesting finding was that prior immunodepletion of neutrophils abrogated the increased infarct size observed in ADAMTS13-deficient mice, suggesting a causal role for acute inflammation for increased brain injury in the setting of ADAMTS13 deficiency.

2.5.6 Clinical evidence for role of VWF- ADAMTS13 axis in arterial thrombosis

Most evidence regarding the role of VWF in arterial thrombosis has been regarding acute myocardial infarction in cardiology literature. There is a decrease in ADAMTS13 activity following acute myocardial infarction, with an observed increase in the VWF- ADAMTS13 activity ratio- hypothesized to indicate that the reduction of plasma ADAMTS13 activity in acute myocardial infarction results from a rapid response to increased VWF activity (Kaikita et al. 2006).

Within stroke literature, the Stroke Prevention in Atrial Fibrillation Trial found VWF to be a significant predictor of both subsequent stroke ($p=0.03$) and vascular events ($p<0.01$), with the highest VWFAg levels corresponding to the greatest clinical risk. After adjusting for age, previous cerebral ischaemia, hypertension, diabetes and left ventricular dysfunction, VWF level remained a significant predictor of subsequent vascular events although non-significant for stroke per se (Conway et al. 2003). Association between VWF and relative risk of stroke was not, however, altered by CRP, ADAMTS13 or VWF genetic variability.

2.5.7 VWF and ADAMTS13 in ischaemic stroke: at presentation

Clinical data regarding the VWF-ADAMTS13 balance, or axis, in ischaemic stroke have burgeoned. An initial case-control study compared levels of VWF (not stated whether antigen or activity, measured via ELISA), fibrinogen, and P-selectin within 48 hours of presentation of patients with acute ischaemic stroke (n=163), primary intracerebral haemorrhage (n=40), and age, gender and race matched-controls (n=33) (Bath et al. 1998). VWF was significantly increased in cortical ischaemic stroke (median 158IU/dl), lacunar ischaemic stroke (median 144IU/dL) and haemorrhagic stroke (median 147IU/dL) compared to controls (median 114IU/dL) respectively. Levels of VWF remained elevated for up to 3 months, and were correlated with functional outcome as reflected by the modified Rankin score. Both VWF and fibrinogen showed a positive correlation with Rankin at 3 months post event: $r=0.371$ ($2P = 0.0006$), and $r=0.195$ ($2P = 0.042$), respectively: ie the higher the VWF, the higher the Rankin score, reflective of increased disability.

High levels of VWF: Ag and VWF:Act were also associated with an increased risk of stroke in a case-control study of 124 patients with first-ever acute ischaemic stroke compared to age- and sex- matched controls (Bongers et al. 2006) Increased risk remained similar after adjustment for inflammation, genetic variation, ADAMTS13 activity and blood group. ADAMTS13 levels were found to be lower in ischaemic stroke patients compared with controls, but not significantly (Bongers et al. 2006). Such potential correlation between VWF levels, ADAMTS13 and ischaemic stroke was also investigated in a paediatric frequency-matched case-control study, with decreased ADAMTS13 activity seen in children with ischaemic stroke but no significant difference in VWF levels (Lambers et al. 2013). However, blood sampling was conducted 6 to 12 months after stroke, so it is unclear what such convalescence levels represent.

2.5.8 VWF and ADAMTS13 in ischaemic stroke: at follow up

A Dutch study addressed this by blood sampling after a median of 95 months (range 23-146) following acute ischaemic stroke, theorising this would avoid an acute phase effect (Andersson et al. 2012). VWF and ADAMTS13 antigen plasma levels were measured in a frequency-matched case-control study of 1018 young (18-49 years) women including 175 IS patients and 205 MI patients. Increased VWF:Ag and decreased ADAMTS13Ag were associated with risk of IS and MI in a dose-dependent manner. Quartile analysis showed having both high VWF:Ag and low ADAMTS13:Ag resulted in an odds ratio (OR) of 6.9 (95% CI 2.0-23.0) for IS. Use of oral contraceptives increased the risk of IS (OR = 12; 95% CI, 5.5-26.2) and MI (OR = 7.5, 95% CI 3.6-15.7) and the risk of IS associated with low ADAMTS13Ag (OR = 5.8, 95% CI 2.7-12.4). Raised VWF:Ag and low ADAMTS13:Ag was associated with increased risk of MI and stroke in young women, compounded by the oral contraceptive pill.

Investigation of how the VWF-ADAMTS13 axis alters in the acute and later phase following stroke or TIA was more closely examined by McCabe et al (McCabe et al. 2015). ADAMTS13:Ac and VWF:Ag levels were quantified in platelet poor plasma in 53 patients in the early phase (<4 weeks) and 34 of these patients in the late phase (>3 months) after TIA or ischaemic stroke (non-TTP related) on aspirin in a prospective pilot observational analytical case-control study. ADAMTS13:Ac was reduced and VWF:Ag expression increased within 4 weeks of TIA or ischaemic stroke onset. VWF:Ag levels were increased in both early and late phases after TIA or stroke onset. There was no significant reduction of ADAMTS13Ac in the late phase, with the authors postulating that it is a dynamic protease with initial decrease in activity and then a return to baseline over time following stroke or TIA.

2.5.9 VWF-ADAMTS13 in ischaemic stroke: prospective data and a temporal relationship

Measuring VWF and ADAMTS13, either Ag or Ac or both, either in the immediate or longer-term aftermath of ischaemic stroke does not allow further extrapolation as to whether increased or decreased values represent cause or consequence; or merely reflect burden of vascular disease. Prospective work allows clearer appraisal of how the VWF-ADAMTS13 axis correlates with risk. One prospective study confirmed the association of VWF with stroke following 3358 men aged 60 to 79 years with no previous diagnosis of myocardial infarction, stroke or atrial fibrillation (Wannamethee et al. 2012). For an average of 9 years follow up, there were 187 incident stroke events. Increased levels of D-dimer and VWF activity were associated with significantly increased risk of major stroke events after adjustment for confounders, including blood pressure (adjusted hazard ratios and 95% confidence interval per standard deviation increase in D-dimer and VWF were 1.24 [95% confidence interval, 1.08-1.44] and 1.25 [95% confidence interval, 1.09-1.45], respectively).

The Rotterdam study is an ongoing prospective population-based cohort, originally designed to investigate the impact of ageing on a variety of disease states, from which a number of publications have arisen regarding ADAMTS13, VWFAg levels and stroke (Hofman et al. 2015). From this cohort, 6250 patients stroke-free at baseline

(from 1997 to 2001) with plasma samples available, and aged 55 years and above, were taken to specifically examine the impact of VWF on ischaemic stroke (Wieberdink et al. 2010). During the average follow up time of 5.0 years, 290 first-ever strokes occurred, of which 197 were ischaemic. The risk of stroke increased with increasing VWF:Ag (age and sex-adjusted hazard ratios per SD increase in VWF level: 1.12 (95% CI, 1.01 to 1.25) for stroke, 1.13 (95% CI, 0.99 to 1.29) for ischaemic stroke, slightly attenuated when adjusted for additional confounders such as ABO blood group and cardiovascular risk factors. SNPs known to be associated with these raised levels of VWF were not associated with stroke (Bongers et al. 2006 & Hanson et al. 2009).

Findings of the Rotterdam study have subsequently been expanded by adding ADAMTS13 levels into the association, and inspecting all-cause mortality (Sonneveld et al. 2016). Over a longer median follow up of 11.3 years, 6130 participants of the Rotterdam study were included. Association between ADAMTS13:Ac, VWF:Ag levels, all-cause and cardiovascular mortality was conducted by Cox proportional hazard regression analysis. Individuals with low ADAMTS13:Ac (<80.7%) had a 1.46-fold higher risk of mortality than individuals with high ADAMTS13:Ac, as measured by FRETS analysis. Those patients with the lowest ADAMTS13 had similar risk estimate for all-cause and cardiovascular mortality (HR 1.46 for both). This association was not demonstrated to be independent of VWF levels.

Vice versa, individuals with the highest VWF:Ag levels showed an increased all-cause mortality and borderline significant association with cardiovascular mortality at a higher risk estimate. Taking both factors into account, individuals with high VWF:Ag and low ADAMTS13:Ac (7.3% of cohort), compared with those with both low VWF levels and high ADAMTS13:Ac (24.1% of cohort), had an increased risk of all-cause and cardiovascular mortality of ≤1.73-fold (Sonneveld et al. 2016). With this combination, the authors postulate that levels of VWF:Ag and ADAMTS13:Ac may represent independent risk factors for cardiovascular mortality, but also demonstrate an additive effect.

The same group went on to specifically examine the longitudinal relationship between VWF:Ag-ADAMTS13 and ischaemic stroke in the same Rotterdam cohort (Sonneveld et al. 2015). Focus on 5941 individuals without history of stroke or TIA followed up for a median of 10.7 years demonstrated those with lowest quartile ADAMTS13:Ac to have a higher risk of ischaemic stroke (absolute risk 7.3%) than those in the reference highest quartile (absolute risk, 3.8%; hazard ratio, 1.65; 95% CI, 1.16-2.32). Furthermore, adding ADAMTS13:Ac to the model to predict ischaemic stroke above traditional risk factors improved accuracy of risk prediction for ischaemic stroke (p= 0.003). The prospective nature of a well-phenotyped population suggests association of reduced ADAMTS13:Ac in the development of ischaemic stroke. Limitations include no young stroke patients involved, a relatively homogenous population (middle-income, predominantly Caucasian), no classification of stroke subtypes or account of other morbidities and perhaps most notably lack of trend of consecutive markers.

In a further prospective longitudinal observational study of 595 patients with peripheral artery disease, blood samples were obtained every 2 months for up to 3 years, with haemostatic markers examined at the closest interval preceding an ischaemic cardiovascular event (Green et al. 2016). A higher VWF:Ag/ADAMTS13:Ag ratio was

seen in cases compared to controls in the 2-month period prior to an event, but significance was lost after adjustment for baseline differences in myocardial infarction, unstable angina and stroke. An important point made by the authors of the study, valid for much other work in this area, is that patients whom are most ill and least able to attend outpatient visits were excluded, and it is these very patients that may demonstrate the "most profound perturbation of haemostatic factors".

Further work examining the sequential nature of VWF:Act and ADAMTS:13 changes following ischaemic stroke suggests VWF:Act levels remain raised for up to 3 months after an acute stroke, possibly related to continued endothelial dysfunction and platelet activation even despite antithrombotic treatment (Nomura et al 2001 & McCabe et al. 2004). An interesting adjunct examined patients with non-valvular atrial fibrillation and found higher VWF:Ag and VWF:Act levels to be associated with left atrial distension and stasis; suggesting an explanation of the link between atrial stasis and thrombotic propensity (Ammash et al. 2011).

2.5.10 VWF and ADAMTS13 in ischaemic stroke: ratios and reciprocity

Focus on the relationship between VWF and ADAMTS13Ac rather than the absolute markers themselves in ischaemic stroke was also supported by a Chinese case-control cohort (Qu et al. 2016). Although a smaller series (94 patients with cerebral infarction- subtype not specified- and 103 controls respectively), an association was found between cerebral infarction and reduced ratios of ADAMTS13Ac-VWF:Ag and ADAMTS13Ac-VWF:Ac. The authors reasonably argued that reflecting on ratios was more precise in respective effect estimates, although commenting further on cause and effect was precluded by the study design.

Reciprocity of VWF and ADAMTS13 activities has been demonstrated in a number of non-TTP disorders so extrapolating to ischaemic stroke seems logical (Scully et al. 2008). In vitro examination of ADAMTS13 clearance has shown it is proteolysed by thrombin and plasmin (Crawley et al. 2005). Crawley et al suggest that this demonstrates a novel procoagulant fucntion of thrombin: as well as the well-characterised cleavage of fibrinogen, feeback activation of clotting factors and activation of platelets; inactivation of ADAMTS13 will promote thrombus growth. Increased thrombin presence during the course of a thrombotic event may cause reduction of ADAMTS13, though association does not prove causality.

2.5.11 ADAMTS13 in thrombolysis

Furthermore the interaction of the VWF-ADAMTS13 axis with thrombolysis has been manipulated in experimental models. Tissue plasminogen activator (tPA) is known to exacerbate ischaemic endothelial injury and blood-brain barrier disruption, highly predictive for subsequent cerebral haemorrhage. Intraventricular injection of either tPA or recombinant VWF (rVWF) into non-ischaemic mice induced a rapid opening of the blood-brain barrier, with recombinant ADAMTS13 (rADAMTS13) blocking loss of cerebrovascular integrity and consequent tPA-related

cerebral haemorrhage. The anti-haemorrhagic effect of rADAMTS13 was reversed by injection of rVWF (Wang et al. 2013). It is therefore postulated that rADAMTS13 could limit tPA-induced haemorrhage following stroke. Muroi et al suggest subarachnoid haemorrhage, with consequent inflammation response and cytokine release decreases ADAMTS13 activity (Muroi et al. 2014). Platelet thrombi form, without VWF proteolysis by ADAMTS13, so that progressive thrombus growth occludes microvasculature. The uncontrolled platelet-VWF string propagates further inflammation, ultimately leading to neuronal injury. Mouse models of subarachnoid haemorrhage with systemic administration of rADAMTS13, have demonstrated decreased microthrombi, decreased brain injury as reflected by microglia activation and improved neurological performance (Muroi et al. 2014; Vergouwen et al. 2014).

One of the main clinical limitations of tPA is the time limit post stroke that must be imposed to prevent delayed tPA leading to intracerebral haemorrhage and worsened neuronal damage. Recent mouse work demonstrated equivalent infarct volume reduction at 2 hours post middle cerebral artery occlusion with rADAMTS13 versus tPA (Nakano et al. 2015). However, at 4 hours post ischaemia, rADAMTS13-treated mice still had reduction of infarct volume and improved cerebral blood flow without haemorrhagic complication, compared to the massive intracerebral haemorrhage witnessed in the tPA-treated mice.

Beyond formation of thrombosis, there is increasing interest into the therapeutic potential of ADAMTS13 in thrombus dissolution. Mice data suggest that VWF is more important than fibrin in arterial thrombus formation, yet tPA uses fibrin as the substrate (Ni et al. 2000). A recent mouse experimental model with VWF-rich thrombi generated in the mice MCA found that while tPA did not wield effect, administration of rADAMTS13 quickly restored MCA patency and reduced consequent infarct size (Denorme et al. 2016). The authors postulate that cerebral thrombus composition may well be important for designing effective therapies- with substantial difference in VWF content noted in thrombi retrieved via thrombectomy- although not apparently correlated with target vessel or severity. The ADAMTS13 mechanism of action was to limit activity of VWF at thrombus surface as well as cleaving ultra-large forms linking platelets together- overall limiting further platelet engagement into thrombus (Denorme and De Meyer 2016). This introduces rADAMTS as a potential antithrombotic agent in the hyperacute setting of acute ischaemic brain injury.

2.5.12 Unanswered questions

Thus far, data regarding the role of VWF and ADAMTS13 in ischaemic stroke have shown associations rather than causality. Baseline reduced ADAMTS13 may predispose to stroke, but may also be consumed as it meets the demand of the acute outpouring of ULVWF multimers in ischaemic insult. There is no particular reason why production should be impaired in stroke, with liver function usually unaffected, so increased clearance seems a more logical hypothesis (Zheng 2015). Further work with desmopressin infusion has shown increased ULVWF multimers being secreted, with consequent steep decline in ADAMTS13 suggesting the protease is consumed as it cleaves the very large multimers (Reiter et al. 2003). Even less well characterised is the functional outcome of those patients with reduced ADAMTS13 activity at baseline, and what behaviour is seen in TIA or those patients with a

more ‘stuttering’ type of stroke phenotype. Further investigation is merited into how the VWF- ADAMTS13 axis behaves and is influenced.

In the era of development of anti-VWF and recombinant ADAMTS13 therapies for TTP, considering therapeutic potential for ischaemic stroke is pivotal. This relates to the development of recombinant ADAMTS13, now in clinical trial use in congenital TTP. The only available ADAMTS13 currently available clinically is limited: either from plasma, which cannot achieve adequate peak levels for the volumes required, or from BPL 8-Y, a plasma-derived FVIII concentrate containing ADAMTS13 (Scully et al. 2006). With both plasma and BPL 8-Y, both contain additional thrombogenic proteins (factor VIII, VWF) obviating use in ischaemic stroke, hence the potential for pure recombinant ADAMTS13. Further work is mandated to understand the mechanisms involved, and explore whether manipulation of the VWF-ADAMTS13 axis could potentially reduce infarct size in acute cerebral ischaemic presentation, make thrombolysis safer by reducing haemorrhagic propensity, regulate VWF- dependent inflammation and microvascular plugging, prevent deterioration of cerebral perfusion in patients presenting with TIA and overall reduce risk of recurrence and perhaps even mortality (Montaner 2015).

2.6 Thrombin generation

2.6.1 Thrombin generation overview

The formation of thrombin in vivo involves three procoagulant vitamin K-dependent enzyme complexes and one anticoagulant vitamin K-dependent complex. Each complex consists of a cofactor protein and a vitamin-K dependent serine protease (FIXa, FXa and FVIIa respectively). Once activated, the complex will assemble on a membrane surface provided by activated platelets or damaged cells (Mann, Butenas, and Brummel 2003). The crucial initiating step for the cascade is the interaction of factor VIIa and tissue factor. Minimal amounts of thrombin are produced during initiation but small amounts of factor Xa and IXa are produced (Lawson and Mann 1991; Mann, Butenas, and Brummel 2003). Ongoing propagation is dependent on this first interaction since the small amount of thrombin will go on to activate platelets, factor V and factor VIII. The factor VIIIa- IXa complex will catalyse factor Xa production (Lawson and Mann 1991). Herein lies the thrombin burst mechanism- the VIIIa-IXa complex is 10^5- 10^6 fold more active than IXa alone and approximately 50 times more active than the VIIa-tissue factor complex at catalysing factor X activation (Ahmad, Rawala-Sheikh, and Walsh 1992). Factor Va and Xa combined on the activated platelet membrane surface together comprise the prothrombinase catalyst, responsible for converting prothrombin to thrombin (Mann, Butenas, and Brummel 2003).

The generation of thrombin is multistep and highly regulated. Specific plasma proteins require activation. The membrane surface supporting the assembly of the complex requires platelet activation and adhesion.(Rosing et al. 1985) There are synergistic dynamic and stoichiometric inhibitory systems which overall act to dampen any potential procoagulant response (van ’t Veer and Mann 1997; Mann, Butenas, and Brummel 2003).

Thrombin generation represents the confluence of multiple haemostatic pathways, together influencing thrombin generation and consequent action on blood and vessels (H. C. Hemker et al. 2002). As proteases responsible for thrombin generation are localised and rapidly amplified at sites of vascular injury, and inhibited elsewhere in the circulation; this limits bleeding and prevents thrombosis. Thrombin generation in the laboratory is an established tool in haemostasis research, made more user-friendly by the advent of calibrated automated thrombography (CAT) (Hemker et al. 2003).

2.6.2 Basis for laboratory thrombin generation testing

The calibrated automated thrombin generation measurement was developed by the Hemker group.(Hemker et al. 2002; 2003) The principle is based on using a slow fluorogenic thrombin substrate and continuously comparing to a simultaneously run calibrator. This monitors thrombin generation automatically and allows the throughput of 100 thrombin generation curves per hour. In outline, a 96-well round bottom plate allows 20 µL of pre-warmed trigger solution (PPP reagent) to be added to one well, 20µL of pre-warmed calibrator (Thrombin Calibrator, α2M-thrombin complex) to another; with 80 µL of plasma added to both wells. Calcium is added together with the fluorogenic substrate immediately before the beginning of the measurement, and readings are done in a microtitre plate fluorometer. At the commencement of the assay, the fluorogenic substrate- calcium solution is dispensed at 20 µL into each well at zero time. The programme compares the readings from the thrombin generation wells and calibrator wells respectively as the measurement continues. Thrombin concentration is calculated and displayed in time. The software produces a curve for each well, demonstrating the calibrator signal and thrombin calculated. This preliminary result is subsequently modified by the software to calculate the newly generated thrombin. As the experiment progresses, an α2M-thrombin complex builds up from the continuous generation of thrombin in the experiment as well as the α2M normally present in any plasma. This has to be subtracted from thrombin generation as measured by the rate of conversion of chromogenic substrate, to reflect the actual amount of generated thrombin (i.e. thrombin minus the thrombin-α2M complex).

Thrombin calibrator presence allows for correction of substrate consumption with time and the colour differences between plasmas from different donors. The fluorescent molecules will also absorb light from other product molecules, thereby the optical signal will not necessarily be linearly-dependent on the concentration of fluorescent product. This 'inner filter effect' will also be compensated by the calibrator.

The CAT® calculates thrombin generation according to the following measures:

i) Lag time: clotting time, the moment at which thrombin generation begins.
ii) Endogenous thrombin potential: ETP, area under the curve, the total amount of thrombin generated in nanomolar/minute.
iii) Peak height: maximum thrombin concentration, in nanomolar thrombin.
iv) Time to peak: time to reach maximum thrombin concentration.

The lag phase will usually represent the initiation state of thrombin generation, below the level of detection of most commonly used substrates. The maximum rate, as the curve lifts off baseline, matches the propagation stage of thrombin generation. This is the thrombin burst occurring on the surface of activated platelets. Free thrombin concentration reaches a peak as thrombin generation slows following the burst. It is this time to peak that reflects the most procoagulant part of the reaction. The curve descends back to baseline as physiological inhibition kicks in and the amount of free thrombin able to cleave fibrinogen declines. The area under the curve, or ETP, reflects the amount of free thrombin present in the reaction from initiation until return to baseline (Hemker et al. 2002).

Overall, thrombin generation assays have become adopted as diagnostic, monitoring and research tools in a variety of clinical scenarios. Since the method represents global haemostasis, it can be used for perioperative management of patients with a bleeding disorder, including estimating the effect of blood products (Cate 2012). Thrombin generation is used in factor deficiencies, to distinguish between bleeding phenotypes in haemophilia and to assess response of haemophilia patients with inhibitors post bypassing agents (Berntorp 2009). At the other end of the scale, thrombin generation can be used to assess recurrent venous thromboembolism risk and hypercoagulability, but is not a standardised tool in clinical practice, mainly because of lack of reproducibility. There is also a lack of proven clinical utility, with the Cambridge Leiden study showing thrombin generation did not predict recurrent venous thromboembolism risk (van Hylckama Vlieg et al. 2015).

2.6.3 Is thrombin generation increased in acute stroke?

In summary, the evidence for increased thrombin generation is ischaemic stroke is conflicting. Firstly, it is important to note that studies thus far may not have been explicit about the mechanism of stroke. The very diverse pathological mechanisms of cardioembolic, atherosclerotic and small vessel disease may well have altered thrombin generation mechanics in place.

Heightened thrombin generation leading to stroke risk was suggested by multivariate analysis of the large Three-City study (a prospective cohort including 9294 subjects), showing high levels of ETP and peak to be associated with an increased risk of acute ischaemic stroke at 4-year follow up (Carcaillon et al. 2011). This was substantiated by a high participation rate at 4-year follow up (92%). It was suggested this linked hypercoagulability in the pathogenesis of acute ischaemic stroke, in line with other studies drawing a positive relationship between haemostatic markers and subsequent stroke risk (Danesh et al 2005 & Folsom et al. 1999). There was no relationship found between thrombin generation and coronary heart disease, the latter reflected by myocardial infarction, angina, coronary dilatation or bypass (Carcaillon et al. 2011). A further prospective cohort study of 205 stroke patients compared with healthy controls supported this, showing an overall higher thrombin generation in patients with both acute cardioembolic and non-cardioembolic stroke/ TIA (Rooth et al. 2013). This was a variable finding however, according to the thrombin generation marker- peak thrombin was significantly higher in stroke compared to controls in both the acute phase and at day-30 follow up. ETP was only higher in stroke than in

healthy controls in the acute phase, and not at follow up. Of note, there was no significant relationship in ETP or peak thrombin with the severity of stroke as measured by NIHSS (Rooth et al. 2013).

Other work suggests the reverse trend with thrombin generation and stroke risk, with a significant but inverse relationship between both ETP and peak with stroke risk in a similar cohort study (Loeffen et al. 2014). No difference in peak thrombin and thrombin potential, and no apparent impact of thrombolytic therapy, was found between patients with acute ischaemic stroke and healthy controls (Balogun et al. 2016). Balogun et al surmised that thrombin generation in platelet poor plasma was not useful in defining hypercoagulability in acute ischaemic stroke. Thrombin generation markers may reflect potential for thrombogenicity, as seen in a recent prospective pilot observational study of patients with carotid stenosis and microembolic signals on transcranial Doppler imaging (Kinsella et al. 2015). Patients with symptomatic carotid stenosis showed greater thrombin generation potential compared to those with asymptomatic disease, which was seen to decrease with time following a TIA or stroke associated with carotid stenosis.

Overall, it seems logical that thrombin generation should reflect hypercoagulability, linking carotid artery plaque lesions to recurrent vascular complications such as ischaemic stroke (ten Cate and Hemker 2016). However, laboratory markers of thrombin generation may not be reliably conveying clinical subtleties of thrombus formation in ischaemic stroke, as perhaps might be expected in such a heterogeneous condition.

2.7 Genetics and ADAMTS13 activity

The *ADAMTS13* gene consists of 29 exons located on chromosome 9. We understand that severe deficiency of ADAMTS13 activity due to variants or acquired autoantibodies inhibiting ADAMTS13 activity leads to TTP. Rare causative variants causing loss-of-function have been identified in patients with congenital TTP (Lotta et al. 2010). If the balance between VWF and its protease ADAMTS13 plays such a pivotal role in platelet aggregation and pathogenic thrombosis, understanding both genetic and acquired influences on plasma ADAMTS13 behaviour is critical.

An association between genetic variation at the *ADAMTS13* locus and ischaemic stroke may relate to ADAMTS13 plasma levels and membrane interaction. Three SNPs in the *ADAMTS13* gene were found to be associated with ischaemic stroke in patients from Western Sweden. A case-control study was performed with 600 patients presenting to stroke units in Sweden, of less than 70 years of age, compared with healthy community controls matched for age, sex and geographical residence. Genotype rs4962153 showed the uncommon A allele increased risk of ischaemic stroke: with the heterozygous state (GA) prevalence 30% (n=179) in the ischaemic stroke cohort compared to controls of 26% (n=179), and homozygous state prevalence 4% (n=21) in the ischaemic stroke cohort compared to controls of 2% (n=12); OR 1.25, 95% CI 1.01-1.54. Decreased stroke risk was seen with the uncommon alleles of two other SNPs: i) rs2285489: with heterozygous state (CT) 47% in ischaemic stroke (n=279) vs controls 50% (n=302) and homozygous state (TT) 13% ischaemic stroke (n=80) vs 16% control (n=97), OR 0.82, 95% CI 0.7-0.97; and ii) rs2301612, with heterozygous state (CG) 48% ischaemic stroke (n=290) vs 50% control (n=302), and homozygous state (GG) ischaemic stroke 20% (n=118) vs controls 23% (n=137); OR 0.85, 95% CI 0.73-1.00) (Hanson et al. 2009)**.**

De Vries et al took a systematic genome-wide assocation approach to investigate genetic variants in *ADAMTS13* that might influence activity by returning to the large prospective population-based Rotterdam study (De Vries et al. 2015). ADAMTS13 activity was measured using FRETS analysis in more than 6000 individuals, with genotyping conducted for genome wide SNPs for common genetic variants and exome-wide SNPs for rare genetic variants. The strongest genetic determinant of ADAMTS13 activity was found to be p.Ala732Val (rs41314453) on exon 18 in *ADAMTS13*; with the minor allele (frequency 1.88%) associated with a decrease of 21.7% activity. A second rarer variant (frequency 0.06%) was p.Arg1060Trp (rs142572218) on exon 24 in *ADAMTS13*; with an associated decrease of 47.6% in ADAMTS13 activity (Miyata 2015; De Vries et al. 2015). Whether these genetic variants and associated influences on ADAMTS13 activity could be found in our own cohort was of key interest, and more specifically whether there might be links with stroke incidence.

2.8 Genetics and secondary stroke prevention

2.8.1 Genetic variation and drug resistance

Clopidogrel is a thienopyridine derivative, and inhibits platelet aggregation secondary to ADP induction (Hulot et al. 2006). Its use as an anti-platelet drug to treat or prevent atherothrombotic events has meant it is used by more than 153 million patients worldwide (United Kingdom government 2013). The active metabolite of clopidogrel binds irreversibly to the platelet ADP receptor P2Y. The prodrug requires biotransformation to an active metabolite by cytochrome P-450 enzymes to achieve the active metabolite, with at least two separate CYP- dependent oxidative steps (Mega et al. 2008).

2.8.2 CYP genes: clopidogrel resistance & *CYP2Y19C*2*

Genes encoding the CYP enzymes are polymorphic, with particular alleles conferring reduced enzymatic function (Ingelman-Sundberg et al. 2007). The logical surmise would be a consequent impact on clopidogrel metabolism and hence platelet inhibition, supported by pharmacokinetic and pharmacodynamic data (Brandt et al. 2007; Kim et al. 2008). Inadequate inhibition of the platelet P2Y12 receptor after treatment with clopidogrel was most strongly predicted by the loss-of-function *CYP2C19*2* allele (rs4244285) in patients undergoing elective coronary stenting (Hochholzer et al. 2010). In terms of potential epidemiological implications, this genetic variant is seen in up to 30% of western Europeans and up to 50% of Asian ancestry (Roberts et al. 2012). A seminal meta-analysis considering its impact on cardiovascular outcome examined 1477 patients with acute coronary syndromes treated with clopidogrel (Mega et al. 2008). Carriers of a reduced-function *CYP2C19* allele had significantly lower levels of the clopidogrel active metabolite, decreased platelet inhibition and an increased rate of major cardiovascular events. Heterozygosity alone for *CYP2C19*2* was associated with a significantly increased risk of adverse cardiovascular events (hazard ratio 1.55) and stent thrombosis (hazard ratio 2.67) (Mega et al. 2008). The US Food and Drug Administration (FDA) consequently issued a boxed warning recommending "consideration of the *CYP2C19* genotype" prior to prescription of clopidogrel (Food and Drug Administration 2010).

Counter-argument came from the American Heart Association and American College of Cardiologists, claiming there was insufficient evidence for this warning (Holmes et al. 2010). Controversy about the utility of genetic testing has continued, with arguments for and against primarily directed toward patients with coronary stenting (Sibbing et al. 2011 & Pare and Eikelboom 2011).

A subsequent systematic review and critical appraisal of the evidence linking the *CYP2C19* genotype with clopidogrel responsiveness identified 32 studies of 42,016 patients reporting 3,545 cardiovascular events and 1,413 bleeding events (Holmes, P., et al. 2011). In treatment-only analysis, individuals with one or more *CYP2C19* alleles associated with lower enzyme activity had less platelet inhibition, lower bleeding rates and increased risk of

cardiovascular disease events (relative risk 1.18; 95% CI, 0.86-1.09). However, the weight of this finding was reduced by evidence of small-study bias, selective outcome reporting and genotype misclassification: overall concluding there was no significant association of genotype with cardiovascular events.

2.8.3 Clopidogrel resistance in stroke

More specifically, the focus of the debate regarding the role of *CYP2C19* in clopidogrel resistance has been firmly on adverse cardiovascular outcomes in the form of myocardial infarction and stent thrombosis. There are very limited data regarding the impact of *CYP2C19* variants on clopidogrel efficacy in stroke and cerebrovascular disease. The Clopidogrel for High Atherothrombotic Risk and Ischaemic Stabilisation, Management and Avoidance (CHARISMA) study genotyped 4819 patients with cardiovascular disease at baseline, including stroke. Rates of ischaemic and bleeding events, were compared between patients carrying alleles for either loss-of-function or gain-of-function in patients randomised to clopidogrel vs placebo (Bhatt et al. 2012). The overall analysis showed no effect of *CYP2C19* genotye on ischaemic outcome in clopidogrel-treated vs placebo-treated patients. Carriers of *CYP2C19* loss-of-function alleles had a significantly lower rate of bleeding when on clopidogrel, suggesting less anti-platelet response to clopidogrel. However, there was no increased rate in ischaemic events, nor did gain-of-function carriers have fewer ischaemic outcomes.

More recent support for examining the impact of genotyping in the efficacy of clopidogrel as secondary prevention came from the CHANCE randomised trial (Wang et al. 2016). *CYP2C19* major alleles were examined in 2933 Chinese patients enrolled with minor ischaemic stroke or TIA. Clopidogrel plus aspirin versus sole-aspirin use reduced the risk of new stroke only in the subgroup of patients not carrying the *CYP2C19* loss-of-function alleles.

2.8.4 Evidence for thrombin generation in stroke population

Using thrombin generation assays in cardiovascular disease research is not as established as in venous thrombosis (Cate 2012). Some sources demonstrated elevation of thrombin generation following acute myocardial infarction but apparently low thrombin generation appears to be associated with recurrence (Orbe et al. 2008 & Smid et al. 2011). Confounders may include vessel injury and acute inflammation. Using thrombin generation as a tool in acute stroke must allow for acute stroke being a far more multifactorial process than myocardial infarction: for example, atherosclerotic thrombosis being a very different process to arterial thromboembolism, such as that caused by atrial fibrillation.

It is also important to consider the difference between thrombin generation in vivo and in vitro (Hemker 2015).The development of thrombin in vivo is a normal phenomenon to some extent, with markers of thrombin activity present including D-dimers as reflecting degraded fibrin, prothrombin fragment 1.2 and thrombin-antithrombin (TAT) complexes. In vitro thrombin generation examines the capacity of blood to form thrombin (ten Cate and

Hemker 2016). Therefore a small child with antithrombin deficiency yet to yield clinical effect may demonstrate high in vitro thrombin generation. An adult with a provoked thrombotic event will show increased in vivo thrombin generation yet a normal capacity to form thrombin in vitro.

Rupture or abrasion of the atherosclerotic lesion provokes haemostasis. If an occlusive thrombus forms to impair downstream blood flow, myocardial infarction or ischaemic stroke may result (ten Cate and Hemker 2016). This plug is comprised of interacting platelets, clotting factors, subendothelial proteins and atheromatous debris. VWF and collagen adhere platelets. Tissue factor exposed in the lesion stimulates plasma coagulation (Davi and Patrono 2007).

A recent review has focused on evidence concerning in vivo thrombin generation via determination of i) fragment 1.2, ii) TAT complexes and/or iii) in vitro thrombin generating capacity and relation to atherothrombotic disease. Some studies showed increased TAT and F1.2 in symptomatic coronary artery disease and recurrent events (Becker et al. 1995; Rho et al. 1995; Szczeklik et al. 1992). Overall there was no clarity over whether there is a predictable relationship between the clinical picture and in vivo thrombin generation markers (Becker et al. 1995; Granger et al. 1998; Rho et al. 1995; Szczeklik et al. 1992). This also appears the case with ex vivo thrombin generation analysis. Smid et al demonstrated acute MI patients to have 7% higher ETP and 15% greater peak thrombin generation compared to controls, attributed to higher factor VIII and prothrombin (Smid et al. 2011). However, lower grade and moderate coronary arterial disease as measured via CT angiography did not link with thrombin generation (Borissoff et al. 2012). Somewhat surprisingly a low ETP versus a high ETP was predictive for recurrence of symptomatic coronary arterial disease with a similar paradoxical significant increase in thrombin generation seen with reduced APC sensitivity (Smid et al. 2011).

2.8.5 Current practice

Overall the concept of tailoring pharmacy according to individual pharmacogenomics is not yet put into practice. Such an approach currently lacks sufficient evidence, with no current randomized controlled trial that compares strategy of modifying treatment based on genetic test results with current standard of care of no testing (Markus 2012). Risks of screening, interpretation of results and influence on clinical implications remain unsubstantiated in practice (Pare and Eikelboom 2011).

3 VWF and ADAMTS13 in acute ischaemic brain injury: methods

3.1 Inclusion and exclusion criteria

The main investigation was a prospective observational investigation of the VWF-ADAMTS13 axis in acute ischaemic brain injury from March 2015 to October 2017. We investigated VWF:Ag, VWF:Act, ADAMTS13Ac, FVIII and thrombin generation in patients presenting with acute ischaemic stroke or TIA, compared with controls, and examined sequentially. This is the largest cohort examined over the hyperacute and convalescent phases, with correlation of these haemostatic markers with clinical outcome, as measured by functional stroke scores and follow up mortality. In addition to this, we examined the impact of thrombolysis, and whether genetic mutations known to influence ADAMTS13 activity had influence.

Inclusion criteria were patients of more than 18 years of age presenting with symptoms suggestive of stroke, of less than 48 hours onset, recruited from the hyperacute stroke service at University College London Hospital. This involved a full investigative pathway of clinical, laboratory and radiological assessment. Imaging consisted of either computed tomography (CT) or magnetic resonance imaging (MRI) of the head, reported by a neuroradiologist. Senior stroke physicians clinically assessed all patients, and further categorised diagnosis as ischaemic stroke, TIA, haemorrhagic stroke or none of the above following this clinical diagnostic pathway. TIA diagnosis followed the time-based definition of full resolution of all symptoms within 24 hours. Control patients were those who presented with symptoms suggestive of stroke or TIA, but for whom the diagnostic pathyway found no evidence of acute ischaemic brain injury, such as those with a subsequent diagnosis of seizure or migraine.

Exclusion criteria included secondary precipitating causes such as known active malignancy and autoimmune disease.

Clinical data were collected on cerebrovascular risk factors, admission medications, stroke subtype, disability scores and mortality. Biomarkers measured included VWF:Ag, VWF:Act, ADAMTS13 :Act and thrombin generation over the hyperacute and convalescent phase of acute ischaemic brain injury to see whether there is correlation with clinical outcome as reflected by the NIHSS and modified Rankin scoring.

3.2 Ethical approval

Prior to commencing patient recruitment, we ensured approval of the study protocol, patient information sheet, consultee information sheet, consent form and supporting documents by a main research ethics committee (REC). The REC reference number for the work was 14/EE/0169 and IRAS ID number 148936. All subsequent and substantial protocol amendments were documented and re-submitted for ethical and regulatory approval prior to implementation. Local NHS permission was obtained from the Trust Research & Development (R&D) department prior to patient recruitment. The UK Clinical Research Network (CRN) ID was 17684 and project ID 14/0088.

3.3 Clinical procedures and data collection

3.3.1 Informed consent procedure

The study adhered to section 32 of the Mental Capacity Act (2005) and the Department of Health's guidance on nominating a consultee for research involving adults who lack capacity (2008).

All patients included in the study had written informed consent. The study design allows for bloods to be taken on admission, as part of routine care, prior to consent. If the patient decided not to take part, blood samples were discarded. Patients were under no obligation to enter the study and could withdraw at any time during the study, without having to give a reason. In such situations, samples were destroyed and data removed from the paper and electronic files.

Consent from the patient directly involved explanation of the aims, methods, anticipated benefits and potential hazards of the study. Adequate time, up to 24 hours if necessary, was given for consideration by the patient before taking part. The patient information sheet (PIS) was given to each patient and consent documented.

In patients who lacked capacity to consent on their own behalf, whether due to the acute stroke or pre-existing difficulty with understanding the information, a consultee information sheet (CIS) and declaration form was made available to the stroke research practitioner. In stroke patients, this was also applicable to those with neurological deficit, such as dysphasia/aphasia, but in whom there is understanding of the consent or loss or reduced use of the dominant hand. The patient could be recruited via this route once the team had taken reasonable steps to identify someone close to the participant (if unable to consent for themselves) like family or friend and not acting in a paid capacity to advise on whether s/he would want to be involved.

To ensure that all participants were given equal opportunity to participate in the study, for participants who lack capacity and have no family or friends willing and able to fulfil the role of personal consultee, the research team nominated a third party unconnected with the research, willing to act as a nominated consultee. For the purposes of this study, a nominated consultee could be a member of the care team in the hospital who is independent of the research team. An independent practitioner information sheet and consent form were developed for this purpose. Signed original consent forms were retained at the study site and logged with the CRN.

3.3.2 Screening Period

Patients reviewed urgently in A&E with acute symptoms, and referred on to the hyperacute stroke unit (HASU), service were all considered for recruitment. Main sites of recruitment for the study were the daily TIA clinic, which general practitioners and emergency physicians could refer directly into, and the HASU in-patient wards.

3.3.3 Clinical data collection and assessment points

At presentation, detailed clinical assessment was undertaken including information regarding vascular risk factors and medications. GCS, NIHSS score and estimated premorbid modified Rankin scores (mRS) were documented on admission (Farrell et al. 1951; NINDS Study Group 1995; Quinn, Dawson, and Walters 2008; Teasdale and Jennett 1974). Investigations were undertaken as per the UCLH stroke management protocol, including routine haematological, coagulation, blood group and biochemical testing, CXR, ECG, brain CT or MRI imaging and carotid Dopplers as indicated. Data were collected on all functional scores at each time point of assessment. Subsequent diagnosis was based on results of combined clinical, laboratory and radiological assessment of a consultant neurologist or stroke physician. Diagnosis was included on all clinical discharge documents, including ischaemic stroke subtype according to the TOAST classification (Adams et al. 1993). Time points of assessment were presentation (time point zero, at point of presentation to the service), 24 hours post presentation (time point 1), 48 hours post presentation (time point 2), 7-14 days post presentation (time point 3) and any time following 6 weeks from the initial event (time point 4). Measurements at each time point were not achieved for every patient, such as those who attended the TIA clinic as outpatients.

Tables 1 and 2 below illustrate the scoring system for the detailed clinical assessments undertaken.

Table 1 outlines the modified Rankin score (mRS). This is a functional assessment tool, which aims to measure the degree of neurological disability or dependence in the daily activities of stroke patients. Table 2 outlines the NIHSS (National Institutes of Health Stroke Scale), which aims to quantitate stroke-related neurological deficit. This was originally designed as a research tool, and has now been validated in predicting lesion size and serving to measure stroke severity.

Table 1: Modified Rankin score (mRS)

Score	Characteristics
0	No symptoms at all
1	No significant disability despite symptoms, able to carry out all usual duties and activities.
2	Slight disability, unable to carry out all previous activities but able to look after own affairs without assistance
3	Moderate disability, requiring some help abut able to walk without assistance
4	Moderately severe disability, unable to walk without assistance and unable to attend to own bodily needs without assistance
5	Severe disability, bedridden, incontinent and requiring constant nursing care and attention
6	Dead

Table 2: NIHSS score (National Institutes of Health Stroke Scale) 0-42

i) Level of consciousness Ability to answer questions Ability to follow commands,	0-3 0-2 0-2 0-3
ii) Gaze	0-2
iii) Visual	0-3 (based on hemianopia)
iii) Facial palsy	0-3
iv) Motor function: arm, leg (drift, timing of drift, effort against gravity)	0-4 for each limb
v) Limb ataxia	0-2
vi) Sensory	0-2
vii) Language (degree of aphasia)	0-3
viii) Articulation (degree of dysarthria)	0-2
ix) Extinction and inattention	0-2

3.4 Research samples

Blood samples were collected at each time point of assessment as outlined above: I) on admission, ii) 24 hours post presentation, iii) 48 hours post presentation, iv) at 7-14 days post presentation if feasible and v) at follow up, typically 6 to 12 weeks post admission. Samples were centrifuged and stored at -80C before assays were performed.

At presentation, an additional EDTA was taken and stored to allow identification of genetic polymorphisms relating to ADAMTS13 and clopidogrel resistance. DNA was extracted and stored for further analysis as above. DNA will be stored once the study has been completed and patients' consent to further use of these samples, but only after the project has been agreed with the investigators and has sought new ethics review. DNA samples will be linked to the UCL Biobank.

3.5 Sample collection and preparation

At each time point, blood collection consisted of 2 citrated samples (each x 4.5ml), 1 serum sample (x 5ml) and 1 EDTA sample (x 4.5ml).

3.5.1 Blood collection protocol:

Venepuncture was performed via standard procedures. Citrated samples were taken first, ensuring the tube was correctly filled to 4.5ml, followed by the serum and EDTA samples. Phlebotomy equipment was discarded according to safety procedures.

3.5.2 Blood processing protocol: centrifugation & aliquotting

Further processing of samples was performed in the Clinical Research Facility Lab, and all standard operating procedures followed as applicable. Citrate and serum samples were centrifuged at 1500g for 10 minutes within 4 hours of sampling, with the citrate samples then double spun for the same duration. EDTA samples were not further processed. Following centrifugation of the citrate and serum samples, the supernatant was stored in labelled microtubes for freezing.

3.5.3 Sample storage

Samples were then stored at -80°C until further processing. The sample storage log was updated with relevant patient details. If a patient subsequently failed to consent, or subsequently withdrew from the study, all samples were disposed.

3.6 VWF analysis- VWF antigen (VWF:Ag)

3.6.1 Principle:

The Sysmex CS-2000i analyser was used for automated analysis. Standard and study plasmas were diluted, and mixed with latex particles coated with polyclonal antibodies to VWF. The addition of VWF- containing samples causes an antigen/ antibody complex, leading to increased turbidity in proportion to the antigen level in the test sample, hence allowing direct measurement.

3.6.2 Equipment

i) Sysmex CS2000i analyser

ii) Citrated plasma (thawed from -80°C in 37°C water bath)

3.6.3 Reagents

i) VWF:Ag kit (VWF:Ag code OPAB03, Siemens Healthcare Diagnostics, Marburg, Germany; distributed in UK by Sysmex UK Ltd), comprising:

- VWF:Ag buffer
- VWF:Ag reagent diluent
- VWF:Ag reagent (reconstituted with 4.0mL Reagent Diluent, allowed to stand at room temperature for 15 minutes before use)

ii) Standard Human Plasma (Code No. ORKL). Reconstituted with 1.0mL distilled water.

iii) Control Plasma N (Code No. ORKE). Reconstituted with 1.0mL distilled water.

iv) Control Plasma P (Code No. OUPZ). Reconstituted with 1.0mL distilled water.

v) CA-Clean I (Sysmex UK code number GSA-500A)

3.6.4 Method

Reagents were placed on the analyser following time after reconstitution. Standard curve dilutions were performed and validated following comparison to previous curves. With new reagent lot numbers, calibration was performed and quality controls run.

Samples were then processed accordingly, with the ‘medium protocol’ used.

VWF:Ag reference range (manufacturer)= 50-150 IU/dL.

Dilutions of samples were adapted accordingly- eg 1 in 2 or 1 in 4 for very high Ag levels and repeated as necessary.

3.7 VWF analysis- activity

3.7.1 Principle

An immunoturbimetric method was used to quantify von Willebrand factor activity (VWF:Act). Latex particles coated with monoclonal antibodies specific for glycoprotein Ib (GPIb) were in suspension and mixed with a blocking agent, recombinant GPIbα (rGPIbα) and dilute sample. The rGPIbα fragment contains mutations G233V and M239V, naturally occurring gain-of-function, which facilitate VWF binding without ristocetin support to the mutant rGPIbα.

Antigen-antibody reaction leads to agglutination of the latex particles, inducing an increase in turbidity. Increased turbidity results in increased absorbance of the reaction medium, the latter measured photometrically, and reflects the level of VWF:Act present in the test sample.

3.7.2 Equipment

i) Sysmex CS2000i analyser

ii) Citrated plasma (thawed from -80°C in 37°C water bath)

3.7.3 Reagents

i) Innovance VWF:Act kit (VWF:Act code OPHL035, Siemens Healthcare Diagnostics, Marburg, distributed in UK by Sysmex UK Ltd), comprising:
   a) Innovance VWF:Act Reagent 1: polystyrene particles coated with anti-GPIb monoclonal antibodies (3 x 2.0mL).
   b) Innovance VWF:Act Reagent 2: buffer, heterophilic blocking reagent (3 x 3.5mL)
   c) Innovance VWF:Act Reagent 3: rGPIb reagent (1 x 2.5mL).

ii) Standard Human Plasma (Code No. ORKL). Reconstituted with 1.0mL distilled water.

iii) Control Plasma N (Code No. ORKE). Reconstituted with 1.0mL distilled water.

iv) Control Plasma P (Code No. OUPZ). Reconstituted with 1.0mL distilled water.

v) CA-Clean I (Sysmex UK code number GSA-500A)

vi) Owren's Veronal Buffer (Code number B4234-25)

3.7.4 Method

Reagents were placed on the analyser following time after reconstitution. Standard curve dilutions were performed and validated following comparison to previous curves. With new reagent lot numbers, calibration was performed and quality controls run. Samples were then processed accordingly, with the 'medium protocol' used. Dilutions of samples were adapted accordingly- eg 1 in 2 or 1 in 4 for very high measurements and repeated as necessary.

Our laboratory verified the manufacturer's ranges using 30 normals. Our data confirmed a blood group independent range of 49-187IU/dL for both VWF:Ag and VWF:Act. For use of standardisation with other factors,

this normal range was rounded up to 50-187IU/dL (or 0.5-1.87IU/ml). A blood group specific range is not reported by our clinical laboratory but taken into consideration when reporting. The manufacturer's reference range for non-O blood groups quotes 63-200IU/dL.

Both control plasmas were supplied in siliconized vials to avoid contact activation of the coagulation system. Control plasma N (Siemens) is obtained from pooled plasma collected from selected healthy blood donors. It is stabilised with HEPES buffer solution (12g/L) and lyophilised. Control plasma P (Siemens) is obtained from pooled plasma collected from selected healthy blood donors. It is stabilised with HEPES buffer solution (12g/L), lyophilised and adjusted to defined factor concentrations.

3.8 ADAMTS13 activity analysis

3.8.1 Principle

ADAMTS13 activity was assessed by a fluorogenic technique, the Fluorescence Resonance Energy Transfer assay (FRETS). A synthetic fluorogenic 73 amino acid peptide (FRETS-VWF73) contains the amino acid naturally occurring in VWF between D1596 and R1668, incorporating the site that ADAMTS13 cleaves. Rather than the q1599 residue at P7 that occurs in the natural VWF peptide, this artificial peptide is converted to a 2, 3 dian-minopropionic residue (A2pr) modified with a 2-(N-methylamino) benzoyl group (Nma). The N1610 residue at the P5' position has been converted to A2pr modified with a 2,4-dinitrophenyl group (Dnp). The Nma group is excited at 340nm, transferring fluorescence resonance energy to the neighbouring quencher: Dnp. With ADAMTS13 presence, the bond is cleaved between Y1605 and M1606, so there is no energy transfer quenching the fluorescence, resulting in fluorescence emission from 440nm from Nma. If there is ADAMTS13 deficiency, there is no cleavage of this bond so that the fluorescence emitted by one modified residue is quenched by the other, with less or no fluorescence emitted. One point of caution is checking the baseline fluorescence reading; if this is very low, it may indicate quenching of the fluorescence signal (eg. due to high bilirubin), which will give a false, lower estimation of ADAMTS13 activity.

3.8.2 Equipment

- Fluoresecent plate reader: FLUOstar Optima (BMG Labtech) with excitation filter: 355nm and emission filter: 460nm.
- Fluoronunc C96 white maxisorp plates (Product Code 402/2011/22, VWR International Ltd)
- Polypropylene tubes
- 37°C water bath

3.8.3 Reagents

- Pooled normal plasma (PNP)
- Heat inactivated PNP (inactivated at 56°C for 30 minutes in water bath, then stored at -80°C)
- Dimethyl sulphoxide (Sigma D-8418)
- Pefabloc SC (Pentapharm: 100mg vial reconstituted with 1 ml distilled water (417.2mM), stored at -80°C. 24 μL added to 10ml Bis-Tris buffer just before use (achieving 1mM final concentration)
- Bis-Tris buffer, pH 6.0:
  Bis-Tris 5mM, 1.05g (Fluka, Sigma-Aldrich; Product 14880)
  $CaCl_2.2H_2O$, 3.68g, (VWR Product 10070)
  Made up to 900ml with deionised water, adjusted to pH 6.0 using 4M HCl, 10ml Tween 20 working solution added, made up to 1L. Addition of 24μL 1mM Pefabloc SC to 10ml Bis-Tris buffer just before use.
- FRETS-VWF73 peptide (Peptide Institute International)
  Reconstituted according to manufacturer's instructions.
- 100μL FRETS-VWF73 added to 2.4ml Bis-Tris/ Pefabloc Buffer to make a 4μM working solution.

3.8.4 Method

1) FLUOstar Optima fluorescent plate reader turned on 30 minutes before use. Fluoronunc C96 white maxisorp plate put into plate reader to warm to 37°C.
2) Bis-Tris buffer aliquotted at 10ml.
3) Patient samples, frozen reagents and QC material thawed in water bath at 37°C (heat inactivated PNP, PNP, Con A, Ph1, Pefabloc).
4) FRETS73- VWF73 peptide prepared by adding DMSO and dH_2O.
5) Pefabloc (24μL) added to 10ml Bis-Tris buffer.
6) Standard curve prepared using PNP and heat inactivated PNP in dilutions as follows: 2%, 5%, 10%, 25%, 50% and 100%.
7) FRETS working solution incubated in the 37°C water bath in the main lab for 10 minutes.
8) 100μL FRETS working solution added to each well with a multi-head pipette to decrease time removed from plate.
9) FRETS analysis for 75 minutes using the FLUOstar Optima software.
10) In house reference range 64-132 IU/dL ADAMTS13 activity was determined using 54 normal controls. Other users have quoted an observed range 46-141% (n = 40). (Hovinga et al. 2007)

3.9 ADAMTS13 antigen analysis

ADAMTS13 antigen (ADAMTS13:Ag) analysis was utilised to further examine those samples with activity less than 60IU/dL. ADAMTS13:Ag levels were quantified using a newly developed in-house ELISA.

1) Plates were covered with 3H9 mouse monoclonal anti-human antibody
2) ADAMTS13 antibody incubated overnight.
3) Following washing, these were blocked with phosphate buffered saline (PBS) and 3% dried milk powder for one hour.
4) A standard curve was created with dilutions of pooled normal plasma. All standards, tests and controls were diluted 1:100 in PBS.
5) Samples, standards and controls were then added to the duplicate wells on the microplate and incubated for 1 hour at 37°C.
6) After washing, two detection biotinylated-conjugated mouse monoclonal anti-human ADAMTS13 antibodies were prepared in PBS, '17G2' biotinylated at 1.5µg/mL, and '19H4' biotinylated at 1.5µg/mL and added to each well and incubated at room temperature for one hour.
7) After washing, Streptavidin-HRP, 1:10000 in PBS was added to each well to bind biotin and incubated at room temperature for 1 hour before a final wash and the addition of 160µl of fresh substrate to each well (15mg ophenylenediaminedihydrochloride - OPD, Sigma-Aldrich + 24ml citrate phosphate buffer + 12µl 6% hydrogen peroxide).
8) The reaction was stopped with 4M sulphuric acid before plates were read using a Dynex spectrophotometer at 490nm.
9) A normal range was determined using 100 normal controls: 74-134% was determined with a sensitivity of 0.5% based on the lower limit of detection. The intra-assay and inter-assay coefficients of variability were 3.57% and 5.04% respectively.

3.10 Thrombin generation

3.10.1 Principle:

We used the Thrombinoscope ® software to measure the capacity of plasma to generate thrombin over time, the assay as previously described by Hemker et al (2002). Tissue factor, phospholipid and calcium are added to citrated plasma to generate thrombin. The development of thrombin generation is monitored via a fluorogenic substrate. The substrate releases the fluorophore AMC (7-amino-4-methylcoumarin) upon cleavage by thrombin. The fluorophore release is measured using excitation at 390-nm and emission detection at 460-nm. Thrombin generation for each sample is compared to the activity of an α-2 macroglobulin thrombin calibrator. The software combines data from the two reactions to illustrate the sample's thrombin generation in comparison to the calibrator. Thrombin concentration generated in real time is displayed.

Thrombin generation, as measured via the fluorogenic method, is quantified using:

- The lag time (LT) from the first addition of reagents until the first burst of thrombin
- The time to peak thrombin generation
- Peak thrombin (the height of the thrombin generation curve or maximal velocity of thrombin generation)
- Endogenous thrombin potential (ETP), which is the total amount of thrombin generated over time and corresponds to the area under the curve.

An example of the thrombin generation curve is shown below.

Figure 1: Example of thrombin generation curve

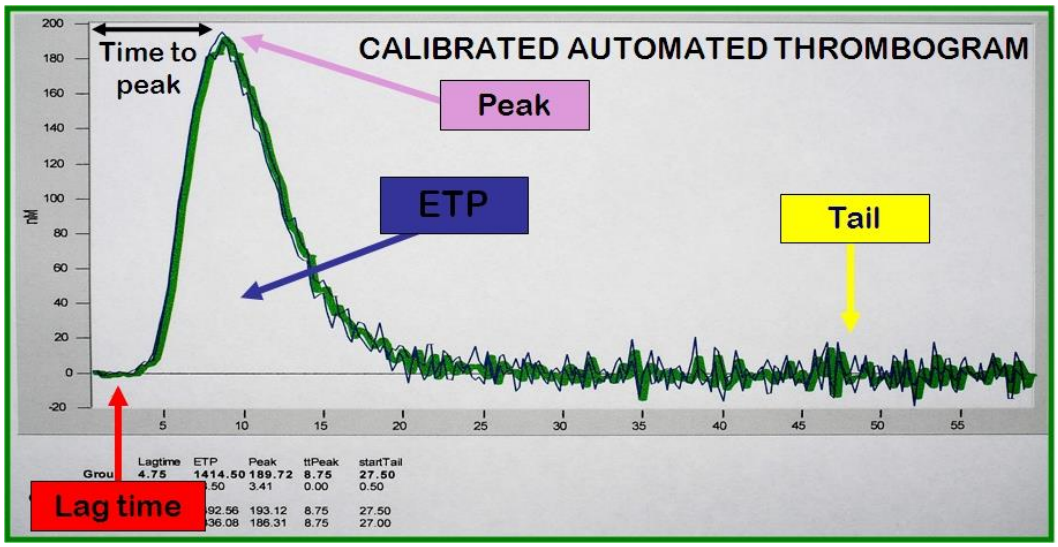


Figure 1 demonstrates a typical thrombin generation curve.
Lag time: time from first addition of reagents until first burst of thrombin.
Peak thrombin: height of thrombin generation curve or maximal velocity of thrombin generation.
ETP (endogenous thrombin generation): total amount of thrombin generated over time corresponding to the area under the curve.
Time to peak thrombin generation: measured in seconds.

Due to the use of a fluorogenic substrate there is no direct correlation between thrombin activity and fluorescent signal intensity. To overcome this, the splitting of the fluorogenic substrate is compared to a constant known thrombin activity in a parallel non-clotting sample, using Thrombin Calibrator (Diagnostica Stago), to correct inner filter and substrate consumption effects. All samples are tested in triplicate and mean values are used in further calculations. All results for each sample are calculated with the Thrombinoscope software.

3.10.2 Equipment

- Thrombinoscope software
- Fluoroscan ascent equipped with a 390nm excitation and 460nm emission filter and reagent injection system.
- 37°C water bath
- Centrifuge
- Immulon 2HB clear U-bottom 96-well plate (Diagnostica Stago Product code: 86175)
- Pipettes range 20 µL to 5mL.

3.10.3 Reagents

- Buffer: 20mM HEPES, 140nM sodium chloride, 0.02% sodium azide at pH 7.35.
- Bovine serum albumin (BSA)- Sigma-Aldrich product code: A-7030.
- 1M Calcium chloride ($CaCl_2$ solution-VWR International Ltd), product code 19046 4K.
- Dimethyl sulphoxide (DMSO)- Sigma Aldrich product code D8418.
- Thrombin fluorogenic substrate: benzyloxycarbonyl-Gly-Gly-Arg-7-amido-4-methylcoumarin (Bachem cat. Nr. I-1140)
- Platelet Poor Plasma (PPP)- Reagent (Diagnostica Stago product code TS 30.00), 5pM tissue factor and 4 microM phospholipids (4µM phospholipid vesicles (phosphatidylserine (20%), phosphatidylethanolamine (20%), and phosphatidylcholine (60%)) and 5pM recombinant human TF) (Diagnostica Stago, UK), in a final volume of 100µl (all concentrations are final).
- Thrombin calibrator (Diagnostica Stago product code TS20.00). Activity 570nM
- SSC thrombin generation reference plasma (Code 061232; NIBSC, South Mimms, UK).

3.10.4 Sample preparation

- Samples were obtained as per protocol outlined above, and stored at -80°C.
- Samples were thawed to 37°C immediately prior to assay.

3.10.5 Working reagents

- Reconstitution of fluorogenic substrate to 100nM:
- 50mg, >99% purity content 90.8%, MW 616.07, to obtain 100mM require 61.607mg/ml.
- Correct for purity (÷0.908) is 67.849mg/ml. 50mg ÷ 67.849 = 0.7369ml of DMSO.
- Wash solution i.e. 10ml deionised water (incubated at 37°C).
- Buffer: 20mM HEPES buffered saline (5mL) + 60mg/mL BSA. Gently mixed for 30 minutes on daisy wheel.
- FluCa reagent: 2.275mL buffer (HEPES/ BSA) + 0.26mL 1M $CaCl_2$ (incubated at 37°C). Immediately prior to use, 65microl of flurogenic substrate was added and vortex-mixed (2.5mM Z-Gly-Gly-Arg-AMC·HCl fluorescent substrate (Bachem, UK) in 2.5% dimethyl sulphoxide, 20mM HEPES pH 7.4 60 mg/ml BSA, and 100mM $CaCl_2$).
- 1mL of deionised water added to the tissue factor/phospholipid reagents and thrombin calibrator reagent, gently mixed.

3.10.6 Assay protocol

- Plate plan created using software
- Assay plate pattern printed and placed under microplate as pipetting guide.
- 20 µL of trigger reagent added to all thrombin generation wells using reverse pipetting technique.
- 20 µL of thrombin calibrator added to all calibrator wells using reverse pipetting technique.
- 80microl of each plasma added to respective thrombin generation and calibrator wells using reverse pipetting technique.
- Microplate incubated in instrument for 10 minutes.
- Dispenser primed with warm distilled water.
- Dispenser then primed with FluCa reagent, with injection tip thereafter placed into position M of the Fluoroscan Plate Reader.
- Plate reader automatically shaken by the microplate, and instrument automatically dispenses 20 µL of FluCa reagent to each well with monitoring for the desired time interval.
- Measurements are taken at 20 second intervals for 60 minutes using the following wavelengths: 390nm (excitation) and 460nm (emission) with a Fluoroscan Ascent FL Plate Reader (Thermo Lab System) in combination with the Thrombinoscope software (Synapse, BV), reactions all performed at 37°C.
- Injection tip transferred to container of distilled water to prevent occlusion of injector tip.
- Following end of assay, data are inspected for standard deviation of each curve. Three thrombin generation curves and three calibration curves are produced for each sample. Standard deviation should be well below 10%; therefore one of three replicates was edited to improve imprecision.
- Modifications saved and data imported into Excel for ongoing calculations.

3.10.7 Precision

Intra-assay precision data were established using lyophilised, commercial plasmas (Technoclone, Vienna). These plasmas were run 6 times each to establish intra-assay variation.

Table 3: Thrombin generation inter-assay variation

Establishment of intra-assay variability: each commercial plasma was tested 6 times to establish intra-assay variation. The thrombin generation assay controls (Technoclone, Vienna) are produced from 15-30 donations, depending on lot size. Interassay variation was calculated using a normal and abnormal plasma in each plate.

	Lag time (min)	ETP (nM/ min)	Peak thrombin (nM)
Abnormal plasma (Technoclone, Vienna**)**			
Average	3.94	1159.8	128.5
SD	0.09	51.4	3.0
CV (%)	2.2	4.4	2.4
Normal plasma (Technoclone, Vienna**)**			
Average	3.80	2530.1	365.1
SD	0.06	93.3	10.5
CV (%)	1.7	3.7	2.9

3.10.8 Calculations, reference plasma and normalisation

For each plate and for each set of samples tested, reference plasma (normal pooled plasma) is included to allow normalisation of the ETP and peak thrombin generation results, necessary to reduce the influence of day- to- day variability in reagents and assay performance. The SSC thrombin generation reference plasma was used (Code 061232; National Institute for Biological Standards and Control, South Mimms, UK). The reference plasma was tested on five different days, three times on each day in triplicate, in order to establish an average value for both ETP and peak thrombin generation. These values are: ETP 2069nM/min, and peak thrombin: 305nM.

3.10.9 Normalisation of the results

Figure 2: Example of patient thrombin generation curves compared to reference

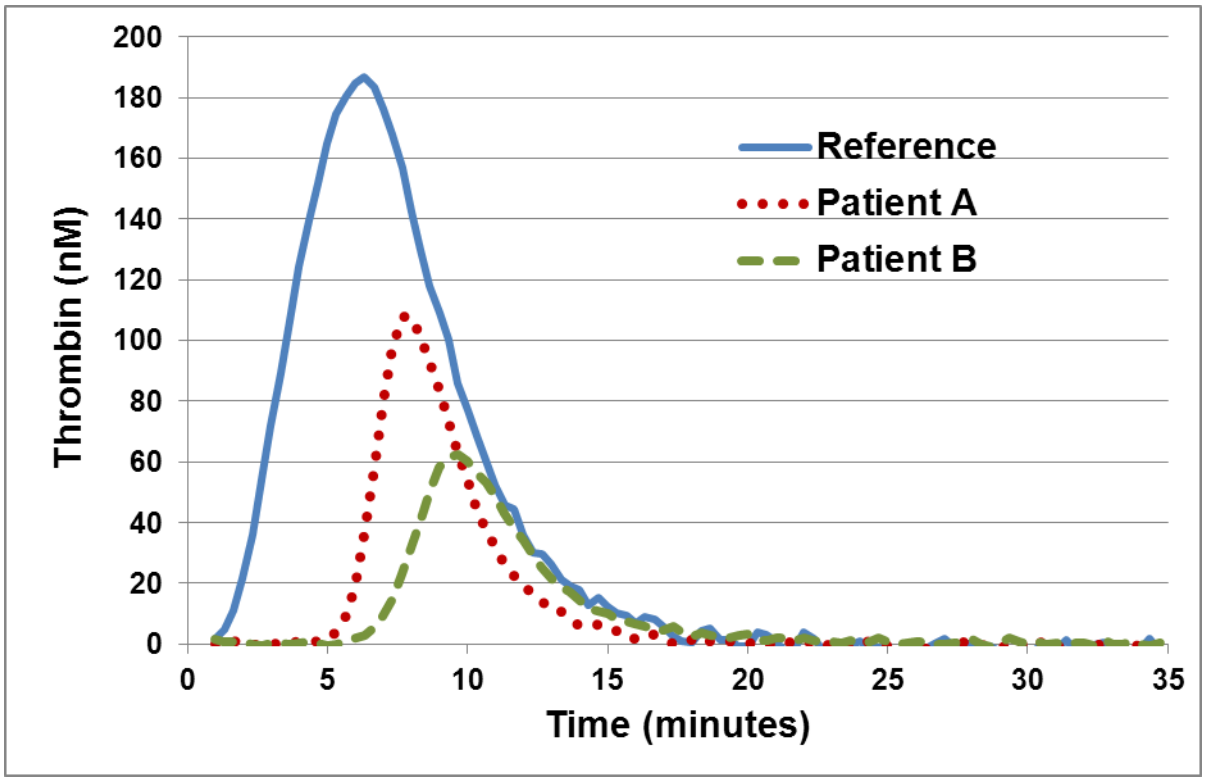


Figure 2 demonstrates a typical thrombin generation curve, illustrating how patient plasma samples are compared to a reference to allow normalisation of results.

Table 4: Example of results on day of evaluation (without normalisation)

Plasma sample	**Thrombin Generation parameters**			
	Lag (min)	ETP (nM /min)	Peak Thrombin (nM)	Time to Peak (min)
Commercial reference plasma	1.98	1239	186	6.32
Patient A	5.7	428	108	8
Patient B	7.2	315	62	9.8

Both ETP and peak thrombin generation for each test subject are normalised as a percentage of the values obtained with the reference plasma on the day of testing and then corrected for between assay drift using the average ETP and peak thrombin values obtained for the reference plasma, using the following equations respectively:

Test plasma ETP x Average reference plasma ETP/ Reference plasma ETP = Normalised ETP.

Test plasma peak thrombin x Average reference plasma peak thrombin / Reference plasma peak thrombin = Normalised peak thrombin.

A similar principle is applied for calculating the normalised peak thrombin, employing the peak thrombin result for the reference plasma obtained that day and the average value.

Example of normalisation: 428 x 100 x 2069 / 1239 x 100 = 715nM/min

Similar equations are used for calculating the normalised peak thrombin, employing the peak thrombin result for the Reference Plasma obtained that day and the average value.

Table 5: Example of results on day of evaluation (post normalisation)

Plasma sample	**Normalised Thrombin Generation Results**			
	ETP (nM/min)	Normalised ETP (nM/min)	Peak Thrombin (nM)	Normalised Peak (nM)
Reference	1239	-	186	-
Patient A	428	715	108	177
Patient B	315	526	62	102

3.10.10 Normal range

The following data, normalised against the SSC reference plasma, were obtained from 50 healthy normal subjects, using non-parametric analysis:

Lag time: 2.85- 5.29 minutes

ETP: 1308-2291 nM/min.

Peak: 173-353nM

Established for PPP- reagent Diagnostica Stago product code TS 30.00 containing 5pM Tissue factor and 4 µM phospholipids.

3.10.11 Potential errors

It has been suggested that the thrombin generation mechanism could be adversely affected by an artificial thrombin substrate acting as a competitive thrombin inhibitor (Butenas and Mann 2007). However, it appears that prothrombin conversion and inhibition of thrombin generation are minimally disturbed by the presence of substrate at the usual concentration. Disturbance of measurements can be erroneously created through incorrect sample preparation, small air bubbles, wrong pipetting and delay in starting measurement following reconstitution of PPP and Thrombin Calibrator reagents.

Standardisation of thrombin generation methodology has not yet been achieved internationally. There is variability in pre-analytical conditions such as blood sampling, storage and centrifugation. Sample quality has been associated with more than 60% of laboratory errors in coagulation tests. (Bonini et al. 2002) Comparison of results from different studies will be confounded by these influences.

Contact activation is a particular concern since haemostasis will be activated during the pre-analytical phase. It has been suggested that this is less of a concern when the tissue factor trigger is of concentration 5pM and above.(Van Veen et al. 2008) The same authors report that using frozen-thawed PPP and fresh PPP gave the same results with thrombin generation. Contact activation has been limited by other studies by performing thrombin generation measurements in PPP under standard conditions: eg using 4μM phospholipids and 5pM tissue factor.(Spronk et al. 2009; Van Veen et al. 2008) PPP has therefore been used in our protocol as the same method in which plasma was prepared for VWF and ADAMTS13 analysis.

3.11 Genetic testing: PCR

3.11.1 ADAMTS13 genetic variants

We examined whether exons 24 and 18, associated with congenital TTP, influence ADAMTS13 antigen and activity. Exon specific polymerase chain reaction (PCR) amplification was performed on each sample for ADAMTS13 exon 24 and exon 18 using Biotaq™ DNA polymerase. This was followed by standard restriction fragment length polymorphism analysis by gel electrophoresis to determine the variants present for these exons in ADAMTS13.

Primers were all supplied by Invitrogen, Thermo Fisher Scientific

Primers for exon 24 were as follows:

(Forward): ADAMTS13 exon 24/ R1060W

5’-GTG-GAC-GAG-GCG-GCC-TGT-GCG-GCG-CTG-3’ (primer name ex24R1060WBsrB1, number L0917H10, length 30, molecular weight 9387 microg/ mole, nmoles per OD: 3.1)

(Reverse): 5'-CAC-ATG-AGG-AGC-CAC-AGC-TGA-T3' (primer name ex24R1060WBsrB1Rev, number L0917H09, length 22, molecular weight 6754.4 microg/mole, nmoles per OD: 4.0)

Primers for exon 18 were as follows:

(Forward): ADAMTS13 exon 18

5'-TCC-AGT-GCC-AAG-GCC-AGC-AGC-CAC (primer name ex18A732MBstU1, number L0917H08, length 30, molecular weight 9173microg/ mole, nmoles per OD 3.1)

(Reverse) 5'GGT-AAT-GAG-AGC-CTG-CAC-AGG-3' (primer name ex18A732MBstU1Rev, number L0917H07, length 21, molecular weight 6521.2 microg/ mole, nmoles per OD: 4.1)

Expected size of ADAMTS13 exon 24 (R1060W) product was 169 base pairs.

Exon 24 digest was performed using enzyme BsrBI (Bioline, #R0102S, lot number 0081607, 10,000IU/ml, 0.1ml)

Exon 18 digest was performed using enzyme BstUI (Bioline, #R0518S, lot number 0121611, 10,000IU/ml, 0.1ml).

Following digestion, products were identified as follows:

i) Uncut- 169 base pairs
ii) Mutant- 169 base pairs
iii) Wild type- 136 base pairs.

3.11.2 CYP2C19 genetic variants

Our cohort was examined for clopidogrel resistance, as reflected by genotyping for the CYP2C19 (*2) allele and thrombin generation. Wild type phenotypes were compared to mutant, both heterozygotes and homozygotes, to investigate potential differences in baseline and follow up VWF and ADAMTS13.

Exon specific polymerase chain reaction (PCR) amplification was performed on each sample for the major allele associated with clopidogrel loss-of-response, CYP2C19 (*2), SNP 681 G>A; rs4244285. Methodology was adapted based on CYP2C19 catalytic efficiency being mainly influenced by the 681G>A polymorphism in exon 5 (CYP2C19*2, allelic frequency 15% in Caucasians) (Hulot et al. 2006). The polymorphism described creates an aberrant splice site and results in a truncated and non-functional enzyme.

PCR was performed with Biotaq™ DNA polymerase, with primers as follows (Invitrogen, Thermo Fisher Custom Primers):-

Forward = 5'-CAA CCA GAG CTT GGC ATA TTG TAT C-3'

(Primer name rs4244285SmaIFor, number L7326H12, length 25)

Molecular weight (Ug/umole): 7642.0

Nmoles per OD: 3.6

Reverse = 5'-TCC GTA GTA AAC ACA AAA CTA GTC AAT G-3'

Primer name: rs4244285SmaIRev, number L7326H11, length 28

Molecular weight (Ug/umole): 8574.6.

Nmoles per OD: 3.0

This was followed by standard restriction fragment length polymorphism analysis with Sma-I by gel electrophoresis to determine the variants present.

3.11.3 Polymerase chain reaction (PCR)

PCR reaction conditions were made up as per BioTAQ DNA polymerase guidelines (for a 20 microl reaction):

i) I microl DNA
ii) 2 microl 10x NH4 reation buffer
iii) 0.6 microl magenesium chloride
iv) 0.4 microl 10mM dNTP mix (cat-No BIO-39028)
v) 1 microl forward primer (100pmol/micol)
vi) 1 microl reverse primer (100pmol/microl)
vii) 0.1 microl Biotaq
viii) 13.9 microl nuclease-free water.

PCR was then conducted on the following cycles:

Denaturation: 94-96˚C

Bioline: 35 cycles at 64 ˚C, 30 cycles at 95 ˚C, 30 cycles at 64 ˚C, 30 cycles at 72 ˚C

Extension: 70-72 ˚C allowing 15-30 second per kb.

3.11.4 Gel electrophoresis

Gels were made up using 2% agarose gel: 50ml of TBE (Tris, Borate Acid and EDTA) to which 1gm of Agarose powder was added. The mixture was microwaved at high-power until translucent. 2.5microl of Gel Red was added and combs added to make up the wells as the gel fixed. Once gels were ready, 5 microl of PCR product was loaded with DNA loading buffer into each well of the gel. Electrophoresis was then run at 140 volts. Once gels were run, results were examined in the UV box/ Gel Doc system.

3.11.5 Restriction digest

Digestion of products was set up as follows:

i) 5 microl PCR product
ii) 1 microl Buffer 4 (#B7004S, 10x concentrate, 1.5ml, lot number 0030901)
iii) 1 microl restriction enzyme (BsrBI for exon 24 digest, BstUI for exon 18 digest, Sma-I for CYP2C19 digest)
iv) 3 microl nuclease free water.

Digestion was run overnight for 12 hours. Products were then run on a 3% agarose gel (1.5mg Agarose added to 50ml TBE) after adding to loading buffer. The smaller DNA molecules migrate faster than the larger fragments, allowing distinction of each variant according to size, matched with the known length expected to travel according to the genetic variant.

3.12 Statistics

Statistical packages used were

i) STATA for multiple regression and receiver operator characteristic (ROC) analysis.
ii) Graphpad PRISM for correlation, Mann-Whitney, Wilcoxon matched-pairs signed rank, Kruskal-Wallis and chi-square testing.

Comparison of presenting haemostatic markers between 2 groups was performed using the Mann-Whitney test if populations tested demonstrated non-parametric distribution, or unpaired *t* test if parametric distribution for unmatched data sets.
The Wilcoxon matched-pairs signed rank test was used for nonparametric matched data sets.
Kruskal Wallis testing was used to compare the medians of 3 or more groups with non-parametric distribution. Multiple linear regression, logistic regression and ROC analysis were used to determine the independent influence of VWF and ADAMTS13 on stroke scores and mortality.

4 VWF and ADAMTS13: results at presentation

4.1 Results at presentation

A total of 308 patients were recruited from March 2015 to July 2017, comprised of 149 males and 159 females, aged from 23 to 100 years (median 71). Patients were categorized into subgroups according to discharge diagnosis: ischaemic stroke (n=103), TIA (n=80), haemorrhagic stroke (n=16) or control (n=109). We attempted to recruit equal numbers to ischaemic stroke, TIA and control groups, in the knowledge that the haemorrhagic stroke cohort would be lesser according to the relative population incidence. The control cohort consisted of patients with symptoms suggestive of stroke or TIA but subsequent investigations identifying other causes such as seizure or infection. The demographic data below display that the control group was significantly younger than the ischaemic stroke and TIA groups, which will be outlined further in the discussion.

TOAST classification was applied to patients whom presented with either IS or TIA. Most patients (55%) were of 'undetermined' aetiology, a classification category that includes stroke with no clear cause or two potential causes of ischaemic brain injury- eg a patient with carotid stenosis and atrial fibrillation. In terms of past medical history, almost one-third (30%) of the entire cohort had a previous history of stroke or TIA. The most frequently occurring cardiovascular risk factor in the entire cohort was hypertension (56.8%), followed by hypercholesterolaemia (40.5%), smoking (31.1%), type 2 diabetes (16.6%) and atrial fibrillation (16.6%). Baseline medications of the entire cohort included aspirin (18.5%), clopidogrel (9.1%) and warfarin (6.1%).

Table 6: Demographic characteristics at presentation

Total cohort	**Ischaemic stroke (n=103)**	**TIA (n=80)**	**Haemorrhagic stroke (n=16)**	**Controls (n=109)**	**TOTAL= 308**
Age in years, median (range)	77 (42-97)	75.5 (25-99)	75.5 (31-88)	55 (23-100)	**71 (23-100)**
Gender: Male	56 (54% IS)	40 (40% TIA)	8 (50% HS)	45 (41% controls)	**147 (48%)**
Female	47 (46% IS)	40 (40% IS)	8 (50% HS)	64 (59% controls)	**159 (52%)**
Blood group: O	52 (50%)	42 (53%)	10 (63%)	45 (42%)	**149** (48%)
Non-O	51 (50%)	36 (45%)	6 (37%)	64 (48%)	**157** (51.7%)
Unknown	0	2 (3%)	0	0	**2**

Table 6 illustrates the baseline demographics of the entire cohort, dividied into ischaemic stroke (IS), TIA, haemorrhagic stroke and controls. The final column shows total numbers. Since the groups did not follow a normal distribution, the median and range for each is quoted.

Table 7: TOAST classification

TOAST classification	**Ischaemic stroke (n=103)**	**TIA (n=80)**	**TOTAL= 308**
Large- artery atherosclerosis	18	10	**28**
Cardioembolic	28	10	**38**
Small vessel disease	5	4	**9**
Other aetiology	4	1	**5**
Undetermined	46	48	**94**

Table 7: TOAST classification is commonly used for categorisation of ischaemic stroke and TIA. The majority of patients fell into the 'undetermined' aetiology due to two potential causes of the ischaemic brain injury (eg carotid artery stenosis and atrial fibrillation), no cause identified or incomplete investigations.

Table 8: Anti-platelet or anticoagulant medications on admission

Medications on admission	***Ischaemic stroke***	***TIA***	***Haemorrhagic stroke***	***Control***	Total number of patients on medication
Aspirin	*22*	*17*	*2*	*14*	55
Clopidogrel	*10*	*10*	*2*	*10*	32
Dipyridamole	*0*	*1*	*1*	*1*	3
Warfarin	*8*	*8*	*1*	*5*	22
Rivaroxaban	*0*	*1*	*1*	*1*	3
Tinzaparin	*0*	*0*	*0*	*1*	1

Table 8 outlines the use of antiplatelet and/ or anticoagulant medication on admission. The numbers listed are how many patients were on each medication, therefore not mutually exclusive, with some patients on more than one medication.

Table 9: Known medical co-morbidities

Co-morbidities (n=number of patients)	**IS (n=103)**	**TIA (n=80)**	**HS (n=16)**	**Control (n=109)**	**Total (n=308)**
Hypertension	*71 (68.9%)*	*47 (58.8%)*	*8 (50%)*	*41 (38%)*	***168***
Hypercholesterolaemia	*41 (39.8%)*	*37 (46.3%)*	*4 (25%)*	*38 (35.2%)*	***120***
Previous ischaemic stroke	*15 (14.6%)*	*12 (15%)*	*1 (6.3%)*	*14 (13.6%)*	***42***
Previous haemorrhagic stroke	*2 (1.9%)*	*1 (1.3%)*	*1 (6.3%)*	*1 (0.9%)*	***5***
Previous TIA	*13 (12.6%)*	*17 (21.3%)*	*3 (18.8%)*	*9 (8.4%)*	***42***
Diabetes type 1	*0*	*2 (2.5%)*	*0*	*3 (2.9%)*	***5***
Diabetes type 2	*24 (23.3%)*	*13 (16.3%)*	*1 (6.3%)*	*11 (10.7%)*	***49***
Previous venous thromboembolism	*4 (3.8%)*	*0*	*1 (6.3%)*	*1 (0.9%)*	***6***
Atrial fibrillation	*25 (24.3%)*	*12 (15%)*	*2 (12.6%)*	*10 (9.7%)*	***49***
Congestive cardiac failure	*6 (5.8%)*	*1 (1.3%)*	*0*	*4 (3.9%)*	***11***
Previous myocardial infarction	*7 (6.8%)*	*3 (3.8%)*	*0*	*2 (1.9%)*	***12***
Peripheral arterial disease	*3 (2.9%)*	*0*	*0*	*0*	***3***
Previous PCI or CABG	*5 (4.9%)*	*5 (6.3%)*	*1 (6.3%)*	*4 (3.9%)*	***15***
Smoker	*27 (26.2%)*	*22 (27.5%)*	*4 (25%)*	*39 (37.9%)*	***92***

Table 9 outlines known co-morbidities of patients included in each group.

Table 10: Functional and disability status scores of patients in each subgroup

Median scores (range)	***Ischaemic stroke***	***TIA***	***Haemorrhagic stroke***	***Controls***
Pre-admission modified Rankin score	***1 (0-5)***	***0 (0-4)***	***0 (0-2)***	***0 (0-5)***
Modified Rankin score	***3.5 (0-5)***	***0 (0-4)***	***4.5 (0-5)***	***0 (0-5)***
Baseline NIHSS	***4.5 (0-28)***	***0 (0-16)***	***12 (2-23)***	***0 (0-15)***
Baseline GCS	***15 (10-15)***	***15 (14-15)***	***14 (7-15)***	***15 (9-15)***

Table 10 outlines the classification of the functional and disability status scores of each patient in each subgroup. IS and HS demonstrated a median higher Rankin and NIHSS score at presentation compared to TIA and control groups.

Clinical classification and scoring of patients according to modified Rankin, NIHSS and GCS was made by either the physician or stroke team research practitioner recruiting to the study. To standardise the functional and stroke-specific scoring of each patient, some compromises were necessary. Firstly, NIHSS scoring is a specific stroke score, and arguably should not be applied to non-stroke patients. Secondly, the modified Rankin score (mRS) is a functional score, which can arguably not be fully assessed in an acute care setting such as resuscitation. The pre-admission mRS was based on the patient's self-description, or on that of their consenting family member. The admission mRS was based on the functional assessment possible at the time of recruitment. The range in ischaemic stroke patients included zero- ie no symptoms at all- which applied to those patients who presented at a time when their symptoms had fully resolved,, but in whom subsequent investigation revealed evidence of ischaemia. The overlap with the TIA population, and potential blur in diagnostic categories, will be discussed furthermore in the discussion.

4.2 VWF, ADAMTS13 and ischaemic stroke

Table 11: Presentation laboratory results

Presentation	Ischaemic stroke	TIA	Haemorrhagic stroke	Controls	Kruskal Wallis testing
Total number of patients= 308 (% of total cohort)	103 (33.6%)	80 (26.1%)	16 (5.2%)	108 (35.2%)	
FVIII (IU/dL) **Median** (range)	**178.6** (43.1- 612.7)	**149.7** (46.6-557.1)	**187.6** (61.3-404.9)	**142.6** (46.3-705.5)	**15.13** **(p=0.0017)**
VWF:Ag (IU/dL) **Median** (range)	**196.9** (75.4-538.1)	**167.8** (46.5-403.4)	**229.3** (63.9-398)	**160.1** (22.6- 600)	**15.74** **(p=0.0013)**
VWF:Act (IU/dL) **Median** (range)	**188.8** (65.5-338.4)	**159.8** (43.9-310.8)	**213.3** (50.2-335.4)	**140.4** (26.7-304.2)	**16.46** **(p=0.0009)**
ADAMTS13 (IU/dL) **Median** (range)	**86.0** (38.8-114.2)	**94.0** (34.5-129)	**82.4** (52.2-109)	**95.6** (38.4-137)	**27.45** **(p<0.0001)**
Ratio VWF:Ag/ ADAMTS13 **Median** (range)	**2.42** (0.79-9.53)	**1.89** (0.41-8.14)	**2.55** (0.74-4.68)	**1.69** (0.25-15.63)	**26.68** **(p<0.0001)**
ETP (nM/min) **Median** (range)	**1696.0** (315.4- 3623.7)	**1612.6** (316.4- 5456.5)	**1420.2** (353.1- 2743.9)	**1561.9** (510.4- 5591.5)	**2.17** **(p=0.539)**
Peak thrombin (nM) **Median** (range)	**264.7** (520.74- 684.2)	**267.5** (37.1- 871.6)	**285.1** (149.5 - 565)	**248.3** (18.3- 980.5)	**1.59** **(p=0.6620)**

Table 11: The laboratory results in each subgroup are outlined above. Calculation of the VWF:Ag- ADAMTS13Ac ratio for each patient was performed to capture the intraindividual balance between the haemostatic markers and examine association between the groups.

The distribution of results in each subgroup was tested, and shown to have a non-Gaussian distribution. Non-parametric testing was therefore adopted. Kruskal Wallis testing was used, as per statistician approval, as a non-parametric test comparing three or more unmatched groups. This was a preferable method to repeated unpaired t-testing. The null hypothesis was that all groups would have equal values. Significant differences were demonstrated between the groups at baseline for FVIII, VWF:Ag, VWF:Act, ADAMTS13Ac and the ratio of VWF:Ag/ ADAMTS13Ac. There was no significant difference in thrombin generation markers ETP and peak thrombin between groups.

4.3 Haemorrhagic stroke

As would be expected from epidemiological trends, the haemorrhagic stroke subgroup was extremely small (n=16). As Table 6 above reveals, the highest VWFAg, VWFAc and FVIII levels and lowest ADAMTS13 levels are seen in the haemorrhagic stroke group. This contributes to the overall significant difference between groups reflected in the Kruskal-Wallis testing. This pattern also supports the concept of a reciprocal relationship between the highest VWFAg and lowest ADAMTS13 activity. Due to the very small sample size, and different pathophysiological mechanisms in haemorrhagic stroke, this group was not included in further analysis with regard to the VWF-ADAMT13 axis in acute ischaemic brain injury.

Figure 3: Comparison of haemostatic markers between groups at presentation

Figure 3.1 VWF:Ag

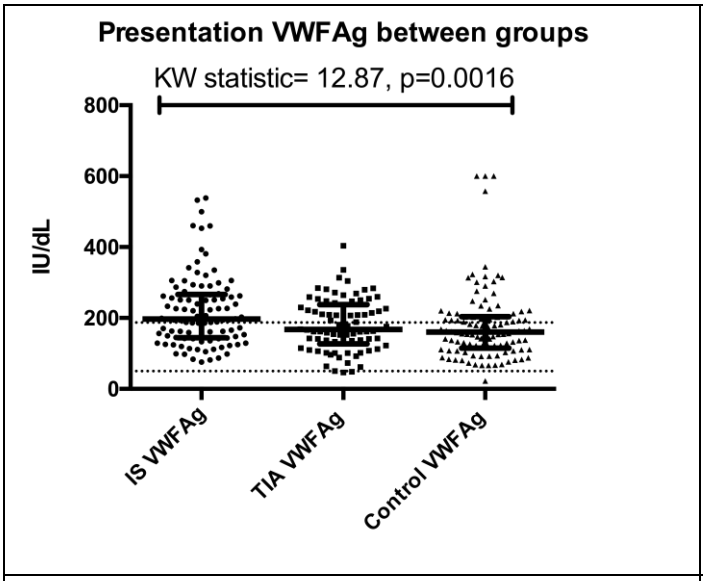
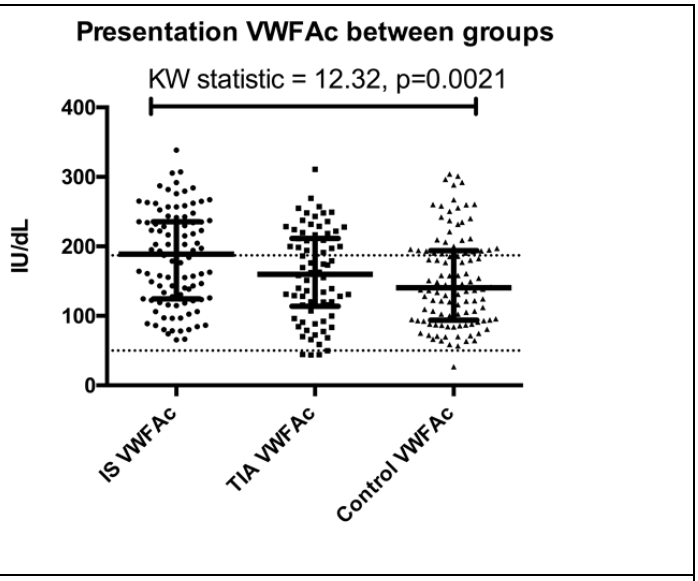


Figure 3.2 VWF:Act



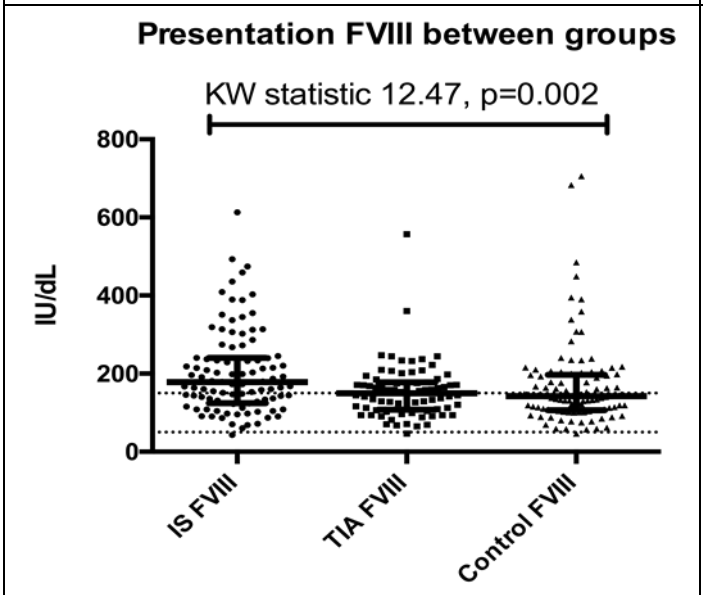


Figure 3.3 FVIII

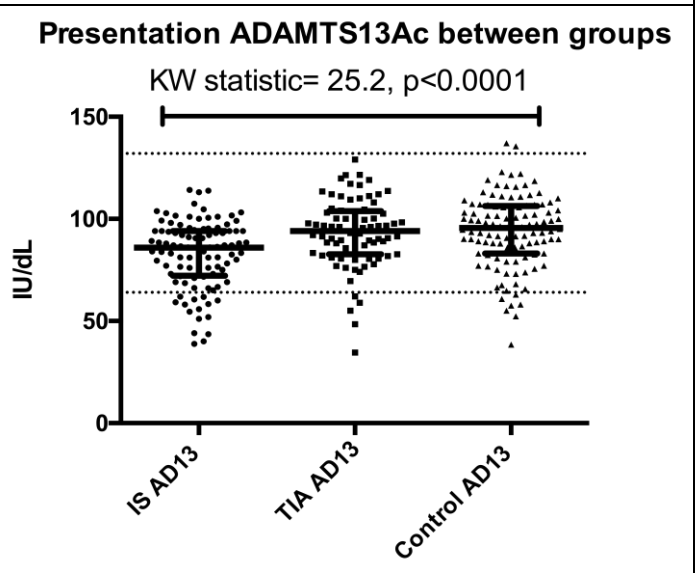


Figure 3.4 ADAMTS13

Figure 3 demonstrates Kruskal Wallis testing: comparing the medians of more than 2 groups with a non-parametric distribution, comparing ischaemic stroke, TIA and control groups at baseline for each haemostatic marker. The dotted lines illustrate the normal ranges for each haemostatic marker. The bars illustrate the median and interquartile range for each haemostatic marker.

Figure 3.1: VWFAg (normal range 50-187IU/dL). Medians: IS 196.9IU/dL (100 values), TIA 167.8IU/dL (79 values) and controls 160.1IU/dL (109 values).

Figure 3.2: VWF:Act (normal range 50-187IU/dl). Medians: IS 188.8IU/dL (99 values), TIA 159.8IU/dL (79 values) and controls 140.4IU/dL (109 values).

Figure 3.3: FVIII (normal range 50-150IU/dl). Medians: IS 178.6IU/dL (99 values), TIA 149.7IU/dL (77 values) and controls 142.6IU/dL (106 values).

Figure 3.4: ADAMTS13Ac (normal range 64-132IU/dL). Medians 86.0IU/dL (100 values), TIA 94.0IU/dL (79 values) and controls 142.6IU/dL (109 values).

Figures 3.1, 3.2 and 3.3 demonstrate significant differences between all groups, with IS demonstrating the highest median VWF:Ag, VWF:Act and FVIII, followed by TIA and the control group, in keeping with a hypothesis of relating

VWF and FVIII to arterial occlusion and hence cerebral ischaemia. The reverse trend was seen for ADAMTS13, as illustrated in Figure 3.4. This heterogeneity demonstrated by Kruskal Wallis testing was also replicated on comparing each group against the other, as illustrated in Table 10 below.

Outliers in the control group with significantly raised VWF:Ag and FVIII (both circa 600IU/dL) had significant other medical issues; including severe infection. In retrospect, despite the control group being deliberately chosen as 'stroke mimics', the group was significantly younger than the ischaemic stroke group, which has been difficult to fully adjust for in the statistical analysis. This will be discussed furthermore as one of the limitations of the study in the discussion.

Figure 4: Comparison of haemostatic markers between groups at presentation: Mann Whitney testing

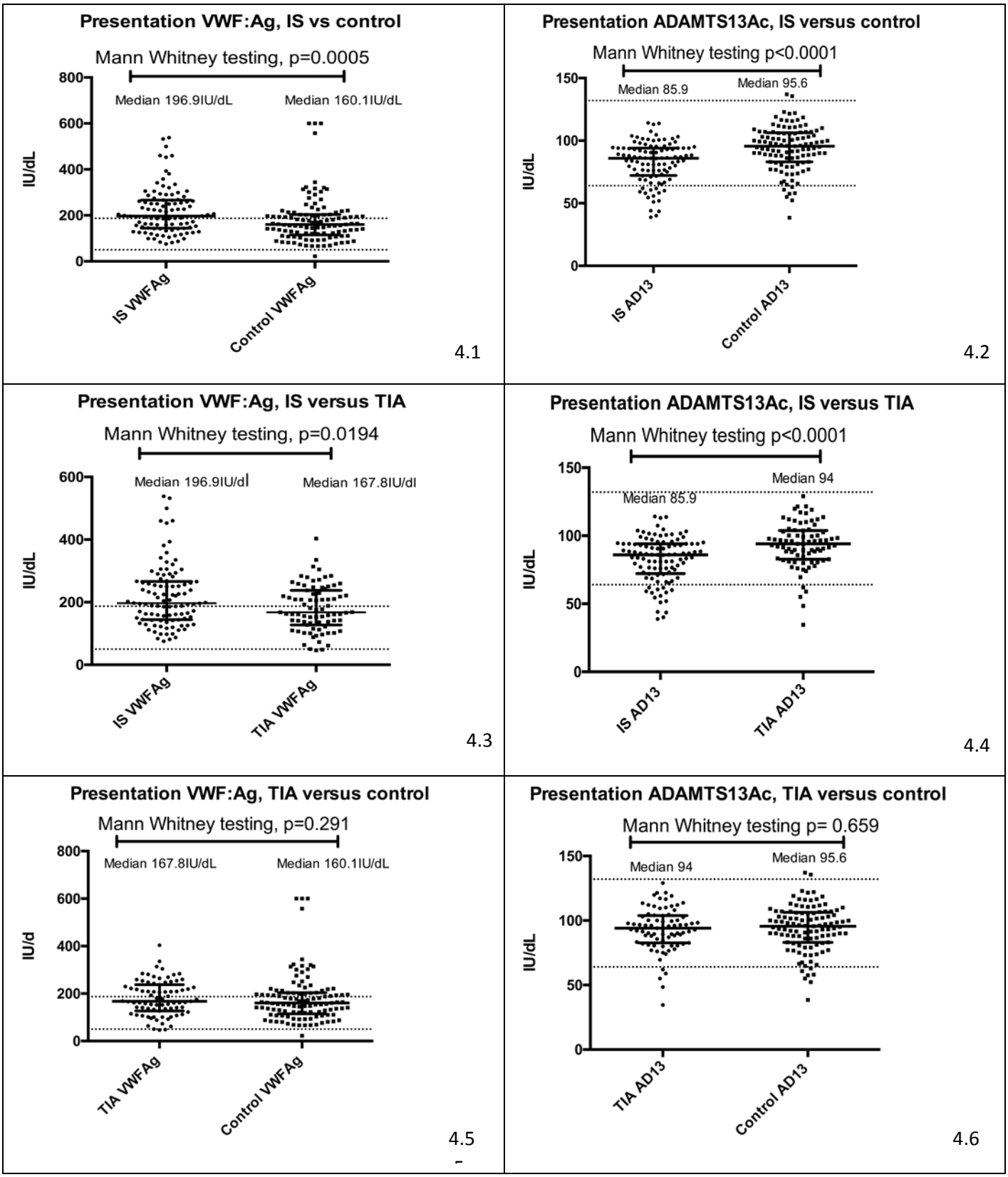


Figure 4 demonstrates Mann Whitney testing: comparing the medians directly between 2 groups: IS vs control (Figures 4.1 and 4.2) , IS vs TIA (Figures 4.3 and 4.4) and TIA vs control (Figures 4.5 and 4.6). There was a significant difference in both VWF:Ag and ADAMTS13Ac between IS and TIA groups, and between IS and control groups. There was no difference in either VWF:Ag or ADAMTS13Ac between the TIA and control groups. This supports the differences seen in the Kruskal Wallis testing above, suggesting ischaemic brain injury is associated with an imbalance of both VWF:Ag and ADAMTS13Ac. As before, the dotted lines illustrate the normal ranges for each haemostatic marker, with bars indicating the median and interquartile range .

4.4 TIA at presentation

To further investigate the TIA group, patients were divided between inpatient (IP, n=26) and outpatient (OP, n=54) cases. Outpatients were patients either seen in the walk-in TIA clinic or diagnosed and discharged immediately from A&E. Whether patients required admission was felt to be a natural distinction between those well enough to go home, or compromised enough to require admission.

Table 12: TIA cohort at presentation

Comparison of TIA patients **All values, median (range)**	**In-patients**	**Out-patients**	**P value** **(significant in bold)**
N=80	**N=26**	**N=54** ***(*clinic =46, A&E =8*)***	
Gender	M=14 F=12	M=26 F=28	
Age	84 (38-99)	71 (25-93)	**0.0288**
Baseline VWF: Ag (IU/dL)	**229.5** (48.3-335.4)	**161.0** (46.5-403.4)	**0.0029**
Baseline VWF: Act (IU/dL)	**199.8** (44.3- 310.8)	**136.3** (43.9-254.7)	**0.0173**
Baseline ADAMTS13 (IU/dl)	**95.3** (34.5- 119.8)	**92.1** (48.4-129)	0.5051
Baseline FVIII (IU/dl)	**177.5** (46.6- 557.1)	**128.9** (65- 207.2)	**<0.0007**
Baseline ETP (nM/min)	**1451.2** (318.2 – 5456.5)	**1614.2** (316.4- 5292.5)	0.263
Baseline peak thrombin (nM)	**280.0** (42.1- 871.6)	**263.9** (37.1-745.6)	0.712

Table 12 shows the comparison of patients with TIA divided into inpatients admitted on the ward versus outpatients seen in A&E or in the daily TIA clinic. Median age was significantly higher for IP vs OP (84 vs 71 years, p=0.0288). Presentation VWF:Ag, VWF:Act and FVIII were higher in IP compared to OP (in IU/dL: VWF:Ag 229.5 vs 161.0, p=0.0029; VWF:Act 199.8 vs 136.3, p=0.0173; FVIII 177.5 vs 131.3, p=0.0007). There was no significant difference in presentation ADAMTS13 activity (inpatients 95.3IU/dL vs outpatients 92.IU/dL, p=0.505).

4.5 Blood group O vs non-O: association with VWF:Ag, VWF:Act, FVIII and ADAMTS13:Ac

Table 13: Comparison of blood group O/ non-O according to presentation haemostatic markers

(Median, IU/dL)	Blood group O	Blood group A/B/AB	P value (unpaired t test)
FVIII	140.0 (43.1-705.5)	164.5 (58.6- 682.9)	**0.0008**
VWF:Ag	161.4 (22.6-600)	193.6 (61.4- 600)	**0.0061**
VWF:Act	148.3 (26.7- 335.4)	170.6 (49.8- 338.4)	**0.0081**
ADAMTS13 Ac	90.7 (34.5-129)	92.0 (38.4-137)	0.545

Table 13 shows the haemostatic markers of all those in the overall cohort according to whether blood group O versus blood group A, B or AB.

It is well recognized that blood group O is associated with a lower VWF:Ag than those who are non- blood group O (A, B or AB). Comparing blood group O versus non-O showed significantly decreased VWF:Ag, VWF:Act and FVIII in the overall cohort, but no difference in ADAMTS13Ac (Table 11), as per previous publication (Alpoim et al. 2011; Rios et al. 2012). The ischaemic stroke, TIA and haemorrhagic stroke subgroups were examined independently, demonstrating no difference in VWF:Ag, FVIII and ADAMTS13 Ac within each group according to whether blood group O versus non-O (Tables 14 to 16 respectively). There was a significant difference in VWF:Act in the TIA group (133.7IU/dL in blood group O compared to 169.6IU/dL in blood groups A, B and AB, p=0.0431), but not in VWF:Ag.

In contrast, the control group showed blood group O versus non-O had significantly decreased VWF:Ag, VWF:Act and FVIII as in the overall cohort (Table 17). This could suggest that an increased VWF and FVIII as acute phase reactants in IS and TIA overrides the usual blood group-derived differences. The usual difference is restored at convalescence (VWF:Ag in groups A/B/AB at final follow up 184.7 vs group O 143.1IU.dL, p=0.0131; FVIII in groups A/B/AB 158.3 vs group O 135.0IU/dL; p=0.0095).

4.5.1 Blood group O vs non-O: association with VWF:Ag, VWF:Act, FVIII and ADAMT13

Table 14: Ischaemic stroke cohort blood group comparison

(Median, IU/dL)	Overall IS	Blood group O (n=52)	Blood group A/B/AB (n=51)	P value (unpaired t test)
FVIII	**178.6** (43.1-612.7)	**160.1** (43.1- 612.7)	**186.7** (68.6- 474.4)	0.179
VWF:Ag	**196.9** (75.4- 538.1)	**191.6** (75.4- 460.2)	**215.3** (81.5- 538.1)	0.135
VWF:Act	**188.7** (65.5- 338.4)	**184.2** (65.5-307)	**196.2** (74.3-338.4)	0.343
ADAMTS13Ac	**86.0** (38.8- 114.2)	**81.0** (38.8-113.8)	**88.0** (43.6-114.2)	0.165

Table 15: TIA cohort blood group comparison

(Median, IU/dL)	Overall TIA	Blood group O (n=42)	Blood group A/B/AB (n=36)	P value (unpaired t test)
FVIII	**149.7** (46.6- 557.1)	**134.3** (46.6- 557.1)	**158.8** (68.8- 246.8)	0.105
VWF:Ag	**167.8** (46.5-403.4)	**148.4** (46.5- 335.3)	**192.7** (61.4-403. 4)	0.0845
VWF:Act	**159.8** (43.9-310.8)	**133.7** (43.9- 257)	**169.6** (49.8-310.8)	**0.0431**
ADAMTS13Ac	**94** (34.5-129)	**95.9** (34.5-129)	**91.4** (55-119.8)	0.497

Table 16: Haemorrhagic stroke cohort blood group comparison

(Median, IU/dL)	Overall HS	Blood group O (n=10)	Blood group A/B/AB (n= 6)	P value (unpaired t test)
FVIII	**187.6** (61.3-404.9)	**220.3** (61.3- 404.9)	**168.2** (100.9- 298.2)	0.535
VWF:Ag	**229.3** (63.9- 398.0)	**242.0** (63.9- 398.0)	**206.0** (127.9- 281.2)	0.468
VWF:Act	**213.3** (50.2- 335.4)	**223.0** (50.2- 335.4)	**184.3** (95.1-275.3)	0.603
ADAMTS13Ac	**82.4** (52.2-109)	**85.6** (52.2- 109)	**79.4** (67.5- 99.3)	0.854

Table 17: Control cohort blood group comparison

(Median, IU/dL)	Overall control	Blood group O (n=45)	Blood group A/B/AB (n=64)	P value (unpaired t test)
FVIII	**142.6** (46.3- 705.5)	**108.5** (46.3- 705.5)	**156.6** (58.6- 682.9)	**<0.0001**
VWF:Ag	**160.1** (22.6- 600)	**141.5** (22.6- 600)	**178.1** (80.9- 600)	**0.0064**
VWF:Act	**140.4** (26.7-304.2)	**106.9** (26.7- 301.1)	**149.9** (70- 304.2)	**0.0038**
ADAMTS13Ac	**95.6** (38.4- 137)	**93.9** (52.3- 122.9)	**97.3** (38.4- 137)	0.994

4.6 Clinical correlation

Simple correlation (Spearman rank) showed positive association of VWF:Ag with age, modified Rankin and NIHSS and negative with GCS. The inverse relationships were seen with ADAMTS13 on correlation (ie negative association with modified Rankin, NIHSS and age; and positive with GCS). Correspondingly, the VWF:Ag-ADAMTS13 ratio showed positive association with age, modified Rankin and NIHSS and negative with GCS: reflective of correlation with disability (see Table 18 below).

Table 18: Correlation of presentation functional scores and laboratory parameters (Spearman rank non-parametric correlation)

IU/dL, **(95% C.I.)**	**Age**	**Modified Rankin**	**NIHSS**	**GCS**
VWF:Ag	**0.531** (0.439- 0.611) p<0.0001	**0.396** (0.289-0.493) p<0.0001	**0.267** (0.152- 0.375) p<0.0001	**-0.222** (-0.332- -0.105) p=0.0002
ADAMTS13Ac	**-0.328** (-0.430- -0.218) p<0.0001	**-0.298** (-0.404- -0.185) p<0.0001	**-0.283** (-0.389- -0.169) p<0.0001	**0.194** (0.0769- 0.306) p=0.0009
VWF:Ag-ADAMTS13Ac	**0.577** (0.491-0.651) p<0.0001	**0.447** (0.345- 0.538) p<0.0001	**0.337** (0.227- 0.439) p<0.0001	**-0.255** (-0.363- -0.140) p<0.0001

4.7 Influence of anticoagulant and antiplatelet therapy

4.7.1 Anticoagulant therapy

In the IS group, numbers were too small to draw firm conclusions about the relative impact of warfarin therapy on the VW:Ag- ADAMTS13 axis. Mann Whitney testing between groups did not demonstrate any difference with warfarin therapy on the presentation VWF:Ag: warfarinised (n=8) vs not (n=94); 238.8 vs 194.3IU/dL, p= 0.0677, ADAMTS13Ac (warfarinised vs not, 82.5 vs 86IU/dL, p=0.8144) or the overall VWF:Ag/ADAMTS13Ac ratio (warfarinised vs not, 3.351 vs 2.308, p=0.0576).

Expected reduction in thrombin generation with anticoagulation was apparent: warfarinised vs not, ETP 790 1730NM/min, p<0.0001; peak thrombin 85.06 vs 271.6nM, p<0.0001).

4.7.2 Anti-platelet therapy

In the IS group, anti-platelet therapy at baseline was also examined, inclusive of aspirin, dipyridamole and clopidogrel. There were no differences noted in patients on anti-platelet therapy (n=32) compared to those not (n=71) respectively using Mann Whitney testing: VWF:Ag 197.4 vs 196.4IU/dL, p=0.969; ADAMTS13 86.0 vs 84.95IU/dL, p=0.662; ratio VWF:Ag-ADAMTS13Ac 2.441 vs 2.401, p=0.91; ETP 1797 vs 1671nM/ min, p= 0.654; peak thrombin 285.6 vs 255.4nM, p=0.526.

4.8 Laboratory markers and VWF-ADAMTS13: multiple linear regression

Further potential relationships between the presentation VWF:Ag-ADAMTS13Ac balance and other laboratory measurements were investigated for the entire cohort. Exploration of the data using scatter graphs did not demonstrate linearity of relationship between the VWF:Ag- ADAMTS13Ac ratio and haemoglobin, platelet count, prothrombin time, activated partial thromboplastin time or fibrinogen. There was linearity seen with creatinine, bilirubin, CRP and ETP when plotted against the VWF:Ag-ADAMTS13Ac ratio on scatter graphs.

Multiple linear regression was then performed with the following assumptions:

1) Explanatory variables were reasonably independent of one another.
2) Any relationship between a given explanatory variable and the outcome (or other dependent variable) is linear.
3) Residual variance is normally distributed.
4) Variance of error terms is similar across all explanatory variables.

Firstly, the following regression model was produced with all laboratory variables, including those not expected to show significance such as haemoglobin and platelet count, to demonstrate the use of the regression modelling.

Table 19: Regression of presentation laboratory markers (independent variables) against VWF:Ag-ADAMTS13 ratio (dependent variable)

Source	SS	df	MS	Number of obs= 206
Model	308.79	7	44.11	F (7,198) = 28.34 Prob > F = 0.00
Residual	308.22	198	1.56	R-squared= 0.501 Adj R squared= 0.483
Total	617.02	205	3.01	Root MSE = 1.25

Stata command: regress RatioVWF:Ag-AD13Ac Age Creatinine Bilirubin CRP Hb Plt ETP					
Ratio VWF:Ag-ADAMTS13Ac	Coefficient	Std error	t	P>t	95% confidence interval
Age	-.027	.00539	5.02	0.000	-.0164 - .0378
Creatinine	.00818	.00174	4.71	0.000	.00476 - .0116
Bilirubin	.0352	.140	2.52	0.013	.00763- .6277
CRP	.046	.00541	8.54	0.000	.355- .0569
Haemoglobin	-.00417	.00575	-0.72	0.469	-.0155- .00718
Platelet count	-.00140	.00110	-1.27	0.204	-.00357- .000769
ETP	.0000279	.000101	0.28	0.783	.000172- .000228
cons	-.0251	1.038	-0.02	0.981	-2.07- 2.02

Key: Regression modelling in Stata:

Source: source of variance, Model, Residual and Total. Total variance is divided into i) the variance which can be explained by the independent variables (Model) and ii) the variance which is not explained by the independent variables (Residual).

SS: Sum of Squares. SS for the Model and Residual add up to Total Variance; **df: degress of freedom** associated with sources of variance.

MS: Mean Squares. Sum of Squares divided by respective df

Table 19: This regression model illustrates regression of the entire cohort at presentation, with the VWF: Ag-ADAMTS13Ac ratio as the dependent or outcome variable; with age, creatinine, bilirubin, CRP, haemoglobin, platelet count and ETP as independent variables. Interpretation of the model is as follows. Firstly, the F test is that the R-square for the model is zero, or the model does not explain variability in the depedent variable. The p value for the F test is zero, which means that the model is significant for some explanatory power of the dependent variable. Secondly, an R square of 1 means the model explains all variability in the dependent variable (the ratio of VW:Ag-ADAMTS13Ac). An R square of 0 means the model explains no variability in the dependent variable, or has no explanatory power. The R-square of the model is 0.501, meaning that the model explains 50% of the overall variability in the VWF: Ag-ADAMTS13 ratio. This is adequate, though a strong model would demonstrate an R-square of 0.7 or 70% plus.

Each of the independent variables can be examined in turn to see their impact on the dependent variable. Age has a negative coefficient in this model, which suggests its association with the VWF:Ag-ADAMTS13Ac ratio to be negative. Previous data suggest that the association with the ratio is positive. The 95% confidence interval (CI) crosses zero so this finding is not significant, and this model does not suggest age contributes to the ratio. Creatinine, bilirubin and CRP all have positive coefficients in the model, with p>t values of <0.05 and 95% CIs not crossing zero. This means that each of these variables are positively associated with the VWF:Ag-ADAMTS13Ac ratio, and have exaplanatory power for variability in the ratio in this model. Haemoglobin and platelet count can be discounted as having explanatory power: both by P values and by 95% CIs crossing zero; and are therefore not relevant for the VWF:Ag-ADAMTS13Ac ratio. ETP similarly demonstrates no significance according to the p value for the t-statistic.

Therefore in summary, this model demonstrates 50% explanatory power for variability in the VWF:Ag-ADAMTS13Ac ratio. Creatinine, bilirubin and CRP are relevant independent variables.

4.9 Entire cohort: multiple linear regression of clinical influences on the VWF:Ag-ADAMTS13 ratio

In order to interrogate the data in more depth, multiple linear regression was used to investigate which clinical variables had most significance with regard to the VWFAg-ADAMTS13Ac ratio. The entire cohort was examined.

Table 20: Regression of diagnosis at presentation, modified Rankin score and laboratory markers against VWF:Ag- ADAMTS13 ratio

Source	SS	df	MS	Number of obs= 204
Model	324.54	7	46.36	F (7,196) = 31.17
Residual	291.51	196	1.49	Prob > F = 0.00
Total	616.05	203	3.035	R-squared= 0.527
				Adj R squared= 0.510
				Root MSE = 1.215

regress Ratio VWFAgAD13Ac Age i.diag1 mRS Creatinine Bilirubin CRP					
Ratio VWF:Ag-ADAMTS13Ac	Coefficient	Std error	t	P>t	95% confidence interval
Diagnosis of IS	-.246	.229	1.07	.284	-.697 - .206
Diagnosis of TIA	-.394	.227	-1.74	0.084	.841 - .054
Age	0.256	0.00590	4.34	0.000	.0140 - .0373
mRS	.144	.0603	2.39	0.018	.0250 - .263
Creatinine	.00907	.00163	5.55	0.000	.00585 - .0123
Bilirubin	.0340	.0131	2.60	0.01	.00820- .05998
CRP	.0424	.00506	8.39	0.000	.0325- .0524
-cons	-.913	.398	-2.3	0.023	-1.70- -.129

Table 20: All potentially explanatory variables were added into a regression model. Stata was programmed to distinguish the data according to diagnostic category. The instruction entered into Stata was: 'regress RatioVWFAgAD13Ac Age i.diag1 Age mRS Creatinine Bilirubin CRP'. We expected there to be a difference in ratio according to diagnosis, age and functional score as reflected by the modified Rankin. Bilirubin and creatinine were employed as variables to reflect potential influence of liver and renal function respectively. CRP was used to reflect the potential role of inflammation on the VWF-ADAMTS13 axis. Missing data sets were removed to allow the analysis.

This command requests regression of the VWF:Ag- ADAMTS13Ac ratio as the dependent variable in the model, with age, diagnosis of stroke/TIA/ control, mRS score at presentation, creatinine, bilirubin and CRP as independent variables. The overall R-squared was 0.527, meaning that the model could be used to explain 53% of the overall variability in the presentation VWFAg- ADAMTS13Ac ratio in all patients. P values demonstrate that age, modified Rankin, creatinine, bilirubin and CRP all had positive associations with the VWF:Ag- ADAMTS13Ac ratio and significance when entered as independent variables into the model.

Dividing the cohort according to respective diagnoses of ischaemic stroke, TIA or control did not demonstrate significance in the regression model- both by examining the p value and the confidence intervals. The Rankin functional score was positively associated with the VWFAg-ADAMTS13 ratio, as with CRP, bilirubin and creatinine. The lack of distinction in the ratio between diagnostic categories may be because the control group included patients with other reasons for imbalance of the haemostatic ratio, such as sepsis or inflammation. The R-square value of 53% means that 47% of the variability in the VWF:Ag-ADAMTS13Ac ratio is unexplained by this model.

4.10 Regression: does diagnosis of ischaemic stroke or TIA (combined) influence the VWF:Ag-ADAMTS13Ac ratio adjusted for age and functional score?

Table 21: Regression of diagnosis of ischaemic stroke or TIA at presentation, modified Rankin score and age (independent variables) against VWF:Ag- ADAMTS13 ratio (dependent variable)

Source	SS	df	MS	Number of obs= 281
Model	180.22	3	60.07	F (3, 277) = 25.65
Residual	648.64	277	2.34	Prob > F = 0.00
Total	828.85	280	2.96	R-squared= 0.217
				Adj R squared= 0.2090
				Root MSE = 1.53

regress Ratio VWF:Ag-ADAMTS13Ac diag1 Age mRS					
Ratio VWF:Ag-AD13Ac	Coefficient	Std error	t	P>t	95% confidence interval
Diagnosis of IS or TIA	-.401	.209	-1.92	0.056	-.813 - .011
Age	.0398	.00604	6.59	0.00	.0279 - .0517
mRS	.171	.0509	3.35	0.001	.0706– 0.271
cons	-0.405	0.371	-1.09	0.277	1.135 - .326

Table 21: Regression of the entire cohort was then repeated to only include key variables believed to have bearing on the VWF:Ag-ADAMTS13Ac ratio: diagnosis of stroke/ TIA vs control, age and mRS score at presentation. Limiting the number of independent variables meant missing data had less impact and 281 observations were inputted into the model. Age and mRS had significance for the VWF:Ag-ADAMTS13 ratio, based on p value and confidence interval. Again, diagnosis of ischaemic stroke or TIA had no significance in this model, since the confidence interval includes zero. However, the overall R-square of the model was 0.217, or 27%; which means it does not well describe variability in the VWF:Ag-ADAMTS13Ac ratio: ie a model based on diagnosis, age and diability scores alone. It is likely there are many more factors involved, as Table 18 would suggest, with the addition of independent variables including CRP, bilirubin and creatinine. An attempt to model out age was made with regression on only those aged 75 years and younger. There was no relationship seen with diagnosis category and the VWF:Ag-ADAMTS13Ac ratio despite this.

4.11 Regression: does diagnosis of ischaemic stroke or TIA (separate diagnoses) influence the VWF:Ag-ADAMTS13Ac ratio adjusted for age and functional score?

Table 22: Regression of diagnosis of ischaemic stroke or TIA (split) at presentation, modified Rankin score and age (independent variables) against VWF:Ag- ADAMTS13 ratio (dependent variable)

Source	SS	df	MS	Number of obs= 281 F (4, 276) = 19.45 Prob > F = 0.00 R-squared= 0.220 Adj R squared= 0.2090 Root MSE = 1.53
Model	182.26	4	45.56	
Residual	646.6	276	2.34	
Total	828.85	280	2.96	

regress Ratio VWF:Ag-ADAMTS13Ac diag1 Age mRS					
Ratio VWF:Ag-AD13Ac	Coefficient	Std error	t	P>t	95% confidence interval
Diagnosis of IS	-.272	.250	-1.09	0.277	-.767 - .220
Diagnosis of TIA	-.518	.244	-2.12	0.035	-.999 - .0376
Age	.0399	.00605	6.61	0.00	.0280 - .0519
mRS	.147	.0571	2.57	0.011	.0342– 0.259
-cons	-0.384	0.372	-1.03	0.303	1.12 - .348

Table 22: Splitting the diagnosis categorisation furthermore, into either "1"= ischaemic stroke or "2"= TIA is shown above in Table 20. Neither diagnosis of stroke or TIA was significant in terms of p value or confidence intervals. As in Table 21, the overall R square for the model was poor at 0.22 (22%), therefore explaining 22% of variability in the dependent variable of VWF:Ag-ADAMTS13Ac. Regression modelling as shown in both Tables 21 and 22 suggest that age, functional score and diagnosis of ischaemic brain injury may contribute to the presenting ratio of VWF:Ag- ADAMTS13. However, the weak R square values suggests there are other influential factors, with Table 18 illustrating that inflammation (as reflected by CRP) and organ dysfunction (as reflected by creatinine and bilirubin) will play a role.

4.12 Regression: In ischaemic brain injury, does age and functional score influence the VWF:Ag-ADAMTS13 ratio?

Table 23: Regression of diagnosis of ischaemic stroke or TIA (split) at presentation, modified Rankin score and age (independent variables) against VWF:Ag- ADAMTS13 ratio (dependent variable)

Source	SS	df	MS	Number of obs= 174 F (2, 171) = 18.37 Prob > F = 0.00 R-squared= 0.177 Adj R squared= 0.167 Root MSE = 1.34
Model	293.864	2	33.21	
Residual	309.20	171	1.81	
Total	375.62	173	2.17	

regress Ratio VWF:Ag-ADAMTS13Ac Age mRS					
Ratio VWF:Ag-AD13Ac	Coefficient	Std error	t	P>t	95% confidence interval
Age	.0308	.00798	3.86	0.00	.0150 - .0465
mRS	.184	.0608	3.03	0.003	.0643 – 0.304
cons	0.149	0.566	-0.26	0.792	-1.266 - .967

Table 23: Linear regression was repeated for just the ischaemic brain injury group (IS and TIA). The model suggests that both age and mRS independently contributed to variation in the VWFAg-ADAMTS13 ratio- as reflected in the p values of <0.05 and confidence intervals not including zero. However, the overall R-square value was weak at 0.177 (18%). The conclusion is that the modelling in Table 23 does not well reflect the true relationship between the dependent and independent variables: ie age and functional score influence the presenting VWF:Ag- ADAMTS13Ac ratio but do not fully explain the variability.

4.13 Regression and the VWF:Ag-ADAMTS13 axis in ischaemic brain injury

Regression was used in an attempt to 'model out' variables which would have bearing on the VWF:Ag-ADAMTS13Ac axis. We expected age and functional scores to demonstrate a positive association with the ratio, as already seen in the correlation. As already published, and as demonstrated in correlation; age has a strongly positive association with the VWF:Ag-ADAMTS13Ac ratio, both by virtue of escalating VWF:Ag levels and decreasing ADAMTS13Ac levels. This was not seen in the first regression model (Table 19), but seen in the subsequent was seen in the subsequent regression models (Tables 20-23).

Reflecting then on clinical status as denoted by functional scores, regression indicates that presentation Rankin is associated with the VWF:Ag-ADAMTS13Ac axis. Lower functional scores (ie improved functional status) are associated with higher ADAMTS13, and vice versa. Similarly, higher VWF:Ag is associated with higher functional scores reflecting more functional compromise.

The regression modelling also demonstrated positive associations of the VWF:Ag-ADAMTS13Ac ratio with creatinine, bilirubin and CRP; which is biologically plausible. A higher creatinine can represent kidney injury, hyperbilirubinaemia can represent liver injury or the haemolysis seen in TTP (reflective of ADAMTS13 deficiency) and a rising CRP is seen in the infected and inflamed state

We were interested in whether splitting diagnosis into either binary- stroke/TIA vs control- or into 3 diagnostic categories of stroke, TIA or controls in regression modelling would independently show significance for the VWF:Ag-ADAMTS13Ac ratio. This was not demonstrated. This suggests that diagnosis alone of ischaemic stroke vs TIA vs control does not independently predict the VWF:Ag-ADAMTS13Ac balance. It is likely there are a myriad of other influences: including age, functional score or disability, inflammation and organ dysfunction. The VWF:Ag-ADAMTS13Ac axis at presentation and its close correlation with disability association is not specifically restricted to those with arterial thrombosis. Later analysis of longitudinal trends showed more convincing suggestions of the role of the presenting VWF:Ag-ADAMTS13Ac axis specifically toward long-term outcome of ischaemic brain injury.

5 VWF and ADAMTS13: longitudinal results

Longer-term follow up was achieved in 101 patients (ischaemic stroke=36, TIA=36, controls=28, haemorrhagic stroke=1): ie follow up clinical assessment and blood samples at a minimum of 6 weeks following presentation (ie t4). Every attempt was made to achieve follow up, including numerous home visits. For the remainder of the cohort, follow up was not achieved at the time of final data capture in October 2017, for the reasons as follows:

i) Declined n=69
ii) Inappropriate (patient now palliated) n=11
iii) Out-of-area n=34
iv) DNA n=13
v) Venepuncture not possible n=1
vi) No contact (either no active telephone number, or no answer to several phone call attempts) n=51
vii) Deceased n=28

Median follow up time, from t0 to t4, was 188 days (range 41 to 889 days).

5.1 Longitudinal changes: ischaemic stroke

The ischaemic stroke group was then analysed separately. Longitudinal changes in all haemostatic markers were measured at presentation (t0), 24 hours later (t1), 48 hours post presentation (t2), 5-7 days post presentation (t3) and final follow up from 6 weeks post presentation (t4). This final follow up timing was planned to represent convalescence from the initial presentation. Median follow up time for ischaemic stroke specifically was 257 days (range 48-889). Data sets were incomplete, particularly in those patients who were not admitted to hospital and therefore not present for consecutive days clinical follow up and blood sampling. The number of values at each time point was as follows: t0= 103 values, t1= 41 values, t2= 30 values, t3= 20 values and t4= 36 days.

Trends in each haemostatic marker are outlined in Figure 5 below.

Figure 5: Longitudinal changes in haemostatic markers in ischaemic stroke

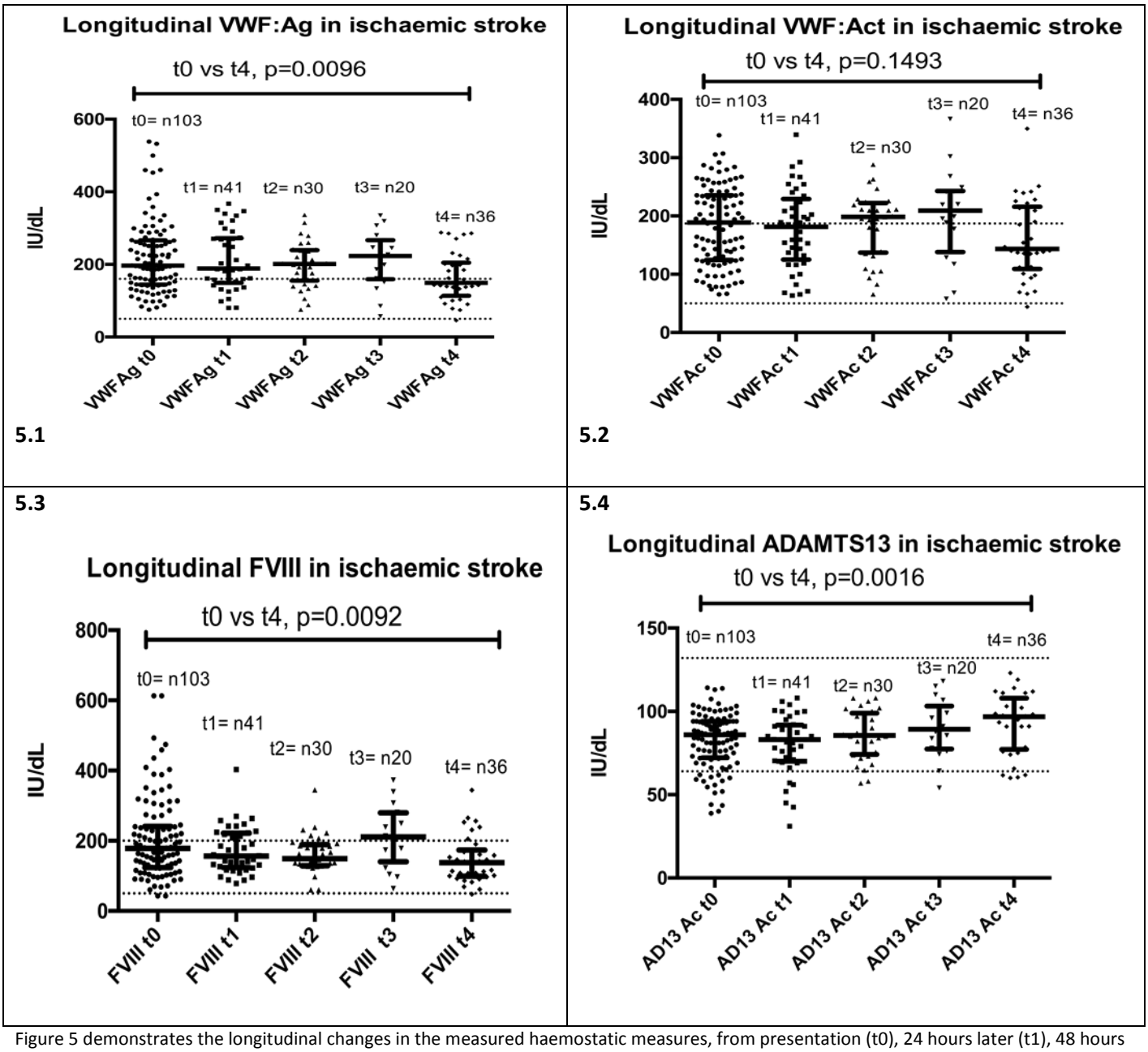


Figure 5 demonstrates the longitudinal changes in the measured haemostatic measures, from presentation (t0), 24 hours later (t1), 48 hours post presentation (t2), 5-7 days post presentation (t3) and final follow up from 6 weeks post presentation (t4). Median follow up time for ischaemic stroke specifically was 257 days (range 48-889). Data sets were incomplete, particularly in those patients who were repatriated to local hospitals and therefore not present for consecutive days clinical follow up and blood sampling. The number of values at each time point was as follows: t0= 103 values, t1= 41 values, t2= 30 values, t3= 20 values and t4= 36 days. P values in the figures refer to Mann-Whitney testing of t0 (all available data) versus t4 (all available data) for each haemostatic marker in ischaemic stroke. The dotted lines illustrate the normal ranges for each haemostatic marker. The bars illustrate the median and interquartile range for each haemostatic marker.

Figure 5.1: VWFAg (normal range 50-187IU/dL). Medians: t0 196.9IU/dL, t1 188.7IU/dL, t2 201.3IU/dL, t3 223.4IU/dL and t4 157.2IU/dL; t0 vs t4 unpaired t-test p=0.0096.

Figure 5.2: VWF:Act (normal range 50-187IU/dl). Medians: t0 188.8IU/dL, t1 181.5IU/dL, t2 198.5IU/dL, t3 209IU/dL and t4 150.4IU/dL; t0 vs t4 unpaired t-test p=0.149.

Figure 5.3: FVIII (normal range 50-150IU/dl). Medians: t0 178.6IU/dL, t1 156.1 IU/dL, t2 148.9IU/dL, t3 211.1IU/dL and t4 140.9; t0 vs t4 unpaired t-test p=0.0092.

Figure 5.4: ADAMTS13Ac (normal range 64-132IU/dL). Medians: t0 86.0IU/dL, t1 83.0IU/dL, t2 85.5IU/dL, t3 89.4IU/dL and t4 96.8IU/dL; unpaired t-test p=0.0016.

Decrease in VWF:Ag from presentation (median 196.9 IU/dL) to final follow up (median 157.2 IU/dL) was observed (p=0.0096; Figure 5.1). The same trend was seen with FVIII (presentation median 178.6 to final follow up 140.9 IU/dL; p=0.0092; Figure 5.3). Interestingly, although VWF:Act decreased from presentation (median 188.7 IU/dL) to final follow up (median 150.4IU/dL, Figure 5.2) this was not significant (p=0.1493), possibly skewed by some outliers.

A clear reverse trend was seen with ADAMTS13Ac in ischaemic stroke (Figure 5.4), demonstrating a significant increase in ADAMTS13Ac from presentation (median 85.9 IU/dL) to final follow up (median 96.8IU/dL) on Mann Whitney testing (p=0.0016). There was a significant increase in median ADAMTS13Ac across all time points (Kruskal Wallis statistic 13.58, p=0.0088).

Thrombin generation similarly decreased over time (presentation vs final follow up respectively, peak: 264.7 to 191.4nM, p=0.0021, ETP: 1695 nM/min to 1329 nM/min, p=0.0016).

With the reciprocal changes in VWF:Ag and ADAMTS13Ac seen in convalescence post ischaemic stroke, the VWFAg-ADAMTS13Ac ratio correspondingly decreased over time , from presentation 2.42 to final follow up 1.66, p=0.0008 (Figure 6).

Figure 6: VWFAg/ ADAMTS13Ac ratio from presentation to convalescence in ischaemic stroke

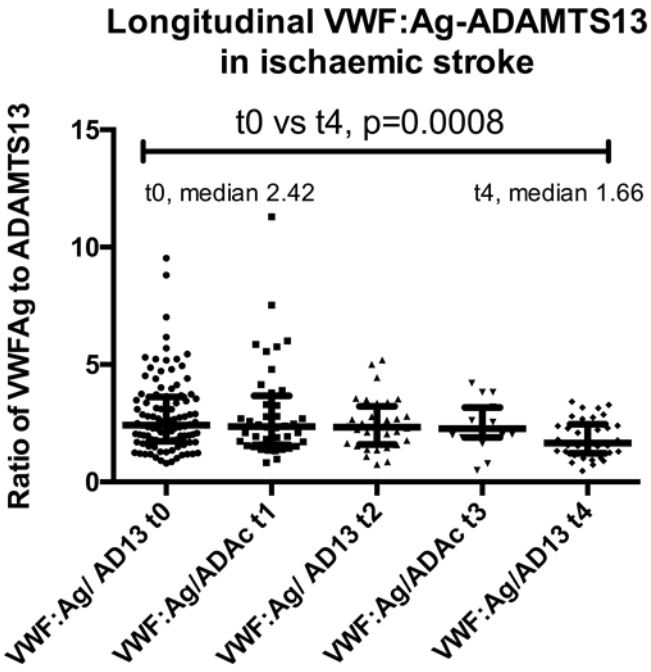


Figure 6 demonstrates the changes in the VWFAg-ADAMTS13 ratio in ischaemic stroke from presentation (median 2.42; range 0.79- 9.53) to final follow up (median 1.66, range 0.47- 3.42) on Mann Whitney testing. Bars indicate medians and interquartile ranges for every measure captured at each time point.

5.2 Longitudinal trends in ischaemic stroke: laboratory variables at every time point

Changes at the other time points were also examined. Data sets were incomplete, particularly in those patients who were repatriated to local hospitals, and therefore not present for consecutive days clinical follow up and blood sampling. The number of values at each time point was as follows: t0= 103 values, t1= 41 values, t2= 30 values, t3= 20 values and t4= 36 values. This limitation of data capture is commented on further in the discussion, but should be acknowledged in examining the results. For the subset of patients with complete follow up from t0 to t4, paired t-tests were later performed.

With the data available, peaks in VWF:Ag, VWF:Act and Factor VIII were seen at time point 3 (days 7-10 post presentation). The ADAMTS13Ac nadir was at time point 1 (24 hours post presentation), which then showed recovery at each time point thereafter until a peak at final follow up. There was a peak VWF:Ag-ADAMTS13Ac ratio at presentation, which steadily decreased throughout follow up. This is summarized in Table 23 below. Mann Whitney testing performed between each baseline marker and consecutive time points (t0 vs t1, t0 vs t2, t0 vs t3) showed no significance for any haemostatic marker.

Table 24: Haemostatic markers at each time point

Median values	T0 (n=103)	T1 (n=41)	T2 (n=30)	T3 (n=20)	T4 (n=36)
Factor VIII (IU/dL)	**178.6** (43.1-612.7)	**156.1** (78.4-402.9)	**148.9** (60.5-344.7)	**211.1** (63.4-372.8)	**140.9** (47.7-344.2)
VWF:Ag (IU/dL)	**196.9** (75.4-538.1)	**188.7** (80-367.5)	**201.3** (75.4-336.1)	**223.4** (57.4-334.9)	**157.2** (45.6-287.9)
VWF:Act (IU/dL)	**188.7** (65.5-338.4)	**181.5** (63.6-339.5)	**198.5** (65.5-288)	**209** (57.4-366.6)	**150.4** (44.1-349.7)
ADAMTS13 Ac (IU/dL)	**86.0** (38.8-114.2)	**83.0** (31-108)	**85.5** (56.9-108)	**89.4** (54-118.2)	**96.8** (60-123)
VWF:Ag/ADAMTS13 Ac ratio	**2.42** (0.79-9.53)	**2.36** (0.82-11.3)	**2.34** (0.74-5.19)	**2.27** (0.5-4.21)	**1.66** (0.47-3.42)
Peak thrombin (nM)	**264.7** (20.7-684.2)	**283.2** (57.6-712.8)	**272.5** (60.5-647.0)	**280.3** (93.1-469.7)	**191.4** (19.8-454.8)
ETP (nM/min)	**1696.0** (315.4-3623.7)	**1806.7** (505.9-4238)	**1742.6** (585.4-3299)	**1737.0** (783.9- 2952)	**1328.8** (321.1-2893.6)

5.3 Longitudinal changes in ischaemic stroke: analysis

Table 25: Longitudinal changes in ischaemic stroke: comparison

Median, IU/dL	**Baseline (t0)**	**Final follow up (t4)**	**Wilcoxon matched-pairs signed rank test (n=34-36 Median of differences**	**Mann Whitney test (baseline vs final follow up)**
FVIII	178.6	140.9	**-21.2 (p=0.0137)**	**37.7 (p=0.0092)**
VWF:Ag	196.9	157.7	**-18.24 (0.0107)**	**39.7 (p=0.0096)**
VWF:Act	188.7	150.4	-8.2 (p=0.0933)	38.8 (p=0.149)
ADAMTS13Ac	85.9	96.8	**4.9 (p=0.0070)**	**10.88 (p=0.0016)**
Ratio VWF:Ag: ADAMTS13Ac	2.42	1.66	**-0.2775 (p=0.0005)**	**0.76 (p=0.008)**
Peak thrombin (nM)	264.7	191.4	**-94.42 (p<0.0001)**	**73.3 (p=0.0021)**
ETP (nM/min)	1696.0	1328.8	**-580.0 (p<0.0001)**	**367.2 (p=0.0016)**

Table 25: Further analysis showed significant changes in IS were demonstrated for each marker in comparison of presentation versus final follow up aside from VWF:Act. This was shown on both Mann Whitney testing (presentation versus final follow up) for all values, and Wilcoxon matched-pairs signed rank testing performed for paired data sets (ie same patient t0 and t4).

Unpaired data analysis (Mann Whitney testing) demonstrated a significant difference in medians for FVIII, VWF:Ag, ADAMTS13Ac, ratio of VWF:Ag-ADAMTS13Ac, peak thrombin and ETP. This was seen in 34 patients with presentation and final follow up measurements taken, and illustrated in Figure 5 below. The median age of these patients was 72 years (range 42 to 92 years).

Incomplete data sets are acknowledged, particularly with regard to drop out at follow up. This could unduly bias the analysis, for example, with failure to capture the sickest patients who were either too unwell to participate in later follow up or may have even died in that time. We have attempted to address this by performing a paired test on those samples where paired data were available, using the Wilcoxon matched-pairs signed rank test.

Figure 7: Paired testing in ischaemic stroke: presentation to final follow up

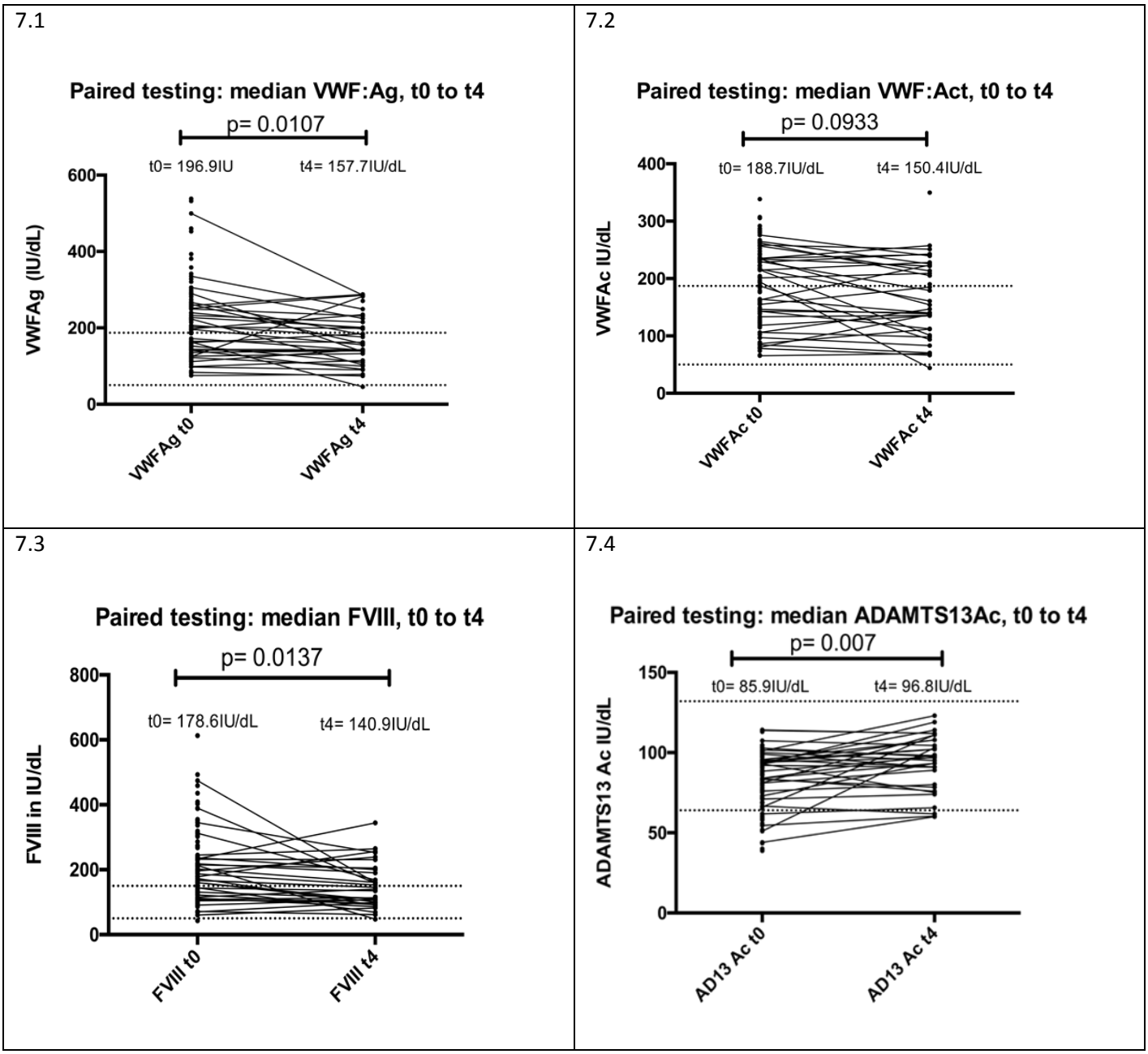


Figure 7: Paired t-testing supported the same longitudinal trends seen with the unpaired testing:

7.1: VWF:Ag decreased difference in medians -18.24, p=0.0107

7.2: VWF:Act decreased difference in medians -8.2, p=0.0933

7.3: FVIII decreased difference in medians -21.2, p=0.0137

7.4: ADAMTS13Ac increased difference in medians 4.9, p=0.007.

Anticoagulation with warfarin or full dose dalteparin did not influence follow up VWF:Ag: anticoagulated (n=5) 215 vs not (n=32) 142.9IU/dL, p=0.0775) or ADAMTS13Ac (anticoagulated vs not, 78.4 vs 96.8IU/dL, p=0.415) on Mann Whitney testing. As seen at baseline, there was again a reduction in thrombin generation seen with anticoagulation at final follow up: warfarinised vs not (ETP 688.3 vs 1329, p=0.04); though not significant for peak thrombin (72.5 vs 191.4 IU/dL, p=0.173).

Once those patients whom were thrombolysed were discounted, there were too few patients with IS at final follow up to meaningfully assess the effect of anti-platelets (n=25) initiated at presentation compared to those who were not on an anti-platelet (n=1). This was also applicable when including all patients who were thrombolysed at baseline, but discounting those anticoagulated: only 2 patients were not on anti-platelets at final follow up.

5.4 Longitudinal patterns in TIA

Follow up was achieved in 36 patients who had originally presented with TIA. Time points 1, 2 and 3 were not captured since these patients did not generally remain as in-patients.

There was a slight reduction in VWF:Ag over time in the overall TIA cohort, but non-significant (167.8 to 151.6IU/dL, p=0.194, Figure 8.1). Similarly, there were no significant changes in VWF:Act or FVIII over time in the TIA group (Figures 8.2 and 8.3 below). In contrast to the ischaemic stroke cohort, there was no difference between baseline and follow up ADAMTS13Ac in the TIA cohort (94 to 97IU/dL, p=0.355, Figure 8.4) overall.

Restricting testing to only those patients with paired data at t0 and t4 was performed with the Wilcoxon matched-pairs signed rank test. This revealed increased ADAMTS13Ac from baseline to final follow up (median of differences 4.0IU/dL, p=0.0404). There was a corresponding decrease in the VWF:Ag- ADAMTS13Ac ratio from 1.89 at presentation (range 0.41 to 8.14) to 0.65 at final follow up (range 0.32-2.03; median of differences -1.175, p<0.0001). The median age of the TIA patients for whom we had final follow up data was 70.5 years, range 25 to 93.

This suggests that the significant difference seen in the VWF:Ag-ADAMTS13Ac ratio from presentation to final follow up in ischaemic stroke is also seen in TIA.

Figure 8: Longitudinal changes in haemostatic markers in TIA

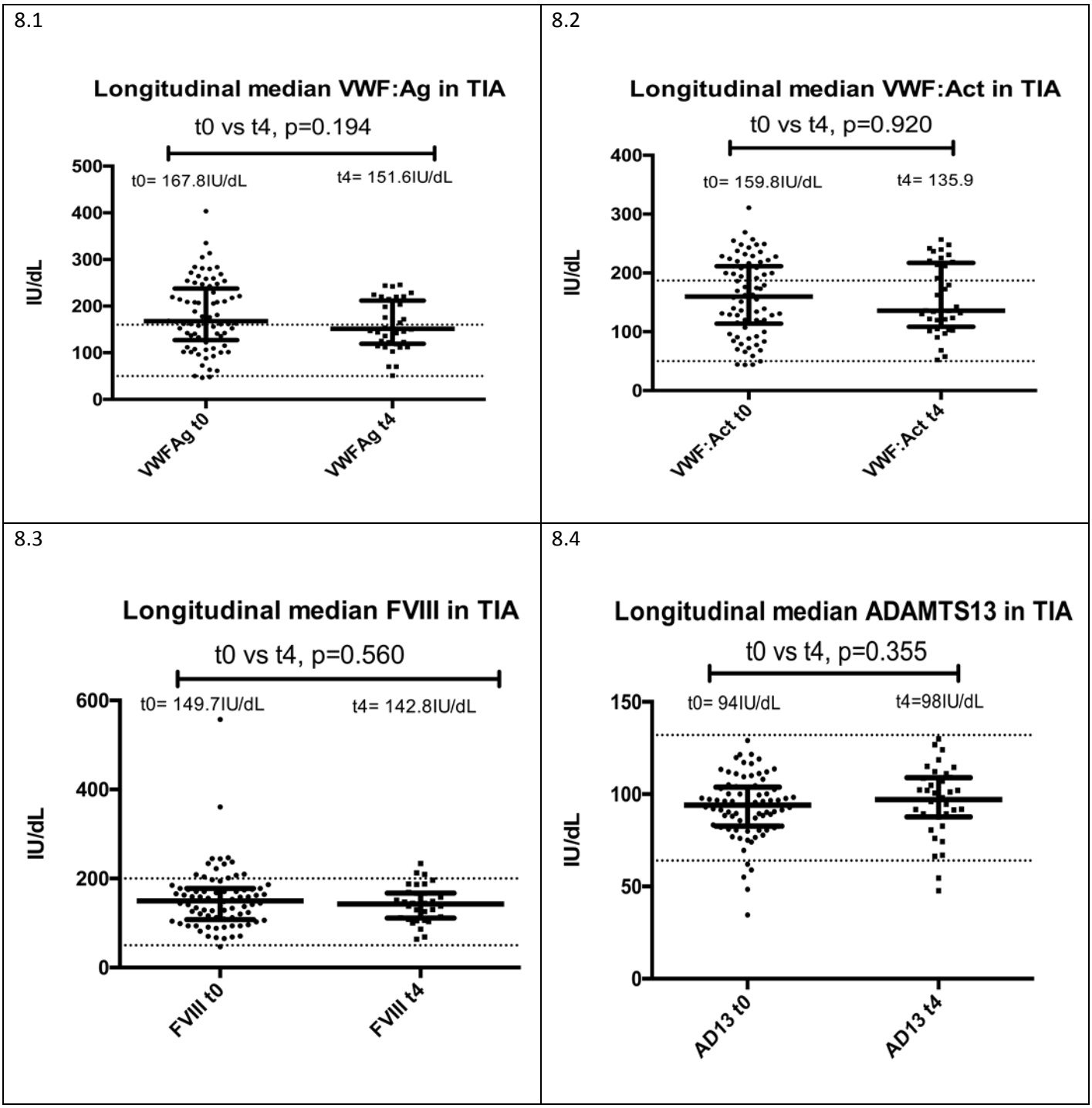


Figure 8 demonstrates Mann Whitney testing comparing the median haemostatic markers of the TIA group at baseline (t0= 79 values) versus final follow up (t4= 36 values). The dotted lines illustrate the normal ranges for each haemostatic marker. The bars illustrate the median and interquartile range for each haemostatic marker.

Figure 8.1: VWFAg (normal range 50-187IU/dL). Medians: t0 167.8IU/dL vs t4 151.6IU/dL (p=0.194).

Figure 8.2: VWF: Act (normal range 50-187IU/dl). Medians: t0 159.8 vs t4 135.9IU/dL (p=0.920).

Figure 8.3: FVIII (normal range 50-150IU/dl). Medians: t0 149.7 vs t4 142.8IU/dL (p=0.569).

Figure 8.4: ADAMTS13Ac (normal range 64-132IU/dL). Medians: t0 94 vs t4 97IU/dL (0.355).

As with admission, the longitudinal behaviour of the TIA cohort was more closely examined by comparing in-patient (IP) and outpatient (OP) admissions. There were no significant differences between IP or OP TIAs in ADAMTS13Ac or VWF:Ag between presentation and final follow up on Mann Whitney testing.

In the overall TIA group, thrombin generation makers showed a significant decrease in ETP (-153.5, p=0.0252) and a similar downward trend in peak thrombin (-51.7, p=0.0230) on Mann Whitney testing.

5.5 Longitudinal changes in controls

Follow up was achieved in 28 control patients (median follow up 204 days, range 44- 566). The control group was heterogeneous in phenotype, inclusive of well patients referred to the TIA clinic with later benign diagnoses, as well as frail in-patients presenting with symptoms suggestive of stroke with alternative resultant diagnoses such as seizure or severe sepsis.

In contrast to the ischaemic stroke group, there were no significant changes in any haemostatic marker as illustrated in Figures 9.1- 9.4 below. However, incomplete data sets limit the interpretation. Mann Whitney tests were performed between presentation and final follow up, and demonstrated no significant longitudinal change in the control group VWF:Ag (160.1 to 131.4IU/dL, p=0.1461; Figure 9.1), VWF:Act (140.4 to 120.6IU/dL, p=0.293; Figure 9.2), FVIII (142.6 to 115.0, p=0.1906; Figure 9.3), ADAMTS13Ac (95.6 to 98.95IU/dL, p=0. 1439; Figure 9.4). Likewise there was no change in markers of thrombin generation (peak thrombin 248.3 to 215.3nM, p=0.1; ETP 1562 nM/min to 1611 nM/min, p=0.1).

For those patients in whom we had paired values available (presentation to final follow up, n=28), there was no significance in any Wilcoxon matched-pairs signed rank testing for any marker. This included the VWF:Ag-ADAMTS13Ac ratio, with no significant longitudinal change (1.69 to 1.21, p=0.074, Wilcoxon matched pairs signed rank test median difference, 0.0362; p=0.562). The median age of the control patients for whom we had final follow up data was 55 years, range 25 to 86. Taking into account this unpaired and paired longitudinal trend analysis suggests that acute phase elevation of the VWF:Ag- ADAMTS13Ac ratio, with normalisation over time, is specific to ischaemic brain injury.

Figure 9: Longitudinal changes in haemostatic markers in controls

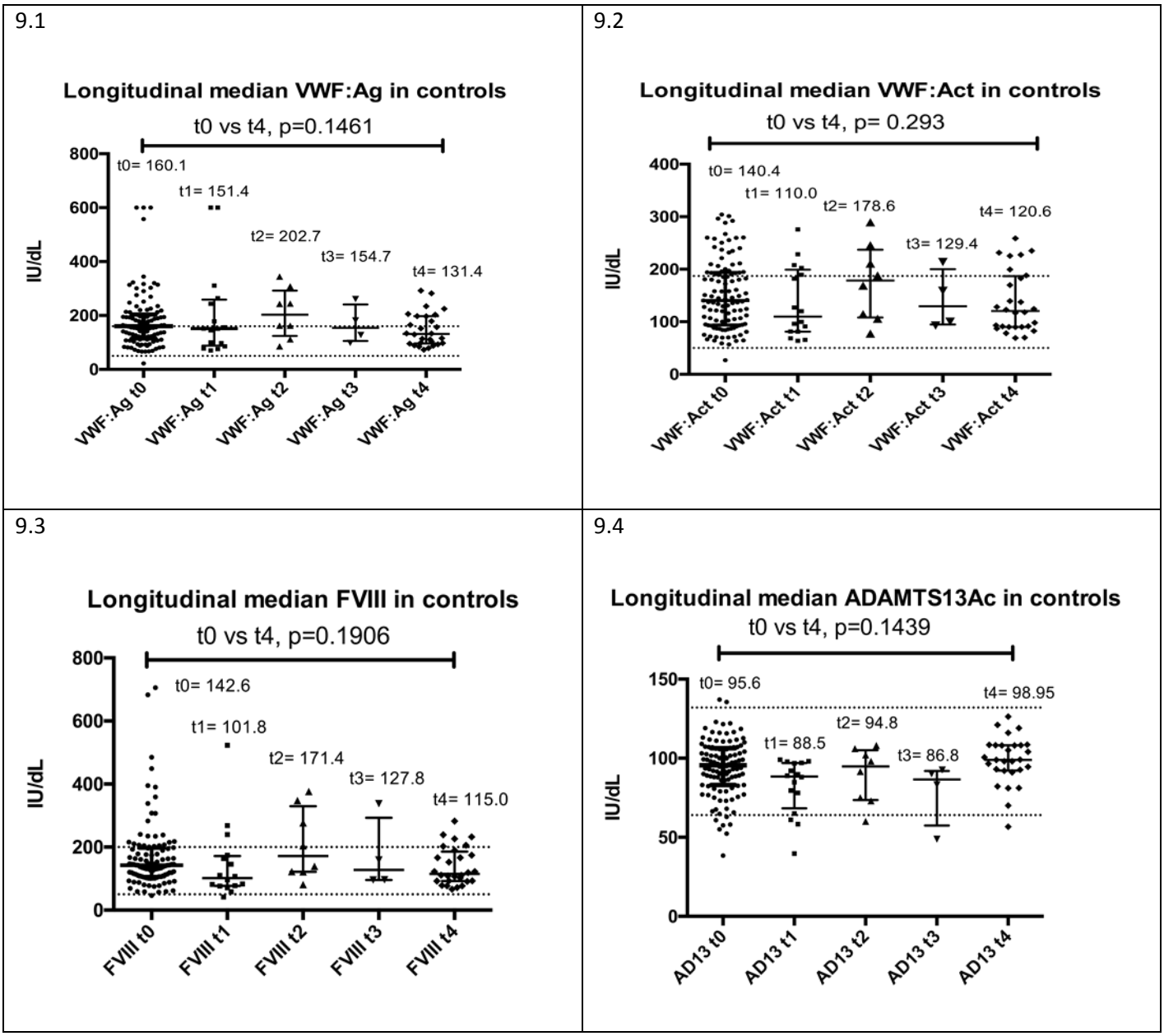


Figure 9 demonstrates the longitudinal changes in the measured haemostatic measures, from presentation (t0), 24 hours later (t1), 48 hours post presentation (t2), 5-7 days post presentation (t3) and final follow up from 6 weeks post presentation (t4). Data sets were incomplete, particularly in those patients who were repatriated to local hospitals and therefore not present for consecutive days clinical follow up and blood sampling. The number of values at each time point was as follows: t0= 109 values, t1= 16 values, t2= 8 values, t3= 41 values and t4= 28 values. P values in the figures refer to Mann Whitney testing of t0 (all available data) to t4 (all available data) for each haemostatic marker in the control group. The dotted lines illustrate the normal ranges for each haemostatic marker. The bars illustrate the median and interquartile range for each haemostatic marker. Paired analysis is not illustrated, but was also performed as outlined in the above text.

Figure 9.1: VWFAg (normal range 50-187IU/dL). Medians: t0 160.1IU/dL, t1 151.4IU/dL, t2 202.7IU/dL, t3 154.7U/dL and t4 131.4U/dL; t0 vs t4, p=0.1461.

Figure 9.2: VWF:Act (normal range 50-187IU/dl). Medians: t0 140.4IU/dL, t1 110.0IU/dL, t2 178.6IU/dL, t3 129.4IU/dL and t4 120.6IU/dL; t0 vs t4, p=0.293.

Figure 9.3: FVIII (normal range 50-150IU/dl). Medians: t0 142.6U/dL, t1 101.8 IU/dL, t2 171.4IU/dL, t3 127.8IU/dL and t4 115.0; t0 vs t4 unpaired t-test p=0.1906.

Figure 9.4: ADAMTS13Ac (normal range 64-132IU/dL). Medians: t0 95.6IU/dL, t1 88.5IU/dL, t2 94.8U/dL, t3 86.8IU/dL and t4 98.95IU/dL; unpaired t-test p=0.1439.

5.6 Comparison of groups at final follow up

There were no significant differences between the ischaemic stroke, TIA and control groups at final follow up in VWF:Ag, VWF:Act, ADAMTS13Ac and the ratio of VWF:Ag-ADAMTS13Ac. Follow up data for haemorrhagic stroke were too limited (n=1) for further analysis.

5.7 Longitudinal analysis: ischaemic brain injury and final follow up clinical scores

Regression was used to investigate whether the VWF:Ag-ADAMTS13Ac axis at presentation of ischaemic stroke correlates with functional recovery. Linear regression was repeated as outlined in Table 25 (univariate) and Table 26 (multiple) below.

Table 26: Regression of modified Rankin score at final follow up as dependent variable, with presenting VWF:Ag-ADAMTS13Ac ratio as independent variable in ischaemic brain injury

Source	SS	df	MS	Number of obs= 54
Model	14.21	1	14.21	F (1, 52) = 5.69
Residual	129.79	52	2.49	Prob > F = 0.0207
Total	144	53	2.72	R-squared= 0.10
				Adj R squared= 0.0813
				Root MSE = 1.58

regress mRS (at t4) RatioVWFAgAD13Ac					
mRS t4	Coefficient	Std error	t	P>t	95% confidence interval
RatioVWFAgAD13Ac	.447	.187	2.39	0.021	0.071- 0.822
cons	0.0058	0.469	0.01	0.990	-0.935 - .946

Table 26: Repeat analysis was performed to determine longitudinal relevance of the VWF:Ag- ADAMTS13Ac ratio in ischaemic brain injury (ischaemic stroke and TIA combined). The final follow up mRS was associated with the presentation ratio according to p value of 0.021 and 95% confidence interval of 0.071- 0.822. The R square is very low at 0.10, suggesting that the model only describes 10% of variability in final follow up mRS in ischaemic stroke. The presenting VWF:Ag-ADAMTS13Ac ratio is positively associated with the final follow up functional score, but this model suggests there are many other influences with explanatory power, as would be expected.

Table 27: Regression of modified Rankin score at final follow up as dependent variable, with presenting VWF:Ag-ADAMTS13Ac ratio and presenting modified Rankin score as independent variables in ischaemic brain injury

Source	SS	df	MS	Number of obs= 53
Model	74.65	3	24.88	F (3, 49) = 17.84 Prob > F = 0.000
Residual	68.34	49	1.39	R-squared= 0.522 Adj R squared= 0.493
Total	143.0	52	2.75	Root MSE = 1.18

regress mRS t4 Age Ratio VWF:Ag-AD13Ac mRS t0					
mRS t4	Coefficient	Std error	t	P>t	95% confidence interval
RatioVWF:Ag-AD13Ac	0.232	0.152	1.53	0.133	-0.0735 - 0.539
Age	0.0134	0.01332	1.01	0.318	-0.0133 - .040
Presenting mRS	0.586	0.0969	6.05	0.00	0.391 – 0.780
cons	-1.44	0.876	-1.64	0.108	-3.195 – 0.324

Table 27: Repeat analysis was performed to determine longitudinal relevance of the VWF:Ag- ADAMTS13Ac ratio in ischaemic brain injury (ischaemic stroke and TIA combined), with age and presenting mRS also introduced into the model. Adding age as a variable alters the relationship between the presenting VWF:Ag- ADAMTS13Ac ratio and follow-up Rankin, but not significantly according to the P value and 95% CI. The presenting mRS has much greater significance, as suggested bu the p value, 95% CI and overall power of the model- much higher at R square 0.522, or 52%.

Table 26 has a low R-square value, suggesting that the presenting VWF:Ag-ADAMTS13Ac ratio is positively associated with the final follow up functional score, but that there are many other influences with explanatory power, as would be expected. Table 27 has a much greater overall explanatory power as a model. Both age and the presenting VWF:Ag-ADAMTS13Ac ratio have a positive association with the final mRS score, but lack significance. The mRS at presentation has far more significance. Our analysis suggests that the presenting VWFAg-ADAMTS13 ratio may be associated with functional outcome in ischaemic brain injury; but further work with increased patient numbers and better matching for confounding effects is warranted.

5.8 ADAMTS13 quartiles and functional scores

Table 28 below explores the ischaemic stroke cohort according to presentation ADAMS13Ac quartiles, and functional score association. Kruskal Wallis and Mann Whitney testing did not demonstrate a significant difference in mRS according to ADAMTS13Ac quartile. There is little relationship apparent with final follow-up functional score recordings (at time point 4). A relative paucity of final follow up data should be considered.

Table 28: ADAMTS13 activity quartiles- is there a correlate with functional scores?

ADAMTS13Ac quartile IU/dL	**1** **38.8-71.8**	**2** **71.9-85.7**	**3** **85.8- 93.9**	**4** **94- 114.2**	**P value** (KW)	Mann Whitney Q1 vs Q4, P value
mRS t0	3 (0-5)	4 (0-5)	4 (1-5)	3 (0-5)	**0.0098** (11.38)	0.404
mRS t4	0 (0-6)	3 (0-6)	2 (2-3)	0 (0-5)	0.5594 (2.197	>0.99
NIHSS t0	5 (0-28)	6 (0-22)	5 (1-19)	4 (0-23)	0.5342 (2.189)	0.396
NIHSS t4	0 (0-0)	0 (0-0)	3 (0-6)	0 (0-0)	0.1636 (4.908)	>0.99
GCS t0	15 (10-15)	15 (12-15)	15 (11-15)	15 (11-15)	0.2042 (4.593)	0.823
GCS t	15 (15-15)	15 (15-15)	3 (14-15)	15 (15-15)	>0.99 (2.667)	>0.99

Table 28: The ischaemic stroke cohort was divided into quartiles according to ADAMTS13Ac level at presentation, to see whether this correlated with mRS as a marker of functional status. Median values are shown, with range in brackets. 'KW' refers to Kruskal Wallis testing, comparing the medians of each quartile at every time point. The seemingly significant difference in median mRS at presentation is likely to be spurious, since the medians are clearly so similar. This reflects a skewed distribution. Mann Whitney testing was performed to compare each quartile at presentation and final follow up, to see whether there was a significant difference in functional score.

4.12 5.9 VWF:Ag-ADAMTS13Ac is associated with functional scores

At presentation, those in the ischaemic stroke group demonstrated median higher mRS and NIHSS scores compared with TIA and control groups. Spearman rank testing showed correlation of the VWF:Ag-ADAMTS13Ac ratio with age (0.577; p<0.0001), mRS score (0.477; p=<0.0001) and NIHSS scores (0.337; p<0.0001). A negative correlation was seen with GCS (-0.255; p<0.0001).

Further exploration of the relationship with functional outcome was performed by categorising ischaemic stroke patients according to degree of disability, to see whether there was a difference in the ratio of VWF:Ag-ADAMTS13Ac.

Division of the IS and TIA groups according to mRS scores at both presentation and final follow up showed prognostic differences in the VWF:Ag-ADAMTS13 ratio. Patients presenting with an mRS scores of 3 to 5 (n=78) compared with patients with an mRS scores of 0 to 2 (n=98) demonstrated a higher median VWF:Ag-ADAMTS13Ac ratio (mRS score, 3-5: median ratio, 1.858: range, 0.415- 9.53:p<0.0001). Despite an overall longitudinal reduction of the VWF:Ag-ADAMTS13Ac ratio, this significant difference persisted at final follow up. Patients with more functional impairment at follow up (n=18), as reflected by mRS score of 3 to 5, maintained a higher VWF:Ag-ADAMTS13Ac ratio of 1.521 (range 0.32-3.42) compared with patients at follow up with an mRS score of 0 to 2 (n=48: median ratio, 0.845: range 0.42- 3.29: p=0.0102). Although the ranges overlapped, this higher VWF:Ag-ADAMTS13Ac ratio was closely linked to increased morbidity. This was not adjusted for age, which together with other variables such as co-morbidities, must be taken into account as a likely confounding effect as well as other variables.

5.9 Control group: longitudinal trends functional scores

. There was no change in the median functional scores from presentation to final follow up, but the maximum scores of both mRS and NIHSS shifted downward from baseline to final follow up (mRS maximum 5 to 3; NIHSS maximum 16 to 10). This could suggest that although there was no change in haemostatic markers of interest in the control group over time, there was an overall trend of functional improvement. This is in contrast to the IS and TIA group: with a downward trend of VWF:Ag, upward trend of ADAMTS13Ac and improved functional status.

5.10 Recurrence and mortality

The ischaemic stroke and TIA groups were re-examined in October 2017 for re-presentation to the Trust with recurrent events. There were 5 patients with recurrent ischaemic strokes (all occurring in patients who had initially presented with IS) and 3 patients with recurrent TIA recorded (all occurring in patients who had initially presented with TIA). This small number of patients re-presenting with recurrence is too small to demonstrate significance: there was no difference in presentation VWFAg-ADAMTS13Ac in those with recurrence at follow up compared to the rest of the cohort.

Within ischaemic stroke and TIA, the mortality rate was 13% (n=24, 21 ischaemic stroke patients and 3 TIA), with median time 152 days from recruitment until death. Median age at presentation of those patients who survived was 75 years (range 25- 99 years); versus 84 years in those who died (range 61-97 years; p<0.0001). Deceased patients (n=24) versus patients alive at final follow up (n=159) showed significant differences in all baseline haemostatic markers (as in Table 27 below). The presenting VWF:Ag- ADAMTS13Ac ratio was significantly higher in those who died (median, 3.68; range 1.70-8.81) compared with those who survived (median, 2.01; range 0.41-9.53).

Table 29: Mortality: comparison of baseline haemostatic markers

	Deceased (n=24)	**Alive (n=159)**	**P value**
Age in years (median, range)	84 (61-97)	75 (25-99)	<0.0001
FVIII (IU/dL)	267.5	153.3	<0.0001
VWF:Ag (IU/dL)	269.1	172.3	<0.0001
VWF:Act (IU/dL)	210.5	160.1	<0.0001
ADAMTS13 Ac (IU/dL)	79.1	90.3	0.014
VWF:Ag-ADAMTS13Ac ratio	3.683	2.014	<0.0001

Table 29 compares the haemostatic markers at baseline in those patients who subsequently died or survivied at latest follow up. There is a significant difference in every marker between the groups, with a consequently raised VWF:Ag- ADAMTS13Ac ratio at presentation of ischaemic stroke in those who later died. There was a significant age difference between the groups, and later analysis has taken this into consideration, proving the difference in ratio at presentation to persist after adjustment for age.

5.11 Presentation VWF:Ag-ADAMTS13Ac axis and subsequent mortality in acute ischaemic brain injury (IS and TIA combined)

Figure 10: Presentation VWF:Ag, VWF:Act, ADAMTS13AC and ratio VWF:Ag-ADAMTS13Ac in ischaemic brain injury according to subsequent mortality

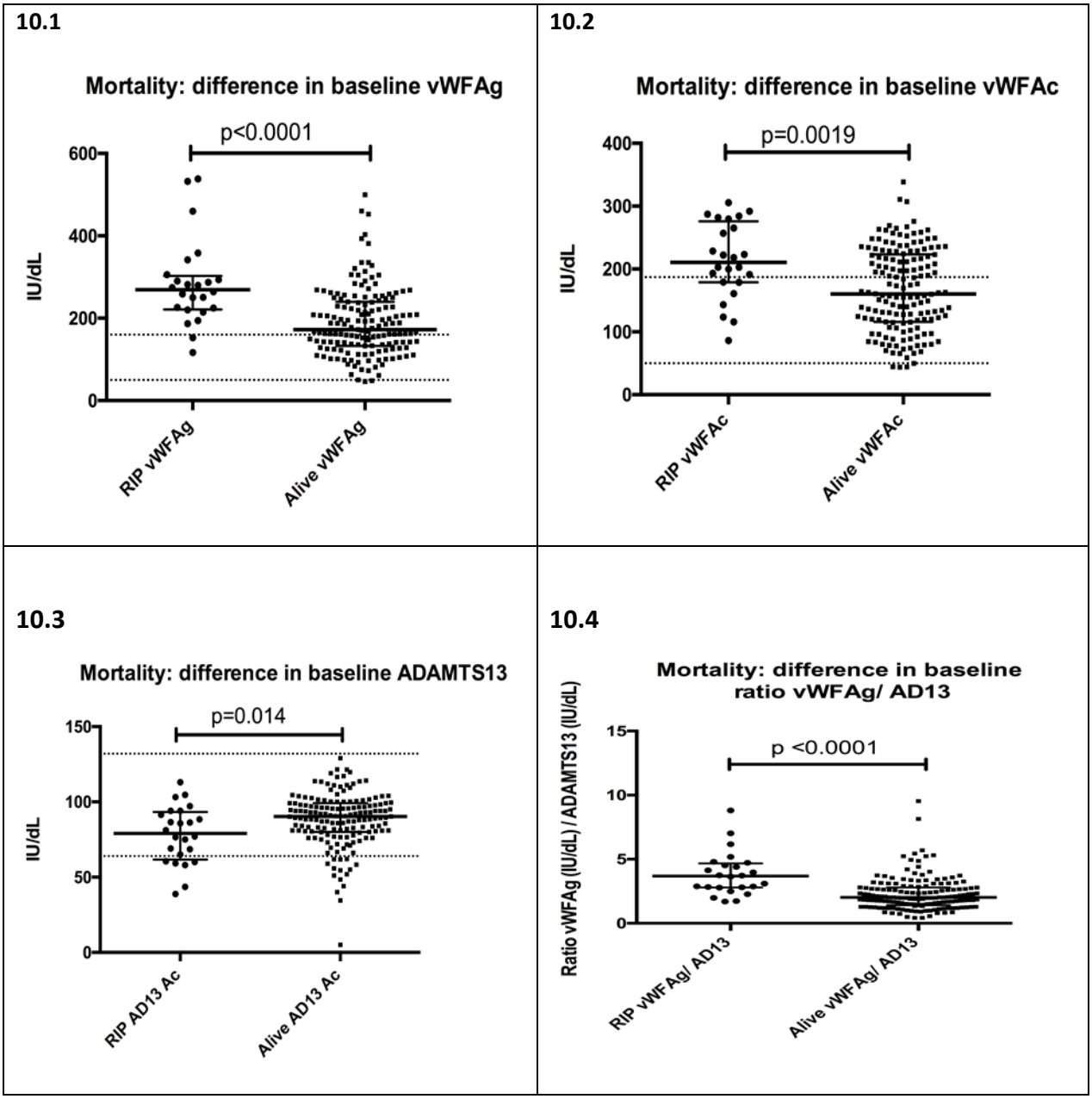


Figure 10: Significant differences were seen in all haemostatic markers at presentation between those patients whom had subsequently died at final follow up (n=24) versus those whom survived (n=159) at a median follow up time of 152 days post initial presentation on Mann Whitney testing. Differences were as follows (died vs survived): VWF:Ag (269.1 vs 172.3IU/dL p<0.0001; Figure 10.1), VWF:Act (210.5 vs 160.1IU/dL p=0.0019; Figure 10.2), ADAMTS13Ac (79.1 vs 90.3IU/dL, p=0.0140; Figure 10.3) and mean VWF:Ag-ADAMTS13Ac ratio (3.683 vs 2.014, p<0.0001; Figure 10.4).

This portrays a striking picture. VWF:Ag, VWF:Act and FVIII are all significantly elevated in the patients who later died. ADAMTS13Ac is significantly lower in this group. The ratio of VWF:Ag-ADAMTS13Ac is correspondingly significantly higher in the deceased group. This would suggest that the VWF:Ag-ADAMTS13Ac axis is associated with not just morbidity relating to acute ischaemic brain injury, but mortality as well.

5.12 Presenting VWF:Ag-ADAMTS13 and mortality: chi square testing

Table 30: Chi squared testing of survival in ischaemic stroke according to presentation VWFAg-ADAMTS13Ac.

Presentation VWF:Ag-ADAMTS13Ac ratio	Alive	Deceased	Total	Chi squared
≤1	15	0	15	2.535 (p=0.1113, NS)
>1	140	24	164	
≤1.1	18	0	18	3.099 (P=0.0784, NS)
>1.1	137	24	161	
≤1.2	27	0	27	4.923 (p=0.0265)
>1.2	128	24	152	OR 10.5 (0.62-177.4)
≤1.3	36	0	36	6.977 (P=0.0083)
>1.3	119	24	143	OR 14.97 (0.89- 252.4)
≤ 1.5	43	0	43	8.763 (p=0.0031)
>1.5	112	24	136	OR 18.95 (1.127-318.6)
≤ 2	77	3	80	11.62 (p=0.0007);
> 2	78	21	99	OR 6.9 (1.98 – 24.1)
≤ 3	10	121	131	14.03 (p=0.0002);
> 3	34	14	48	OR 4.98 (2.033-12.2)
≤ 4	143	15	158	17.7 (p< 0.0001)
> 4	12	9	21	OR 7.15 (2.59- 19.7)

Table 30: chi squared testing demonstrated a difference in mortality according to presenting VWF:Ag-ADAMTS13Ac in ischaemic stroke. With escalation of VWF:Ag-ADAMTS13Ac ratio, there was an increasingly significant difference in survival; with corresponding odds ratios and related confidence interval. Comparing those with a baseline ratio of less than or equal to 1.2 to those greater than 1.2 shows a significant difference in survival (chi-square 4.923, odds ratio 10.5 p=0.0265). From a baseline ratio of 2 upward, there is an increasing significance of not just the chi-square testing of the difference in those whom survived versus those who died, but the odds ratio. The risk of patients with a presentation VWF:Ag-ADAMTS13Ac ratio of greater than 2 dying is 6.9 (95% C.I. 1.98- 24.1) compared to patients with a baseline ratio of less than or equal to 2. Escalating upward, the risk of patients with a presentation VWF:Ag-ADAMTS13Ac ratio of greater than 3 dying is 4.98 (95% C.I. 2.033- 12.2) compared to those with a ratio of less than or equal to 3. The risk of patients with a presentation VWF:Ag-ADAMTS13Ac ratio of greater than 4 dying is 7.15 (95% C.I. 2.59- 19.7) compared to those with a ratio of less than or equal to 4.

5.13 Presenting VWF:Ag-ADAMTS13 quartile analysis

Difference in mortality was also illustrated by splitting the ischaemic stroke and TIA cohort into quartiles according to presentation VWF:Ag-ADAMTS13Ac ratio.

Table 31: Mortality according to VWF:Ag-ADAMTS13Ac ratio quartiles in ischaemic stroke and TIA

Quartile	VWF:Ag-ADAMTS13Ac ratio	Survived (n)	Died (n)	Total number	Mortality rate
1	0.41-1.55	45	0	45	0%
2	1.551-2.11	42	3	45	6.7%
3	2.111-3.09	37	7	44	15.9%
4	3.091-9.53	31	14	45	31.1%

Table 31: those patients in in the highest quartile (VWF:Ag-ADAMTS13Ac ratio 3.09- 9.53) had a 31% mortality rate (n=14) compared to those in the lowest quartile (VWF:Ag-ADAMTS13Ac ratio 0.41-1.55) where no deaths occurred ($p<0.0001$).

In order to be able to adjust for age, receiver-operator curve (ROC) analysis was used to determine sensitivity of the presenting VWF:Ag- ADAMTS13Ac ratio for mortality, demonstrating clear predictive value (area under curve, Figure 11, 0.765; 95% confidence interval [CI] 0.664- 0.886). In comparison, in the ROC analysis for sensitivity of presenting age for mortality, the predictive value was slightly lower (area under curve, 0.75; 95% CI, 0.665- 0.845).

Figure 11: ROC curve analysis: VWFAg-ADAMTS13Ac ratio as predictive for mortality

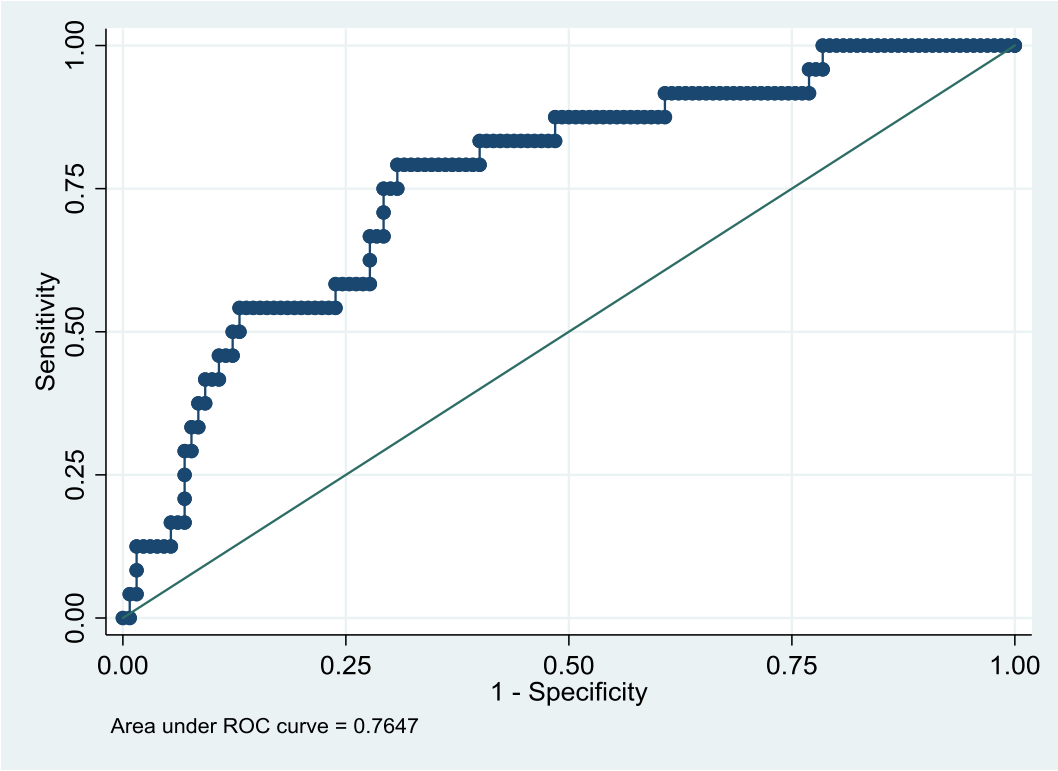


Figure 11 is a graphical representation of the receiver operator characteristic (ROC) curve analysis demonstrating sensitivity and specificity of the presenting VWFAg-ADAMTS13Ac ratio for mortality. The area under the curve (AUC) measures how well the presenting VWFAg-ADAMTS13Ac ratio can distinguish those patients who died and those who survived at follow up. The AUC of 0.765 (a perfect test being 1, a worthless test being 0.5) demonstrates predictive value of the ratio (95% confidence interval 0.664- 0.866).

Using ROC analysis to determine sensitivity and specificity of the VWFAg-ADAMTS13Ac ratio for mortality, a ratio of 2.6 was considered a suitable cutoff, because it provided a good balance of sensitivity (79.2%) and specificity (62.3%). This cutoff ratio was then applied to chi testing of the IS and TIA groups split according to presentation VWFAg/ADAMTS13Ac ratio. Of patients presenting with a VWFAg/ADAMTS13Ac ratio <2.6, 80 were alive and 5 dead at follow-up (mortality, 6%). Of those presenting with a VWFAg/ADAMTS13Ac ratio >2.6, 48 were alive at follow-up, with 19 dead (mortality, 28%). Chi square testing applied showed that those patients with a VWFAg/ADAMTS13Ac ratio of 2.6 or greater were significantly more likely to die (chi-square test, 14.2; P = 0.002; odds ratio [OR], 6.33; range, 2.22-18.1).

Following on from the ROC analysis, logistic regression demonstrated that the association of the presenting VWF:Ag-ADAMTS13Ac ratio with mortality was independent of age. In an unadjusted logistic regression model, the ratio (as independent variable) was associated with mortality (as categorical dependent variable, number of observations= 154, OR 1.67; 95% CI, 1.25-2.22; p 0.00). We then adjusted for age, by adding it as an additional independent variable. There was still a significant association between the ratio and mortality, although slightly reduced in magnitude (number of observations= 154, OR 1.50; 95% CI, 1.11-2.02; P=0.008).

Defining the VWF:Ag-ADAMTS13Ac ratio of 2.6 as predictive of mortality was then extrapolated to morbidity. Patients with a presenting ratio <2.6 (n=109) had a significantly lower mRS score (median, 1; range, 0-5) compared with those with a presenting ratio of ≥2.6 (n=65; median mRS score, 3; range 0-5; p=0.0001). This was also reproduced with NIHSS scoring. Patients presenting with a ratio of <2.6 (n=111) had a significantly lower NIHSS scores (median, 1; range 0-28) compared with those with a presenting ratio of ≥2.6 (n=66; median NIHSS score, 4; range, 0-22, p<0.0001). Since logistic regression was not possible with a continuous variable, this could not be adjusted in ROC analysis for age as mortality was. Again, age should be noted as a confounding effect in examining the effect of the VWF:Ag-ADAMTS13Ac ratio on morbidity.

5.14 Role of thrombin generation in stroke and TIAs

To further investigate haemostatic trends, thrombin generation was also investigated at the various time points. Thrombin generation was assessed via peak thrombin and endogenous thrombin potential (ETP) using the calibrated automated measurement developed by the Hemker group (CAT) (Hemker et al. 2002; 2003).

Figure 12: Comparison of thrombin generation between subgroups at presentation

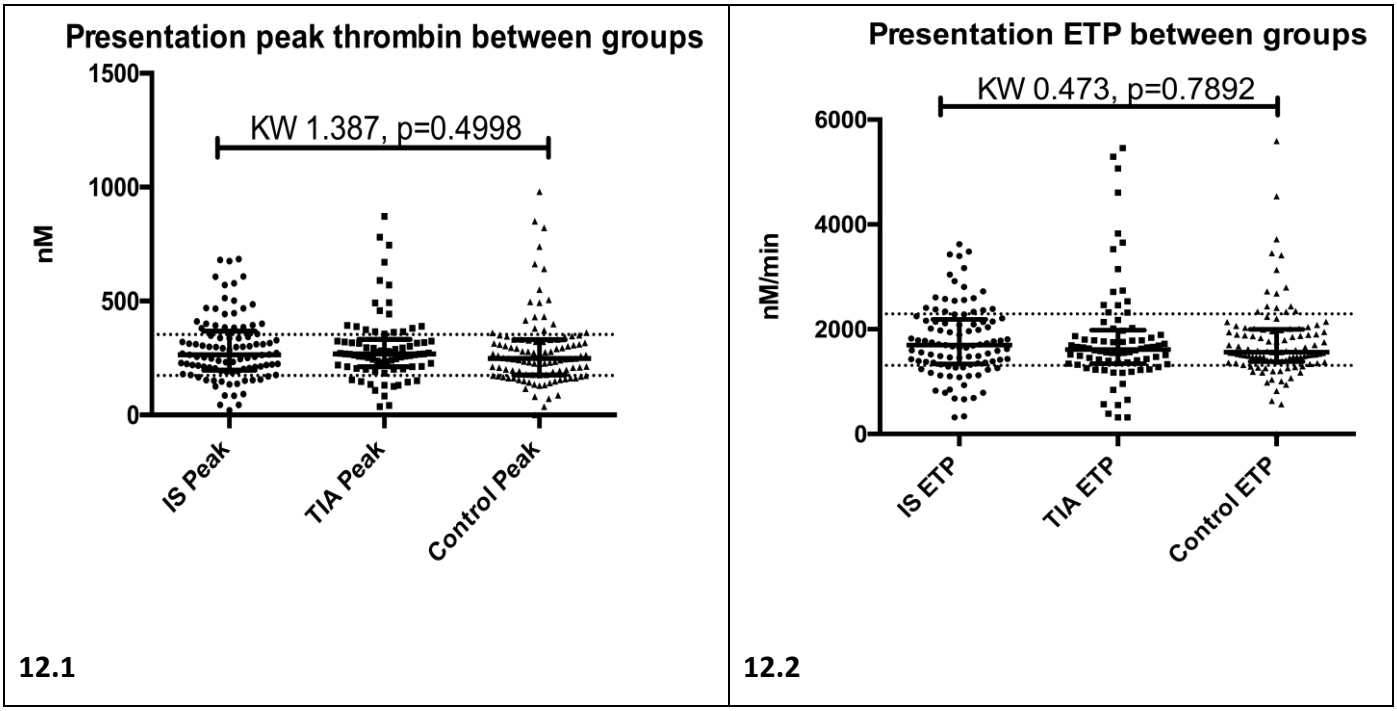


Figure 12: Presentation peak thrombin (Figure 12.1) and presentation ETP (Figure 12.2) showed no significant differences between ischaemic stroke (n=103), TIA (n=80) and control groups (n=108). Dotted lines demonstrate normal ranges, and bars represent median and interquartile range for each value.

Median peak thrombin was measured at 264.7nM in IS, 267.5nM in TIA and 248.3nM in controls. Kruskal Wallis testing of the three medians showed no significant difference (KW 1.387, p=0.4998).

Median ETP was measured at 1695.0nM/min in IS, 1612.6nM/min in TIA and 1561.9nM/min in controls. Kruskal Wallis testing of the three medians showed no significant difference (KW 0.473, p=0.7892).

Longitudinal data showed more distinct trends, as illustrated in Figure 13

Figure 13: Longitudinal trends of thrombin generation: peak thrombin and ETP

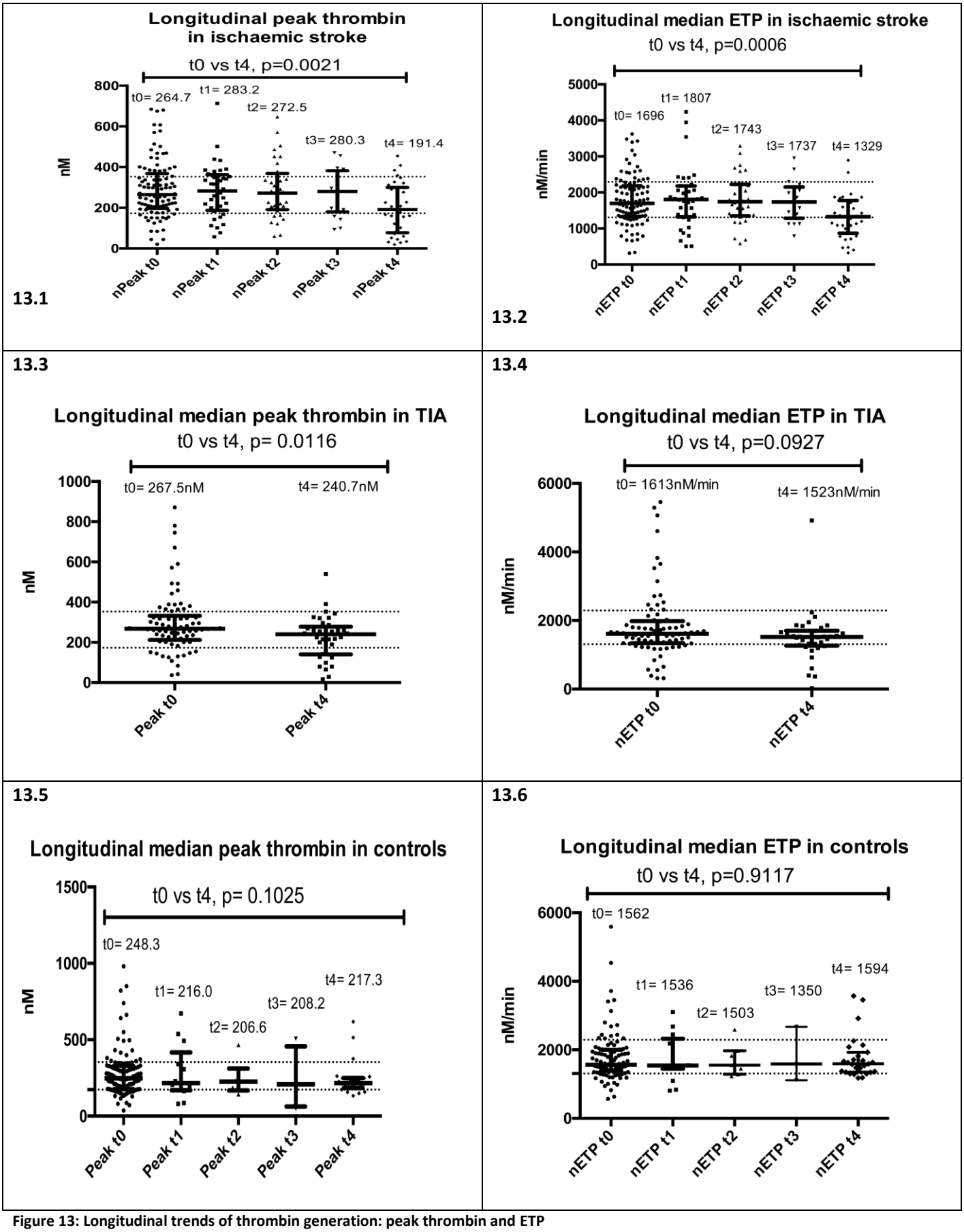


Figure 13: Longitudinal trends of thrombin generation: peak thrombin and ETP

The graphs in Figure 13 demonstrate differences in thrombin generation over longitudinal follow up in ischaemic stroke, TIA and control groups. Mann Whitney tests were performed as for unpaired, non-parametric data analysis. The dotted lines demonstrate the normal ranges, and the bars illustrate the median and interquartile range for each set of values. Data sets were limited over the longitudinal follow up, a limitation of the study as previously discussed. The number of patients included at each point are as follows: IS t0 n=97, t1 n=36, t2 n=32, t3 n=19, t4 n=35; TIA t0 n=78, t4 n=37; control t0 n=105, t1 n=15, t2 n=7, t3 n=4 and t4 n=27

In ischaemic stroke, heightened thrombin generation at presentation showed decrease at final follow up (presentation vs final follow up respectively, peak: 264.7 to 191.4nM, p=0.0021, Figure 13.1; ETP: 1695 nM/min to 1329 nM/min, p=0.0006, Figure 13.2).

The same trend was followed by the TIA group, which demonstrated a decrease in both thrombin generation markers, which reached significance with peak thrombin (presentation vs final follow up respectively, peak: 267.5 to 240.7nM, p=0.0116, Figure 13.3; ETP 1613 to 1523nM/min, p=0.0927, Figure 13.4).

This was in comparison to the control group, which demonstrated no significant change in markers of thrombin generation (peak thrombin 248.3 to 217.3nM, p=0.1025, Figure 13.5; ETP 1562 nM/min to 1594 nM/min, p=0.9117, Figure 13.6).

5.15 Longitudinal changes in IS: impact of thrombolysis

As part of hyperacute stroke management, an expected number of patients were thrombolysed following admission. Baseline bloods were taken beforehand, so time point zero represents pre-thrombolysis state. Time point 1 and thereafter represents post-thrombolysis. Overall, 42 patients were thrombolysed. Characteristics are outlined below:

Table 32: Thrombolysis comparison

	Thrombolysed (n=42)	**Non-thrombolysed (n=266)**
Gender	M=24 F=18	M=125 F=141
Diagnosis: IS	IS= 38 C= 4	IS=65 C=105 HS=16 TIA=80
Age, median (range)	73.5 (42-94)	71 (23-100)

Table 32: Comparison of the patient cohort according to whether thrombolysed or not (demographics at presentation).

For fair comparison, those subsequently confirmed with IS (n=38) were compared with non-thrombolysed IS (n=65). Follow up periods were 236 days (range 49- 735 days) in the thrombolysed group, and 298 days (range 48-889 days) in the non-thrombolysed group.

Table 33: Thrombolysis in ischaemic stroke: comparison of thrombolysed and non-thrombolysed groups at presentation: clinical scores and haemostatic markers at presentation

Ischaemic Stroke	**THROMBOLYSED (N=38)**	**NON-THROMBOLYSED (N=65)**	**Mann Whitney testing (p value)**
Gender	M: 23; F: 15	M: 33; F: 32	xx
Age (median, range)	74.5 (42- 94)	77 (42-97)	0.397
Pre-admission mRS (median, range)	0 (0-5)	1 (0-5)	0.013
mRS at presentation (t0; median, range)	3.5 (0-5)	3 (0-5)	0.587
NIHSS at presentation (t0; median, range)	6 (0-22)	3 (0-28)	0.005
GCS at presentation (T0; median, range)	15 (11-15)	15 (10-15)	0.479
VWF: Ag (t0; IU/dL, median, range)	187.9 (75.4- 152.8)	227.2 (87.2- 538.1)	0.0352
ADAMTS13Ac (t0; median, range)	84 (43.6- 113.8)	86 (38.8- 114.2)	0.860
Ratio VWF:Ag-ADAMTS13Ac (t0; median, range)	**2.02 (0.79- 5.69)**	**2.67 (1.00-9.53)**	**0.066**

Table 33: Comparing the two groups, VWF:Ag was higher in the non-thrombolysed group at presentation on Mann Whitney testing (see text below). There were were no differences in any other haemostatic marker at presentation on Mann Whitney testing, including the overall VWF:Ag- ADAMTS13Ac ratio.

Table 34: Thrombolysis in ischaemic stroke: comparison of thrombolysed and non-thrombolysed groups over longitudinal follow up

Ischaemic Stroke	**THROMBOLYSED (N=38)**	**NON-THROMBOLYSED (N=65)**	**Mann Whitney testing (p value)**
mRS t1 (median, range)	3 (0-5)	4 (0-5)	0.187
NIHSS t1 (median, range)	3 (0-14)	2 (0-26)	0.913
VWFAg t1 (median, range)	159.7 (80- 334)	216.9 (80.8- 367.5)	0.0949
ADAMTS13 t1 (median, range)	83 (45- 105.9)	83 (31-108)	0.614
Ratio VWF:Ag- ADAMTS13Ac t1 (median, range)	**1.90 (0.82- 6.00)**	**2.69 (0.97- 11.3)**	**0.154**
mRS t2 (median, range)	4 (0-5)	4 (1-5)	0.553
NIHSS t2 (median, range)	3 (0-19)	3 (0-14)	0.787
VWFAg t2 (median, range)	192.1 (75.4- 314.8)	202.5 (88.2- 336.1)	0.5507
ADAMTS13 t2 (median, range)	88 (58 – 107.9)	85 (56.9- 108)	0.860
Ratio VWF:Ag- ADAMTS13Ac t2 (median, range)	**2.13 (0.74- 4.44)**	**2.46 (0.85- 5.19)**	**0.609**
mRS t3 (median, range)	4 (0-5)	4 (0-5)	0.50
NIHSS t3 (median, range)	7 (0-26)	5.5 (0-24)	0.816
VWFAg t3 (median, range)	199.5 (57.4- 280)	236.9 (133.8- 334.9)	0.1117
ADAMTS13 t3 (median, range)	95.8 (64.3- 115.2)	85.5 (54- 118.2)	0.428
Ratio VWF:Ag- ADAMTS13Ac t3 (median, range)	**1.99 (0.50- 3.82)**	**2.61 (1.63- 4.21)**	**0.293**
mRS t4 (median, range)	0 (0-5)	2 (0-6)	0.734
NIHSS t4 (median, range)	0 (0-8)	0 (0-6)	0.557
VWFAg t4 (median, range)	141.1 (45.6 – 283.0)	190.9 (90.0- 287.9)	0.0747
ADAMTS13Ac t4 (median, range)	100.8 (60.4- 123)	91 (60-119)	0.0758
Ratio VWF:Ag- ADAMTS13Ac t4 (median, range)	**1.35 (0.47- 3.42)**	**2.19 (0.9- 3.29)**	**0.063**

Table 34: Ischaemic stroke clinical and haemostatic measurements at each time point over longitudinal follow up, according to whether thrombolysed or not. Mann Whitney testing was utilised to compare the two groups (thrombolysed versus non-thrombolysed) for each haemostatic marker at each time point, with no significant differences demonstrated. Differences between the groups are discussed further in the text below.

5.16 Thrombolysis and VWF:Ag-ADAMTS13Ac in ischaemic stroke

Mann Whitney testing was utilised to compared the thrombolysed ischaemic stroke group with the non-thrombolysed ischaemic stroke group, at presentation and longitudinally; as well as to compare trends within each group. Presentation ADAMTS13Ac was comparable between the groups but the thrombolysed group showed increased ADAMTS13Ac at follow up (t0 84 to t4 100.8IU/dL, p= 0.0025, median follow up 221 days) with no change in the non-thrombolysed group (t0 86 to t4 91IU/dL, p=0.285, median follow up n= 286 days).

Presentation VWF:Ag was lower in the thrombolysed group compared with the non-thrombolysed group at presentation (median 187.9 vs 227.2IU/dl: p=0.0352) with no differences between the groups at any point in later follow up. A potential reason for this is that VWF was higher in the group of patients not subsequently thrombolysed, because these patients had reached their ischaemic peak, reflected in the VWF levels, but timing of symptom onset meant they were excluded from being safely thrombolysed (ie >4.5 hours from symptom onset).

The thrombolysed group showed decreased VWF:Ag at follow up (187.9IU/dL to 141.1IU/dL, p=0.0187) with no change in the non-thrombolysed IS group (227.2 to 190.9IU/dL**,** p=0.0747). The thrombolysed group had 37 values at presentation to 16 at final follow up; the non-thrombolysed IS group had 63 values at presentation to 19 at final follow up.

Correspondingly, the difference in VWF:Ag- ADAMTS13Ac ratio from presentation to final follow-up was far more marked in the thrombolysed group compared to the non-thrombolysed (2.024 to 1.355 in thrombolysed group, p=0.0052; 2.672 to 2.091 in non-thrombolysed group, p= 0.0339). This suggests that normalisation of the VWF:Ag-ADAMTS13Ac balance is better achieved in the thrombolysed group compared to the non-thrombolysed.

Figure 14 below illustrates the resolution of ADAMTS13Ac over time in the thrombolysed IS cohort. Three low values are notable at final follow up (ADAMTS13Ac 65.7, 60.4 and 61.7 IU/dL respectively), despite the overall upward trend. Despite being below the normal range, these values still represent an improvement from lower ADAMTS13 at presentation (61.8, 54.6 and one not available). One of these patients was a heterozygote for ADAMTS13 exon 24 (SNP p.Arg1060Trp), known to be associated with lower ADAMTS13Ac levels.

Figure 14: ADAMTS13 and ratio VWF:Ag- ADAMTS13 in thrombolysed ischaemic stroke versus non-thrombolysed ischaemic stroke

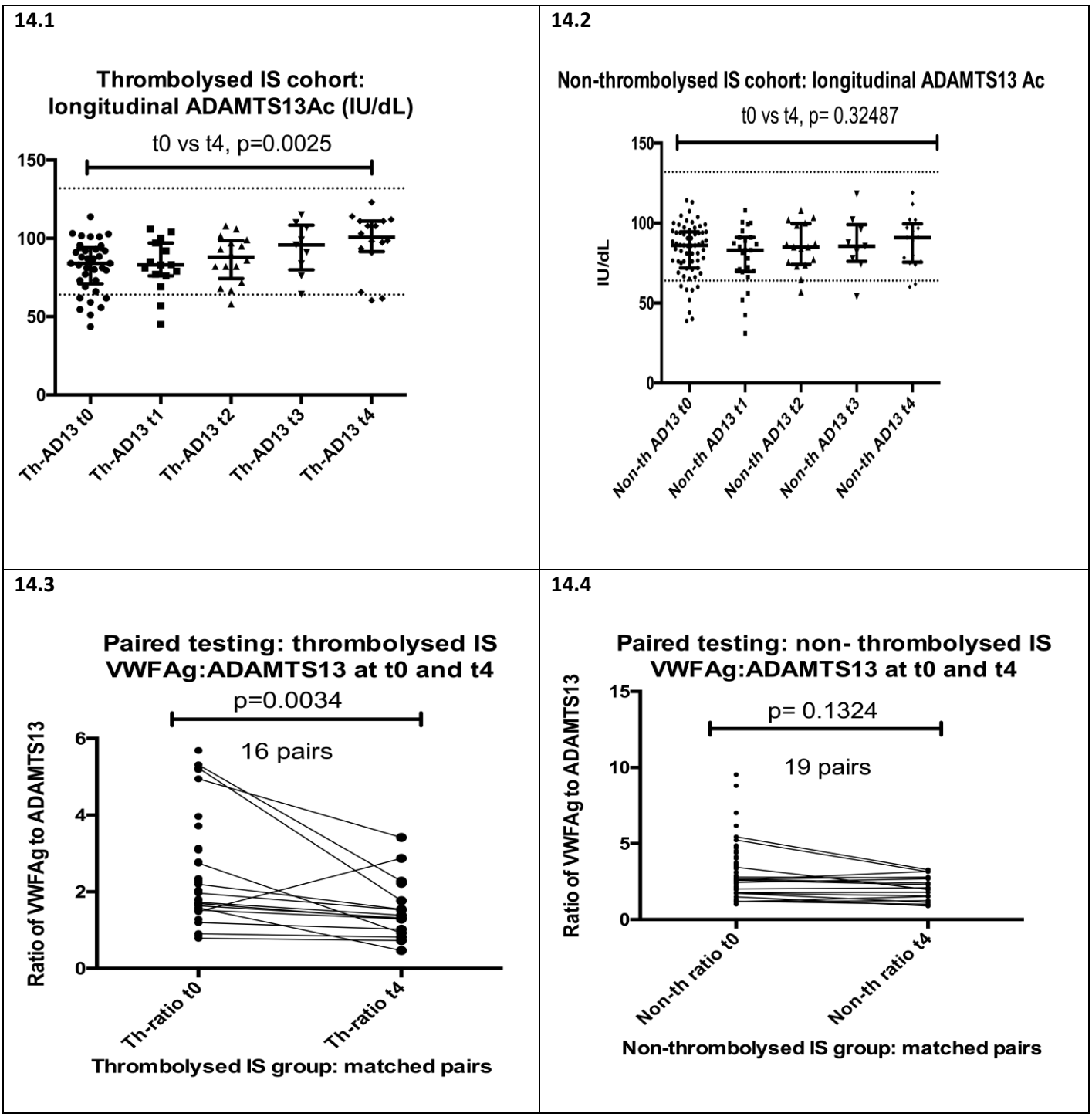


Figure 14: Longitudinal analysis shows increasing ADAMTS13Ac and decreasing VWF:Ag in the thrombolysed group over the weeks of follow up, yet absent in the non- thrombolysed group (thrombolysed group t0 VWFAg 187.9 to t4 141.1IU/dL, p=0.0187; t0 ADAMTS13 84 to 100.8IU/dL, p=0.0025; versus non-thrombolysed group t0 VWFAg 227.2 to 190.9IU/dL, p=0.0944; t0 ADAMTS13 86 to 90.9, p=0.285). There was a corresponding marked difference in VWF:Ag-ADAMTS13Ac ratio from presentation to final follow-up in the thrombolysed IS group (t0 2.024 to t4 1.355, p=0.0052) compared to the non-thrombolysed IS group (2.672 to 2.091 in non-thrombolysed group, p= 0.0339). This was demonstrated in both Mann Whitney testing to capture all values, and paired testing (thrombolysed group: 16 pairs, change in ratio 0.67 p= 0.0034; non-thrombolysed groups: 19 pairs, change in ratio 0.58 p=0.1324. There was therefore a more marked difference in the thrombolysed group. This contrast may reflect infarct extent and consequent stroke severity.

Normalisation of the VWF:Ag-ADAMTS13Ac axis was more effectively achieved with thrombolysis. Thrombolysis was also associated with the expected resolution in clinical scores, with a marked difference seen in the thrombolysed group mRS from presentation to follow up (3.5 to 0, p=0.002) compared to the non-thrombolysed group (3 to 2, p=0.062).

To try to adjust for other patient factors that could confound this interpretation, we considered patient age and stroke severity. Firstly, there was no significant difference in the age of the thrombolysed IS group vs the non-thrombolysed IS group (74.5 vs 77 years, p= 0.397). Secondly, patients were then grouped by stroke severity to see if this altered the VWF:Ag- ADAMTS13Ac differences. The ischaemic stroke cohort was split into presentation mRS 0-3 vs 4-5, to achieve a meaningful split in functional score and balanced patient numbers (mRS 0-3 n=51, mRS 4-5 n=49). For the mRS 0-3 group, the same trend was demonstrated: a significant decrease in VWF:Ag- ADAMTS13Ac ratio from presentation to final follow-up in the thrombolysed group compared to the non-thrombolysed (ratio 1.97 to 1.46 in the thrombolysed group, n=19, p=0.02; with an increased ratio of 2.05 to 2.24 in the non-thrombolysed group, n= 32, p= 0.78). For the mRS 4-5 group, the trend was repeated but did not reach significance for the thrombolysed group- potentially because of a relatively reduced patient number (thrombolysed group VWF:Ag- ADAMTS13Ac ratio at t0 2.24 to t4 1.29, n=19, p=0.17; vs non-thrombolysed group ratio t0 2.88 to t4 2.20, n=30, p=0.01).

Table 35: Ischaemic stroke mRS 0-3 at presentation: comparison of VWF:Ag-ADAMTS13Ac ratio at each time point according to whether thrombolysed or not.

mRS 0-3 n=51	Thrombolysed IS n=19	Non-thrombolysed IS n=32	Mann Whitney test
Age	69 (42-94)	76 (42-92)	p=0.19
T0	1.97	2.05	p=0.57
T1	1.82	2.10	p=0.92
T2	2.13	1.53	p=0.35
T3	2.13	3.41	p=0.14
T4	1.46	2.24	p=0.07
T0 to T1	p-0.84 (unpaired t-test) p=0.50 on paired t-testing (9 pairs)	p=0.99 (unpaired t-test) p=0.28 on paired t-testing (10 pairs)	
T0 to T4	p-0.02 (unpaired t-test) p=0.001 on paired t-testing (11 pairs)	p=0.78 (unpaired t-test) p=0.28 on paired t-testing (10 pairs)	

Table 36: Ischaemic stroke mRS 4-5 at presentation: comparison of VWF:Ag-ADAMTS13Ac ratio at each time point according to whether thrombolysed or not.

mRS 4-5 (n=49)	Thrombolysed IS (n=19)	Non-thrombolysed IS (n= 30)	Mann Whitney test
Age	81 (41-94)	79.5 (60- 97)	p= 0.397
T0	2.24	2.88	p= 0.0513
T1	1.93	2.74	p= 0.067
T2	2.23	2.66	p= 0.276
T3	2.00	2.52	p= 0.715
T4	1.29	2.30	p= 0.47
T0 to t1	0.54 on unpaired t-test 0.813 (matched pairs n=7)	>0.99 on unpaired t-test >0.909 (matched pairs n=12)	
T0 to T4	P=0.17 on unpaired t-test P=0.313 on matched pairs (n=5)	P=0.01 on unpaired t-test P=0.02 on matched pairs (n=8)	

Tables 35 and 36: The ischaemic stroke cohort was split into presentation mRS 0-3 vs 4-5, to achieve a meaningful split in functional score and balanced patient numbers (mRS 0-3 n=51, mRS 4-5 n=49). For the mRS 0-3 group, the same trend was demonstrated: a significant decrease in VWF:Ag- ADAMTS13Ac ratio from presentation to final follow-up in the thrombolysed group compared to the non-thrombolysed (ratio 1.97 to 1.46 in the thrombolysed group, n=19, p=0.02; with an increased ratio of 2.05 to 2.24 in the non-thrombolysed group, n= 32, p= 0.78; Table 35). For the mRS 4-5 group, the trend was repeated but did not reach significance for the thrombolysed group- potentially because of a relatively reduced patient number (thrombolysed group VWF:Ag- ADAMTS13Ac ratio at t0 2.24 to t4 1.29, n=19, p=0.17; vs non-thrombolysed group ratio t0 2.88 to t4 2.20, n=30, p=0.01; Table 36).

5.17 Thrombolysis and thrombin generation

Markers of thrombin generation (both ETP and peak) were significantly higher in the thrombolysed group compared to the non-thrombolysed group at presentation and at 24 hours post presentation (see table 34 below). As discussed above pertaining to the VWF:Ag-ADAMTS13Ac differences between the groups at presentation, this may be related to the earlier timing of presentation of those patients who could be eligible for thrombolysis, potentially at the peak of thrombin generation in vivo. However, there was no significant difference between the groups in later time points.

Table 37: Thrombin generation in ischaemic stroke: comparison according to whether thrombolysed or not

Ischaemic stroke	Thrombolysed IS (n=35)	Non-thrombolysed IS (n= 62)	Mann Whitney test
Age	74.5 (42-97)	77 (42- 94)	p=0.397
T0 ETP	2092	1592	p=0.0018
T0 Peak	320.6	249.1	p= 0.0046
T1 ETP	2062	1650	p=0.0095
T1 Peak	351.2	220.8	p=0.0117
T2 ETP	1963	1585	p=0.079
T2 Peak	287	215.4	p= 0.195
T3 ETP	1985	1455	p=0.0943
T3 Peak	330.6	224.4	p=0.112
T4 ETP	1394	1196	p=0.702
T4 Peak	191.6	191.4	p= 0.728
T0 to t1	ETP 0.576 on unpaired t-test, 0.677 on matched pairs (n=12) Peak 0.610 on unpaired t-test, 0.148 on matched pairs (n=12)	ETP 0.792 on unpaired t-test, 0.648 on matched pairs (n=20) Peak 0.663 on unpaired t-test, 0.927 on matched pairs (n=20)	
T0 to T4	ETP p=0.0003 on unpaired t-test, p=0.0034 on matched pairs (n=15). Peak p=0.0010 on unpaired t-test, p=0.0020 on matched pairs (n=15)	ETP 0.0676 on unpaired t-test, p=0.0052 on matched pairs (n=16) Peak p=0.0623 on unpaired t-test, p=0.0063 on matched pairs (n=16)	

Table 37: There was no change from presentation to day 1 in either group in markers of thrombin generation, whether thrombolysed or not (t0 to t1, see table above). Longitudinal changes from presentation to final follow up were significant in the thrombolysed group and almost significant in the non-thrombolysed IS group on Mann Whitney testing (t0 to t4).

Figure 15: Longitudinal changes in thrombin generation in thrombolysed compared to non-thrombolysed ischaemic stroke

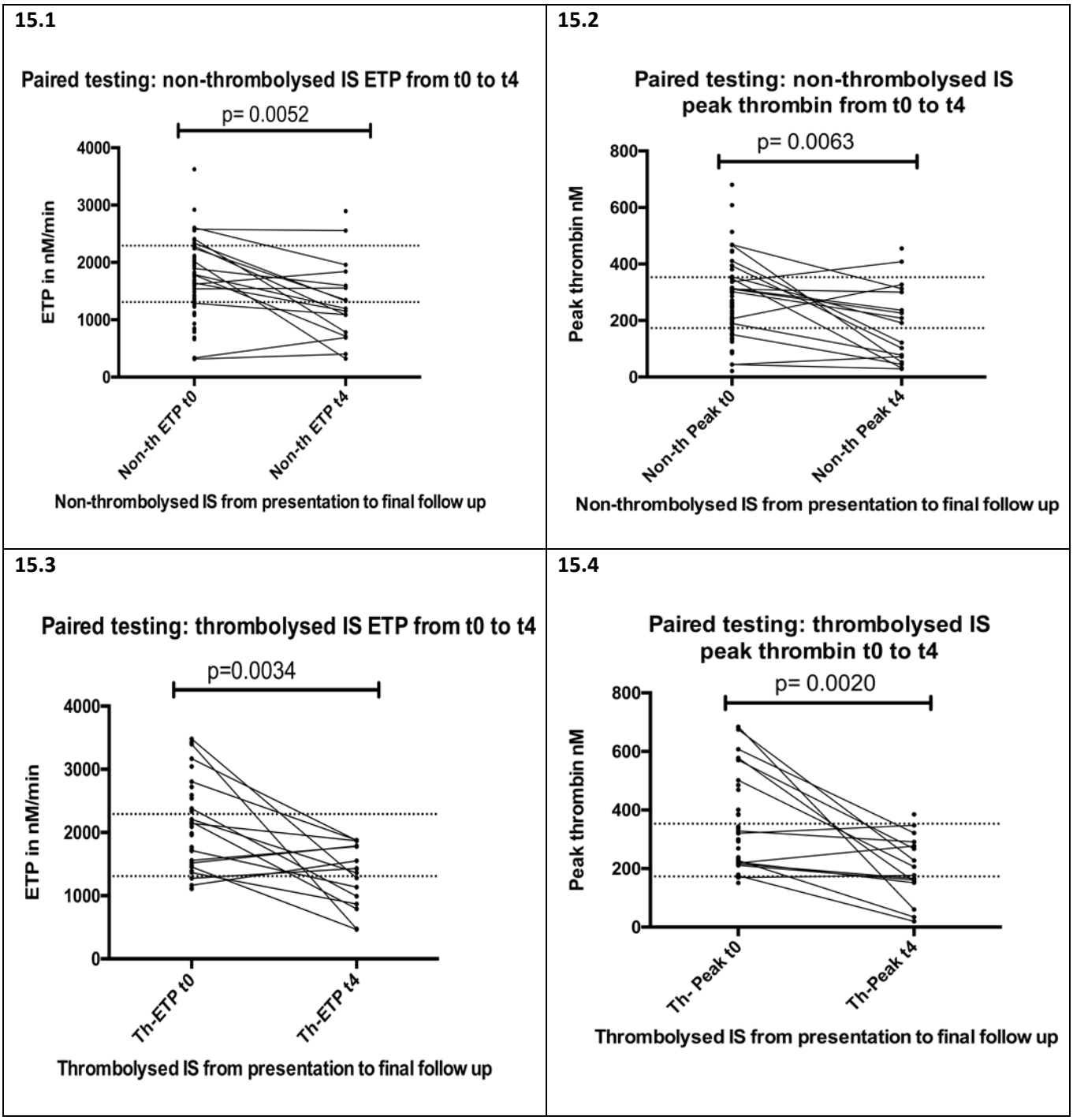


Figure 15: For patients with paired data sets available from presentation to final follow up, Wilcoxon matched-pairs signed rank testing for both groups showed significant resolution. The non-thrombolysed group showed ETP 1592 to 1196 nM/min, p=0.0052 (Figure 15.1) and peak thrombin 249.1 to 191.4 nM, p=0.0063 in 16 matched pairs (Figure 15.2). The thrombolysed group showed ETP 2092nM/min to 1394 nM/min, p=0.00034 (Figure 15.3) and peak thrombin 320.6 to 191.6nM, p=0.0020 in 15 matched pairs (Figure 15.4).

Overall, the difference between the thrombolysed and non-thrombolysed groups in markers of thrombin generation almost resolved between presentation and final follow up (differences between thrombolysed and non-thrombolysed groups in peak thrombin 71.5nM and ETP 500nM/min at baseline, to peak thrombin 0.2nM and ETP 198nM/min at final follow up).

To test this furthermore, as with the VWF:Ag- ADAMTS13Ac axis, potential confounders were considerd. There was no significant difference in age between the thrombolysed ischaemic stroke group and non-thrombolysed ischaemic stroke group, as previously described (see table 35 below). The ischaemic stroke group was split according to presenting mRS 0-3 vs 4-5, to achieve a meaningful split in functional score and balanced patient numbers (mRS 0-3 n=51, mRS 4-5 n=49).

For the mRS 0-3 group, thrombolysis was associated with a significant reduction in ETP from t0 to t4 on Mann Whitney testing (t0 1866 to t4 1431, p=0.0388) though not on matched pairs (p=0.084, n=10); and a significant reduction in peak thrombin (t0 314.0 to 176.9, p=0.024) repeated on matched paired testing (p=0.0195, n=10).

The non-thrombolysed group did not demonstrate a significant reduction on Mann Whitney testing of the whole group (ETP t0 1723 to t4 1345, p=0.1135; peak thrombin t0 300.4 to t4 226.3, p=0.231); but interestingly paired testing did show decreases in both markers of thrombin generation (10 pairs, ETP p=0.0137, peak thrombin p=0.0488).

Table 38: Ischaemic stroke mRS 0-3 at presentation: comparison of thrombin generation at presentation and final follow up according to whether thrombolysed or not.

mRS 0-3 n=51	Thrombolysed IS n=19	Non-thrombolysed IS n=32	Mann Whitney test
Age	69 (42-94)	76 (42-92)	p=0.19
T0 Peak thrombin	314.0	300.4	p=0.439
T0 ETP	1866	1723	p=0.439
T4 Peak thrombin	176.9	300.4	p=0.596
T4 ETP	1431	1345	p=0.931
T0 to T4 peak thrombin	P=0.024 on unpaired t-test P=0.0195 on matched pairs (n=10)	P=0.231 on unpaired t-test P=0.0488 on matched pairs (n=10)	
T0 to T4 ETP	p=0.0388 on unpaired t-test p=0.084 on matched pairs (n=10)	p=0.1135 on unpaired t-test p=0.0137 on matched pairs (n=10)	

Table 38: Thrombin generation markers in ischaemic stroke with presenting mRS of 0-3.

For the mRS 4-5 group, the picture was more mixed. Both the thrombolysed and non-thrombolysed groups demonstrated a downward trend in markers of thrombin generation with various levels of significance. Thrombolysis was associated with a significant reduction in ETP from t0 to t4 (t0 2143 to t4 1134, p=0.0045) though not on matched pairs (p=0.0625, n=5), arguably related to a limited number of pairs. This was also seen in peak thrombin (t0 320.6 to 206.3, p=0.0294; matched pair testing p=0.125, n=5). The non-thrombolysed group showed significant reduction of markers on Mann Whitney testing of the whole group (ETP t0 1377 to t4 886, p=0.0332; peak thrombin t0 204.7 to t4 62.3, p=0.0012); but not when limited to matched pairs only (n=6, ETP p=0.438 and peak thrombin p=0.219).

Table 39: Ischaemic stroke mRS 4-5 at presentation: comparison of thrombin generation at presentation and final follow up according to whether thrombolysed or not.

mRS 4-5 (n=49)	Thrombolysed IS (n=19)	Non-thrombolysed IS (n= 30)	Mann Whitney test
Age	81 (41-94)	79.5 (60- 97)	P=0.397
T0 Peak thrombin	320.6	204.7	p=0.0294
T0 ETP	2143	1377	p=0.0003
T4 Peak thrombin	206.3	62.3	p=0.126
T4 ETP	1134	886	p=0.247
T0 to T4 peak thrombin	P=0.0294 on unpaired t-test P=0.125 on matched pairs (n=5)	P=0.0012 on unpaired t-test P=0.219 on matched pairs (n=6)	
T0 to T4 ETP	p=0.0045 on unpaired t-test p=0.0625 on matched pairs (n=5)	p=0.0332 on unpaired t-test p=0.4375 on matched pairs (n=6)	

Table 38: Thrombin generation markers in ischaemic stroke with presenting mRS of 4-5.

Overall, reduction in markers of thrombin generation, and hence thrombogenic potential, may be associated with convalescence following ischaemic stroke. The overall ischaemic stroke group data suggest this is expedited by thrombolysis, but splitting the cohort according to disability score is less supportive of this conclusion. Less significance in splitting the cohort could be related to consequent loss of patient numbers in each group, and further work with an increased cohort size and improved follow up data would be of interest.

5.18 Impact of genetic variation and ischaemic stroke: results and comment

5.18.1 ADAMTS13 mutations

PCR was performed on 304 patients for whom DNA was available aiming to investigate whether the frequency of the SNPs noted by De Vries et al would be repeated, and whether this could be linked to stroke incidence. (De Vries et al. 2015) The *ADAMTS13* exon 24 SNP (p.Arg1060Trp) was found in 2 patients (heterozygous), with the remaining 302 were wild type. Exon 18 SNP (p.ALA732Val) was found in 8 patients (heterozygous), with the remaining 296 were wild type. The characteristics of these 10 patients are outlined in Table 37 below:

Table 40: Patients with *ADAMTS13* mutations

Clinical and laboratory characteristics	Patients with *ADAMTS13* mutations: i) *ADAMTS13* exon 24 SNP (p.Arg1060Trp) ii) *ADAMTS13* exon 18 SNP (p.Ala732Val)	Patients without mutations in exon 24 or exon 18	Mann Whitney testing p value
Gender	M: 6, F: 4	M: 141, F: 153	xx
Age in years, median (range)	74.5 (34- 87)	72 (23-100)	p= 0.680
Subgroup	IS= 4, TIA= 2, Control= 3, HS= 1	IS=94, TIA=76, Control=104, HS=15	xx
mRS	2.5 (0-5)	1 (0-5)	p= 0.729
NIHSS	2.5 (0-21)	1 (0-28)	p=0.690
GCS	15 (13-15)	15 (7-15)	p=0.878
VWF:Ag	198.8 (115.2- 341.8)	177.5 (22.6- 600)	p=0.22
VWF:Act	200.1 (115.4- 287)	158.3 (26.7- 338.4)	p=0.138
ADAMTS13Ac	76 (38.38- 98)	91.5 (34.5- 137)	p=0.0117
Ratio VWF:Ag-ADAMTS13Ac	2.35 (2.77-8.81)	1.98 (0.25-15.6)	p=0.0481
FVIII	192.5 (93.7- 313.9)	152.9 (43.1- 705.5)	p=0.266
ETP (nM/min)	1403.3 (550.4- 2408.3)	1597 (0-5592)	p=0.229
Peak (nM)	267.1 (85.1-505.4)	262.6 (0- 980.5)	p=0.836

Table 40: VWF was higher in the *ADAMTS13* mutated group compared to the wild type population (both exons 24 and 18), though not significantly (VWF:Ag 198.8 IU/dL vs 177.5IU/dL, p=0.22; VWF:Act 200.1 vs 158.3IU/dL, p= 0.138). ADAMTS13 activity in the 10 patients with *ADAMTS13* mutations was significantly lower compared to those with wild type (76 IU/dL vs 91.5 IU/dL, p=0.0117). This was also reflected in the VWF:Ag-ADAMTS13Ac ratio, higher in the *ADAMTS13* mutated group: 2.35 vs 1.98, p=0.0481. There was no significant difference in thrombin generation markers or clinical scores of disability between the *ADAMTS13* mutated group and wild type group.

Investigation of the cohort for mutations known to influence ADAMTS13 activity was done as a matter of interest, to consider whether a significant reduction in ADAMTS13 and consequent heightening of the VWF:Ag- ADAMTS13Ac ratio was relevant in the ischaemic brain injury groups. Overall the presence of the

SNPs at equal frequency in the control groups suggest irrelevance; which could be investigated further in cohort studies of far greater patient numbers.

5.18.2 CYP 2Y19C*2, clopidogrel resistance and secondary stroke prevention

A potential mechanism for platelet resistance could be reflected in VWF levels as a marker of endothelial activation. Examining for the most commonly known clopidogrel 'resistance' gene, PCR analysis was performed in the same 304 patients for whom DNA was available. Analysis failed in one patient on 2 occasions. Overall the 681G>A polymorphism associated with the CYP2C19 (*2) allele and clopidogrel resistance was seen in 10 patients homozygous for the allele and 103 patients heterozygous for the allele. The remaining 190 patients were wild type for the polymorphism. Comparison of patients at baseline according to CYP 2Y19C*2 status showed no difference in ratio of VWF:Ag-ADAMTS13Ac between those homozygous for the mutation (AA) and homozygous for wild type (GG), nor in those heterozygous for the mutation (AG) compared to wild type (GG). .

Further analysis focused on those either heterozygous or homozygous for the mutation, dividing those who were established on clopidogrel at baseline compared to those whom are not. Previous evidence had suggested carriers of a reduced-function CYP2C19 allele had significantly lower levels of the clopidogrel active metabolite, decreased platelet inhibition and an increased rate of major cardiovascular events (Mega et al. 2008). We hypothesised that this could be manifest in thrombin generation and potentially in the VWF-ADAMTS13 axis.

There were no differences in VWF, ADAMTS13, VWFAg- ADAMTS13Ac or thrombin generation at baseline or final follow up in the overall population in those with 'clopidogrel resistance' alleles according to whether they were on clopidogrel or not. This also applied when restricted to those with acute ischaemic brain injury, whether ischaemic stroke or TIA.

In the group without the CYP2C19 genetic variant associated with reduced enzyme function (*2), there was similarly no difference in VWF, ADAMTS13, ratio of VWF:Ag-ADAMTS13 or thrombin generation at baseline or final follow up in the overall population. Again, this also applied when restricted to those with acute ischaemic brain injury only, whether ischaemic stroke or TIA.

In those patients taking clopidogrel (n=32), there was no difference in VWF, ADAMTS13, VWFAg- ADAMTS13Ac or thrombin generation according to whether homozygous or heterozygous for the 681G>A polymorphism associated with the CYP2C19 (*2) allele versus those wild type.

Table 41: CYP2C19 (*2) polymorphism and relationship with haemostatic markers

Clinical and laboratory characteristics	CYP2C19 (*2) allele 681G>A polymorphism Heterozygous or homozygous	Wild type CYP2C19 allele	Significance
Age in years, median (range)	75 (50-90)	75 (25-93)	0.6341
VWF:Ag	202.7 (88.3- 316.7)	188.7 (63.4- 381.2)	p=0.730
VWF:Act	182.5 (69.7- 310.8)	155.3 (58.7- 307)	p=0.290
ADAMTS13Ac	89.3 (55-117.2)	90.2 (40.0- 129.0)	p=0.622
Ratio VWF:Ag-ADAMTS13Ac	2.267 (0.79-4.19)	2.264 (0.49- 9.53)	p=0.991
FVIII	158.4 (88.1- 344.9)	144 (85.8- 493.3)	p=0.614
nETP (nM/min)	1710 (1353- 2724)	1447 (1080- 2920)	p=0.153
nPeak (nM)	302.2 (134.7-850.4)	251.2 (150.8- 608.6)	p=0.419

6. VWF AND ADAMTS13: Discussion

6.1 The VWF:Ag-ADAMTS13Ac axis in acute ischaemic brain injury

We sought to examine the VWF:Ag-ADAMTS13 axis in ischaemic brain injury, both at presentation and longitudinally, in conjunction with clinical outcome. Heightened VWF levels are recognized as a thrombotic risk factor. Decreased ADAMTS13Ac, whether independently or synergistically, is gaining momentum as a risk factor in itself. We have summarised the relationship by focusing on the VWF:Ag-ADAMTS13Ac ratio as a biological measure of the disturbed haemostatic balance after ischaemic stroke or TIA, incorporating both the elevation of VWF and reduction of ADAMTS13. In addition, our observations suggest an intriguing relationship with thrombolysis and later clinical outcome, with implications for future therapeutic considerations.

6.2 Presentation

Reduction of ADAMTS13Ac at presentation of ischaemic stroke, compared to both TIA and control groups, supports association of presentation ADAMTS13Ac with clinical severity of ischaemic brain injury. Confirmed ischaemic stroke demonstrated the lowest ADAMTS13Ac, whereas the TIA and control groups had a more comparable and higher ADAMTS13Ac level.

Conversely, VWF:Ag was significantly higher in ischaemic stroke compared to both TIA and control groups, with similar trends seen with VWF:Act (see Table 11, Figures 4 and 5) . Differences in the VWF:Ag-ADAMTS13Ac axis between groups persisted following adjustment for ABO blood group (see Table 13). Unlike VWF:Ag-ADAMTS13Ac, thrombin generation did not show significant differences between groups at baseline (see Figure 12).

Increased VWF and reduced ADAMTS13 in stroke supports previously published work (Bath et al. 1998; Bongers et al. 2006; Green et al. 2016; Lambers et al. 2013; Wannamethee et al. 2012). We examined the individual haemostatic markers and overall VWF:Ag to ADAMTS13Ac balance, to ensure we focused on the clinical associations of the biological axis between the two.. As with the aforementioned Chinese case-control cohort, we found strong association between the VWF:Ag- ADAMTS13Ac ratio and cerebral infarction (Qu et al. 2016).

Recruitment of patients at time of ischaemic stroke or TIA means we cannot prove causality of the VWF:Ag-ADAMTS13Ac axis as a risk factor for ischaemic stroke, although prospective data certainly support this (Sonneveld et al. 2015, 2016; Wieberdink et al. 2010). Ischaemic brain injury results in increased ULVWF being released to the damaged vascular endothelium, whereupon it is cleaved by ADAMTS13 to smaller, less haemostatically active VWF multimers- hence ADAMTS13 decreased and VWF increased (Sonneveld et al. 2015; Wannamethee et al. 2012; Wieberdink et al. 2010). Moreover, discrepancies between IS, TIA and

controls support that clinical severity of arterial thrombosis and consequent ischaemic brain injury can be associated with higher VWF and lower ADAMTS13Ac.

We included a haemorrhagic stroke subgroup in our analysis and found this group to have the highest baseline VWF:Ag, VWF:Act and FVIII and lowest ADAMTS13Ac. It is difficult to comment further since this group was so small but this potentially represents an immediate acute phase response in order to secure haemostasis. Further prospective work is warranted to investigate the VWF:Ag-ADAMTS13Ac axis changes in this group, ideally from prior to the event, particularly since the aetiology and pathophysiology is different to ischaemic brain injury.

To further examine the link of the VWF:Ag-ADAMTS13Ac axis with clinical severity at presentation, the TIA group was split by mode of presentation- whether in-patients or outpatients. The inpatient group had expected significantly higher median age and functional scores compared to the outpatient group, in keeping with hospital admission being necessitated. Presentation VWF:Ag, VWF:Act and FVIII were also significantly higher in in-patients compared to outpatients, but ADAMTS13Ac was comparable- ie even in those warranting in-patient admission. Since the overall TIA and control groups had similar ADAMTS13Ac levels, this could suggest ADAMTS13 is maintained until significant ischaemia, manifest clinically by a 'full-blown' ischaemic stroke.

At study design, we deliberately chose 'stroke mimics' to be our control group, as a sensible way of recruiting appropriate controls to the study- ie exactly the same inclusion and exclusion criteria. However, it is clear that our control group was significantly younger than the stroke group, which must be taken into account in examining the results. We have sought statistical guidance for the analysis, and taken care to adjust for age as much as possible.

To examine functional implications of the disordered VWF:Ag-ADAMTS13Ac balance in ischaemic stroke, we first used correlation to demonstrate a positive association of the VWF:Ag-ADAMTS13 ratio with age, Rankin and NIHSS; and negative association with GCS, reflective of correlation with disability.

Regression was used to investigate association of laboratory and clinical markers at presentation with the VWF:Ag- ADAMTS13Ac ratio. A positive association was seen with creatinine, bilirubin and CRP; which is biologically plausible; in keeping with infection and inflammation being linked with thrombogenicity. Regression was used in an attempt to model out the impact of age and disability scores on the VWF:Ag-ADAMTS13Ac ratio, to see whether diagnosis alone could independently predict the ratio. Several models have been included to illustrate the work suggested by statisticians. Regression modelling did not show that diagnosis of ischaemic stroke or TIA predicts the VWF:Ag-ADAMTS13 ratio, nor can the ratio predict the diagnosis, particularly with difficulty adjusting for the confounding influences of age, disability, infection and

organ dysfunction. The models suggested by statisticians lacked overall power- ie did not offer full explanations of variability in presenting VWF:Ag-ADAMTS13 ratio. It is likely there are a myriad of other influences: including age, functional score or disability, inflammation and organ dysfunction. Diagnosis alone of ischaemic stroke vs TIA vs control does not independently predict the VWF:Ag-ADAMTS13Ac balance, and we acknowledge that a disordered VWF:Ag-ADAMTS13Ac axis is not specifically restricted to those with arterial thrombosis. Later analysis of longitudinal trends showed more convincing suggestions of the role of the presenting VWF:Ag-ADAMTS13Ac axis specifically toward long-term outcome of ischaemic brain injury.

The nature of our study means we cannot explain whether ischaemic stroke causes ADAMTS13Ac reduction, or whether decreased ADAMTS13Ac boosts risk or severity of stroke. Complexity of thrombus formation and numerous confounding influences on cardiovascular risk means that simple cause or effect is improbable, although a chronic disturbance of haemostasis seems likely as a risk factor ischaemic stroke. Consumption of ADAMTS13Ac seems plausible, with the metalloproteinase avidly used by heightened ULVWF in the ischaemic insult. However, this does not negate reduced ADAMTS13Ac heightening risk of initial arterial thrombosis. Prospective data regarding VWF:Ag-ADAMTS13Ac and stroke have demonstrated raised VWF:Ag and reduced ADAMTS13Ac to be associated with increased risk of stroke and increased all-cause mortality when followed up over median periods of 3 to over 11 years (Green et al. 2016; Qu et al. 2016; Sonneveld et al. 2015, 2016; Wieberdink et al. 2010). As the longitudinal data below suggest, our data do support a dynamic response of the VWF:Ag-ADAMTS13Ac axis at the time of ischaemic stroke and in convalescence.

6.3 Longitudinal

Our data demonstrate incremental ADAMTS13 recovery in the aftermath of acute ischaemic stroke or TIA in the largest cohort of patients yet published, with no such longitudinal changes seen in controls. Earlier work compared early and late phases post stroke, demonstrating significant early phase ADAMTS13 reduction compared to late; with an accompanying significant inverse relationship between early phase ADAMTS13 activity and VWF:Ag (McCabe et al. 2015). Our data support this incremental ADAMTS13 recovery in the aftermath of ischaemic stroke compared to controls, and also supports that the corresponding VWF:Ag-ADAMTS13Ac ratio can be used as a biomarker for clinical outcome in acute IS and TIA.

From presentation to final follow-up, IS demonstrated ADAMTS13 increase, VWF:Ag decrease and overall reduction of VWF:Ag- ADAMTS13Ac on both Mann Whitney and paired (Wilcoxon matched-pairs signed rank) testing. In TIA, a significant ADAMTS13 increase and corresponding VWF:Ag-ADAMTS13Ac decrease was seen on paired testing. No such longitudinal changes were seen in controls. Therefore although baseline VWF-ADAMTS13 perturbations are witnessed in general sickness and linked with consequent disability,

normalisation was only seen in ischaemic stroke. Resolution of VWF:Ag-ADAMTS 13 in later ischaemic stroke lends further support to the ischaemic insult provoking heightened VWF and ADAMTS13 consumption. We acknowledge that our recurrence data were limited, and further work is merited to investigate whether persistently depleted ADAMTS13 heightens risk of further TIA or stroke; via increased thrombus propensity and increased cardiovascular mortality.

Thrombin generation markers did not correlate with clinical characteristics as reflected by stroke scores, but showed resolution in ischaemic stroke and TIA. This could suggest that raised thrombin generation is seen in the acute aftermath of ischaemic stroke and lessens in convalescence. Further work is warranted particularly in view of the conflicting data currently published regarding the role of thrombin generation in acute ischaemic brain injury.

6.4 Morbidity and mortality

We demonstrate association of longitudinal clinical scoring with the VWF-ADAMTS13 axis. At presentation, the VWF:Ag-ADAMTS13Ac ratio was correlated with functional stroke scores across the entire cohort, with little differentiation between IS, TIA and controls, unsurprisingly as a potential acute phase response. This supports previous data suggesting the balance of VWF:Ag and ADAMTS13 is critical in cerebral infarction (Qu et al. 2016). As the authors postulate, a higher plasma level of VWF:Ag in patients with stroke will increase VWF:Act, with corresponding ADAMTS13 activity reduced by the high molecular weight multimers being released to the damaged vascular wall: hence the HMWMs not easily cleaved, and thrombosis risk heightened. Beyond this, our data support recovery of ADAMTS13 in ischaemic stroke and TIA paired values, without this trend in controls; suggesting convalescence post ischaemic brain injury is specifically associated with a resolution of VWF-ADAMTS13.

Although we have shown resolution of the VWF-ADAMTS13 balance over convalescence, a paucity of follow up data limits clear conclusions as to whether the presenting VWF:Ag-ADAMTS13Ac ratio can predict disability score at final follow up. We used regression to explore which variables might provide explanatory power for follow up disability scores in ischaemic brain injury, including the presenting VWF:Ag-ADAMTS13Ac ratio, age and presenting disability score. This suggested that the presenting VWF:Ag-ADAMTS13Ac ratio is positively associated with follow up disability score, but that the presenting disability score had far more weight in the model's explanatory power.

We then adjusted for the disability score to see if we can extricate whether the presenting VWF:Ag-ADAMTS13Ac ratio had independent power for predicting follow up disability score. Ischaemic stroke and TIA

patients were categorised according to degree of disability, using mRS scores at both presentation and final follow up. This suggested prognostic differences in the VWF:Ag-ADAMTS13 ratio. Patients presenting with an mRS score of 3 to 5 (n=78) compared with patients with mRS scores of 0 to 2 (n=98) demonstrated a higher median VWF:Ag-ADAMTS13Ac ratio (mRS score, 3-5: median ratio, 1.858: range, 0.415- 9.53:p<0.0001). Despite an overall longitudinal reduction of the VWF:Ag-ADAMTS13Ac ratio, this significant difference persisted at final follow up. Patients with more functional impairment at follow up (n=18), as reflected by mRS score of 3 to 5, maintained a higher VWF:Ag-ADAMTS13Ac ratio of 1.521 (range 0.32-3.42) compared with patients at follow up with an mRS score of 0 to 2 (n=48: median ratio, 0.845: range 0.42- 3.29: p=0.0102). Although the ranges overlapped, this higher VWF:Ag-ADAMTS13Ac ratio was closely linked to increased morbidity. This was not adjusted for age, which must also be taken into account as a likely confounding effect.

Our findings imply that the VWF-ADAMTS13 axis is associated with consequential outcome in patients presenting with acute ischaemic brain injury, allowing for the multitude of other influences such as age and functional status at presentation. Potential mechanisms include ADAMTS13 in post-ischaemic angiogenesis, with recombinant ADAMTS13 markedly boosting neovascularization, vascular repair and functional recovery at 2 weeks post ischaemic insult in recent murine data (Xu et al. 2017).

Moving on from morbidity to mortality, we compared presentation measures in ischaemic stroke and TIA patients who subsequently died with those whom survived. A striking elevation of VWF:Ag, VWF:Act and FVIII was seen in the patients who later died, with ADAMTS13 significantly lower in this group. The ratio of VWF:Ag-ADAMTS13Ac was correspondingly significantly higher in the deceased group (deceased 3.683 vs alive 2.014).

Chi squared testing was used to further investigate the relevance of the presentation VWF:Ag-ADAMTS13Ac ratio in relation to future mortality. As the presenting VWF:Ag-ADAMTS13Ac ratio increased, the chi square difference strengthened alongside odds ratios of survival and related confidence intervals. Comparison of those with a presenting VWF:Ag-ADAMTS13Ac ratio of less than or equal to 1.2, to those greater than 1.2 shows a significant difference in survival (chi-square, p=0.0265). The risk of patients with a presenting VWF:Ag-ADAMTS13Ac ratio of greater than 2 dying is 6.9 (95% C.I. 1.98- 24.1) compared to patients with a baseline ratio of less than or equal to 2.

Quartile analysis of the ischaemic stroke and TIA cohort also supported this. Those patients in in the highest quartile (VWF:Ag-ADAMTS13Ac ratio 3.09- 9.53) had a 31% mortality rate (n=14) compared to those in the lowest quartile (VWF:Ag-ADAMTS13Ac ratio 0.41-1.55) where no deaths occurred (p<0.0001).

In order to be able to adjust for age, receiver-operator curve (ROC) analysis was used to determine sensitivity of the presenting VWF:Ag- ADAMTS13Ac ratio for mortality. The ratio demonstrated clear predictive value

(area under curve 0.765; 95% CI 0.664- 0.886). In comparison, in the ROC analysis for sensitivity of presenting age for mortality, the predictive value was slightly lower (area under curve, 0.75; 95% CI, 0.665- 0.845).

Age was successfully adjusted for in the receiver operator characteristic curve analysis, demonstrating that the presenting VWF:Ag-ADAMTS13Ac ratio was significant in predictive value for mortality in ischaemic stroke. We then checked this with corresponding logistic regression. When unadjusted for age, logistic regression demonstrated the VWF:Ag-ADAMTS13Ac ratio to be associated with mortality (OR 1.67; 95% CI, 1.25-2.22; p=0.00). Adjusting for age slightly reduced the magnitude of the association between ratio and mortality (OR 1.50; 95% CI, 1.11- 2.02; p=0.008), but it persisted nonetheless.

This analysis not only supports that the presenting VWF:Ag-ADAMTS13Ac ratio is heightened in ischaemic brain injury, but that it can be used to predict outcome. This endorses that beyond morbidity relating to acute ischaemic brain injury, the VWF-ADAMTS13 axis is critical in association with eventual mortality as well, in keeping with previous work (Sonneveld et al. 2016). Further work is necessary to extricate the association of the VWF:Ag-ADAMTS13Ac ratio from functional status at presentation, to see whether it can be used as an independent laboratory marker of thrombogenicity in arterial thrombosis and determine extent of ischaemic impact in stroke. Resolution of the VWF:Ag-ADAMTS13 axis in convalescence, in alliance with clinical improvement not seen in the control population, further supports that it is a biomarker of clinical outcome.

6.5 Thrombolysis

The impact of thrombolysis on the longitudinal VWF:Ag-ADAMTS13Ac axis has not been previously investigated. We have demonstrated thrombolysis to be associated with ADAMTS13 recovery, and resolution of VWF and thrombin generation, further reinforcing a trend between the VWF-ADAMTS13 axis and arterial thrombosis. Thrombolysis was also associated with the expected resolution in clinical scores, with a marked difference seen in the thrombolysed group mRS from presentation to follow up (3.5 to 0, p=0.002) compared to the non-thrombolysed group (3 to 2, p=0.062). There was no significant difference in the age of the thrombolysed IS group vs the non-thrombolysed IS group (74.5 vs 77 years, p= 0.397).

We then grouped patients by stroke severity to adjust for a potential confounding effect. The ischaemic stroke cohort was split into presentation mRS 0-3 vs 4-5, to achieve a meaningful split in functional score and balanced patient numbers (mRS 0-3 n=51, mRS 4-5 n=49). For the mRS 0-3 group, the same trend was demonstrated: a significant decrease in VWF:Ag- ADAMTS13Ac ratio from presentation to final follow-up in the thrombolysed group (1.97 to 1.46, p=0.02) compared to the non-thrombolysed (ratio 2.05 to 2.24, p=0.78). For the mRS 4-5 group, the same trend was repeated but did not reach significance, potentially because of a relatively reduced patient number.

This supports clinical work suggesting ADAMTS13 predicts response to thrombolysis in patients with acute stroke, higher levels associated with arterial recanalization (Bustamante et al. 2015). Similarly, recombinant ADAMTS13 treatment of cerebrovascular occlusion in mice versus thrombolysis with tPA, reduces infarct volume and improves blood flow, without risk of massive intracerebral haemorrhage associated with tPA (Nakano et al. 2015). The postulated mechanism was the specificity of ADAMTS13 for ULVWF in conditions of high shear stress and thus pathological thrombi, rather than targeting the VWF-platelet primary haemostatic thrombi and inducing haemorrhage following the dissolution of thrombi post ischaemia (Nakano et al. 2015; Shida et al. 2008).

Resolution of the VWF:Ag-ADAMTS13Ac axis over convalescence links with clinical recovery. Given the limited acute treatments available for ischaemic stroke and TIA, there may be potential for the VWF:Ag-ADAMTS13Ac ratio beyond a biomarker. Manipulation of the axis via reducing raised VWF levels and normalising ADAMTS13 activity in acute ischaemic stroke and TIA offers important therapeutic potential.

6.6 Genetics

6.6.1 ADAMTS13 mutations: discussion

Numbers in the ADAMTS13 mutated group were too small for meaningful analysis of follow up data. As would be expected, those patients with exon 24 or exon 18 mutations did demonstrate lower ADAMTS13 activity. Since it would be predicated that ADAMTS13 activity is lower in such patients in anticipation of an acute ischaemic event, rather than being consumed, this could therefore confer increased risk of a poorer outcome in cardiovascular disease. The clinical significance of ADAMTS13 mutations in acute ischaemic brain injury merits further investigation.

6.6.2 Clopidogrel and CYP2C19 genotyping: discussion

Overall our data do not show that the CYP 2Y19C*2 allele demonstrated impact on haemostatic markers. There was no link with the VWF-ADAMTS13 axis, nor was there association with thrombin generation, according to whether either heterozygous or homozygous for the loss-of-function allele and whether on clopidogrel or not at presentation. Our recurrence data were too limited to comment further on risk of later adverse outcome as reflected by recurrent stroke.

Examining the role of genotyping specifically for clopidogrel resistance, one of the concerns raised by Holmes et al was genotype misclassification in their critical appraisal of the evidence linking the *CYP2C19* genotype with clopidogrel responsiveness.(M. V. Holmes, Perel, et al. 2011) In some of the clopidogrel treatment-only

studies included, if a particular *CYP2C19* allele was not typed, the participant was presumed to lack the variant allele in that position. The *CYP2C19* genetic variants associated with reduced enzyme function include *3, *4, *5, *6, *7 and *8 as well as *2. Individuals were therefore assigned to the *1 category, seemingly lacking all alternative alleles, because genotyping was not conducted for some * alleles. Overall this undermines directly linking the *CYP2C19* genotype with clinical cardiovascular end points, and suggests that far more needs to be taken into account before individual pharmacological tailoring is possible. Such an approach currently lacks sufficient evidence, with no current randomized controlled trial that compares strategy of modifying treatment based on genetic test results with current standard of care of no testing (Markus 2012). Risks of screening, interpretation of results and influence on clinical implications remain unsubstantiated in practice.(Pare and Eikelboom 2011) Best estimates suggest the *CYP2C19* loss-of-function alleles account for 12% of response to clopidogrel variability whereas overall heritability accounts for 72% of variability (Shuldiner et al. 2009). Personalisation according to genetics may be more fruitful in future when testing more heritability variants is possible, with the clinical ramifications clearer (Markus 2012).

6.7 Limitations and future improvements

Limitations of our study include firstly the 48-hour allowance of symptom onset before recruitment and how this may have potentially affected biomarkers in the hyperacute phase and eventual outcome. Secondly, those patients who were thrombolysed will have presented within 4.5 hours of symptom onset, which may have influenced results independently of thrombolytic therapy. Thirdly, cause of death in our cohort was not documented (eg whether vascular/ cardiovascular) so we cannot comment on how aberrations in the VWF/ ADAMTS13 axis may have contributed, and what trends occurred before recurrent stroke or death. Another caveat is that patients may have re-presented to other trusts, so recurrence may not be fully captured.

Changes at the other time points were also examined. Data sets were incomplete, particularly in those patients who were repatriated to local hospitals, and therefore not present for consecutive days clinical follow up and blood sampling. The number of values at each time point was as follows: t0= 103 values, t1= 41 values, t2= 30 values, t3= 20 values and t4= 36 days. It is imperative to acknowledge this limitation of data capture in examining the results. A potential scenario is that the sicker patients at presentation, with higher VWF:Ag, VWF:Act and FVIII were more likely to drop out of later follow up. We attempted to mitigate against this by conducting home visits, but many would be too frail even for this, or may well have died in the time since first recruitment. In the statistical analysis of our work, missing data limited analysis. In the longitudinal analysis, we used paired testing from presentation to final follow up to examine only those with full data sets. For regression, including patients only with full data sets reduced the power of the statistical analysis.

The most significant limitation our study faced was that the control population was younger and fitter than the ischaemic stroke population. Although we successfully adjusted for age in the logistic regression investigating the impact of the VWF:Ag-ADAMTS13Ac ratio in predicting mortality, this was not possible in comparison of the presenting ratio between the groups and in consideration of the functional outcome of the ratio. We deliberately chose 'stroke mimics' as our control group, and this allowed for straightforward patient recruitment since the inclusion criteria were exactly the same. Beyond matching for age, matching for disability or functional compromise at presentation is another important consideration. Time and feasibility constraints make this difficult to achieve in a single centre, but would certainly be possible in a larger study potentially collaborating across hyperacute stroke units.

Further work is needed to investigate whether persistently high VWF and low ADAMTS13 are associated with recurrence risk of acute ischaemic brain injury, helping to clarify whether the VWF-ADAMTS13 axis is causal or reactive in development of arterial thrombosis. Similarly, there is much further scope for investigation of the link between inflammation and the VWF-ADAMTS13 axis. We demonstrated a positive association with the VWF:Ag-ADAMTS13Ac ratio and CRP, supporting a significant interaction with inflammation and infection. There is likely to be far more complexity of the leucocyte-endothelial-platelet interaction combined with cytokine cascade worthy of future investigation.

Examination of whether the VWF axis correlates with volume of stroke on imaging would be of keen interest. Our study unfortunately did not allow for this since we did not achieve diffusion weighted image MRI scans for every patient due to clinical constraints, nor was the necessary neuroradiology involvement possible.

A further consideration would be using the 'tissue based' definition of TIA rather than the 'time based' definition we followed. For meaningful comparison of patient imaging, this would be best achieved by DWI MRI scans for all patients included, which has obvious clinical implications.

A prospective study prior to onset of acute ischaemic injury, such as the Rotterdam cohort described previously, could define a potential causal role of ADAMTS13 in stroke. Laboratory based investigation for the future would include in vitro addition of recombinant ADAMTS13 to plasma samples from patients with acute ischaemic stroke and confirming the normalisation of VWF activity and multimer pattern. Further laboratory and clinical work indicated should examine inflammation and the VWF – ADAMTS13 axis. As mechanical thrombectomy becomes more employed as a treatment choice, investigating the impact on the VWF-ADAMTS13 axis, and comparing to the impact of thrombolysis, would be worthwhile.

6.8 Conclusion: VWF and ADAMTS13 in ischaemic brain injury

This prospective investigation has demonstrated higher VWF and lower ADAMTS 13 at presentation of acute ischaemic brain injury to be associated with severity of phenotype, with resolution of the axis over time. We have, for the first time, shown a strong association between the levels of VWF and ADAMTS13 with respect to the severity, functional outcome and mortality of acute ischaemic stroke. We have summarized the relationship by focusing on the VWF:Ag-ADAMTS13Ac ratio as a biological measure of the disturbed haemostatic balance following ischaemic stroke and TIA, incorporating both the elevation of VWF and reduction of ADAMTS13. Longitudinal changes in those presenting with acute cerebral ischaemia supports incremental ADAMTS13Ac recovery in the aftermath of acute ischaemic stroke and TIA in the largest cohort of patients yet published, with no such longitudinal changes seen in controls.

We suggest that presentation VWF:Ag-ADAMTS13Ac ratio is a surrogate biomarker for extent of brain injury, hence in keeping with consequential clinical recovery, potentially far more pivotal in thrombogenicity and infarct volume than previously appreciated. As reflective of clinical recovery, it may be used for future prognostication and guidance of therapy. In the era of both anti-VWF therapies and recombinant ADAMTS13, therapeutic implications of limiting raised VWF levels and normalising ADAMTS13 activity could be critical in optimizing outcomes from ischaemic brain injury.

7 Congenital TTP and pharmacokinetics of plasma infusion

7.1 Introduction

Acquired TTP, by which autoantibodies bind to ADAMTS13 and impair function is the far more commonly recognised clinical entity (Zheng 2015). Congenital TTP, originally thought to be a disease of childhood, is a diagnosis increasingly made in adulthood, especially described in pregnancy (Scully et al 2014; Scully 2016). Congenital TTP is defined by a persistent severe deficiency of ADAMTS13 in the absence of anti-ADAMTS13 inhibitory antibodies, confirmed by mutational analysis. It is caused by biallelic mutations present in the ADAMTS13 gene, with two loss-of-function mutations required to fully inactivate the ADAMTS13 gene product. Compound heterozygous mutations are most common at 65%, with homozygous mutations in the remainder (Lotta et al. 2010). There is considerable variation in the age of onset, severity and frequency of acute episodes, reflecting genetic heterogeneity and susceptibility to precipitants such as surgery, pregnancy, infections, ageing, hormonal changes and comorbidities.

Congenital TTP has been increasingly recognised over the last decade with improved diagnostic techniques. Treatment of congenital TTP is based on the use of plasma infusion (PI) or intermediate purity factor VIII concentrates, which contain ADAMTS13. Both treatment modalities provide functional ADAMTS13 levels, which promote the irreversible cleavage of the high molecular weight von Willebrand factor multimers (HMW VWF; Allford et al. 2000; Karpman et al. 1996; Lester et al. 2002). Regular plasma infusion replenishes the missing protease in order to prevent relapse of TTP, a true haematological emergency that can result in the most life threatening of arterial thrombosis, whether cardiac or cerebrovascular.

In addition, both therapies have demonstrated efficacy in incrementing platelets acutely and as prophylactic treatment (Allford et al. 2000; Lester et al. 2002; Naik and Mahoney 2013; Pandey et al. 2015; Scully et al. 2006). However, there is little available evidence regarding the appropriate dosing requirements and interval between plasma infusions, with dose and dosing interval being selected empirically for each patient. Consequently, some patients are treated on demand due to circumstances such as pregnancy and infection, whereas others need regular infusions to prevent TTP (Blombery and Scully 2014).

Studies of clearance mechanisms of VWF have demonstrated a half-life of 8-15 hours, but with interindividual variation according to blood group, glycosylation and genetic mutations (Lenting, Christophe, and Denis 2015). The behaviour of ADAMTS13 has been less explored. An early study demonstrated half-life activity of von Willebrand factor (VWF)-cleaving protease, now known as ADAMTS13, in 2 brothers with congenital TTP, suggesting a plasma half-life of 2-3 days (Furlan et al. 1999). A typical dosing interval of every 3 weeks would therefore appear too long, given such a half-life of ADAMTS13, with a direct link between its levels and pharmacological activity.

In current practice, dosing and dose interval of plasma therapy is guided by clinical symptoms, platelet count and lactate dehydrogenase levels. It is widely known that the systemic clearance of a drug determines its exposure at steady-state. It also determines the elimination half-life in plasma and consequently informs the dosing rationale for therapies which show immediate pharmacodynamic effects (Rowland and Tozer 2011).

We hypothesised that the individualisation of treatment, both dose and dosing interval, can be guided by further evaluation of inter-individual differences in ADAMTS13 clearance. Such an approach will allow for a more effective clinical management of the individual patient, which may not be predicted by empirically monitoring ADAMTS13 levels. We therefore investigated the pharmacokinetics and interindividual variability in the elimination of ADAMTS13 replacement using plasma infusion in six patients with confirmed congenital TTP on the established regime for each individual. Since a recombinant ADAMTS13 molecule is now in clinical trial for congenital TTP patients, being able to deliver ADAMTS13 in a 'pure' form, without proteins that potentially interact such as VWF and factor VIII, is likely to generate higher peak levels than plasma (Scully et al. 2017). Understanding the comparative pharmacokinetics to plasma infusion will be key in therapeutic optimisation in future. Pharmacokinetic modelling provides a framework to describe and understand the time-course of drug exposure and response after the administration of different doses or formulations of a drug to individuals (Mould and Upton 2012). This allows estimation of associated parameters, such as clearance and volume of distribution of a drug, via development of a mathematical function.

TTP remains a rare disease, with a reported incidence of 6 per million cases per year (Scully et al. 2008). However, an improved comprehension of the pivotal VWF-ADAMTS13 relationship has suggested that it may also play a role in other diseases of disordered haemostasis, with far greater epidemiological impact. Applying the haematological concept to a neurological disease, has arguably no greater potential than in arterial stroke.

7.2 Congenital TTP and pharmacokinetics of plasma infusion: methods

To further our understanding of the VWF-ADAMTS13 axis in the primary disease of congenital TTP, an additional project was performed to investigate the pharmacokinetics of ADAMTS13 and thereby optimal dose and frequency of treatment.

Six congenital TTP cases on a regular plasma infusion regime were selected from our institution. Congenital TTP was confirmed by the presence of microangiopathic haemolytic anaemia and thrombocytopenia at presentation, ADAMTS13 activity of less than 10%, no detectable anti-ADAMTS13 IgG antibodies performed in both the acute phase and in remission after treatment, and confirmation by mutational analysis of the *ADAMTS13* gene (Scully et al. 2017). Patients with previous history of end organ damage, such as stroke, or those who had a normal platelet count, but experiencing severe headaches; were treated with plasma infusion to improve their symptomatology. All patients were clinically well at the time of pharmacokinetic

measurement, with no suggestion of active thrombotic microangiopathy as reflected by their full blood counts and LDH. All consented to the study.

All patients received Octaplas LG which contains mean ADAMTS13 content is 1.13 IU/ml +/-0.17 (ADAMTS13 104%). The mean VWF:Act to VWF:Ag ratio was 0.55 in this product, consistent with a reduction in the number of HMW bands seen in the VWF multimer gels, typical of this product (Lawrie et al. 2010). ADAMTS13 dosing was determined by Octaplas content, and therefore volume of plasma. Each patient was on an established regime of dose, and frequency of treatment was dictated by normalised platelet counts and LDH, ranging from 15.3ml/kg weekly to 7.2ml/kg every 3 weeks. Pharmacokinetic analysis was performed after a typical treatment dose and regimen for each patient.

7.2.1 Plasma samples

Citrated plasma samples were taken at pre-infusion of plasma, 1 hour and between 2-3 hours post infusion. Subsequent samples were taken daily for 7 days, with additional samples at day 10, 14 and 21 according to frequency of infusion.

VWF:Ag, VWF:Act and Factor VIII: VWF:Ag, VWF:Act and FVIII were performed via Sysmex analysis, using Siemens kit (INNOVANCE®, VWF:Act. Siemens Healthcare Diagnostics, Marburg, Germany; Lawrie et al. 2013).

ADAMTS13:Act: ADAMTS13 activity was performed using an in house FRETS- ADAMTS13 activity assay (Fluorescence Resonance Energy Transfer), normal range established at 64-132IU/dl; Groot et al. 2006; Kokame et al. 2005).

ADAMTS13:Ag: A novel ELISA was developed to determine the concentration of ADAMTS13 antigen (Alwan et al. 2017). A monoclonal antibody directed against the metalloprotease domain (3H9, kindly provided by K Vanhoorelbeke) was used to capture the antigen, and two biotinylated monoclonal antibodies directed against the 17G2 and the 19H4 domains used for detection. Dilutions of pooled normal plasma were used as the standard (Alwan et al. 2017).

7.2.2 Pharmacokinetic data analysis

Population pharmacokinetic modelling was used to characterize the pharmacokinetics of ADAMTS13 following intravenous infusion. Expert support was provided from clinical pharmacology colleagues Sean Oosterholt and Professor Oscar della Pasqua, based in the University College London Hospital Clinical Pharmacology and Therapeutics Group, who performed the pharmacokinetic analysis.

Characterisation of the ADAMTS13 pharmacokinetic parameters was performed using a nonlinear mixed effects approach, as implemented in NONMEM (NONlinear Mixed Effects Modeling) version 7.3 and PsN (Perl-

speaks NONMEM). NONMEN is a statistical software package used in population pharmacokinetic-pharmacodynamic analysis, allowing modelling of the raw data, accounting for both unexplainable between and within subject effects (random effects) as well as measured concomitant effects (fixed effects). PsN (Perl-speaks-NONMEM) is an interface package for NONMEM. The pharmacokinetics of ADAMTS13 were described in terms of population parameter values (fixed effects) and between subject variability (mixed effects). Modelling was performed using NONMEM version 7.3 and PsN version 4.6.0. Statistical and graphical summaries were implemented in R version 3.3.2. The input data included patient age, weight, volume of plasma infused (and thereby dose of ADAMTS13), frequency of dosing and the observed concentrations of ADAMTS13 at different time points (as shown in Table 39).

Model building criteria included: (i) successful minimisation, (ii) standard error of estimates, (iii) termination of the covariance step, (iv) correlation between model parameters and (v) acceptable gradients at the last iteration. Fixed and random effects were introduced into the model in a stepwise manner. Inter-individual variability in pharmacokinetic parameters was assumed to be log-normally distributed. Goodness of fit was assessed by graphical methods, including population and individual predicted vs. observed concentrations, conditional weighted residual vs. observed concentrations and time. Comparison of hierarchical models was based on the likelihood ratio test (Bentler and Bonett 1980). Secondary pharmacokinetic parameters (AUC, Cmax and half-life) were then derived using non-compartmental principles. Modelling was performed using NONMEM version 7.3 and PsN 4.6.0. Statistical and graphical summaries were implemented in R version 3.3.2.

Dose individualisation: Using the individual pharmacokinetic parameters obtained for each patient, simulations were performed to explore the feasibility of optimising dose and dosing regimens. Since making adjustments to the dosing interval based on just the observed data from 6 patients is limited, modelling allows for the characterisation of the pharmacokinetics and in turn makes appropriate statistical predictions of the ADAMTS13 profiles. Such processes have been used to determine appropriate dosing strategies in paediatrics, therapeutic drug monitoring or selecting the starting dose in a first-in-human trial (Agoram 2009; Ternant et al. 2018; Völler et al. 2017).

NONMEN interrogates the data, with questions such as “what ADAMTS13 levels can I expect for a patient dosed 20units/kg once a week?”, based on the pharmacokinetic model. A hypothetical target ADAMTS13 activity of 10IU/dl and 50IU/dl respectively were set as references for the purpose of this analysis. Total plasma volume and dosing intervals were then calculated from the simulated scenarios in which both dosing interval and amount were varied. Estimation is a multi-step process performed by the software, minimising differences between estimates and observed values; while simulations use the developed model to extrapolate ADAMTS13 exposure to other scenarios, e.g. a different dosing scheme.

7.3 Congenital TTP and pharmacokinetics of plasma infusion: results

Six cases of congenital TTP were studied, 5 of which presented *de novo* in pregnancy and one in childhood. The indications for regular plasma therapy were previous strokes (4 cases) and severe persistent headaches/migraine and lethargy (2 cases), which were significantly improved following initiation of plasma infusion (PI). Each of these 6 patients had ADAMTS13 activity of less than 5 IU/dL at diagnosis (normal range 64-132IU/dL) and mutational analysis of ADAMTS13 gene to confirm the diagnosis of congenital TTP.

Patient	1	2	3	4	5	6
Age at sample collection (years)	33	41	31	35	68	17
Age at diagnosis	21	39	28	33	21	4
Weight in kg	52.2	93.3	80	50.8	83.2	72
Clinical details	MAHA in pregnancy Right middle cerebral infarction. Complex seizures	Migraines. MAHA in pregnancy, intrauterine fetal death	Strokes, MAHA in pregnancy	MAHA in pregnancy	MAHA in pregnancy, with multiple IUFD. Bilateral MCA ischaemic stroke 2010, recurrent TIAs	MAHA, fever, petechial rash, diplopia at diagnosis.
ADAMTS13 antigen (%)	2.9	4	4.2	6	4.1	2.7
Rationale for PI	End organ damage.	Headaches, lethargy	End organ damage	Headaches, lethargy	End organ damage	Headaches, lethargy
Genetic mutation	Exon 24 R1060W Het, Exon28 c.3015delA	Exon 24 R1060W Hom, Exon24 A1033T Hom	Exon 24 R1060W Het, Exon24 A1033T Het	Exon 24 R1060W Hom, Exon 24 A1033T Hom	Compound Het: exon 7 (Het c719_724del), exon 17 (Het A690T).	Exon 6: D217H Het, Exon 24: R1060W Het
Current treatment (Plasma in ml/kg)	800ml plasma weekly (15.3ml/kg)	800ml plasma every 3 weeks (8.6 ml/kg)	800ml plasma weekly (10 ml/kg)	600ml plasma every 3 weeks (11.8ml/kg).	600ml plasma every 3 weeks (7.2ml/kg)	600ml plasma every 2 weeks (8.3ml/kg)
Plasa sampling frequency	Baseline 1, 3, 5 hours post. Daily for 1 week	Baseline 1 and 3 hours post Daily for 1 week	Baseline 1 and 3 hours post Daily for 1 week	Baseline 1 and 3 hours post Daily for 1 week, then day 11, 14, 21.	Baseline 1 and 3 hours post Daily for 1 week, then day 10, 12, 14, 23	Baseline, 13 and 30 minutes post. Day 1, 2, 4, 7, 8, 9, 10, 11, 12 and 14.

Table 39: Congenital TTP: summary of patient characteristics.

Key: 'Het'= heterozygous mutation; 'Hom'= homozygous mutation.

Table 42: Congenital TTP: raw patient data.

Normal range: FVIII 50-200IU/dL, VWF:Ag 50-160IU/dl, VWF:Act 50-187IU/dL, ADAMTS13 64-132IU/dL, ADAMTS13Ag 74-134%.

Patient 1	Weight 52.2kg, 15.3ml/kg. ADAMTS13 17.3IU/kg weekly, based on Octaplas mean content 1.13 IU/ml						
Status	**Date of sample**	**Hours post plasma**	**VIII %**	**VWF:Ag**	**VWF:Act**	**ADAMTS13 Ac**	**ADAMTS13 Ag**
Baseline	26/06/15	0	98.4	96.1	92.2	**4**	**2.9**
1 hour post	28/01/16	1	167.2	107.91	98.8	**32**	**30.1**
3 hours post	28/01/16	3	168.8	106.72	96	**34**	**27.2**
5 hours post	28/01/16	5	164.1	103.14	94.8	**29**	**30.7**
Day +1	29/01/16	22	178.6	107.12	106.1	**30**	**22.9**
Day +2	30/01/16	46	167.2	98.76	95.3	**22**	**18.4**
Day +4	01/02/16	94	148.1	94.58	97.4	**16**	**12.5**
Day +5	02/02/16	118	135.2	82.05	88.7	**14**	**7.1**
Day +6	03/02/16	142	153.7	98.96	97.4	**14**	**6.8**
Day +7	04/02/16	162	150.9	97.57	103.6	**11**	**5.0**
Day +8	05/02/16	191	212.3	124.82	157.1	**10**	**3.8**
Day +10	18/04/16	239	129.9	91.2	95	**6**	**3.2**
Day +12	20/04/16	287	122.3	88.02	96.1	**4**	**2.4**
Baseline (2)	08/04/16	0	115.2	77.68	81.4	**6**	**3.0**

Patient 2	Weight: 93.3kg, (8.6ml/kg), ADAMTS13 9.7 IU/kg every 3 weekly, based on Octaplas mean content 1.13 IU/ml						
Status	**Date of sample**	**Hours post plasma**	**VIII %**	**VWF:Ag**	**VWF:Act**	**ADAMTS13 Ac**	**ADAMTS13 Ag**
Baseline	05/02/16	0	259.1	298.9	305.6	**3**	**4.0**
1 hour post	05/02/16	1	280.3	284.94	271.7	**26**	**26.0**
3 hours post	05/02/16	3	286	286.93	271	**22**	**16.4**
Day +1	06/02/16	22	286	293.3	282.8	**19**	**16.8**
Day +2	07/02/16	46	347.8	321.94	290.3	**16**	**6.0**
Day +3	08/02/16	73	294.9	283.15	277.9	**13**	**7.1**
Day +4	09/02/16	93	307.2	282.36	271.5	**14**	**6.7**
Day +5	10/02/16	116	280.3	258.49	274.2	**12**	**7.1**
Day +6	11/02/16	141	258.6	250.13	270.7	**12**	**5.0**
Day +7	12/02/16	164	248.5	256.1	268.3	**11**	**6.0**
Day +11	27/02/15	260	283.8	274.36	320.4	**1.3**	**4.1**
Day +14	25/11/16	336	238.1	237.45	309.2	**6.9**	**5.3**
Day +21	11/11/16	500	208.4	219.93	295.6	**2.4**	**4.9**

Patient 3	Weight: 80kg, (10ml/kg), ADAMTS13 11.3 IU/ kg weekly, based on Octaplas mean content 1.13 IU/ml						
Status	**Date of sample**	**Hours post plasma**	**VIII %**	**VWF:Ag**	**VWF:Act**	**ADAMTS13 Ac**	**ADAMTS13 Ag**
Baseline	03/07/15	0	122.2	163.2	204.6	**4**	**4.2**
1 hour post	22/02/16	1	162.6	165.8	180.3	**30**	**28.3**
3 hrs 20 mins post	22/02/16	3	198.4	177.13	180.4	**34**	**40.2**
Day +1	23/02/16	22	225.1	192.05	200.5	**30**	**27.3**
Day +2	24/02/16	46	189.1	164.2	184.3	**23**	**20.0**
Day +3	25/02/16	70	168.8	154.06	177.9	**22**	**14.9**
Day +4	26/02/16	94	185.5	155.65	186	**17**	**11.6**
Day +5	27/02/16	120	192.8	164.8	188.8	**17**	**12.4**
Day +6	28/02/16	144	204.2	172.96	206.4	**15**	**13.4**
Day +7	29/02/16	166	176.9	175.74	197.2	**6**	**8.8**

Patient 4	Weight: 50.8kg, (11.8ml/kg), ADAMTS13 13.3 IU/ kg every 3-weekly, based on Octaplas mean content 1.13 IU/ml						
Status		**Hours post plasma**	**VIII (%)**	**VWF:Ag**	**VWF:Act**	**ADAMTS13 Ac**	**ADAMTS13 Ag**
Baseline	17/06/16	0	145.3	124.42	166.7	**3.6**	**6.0**
1 hour post	17/06/16	1	135.2	137.15	143.2	**24.4**	**40.4**
2 hrs post	17/06/16	2	136.5	138.15	140.4	**23.5**	**28.3**
Day +1	18/06/16	25	153.5	131.58	145.3	**19.9**	**28.6**
Day +2	19/06/16	45	136.5	133.57	152.8	**17.1**	**29.0**
Day +3	20/06/16	72	152.1	125.62	147.5	**13.4**	**12.0**
Day +4	21/06/16	96	165.2	131.18	169.7	**11.8**	**11.5**
Day +5	22/06/16	120	149.4	125.02	127.8	**11**	**13.3**
Day +7	24/06/16	166	142.7	118.06	128.7	**8.3**	**6.4**
Day +11	28/06/16	262	145.3	131.98	175.2	**5.9**	**4.7**
Day +14	01/07/16	335	141.5	117.46	140	**4.6**	**4.2**
Day +21	08/07/16	503	144	127.21	153.2	**5**	**5.2**

Patient 5	Weight: 83.2kg, (7.2ml/kg), ADAMTS13 8.1IU/ kg every 3-weekly, based on Octaplas mean content 1.13 IU/ml						
Status	**Date of sample**	**Hours post plasma**	**VIII %**	**VWF:Ag**	**VWF:Act**	**ADAMTS13 Ac**	**ADAMTS13 Ag**
Baseline	06/11/17	0	169.6	107.7	161.2	0	2.7
15 mins post	06/11/17	0.25	171.4	124.1	142.1	21.7	17.7
30 mins post	06/11/17	0.50	175.1	121.4	137.5	22.4	16.5
Day +1	07/11/17	22	184.7	115	140.8	14.8	11.9
Day +2	08/11/17	46	175.1	111.1	138.1	11.2	8.8
Day +4	10/11/17	94	159.2	104.6	136.2	5.5	6.1
Day +7	13/11/17	166	159.2	103.4	138.7	2.2	4.4
Day +8	14/11/17	192	159.2	110.3	161.8	1.6	3.8
Day +9	15/11/17	216	175.1	117.6	185.5	0.9	3.1
Day +10	16/11/17	239	177	118.2	181.6	0	3.1
Day +11	17/11/17	267	186.7	120	195.4	0	3.1
Day +12	18/11/17	285	199.1	125.7	218.3	0	2.9

Patient 6	Weight: 72kg, (8.3ml/kg), ADAMTS13 9.38IU/ kg every 2-weekly, based on Octaplas mean content 1.13 IU/ml						
Status	**Date of sample**	**Hours post plasma**	**VIII %**	**VWF:Ag**	**VWF:Act**	**ADAMTS13 Ac**	**ADAMTS13 Ag**
Baseline	06/11/17	0	169.6	107.7	161.2	0	2.7
15 mins post	06/11/17	0.25	171.4	124.1	142.1	21.7	17.7
30 mins post	06/11/17	0.50	175.1	121.4	137.5	22.4	16.5
Day +1	07/11/17	22	184.7	115	140.8	14.8	11.9
Day +2	08/11/17	46	175.1	111.1	138.1	11.2	8.8
Day +4	10/11/17	94	159.2	104.6	136.2	5.5	6.1
Day +7	13/11/17	166	159.2	103.4	138.7	2.2	4.4
Day +8	14/11/17	192	159.2	110.3	161.8	1.6	3.8
Day +9	15/11/17	216	175.1	117.6	185.5	0.9	3.1
Day +10	16/11/17	239	177	118.2	181.6	0	3.1
Day +11	17/11/17	267	186.7	120	195.4	0	3.1
Day +12	18/11/17	285	199.1	125.7	218.3	0	2.9
Day +14	20/11/17	335	167.8	120.8	209.3	0	2.4

The clinical details, baseline ADAMTS13 results, genetic mutations, indication for PI and individual regimens are shown in Tables 39 and 40. The median plasma infused was 10mls/kg, whereas plasma infusion intervals range from 1-3 weeks. ADAMTS13 activity was measured for each individual over time to allow pharmacokinetic modelling (Tables 40). The median incremental recovery (IR) was 0.023 IU/ml/IU/Kg. To exclude the impact of VWF contained in PI on ADAMTS13 levels and clearance, there was no significant increase in VWF activity from baseline, measured throughout the timepoints (Figure 15).

7.4 Pharmacokinetics of ADAMTS13

ADAMTS13 antigen showed a similar increase and subsequent decrease over the same period as ADAMTS13 activity (Table 40). ADAMTS13 activity was best described by a two-compartment pharmacokinetic model with linear elimination. Inter-individual variability was identified on clearance, central volume of distribution, intercompartmental clearance and the peripheral volume of distribution. The population clearance was found to be 25.41 ml/h (Table 41). All patients reached baseline ADAMTS13 activity levels within 7-10 days of PI. The median Cmax and AUC were 24.05 IU/dl and 31.5IU*h/ml respectively (Table 42). Given the inter-individual differences in clearance, the half-life of ADAMTS13 in these patients ranged from 82.6 to 189.5 hours (3.4 – 7.9 days), with a median value of 130.3 hours (Table 42). Secondary pharmacokinetic parameters were calculated (Table 43).

Dose individualisation

The attained levels of ADAMTS13 in these patients were below circulating levels seen in non-TTP patients in the general population. We simulated an individualised dose appropriate for each patient based on a hypothetical target trough ADAMTS13 activity of i) 10IU/dl (Figure 13) and ii) 50IU/dL (Figure 16). Simulations were performed for each patient to compare dose necessary to maintain this target trough given their current dosing interval, or optimal dosing interval for each patient based on their current dose.

For dose amount, or plasma volume, our simulations show that ADAMTS13 amount of 59.2- 502IU (mean 241.3) would be required to achieve a target ADAMTS13 activity of 50IU/dL, considerably reduced at 11.8-100.4IU (mean 48.3) to target a trough ADAMTS13 of 10IU/dL using plasma.

Similarly, our simulations show that plasma treatment every 1.5-3 days (mean 2.3) would be required to achieve target ADAMTS13 activity of 50IU/dL but only every 4-10 days (mean 7.3) to target a trough ADAMTS13 of 10IU/dL using plasma.

Figure 16: Change in von Willebrand factor activity over time.

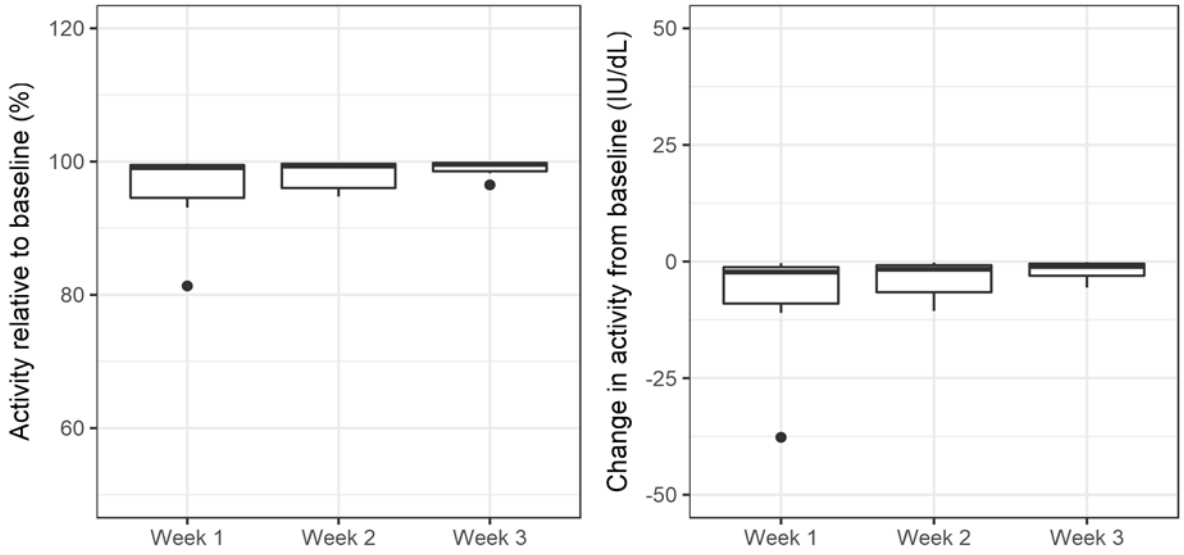


Figure 16: The box-and-whisker plots above demonstrate changes in VWF activity relative to that at baseline (left panel), as well as absolute change in activity from baseline (right panel). In both panels, the box reflects interquartile range (IQR), extending from the 25^{th} (bottom) to 75^{th} (top) percentile. The whiskers below, and just visualised above, reflect the datapoints outwith the 25^{th}- 75^{th} percentile range. The upper whisker extends to the 'maximum', which is Q3 + 1.5* IQR. The lower whisker extends to the minimum, which is Q1-1.5* IQR. The single dots seen represent individual outliers from the end of the whiskers, data points below the limit of the interquartile range, minus 1.5x the range of the box.

Table 43: Estimated model parameter values for a two-compartment model with linear elimination (ADAMTS13 activity)

The parameters outlined in Table 43: data inputted into NON-MEM.

	Parameter	Estimate
Population parameters	Clearance (L/h)	0.0254
	Central volume of distribution (L)	3.61
	Intercompartmental clearance (L/h)	0.00591
	Peripheral volume of distribution (L)	1.05
Inter-individual variability	IIV Clearance (CV%)	27.30%
	IIV Central volume of distribution (CV%)	5.30%
	IIV Intercompartmental clearance (CV%)	187.30%
Residual error	Proportional error (CV%)	3.10%
	Additive error (IU/dL)	4.16

Table 43: Estimated model parameter values: Inter-individual variability was identified on clearance, central volume of distribution, intercompartmental clearance and the peripheral volume of distribution. The population clearance was found to be 25.41 ml/h.

Table 44: Median, 5th and 95th percentiles of secondary pharmacokinetic parameters (ADAMTS13 activity)

Dose (IU/kg)	AUC (IU*h/mL)	Cmax (IU/dL)	Half-life (h)	MRT (h)
10.5 (8.4-16.3)	31.5 (21.0-40.7)	24.05 (19.7-33.9)	130.3 (89.9-179.4)	187.4 (129.1-258.3)

Table 44: All patients reached baseline ADAMTS13 activity levels within 7-10 days of PI. The median Cmax and AUC were 24.05 IU/dl and 31.5IU*h/ml respectively. Given the inter-individual differences in clearance, the half-life of ADAMTS13 in these patients ranged from 82.6 to 189.5 hours (3.4 – 7.9 days), with a median value of 130.3 hours.

Table 45: Individual secondary pharmacokinetic parameters

ID	Dose (IU/kg)	Weight (kg)	AUC (IU*h/mL)	Systemic Clearance (mL/h)	IR ((IU/mL)/(IU/kg))	Cmax (IU/dL)	Half life (h)	Mean Residence time (h)
1	17.3	52.2	30.9	29.2	0.019	33.4	111.9	160.8
2	9.7	93.3	39.8	22.8	0.027	25.9	144.6	207.9
3	11.3	80	32.2	28.1	0.03	34.1	116.2	166.9
4	13.3	50.8	41	16.5	0.017	22.2	189.5	272.8
5	8.1	83.2	30.6	22.1	0.024	19.5	149.4	215
6	9.4	72	17.8	37.8	0.022	20.3	82.6	118.6

Table 45: Secondary pharmacokinetic parameters were calculated.

Figure 17: Visual predictive plot of ADAMTS-13 activity over time.

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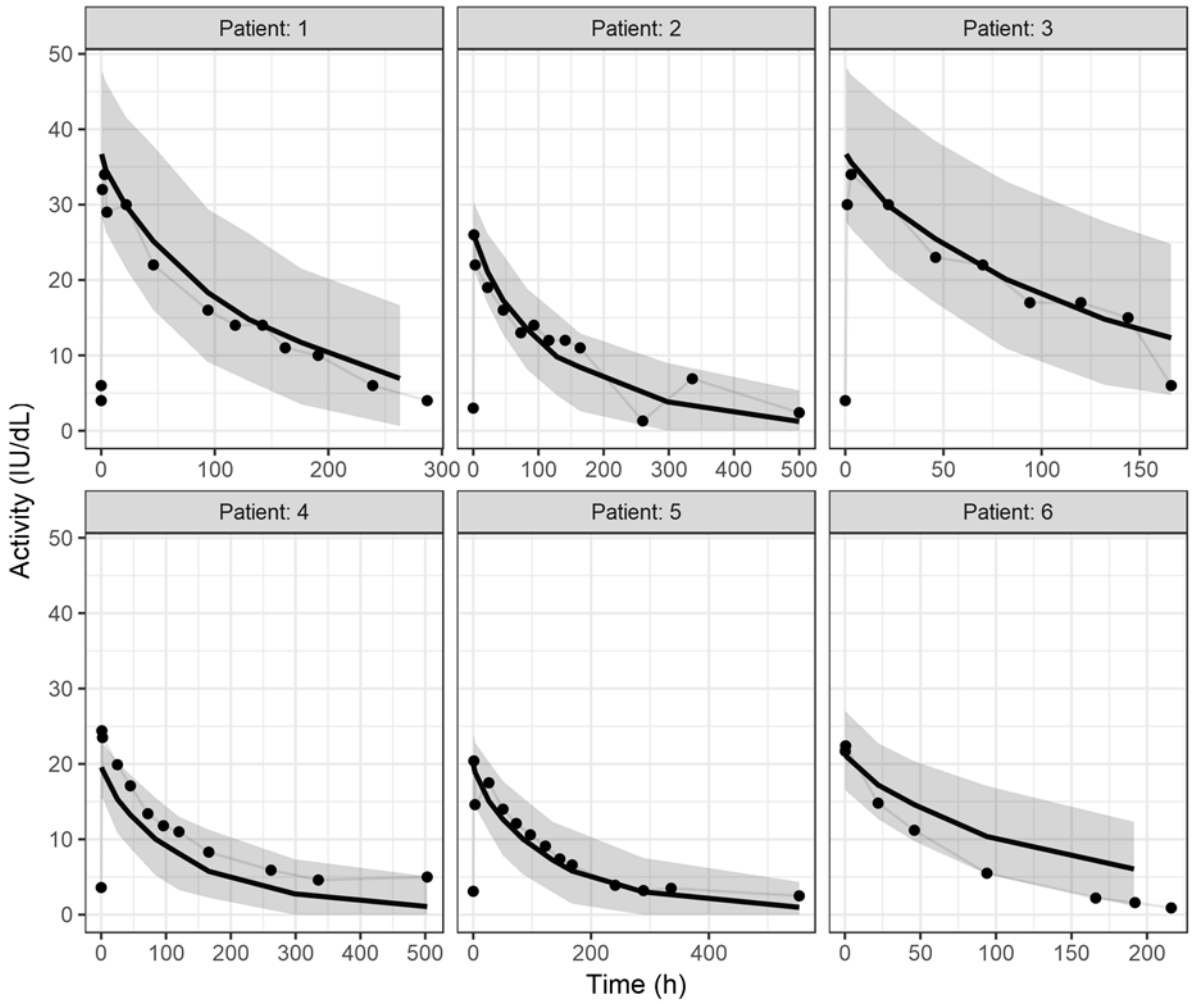


Figure 17: Solid line represents the median predicted individual activity, shaded line represents the 90% confidence interval. Observed values are shown as dots.

The graphs represent the visual predictive check of the data and the corresponding model built. The graph demonstrates that the input data used about the individuals – dosing time, amount and frequency as covariates- can be used to create a model to describe the individuals. The grey shaded area indicates where 90% of the *simulated* profiles would be for any patients with the same input variables. Therefore, patient 6's observed values being toward the edge of the shaded area (but still within) indicates that patient 6 has (one or more) PK parameters that are different from the typical population values. As illustrated in Table 43, patient 6 has a higher clearance than the others, which explains why the observed values are on the lower end of the model predictions.

Overall the visual predictive check graphs illustrate that the model predictions match the observations

Figure 18: Individualised simulations of maintaining target trough ADAMTS13 >10IU/dL with total dose amount (fixed frequency) or dosing interval (fixed dose amount)

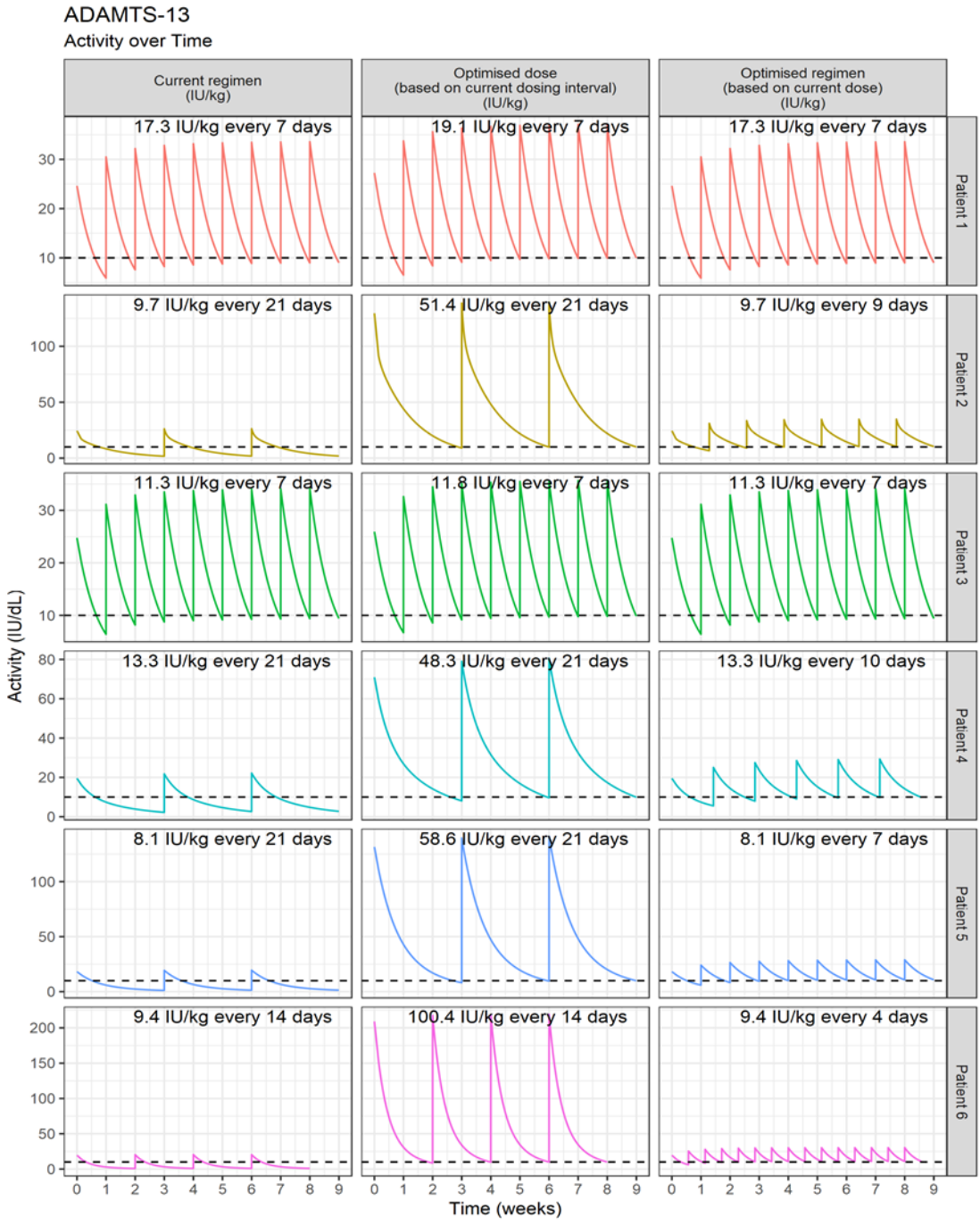


Figure 18 shows each patient's simulation based on a target trough ADAMTS13 level of >10IU/dL. Simulations were firstly based on dose amount (at fixed frequency), to continue at current dose frequency of weekly up to 3-weekly. Dosing ranged from 11.8IU/kg weekly to 100.4IU/kg every 2 weeks accordingly. Simulations were secondly based on dose frequency (at fixed dose), to continue at current dose amount. Treatment would be required every 4 to 10 days for our cohort with dose amount kept same as current.

Figure 19: Individualised simulations of maintaining target trough ADAMTS13 >50IU/dL with total dose amount (fixed frequency) or dosing interval (fixed dose amount)

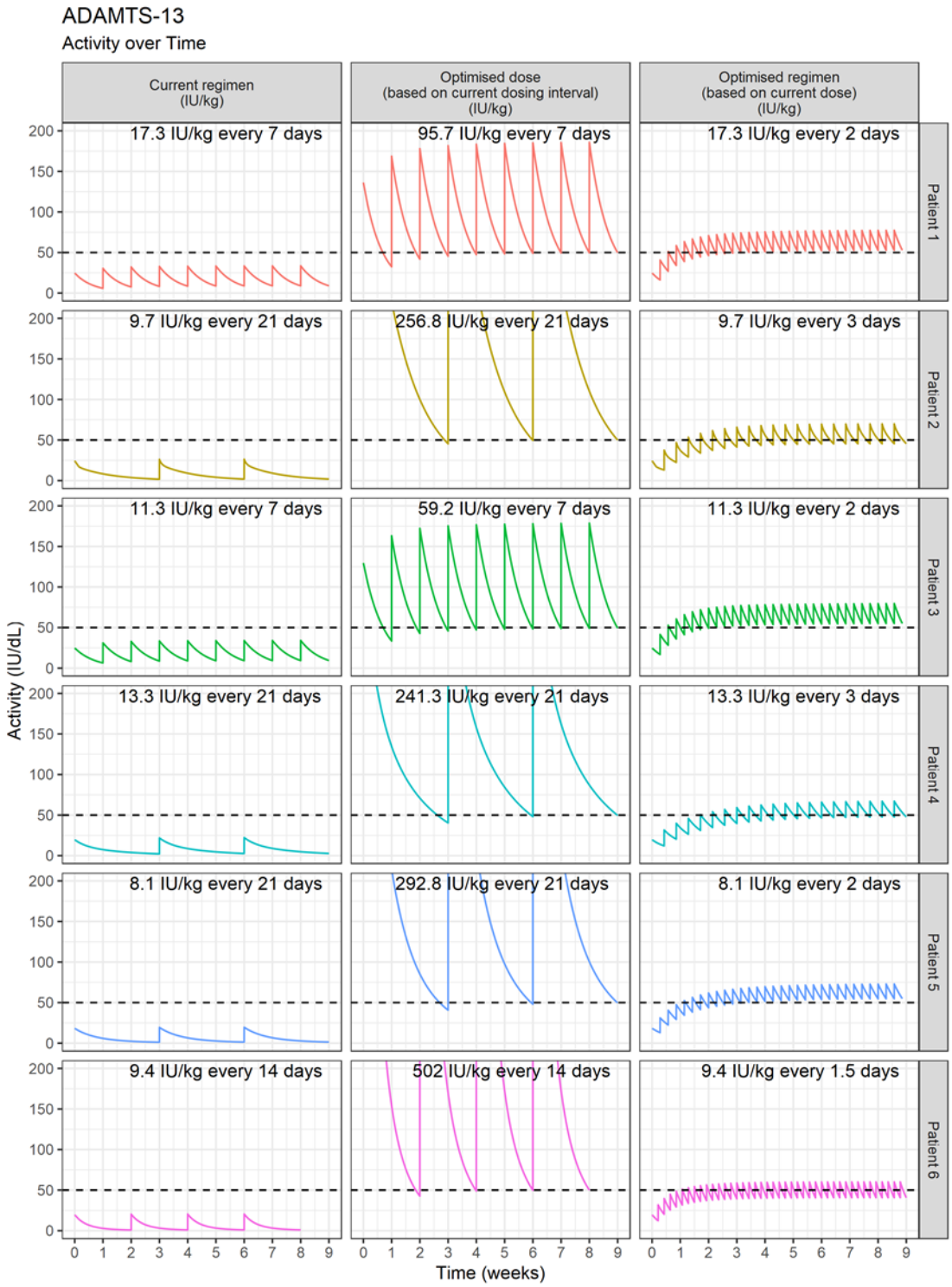


Figure 19 shows each patient's simulation based on a target trough ADAMTS13 level of >50IU/dL. Simulations were firstly based on dose amount (at fixed frequency) to continue at current dose frequency of weekly up to 3-weekly. Dosing ranged from 59.2IU/kg weekly to 502 IU/kg every 2 weeks accordingly. Simulations were secondly based on dosing frequency (at fixed dose). Treatment would be required every 1.5 to 3 days for our cohort with dose amount kept same as current.

7.5 Discussion: congenital TTP and pharmacokinetics of ADAMTS13

In conjunction with the work on the VWF:Ag-ADAMTS13Ac axis in the pathogenesis of ischaemic stroke, we have investigated congenital TTP, which is defined by absence of ADAMTS13. Regular plasma infusion replenishes the missing protease in order to prevent relapse of TTP, a true haematological emergency that can result in the most life threatening of arterial thrombosis, whether cardiac or cerebrovascular. Clinical practice has until now been empirically driven, based on symptom avoidance long before biochemical suggestion of TTP relapse. Our pharmacokinetic analysis of 6 patients with congenital TTP has shown enormous variability in half-life, suggesting that investigation of interindividual clearance of ADAMTS13 is necessary for future optimisation of treatment, to enable rationale for dose and frequency of prophylaxis.

Our assessment of ADAMTS13 levels in six patients with confirmed congenital TTP post plasma infusion found a median clearance of 25.41 ml/h and half-life of 130 hours, ranging between 82.6 and 189.5 hours (3.4 to 7.9 days respectively), with all patients reaching a baseline ADAMTS13 level within 7-10 days post plasma (Figure 17) . Median ADAMTS13 activity peak post PI was 24.05 IU/dL, with variation related to differences in the elimination rate, which appears to be affected by weight and metabolism, but not to von Willebrand factor antigen (VWFAg) or activity (VWF:Act) levels.

Simulations for each patient based on the individual pharmacokinetic parameters were performed for either dose or frequency of plasma infusion to target hypothetical optimal plasma levels of ADAMTS13 of 10 and 50IU/dL respectively (Figures 18 and 19). Results suggest a target trough ADAMTS13 of 50IU/dL would not be achievable with plasma taking into account volume required, but aiming for an ADAMTS13 trough level of 10IU/dL is feasible.

This is the largest formal study investigating the pharmacokinetics of ADAMTS13 in patients receiving PI for congenital TTP. The structural and functional relationship of ADAMTS13 with ultra-large VWF has been well described, with consequent cleavage of VWF (Zheng 2013). However, there is a paucity of data regarding the proteolysis, degradation and/or clearance of ADAMTS13.

We have investigated the impact of PI on ADAMTS13 activity, VWF and Factor VIII in 6 females with congenital TTP. All but one was diagnosed after de novo episodes in pregnancy. Patients had a heterogeneous clinical picture; with therapy initiated for end organ damage, primarily stroke; or to prevent symptomatology, such as headaches/migraines, despite normal platelet and LDH levels.

Our data demonstrate an ADAMTS13 elimination half-life of 82.6 to 189.5 hours (3.4 to 7.9 days) following plasma therapy for congenital TTP. In addition, our data show that ADAMTS13 clearance is not linked to higher VWF:Ag or VWF:Act levels, as two patients with the highest VWF:Ag and VWF:Act levels had low clearance and consequently long half-lives for ADAMTS13 activity (144.6 and 149.4 hours). Ultimately, it is the individual

clearance of ADAMTS13 that should guide dose and dosing interval. Such half-life and clearance data should clarify individual treatment rationale.

All 6 patients demonstrate a remarkably similar trend of ADAMTS13 antigen level in relation to ADAMTS13 activity. This trend would be expected in congenital TTP, since there is no inhibitory activity interfering as there would be in acquired TTP, and provides a useful confirmation of ADAMTS13 activity. Our data support the close correlation of ADAMTS13 activity and antigen as they peak post plasma infusion, and degrade at a relatively constant rate despite variations in the impact of mutations in congenital TTP.

Furlan *et al* published data regarding 2 brothers, both with a "constitutional deficiency of von Willebrand factor-cleaving protease and no inhibitor" (Furlan et al. 1999). Following daily plasma exchange for 4 and 3 days respectively, the protease, or ADAMTS13, was analysed by VWF multimer assay, describing half-lives of 3.3 days and 2.1 days in each patient respectively. Four further patients had half-life estimation following 3-5 mls/kg of plasma, and similar results were identified, i.e. a mean half-life of 2.8 days (Fujimura et al. 2015) Overall it was considered that such half-lives were uniquely long for a proteolytic enzyme, since these are usually rapidly inactivated. Subsequent work has suggested that duration of ADAMTS13 effect is firstly due to resistance of its VWF substrate to cleavage due to circulation in a closed conformation and secondly, the requirement for shear-induced unfolding of VWF for ADAMTS13 to access the cleavage site (Crawley et al. 2011; Siedlecki et al. 1996 & Feys et al. 2009). It is also noted that multimeric assays are sensitive to low ADAMTS13 activity levels but will be less accurate in the mid-normal range with recognition of this being a cumbersome method requiring expertise (Furlan and Lämmle 2002).

ADAMTS13 is recognised as a plasma reprolysin-like metalloproteinase, primarily synthesised by the liver (Feys et al. 2009; Shelat, Ai, and Zheng 2005). Our estimated model parameters are in accordance with ADAMTS13 as a plasma protein, i.e., its central volume of distribution at 3.61L indicates that it is constrained to the plasma compartment. Clearance estimates (25.4mL/h) seem to reflect the range of values observed for other plasma proteins, including coagulation factors and immunoglobulin (Collins et al. 2011; Keizer et al. 2010; Koleba and Ensom 2006; Landersdorfer et al. 2013).

We demonstrate a large interindividual variability in elimination half-life observed in the currently presented cohort (ranging between 82.6 and 189.5 hours, or 3.4 to 7.9 days). This is clearly higher than that observed by Furlan et al but difficult to extract meaning from direct comparison due to small patient numbers in both studies and different means of analysis.

Regulation of ADAMTS13 in physiological conditions is poorly understood. Previous work has shown that VWF levels are not correlated to ADAMTS13 activity in individuals with normal VWF levels and likely normal multimer distributions. In VWF deficiency however, such as type 3 von Willebrand disease, ADAMTS13 levels

may be up to 30% higher. In congenital TTP, it is more plausible that variability in ADAMTS13 half-life is related to clearance mechanisms rather than plasma VWF concentrations. Clearance itself will reflect the ratio between elimination rate and ADAMTS13 plasma concentration.

Our data show that the population clearance of ADAMTS13 is 25.4ml/hour, resulting in a median half-life of 130 hours (or 5.4 days). Shorter half-lives are therefore a consequence of faster rate of elimination from plasma, which in turn may be caused by differences in body weight and basal metabolism.

Review of VWF levels in our cohort, whether activity or antigen, shows a less consistent pattern, matched by Factor VIII. The most direct effect of ADAMTS13 is on proteolysis of high molecular weight VWF, which we did not directly measure. Furthermore, the plasma used in these cases does not have significant HMW VWF multimers. ADAMTS13 should not unduly affect the measured antigen and activity, as reflected by our results. Future work could include methods to examine VWF turnover, such as VWF polypeptide measures; and increased patient numbers could give a clearer idea of whether blood group, known to reflect VWF clearance, influences ADAMTS13 clearance.

There were minimal changes in VWF activity, either absolute or relative to baseline, suggesting that it is high molecular weight VWF turnover that may determine duration of ADAMTS13 effect. This is consistent with Furlan's earlier data: both the purported half-life of ADAMTS13 and enduring effect related to being a plasma-bound protein (Furlan et al. 1999).

A recent phase I study has examined the PK profile of BAX-930, a recombinant ADAMTS13 (Scully et al. 2017). The study confirmed the safety and tolerability of the product during its first in-human use, with PK results suggesting higher peak ADAMTS13 levels can be achieved with the recombinant ADAMTS13 than with plasma infusions of the naturally occurring metalloproteinase. The recombinant ADAMTS13, however, has a clearance twice as high as that of naturally occurring ADAMTS13.

An immediate implication of such differences is that significantly higher doses of the recombinant ADAMTS13 are required to attain the same levels observed at steady state with the natural moiety. Clearance values for the recombinant ADAMTS13 ranged from 59.3ml/h (after 40U/kg dose) to 64.8ml/h (after 20U/kg dose) versus ADAMTS13 systemic clearance of 16.5 to 37.7ml/hour observed in this study. Despite the requirement for higher doses, this may not represent a limitation, given that recombinant ADAMTS13 can achieve higher ADAMTS13 peak levels than PI, whose dose and dosing regimen is primarily restricted by the maximum allowable infusion volumes.

The observed ADAMTS13 half-life in our patient group was higher than expected based on previous PI data, and higher than that determined with recombinant ADAMTS13. Further work is necessary to define the optimal target ADAMTS13 during the clinical management of TTP patients. We have used simulations to

explore the feasibility of individualised protocols for each patient based on either dose or frequency of PI to target a plasma level of ADAMTS13 of 10IU/dL or 50 IU/dL respectively. Targeting a hypothetical optimal ADAMTS13 of >10IU/dL with either dose or interval to prevent symptoms potentially associated with TTP may be sufficient as well as clinically practical. Achieving a level of >50 IU/dL represents a 'normal' level in the non-TTP population, but such a level was not witnessed in our cohort receiving PI, even at peak levels. The simulation models suggest that either scheme is not possible in clinical terms to achieve a level of 50IU/dL: either the plasma volume required is too high or the frequency of PI would be too difficult to safely achieve the dose needed.

Future attempts to individualise and optimise therapy with either naturally occurring ADAMTS13 in allogeneic plasma and/or recombinant ADAMTS13, will need to consider whether clearance is indeed the best surrogate for the duration of effect of ADAMTS13. If our therapeutic aim is to ensure continued presence of ADAMTS13, we firstly need to investigate whether we should treat more frequently than every 3-4 weeks, which has been derived from the historic estimate of half-life of ADAMTS13 of 2-3 days. A dose interval of less than 5 half-lives of ADAMTS13 may be more clinically appropriate. Secondly, given the varied values in this current cohort of between 3.4 and 7.9 days, we should consider undertaking half-life analysis in patients, to maintain a higher ADAMTS13 level in between infusions, which would require more frequent dosing, eg every 1-2 weeks as in some of these cases. Limitations of our study include the small number of patients studied, performing PK analysis after one PI per patient- albeit in steady state- and rarity of the disease. It should also be noted that PK results are heterogeneous, with clear individual variation. With a larger study number, future work could include multivariate analysis of the various factors affecting the individual PK such as age, weight, platelet count, VWF level, LDH and genetic mutations. The FRETS assay results are less sensitive at the lower end of range, which should be taken into account. There is also the possibility of ADAMTS13-binding antibodies enhancing clearance, but not detected in the anti-ADAMTS13 IgG ELISA.

In optimising the treatment of congenital TTP, further research is required to explore the individual determinants of catabolism and clearance. Moreover, one needs to identify metrics for the long-term protective effects of ADAMTS13. Without allowing overt disease relapse, judging symptomatology of TTP can be subtle. Further work is required to establish objective measures of whether treatment is warranted, and how we calculate rationale for dose and frequency of prophylaxis requires further investigation. This is particularly pertinent and achievable in the upcoming era of recombinant ADAMTS13 as an alternative to plasma infusion.

8 Haematology in young stroke: relevance of the full blood count

8.1 Introduction

The aetiology of stroke in younger patients is diverse; including cardiac anomalies, autoimmune disorders and thrombophilia as well as the traditional vascular risk factors. Despite extensive investigations there is no clear cause for around 25- 40% of all stroke events (Jaffre et al. 2017; Larrue et al. 2011; Putaala et al. 2009; Rolfs et al. 2013). Complex and costly thrombophilia testing is routinely sent despite controversy regarding evidence linking to stroke caused by arterial disease (Keeling et al. 2012; Meschia et al. 2014; Morris et al. 2010). The panel will often include protein C, protein S and antithrombin activity levels, factor II mutation (prothrombin *G20210A* mutation), Factor V Leiden, homocysteine, and antiphospholipid antibody testing. Data support the role of antiphospholipid antibodies, or antiphospholipid syndrome, in the aetiology of young stroke (Brey et al. 2002; Cervera et al. 2002; Urbanus et al. 2009). However, evidence linking other potential thrombophilia markers with arterial thrombosis is less robust (Juul et al. 2002; Keeling et al. 2012; Morris et al. 2010). Thrombophilia testing protocols are also often poorly applied and followed up.

In contrast, analysis of the basic full blood count may be more helpful in signifying the presence of diseases such as myeloproliferative diseases (MPD) or thrombocytopenic purpura (TTP) which have been shown to be causative. In the former, there is typically an elevated haematocrit, thrombocytosis and/ or leucocytosis. TTP is characterised by a severe thrombocytopaenia and microangiopathic haemolytic anaemia.

MPDs are heterogeneous but linked by a shared abnormality of haematopoiesis with over-production of one or more myeloid-derived cell lines. Polycythaemia vera (PV), essential thrombocythaemia (ET) and primary idiopathic myleofibrosis (PMF) are included within this diagnostic group (WHO, 2008). There is a clear link with arterial thrombosis with an incidence of 9% in an international ET cohort followed up over 6 years (Carobbio et al. 2011; Elliott and Tefferi 2005; Passamonti et al. 2004; De Stefano et al. 2008; Tefferi and Barbui 2015).

The JAK II *V617F* mutation is seen in 95% of PV and thus the diagnosis is unlikely in the absence of this molecular marker, particularly combined with a normal or increased serum erythropoietin. Overall molecular markers in ET include JAK II (55% incidence), CALR (25%) and MPL (3%), with ‘triple negative’ disease seen in approximately 17% as described in one review (Tefferi and Barbui 2015).

As already discussed, TTP is due to a deficiency of ADAMTS13, the VWF cleaving protein which controls the ultra large multimers of VWF released from the endothelium (Scully et al. 2012). Although a rare disorder, with a reported incidence of 6 cases per million in the UK, the untreated mortality is 90% (Scully et al. 2008). TTP can present as an ischaemic stroke- albeit usually with a severely reduced platelet count- and is a crucial

diagnosis to make to institute potential life-saving plasma therapy to remove autoantibody and replace ADAMTS13.

In a separate cohort of patients under the age of 60 years presenting the hyperacute stroke unit, we retrospectively reviewed full blood counts, specifically haematocrit and platelet count, and whether these were documented and further investigated if outside of the normal laboratory range. We examined whether less common primary haematological disorders known to cause stroke were considered and investigated: for example molecular diagnosis for myeloproliferative diseases such as polycythaemia vera (PV) and essential thrombocythaemia (ET), and ADAMTS13 analysis for TTP.

8.2 Haematology in young stroke: thrombophilia screening for antiphospholipid antibody status

Thrombophilias are broadly defined as inherited or acquired coagulations disorders predisposing to thrombosis (Boekholdt and Kramer 2007; Weston-Smith et al. 1989). 'Young stroke' blood test panels will often include protein C, protein S and antithrombin activity levels, factor II mutation (prothrombin *G202010A* mutation), Factor V Leiden, homocysteine, and antiphospholipid antibody testing.

Although best practice is contentious, the most robust evidence for screening for thrombophilia in the young stroke population pertains to antiphospholipid antibody testing (Brey et al. 2002; Cervera et al. 2002; Urbanus et al. 2009).

8.2.1 Antiphospholipid syndrome

Antiphospholipid antibodies arise in the autoimmune condition antiphospholipid syndrome (APLS). First dubbed 'Hughes' syndrome', APLS is clinically characterised by arterial or venous thrombosis, or defined obstetric complications such as recurrent miscarriage or intrauterine death. Arterial thrombosis in APLS is most frequently in the cerebral vasculature, hence resulting in acute ischaemic brain injury- transient ischaemic accident or stroke. Myocardial infarction is less common in APLS.

Consensus criteria for diagnosis of APLS has been made stricter in the past decade, in an attempt to standardise laboratory practice and ensure consistency in research (Keeling et al. 2012). Commonly used definitions are as follows (Miyakis et al. 2006):

8.2.2 Clinical:

1) Vascular thrombosis- one or more clinical episodes of arterial, venousm or small vessel thrombosis.

2) Pregnancy morbidity:

   i) One or more unexplained deaths of a morphologically normal fetus at or beyond the 10th week of gestation.

   ii) One or more pre-term births of a morphologically normal neonate before the 34th week of gestation due to eclampsia, pre-eclampsia or recognised features of placental insufficiency.

   iii) Three or more unexplained consecutive spontaneous miscarriages before the 10th week of gestation, with exclusion of maternal anatomic or hormonal abnormalities, and maternal or paternal chromosomal causes.

8.2.3 Laboratory:

1) Lupus anticoagulant (LA) present in plasma, on two or more occasions at least 12 weeks apart.

2) Anticardiolipin (aCL) antibody of immunoglobulin (IgG and/or IgM isotype in serum or plasma, present in medium or high titre (ie >40GPL units or MPL units, or >99th centile), on 2 or more occasions, at least 12 weeks apart.

3) Anti β2-glycoprotein I antibody (aβ2GPI) of IgG and/or IgM isotype in serum of plasma (in titre >99th centile), present on 2 or more occasions at least 12 weeks apart.

It is the laboratory criteria that have previously shown variation in methodology and diagnosis of APLS. APLs have been historically detected as either LA or ACL. LA testing is in vitro, and examines for a prolongation of a phospholipid-dependent coagulation test, not related to an inhibitor specific to a coagulation factor. It was originally thought that the LA phenomenon was due to autoantibodies against anionic phospholipids, hence interfering with tenase and prothrombinase complex assembly.

Revisions to lab criteria are the addition of the aβ2GPI antibody test, persistence of antibodies for 12 weeks minimum and definitive cut-off values for the ELISA testing.

8.2.4 Antiphospholipid syndrome- evidence linking to young stroke:

A link between antiphospholipid antibodies and thrombosis is clear, but the overall contribution of antiphospholipid antibodies to venous thrombosis and stroke is deemed to be small (Keeling et al. 2012). The APASS study did not support testing in standard stroke patients; with antiphospholipid antibodies not

predicting either increased risk for subsequent vascular occlusive events over 2 years or a differential response to aspirin or warfarin therapy (Levine 2004). There is a general consensus that the role of antiphospholipid antibodies should be examined more carefully in younger patients with ischaemic stroke with recommendations for screening in patients presenting under the age of 50 years (Keeling et al. 2012).

Laboratory methodology needs to be conducted carefully, as outlined above. The interpretation of what constitutes a 'positive' screen needs to examine antibody type, titre and isotype, in the individual clinical context. Confusion can easily arise, such as with a positive LA test in the presence of aβ2GPI and anti-prothrombin antibodies, a positive aCL ELISA in the presence of antibodies directed against cardiolipin-binding proteins and the aβ2GPI ELISA identifying antibodies that are distinct to those relevant to the syndrome (Miyakis et al. 2006). There is an overall paucity of literature strictly pertaining to the Miyakis criteria for APLS, with difference in laboratory methodology leading to low sensitivity (Pengo et al. 2007).

Arterial thrombosis in APLS has been found to be associated with both LA and IgG aCL in previous reviews, but not IgM aCL (Galli et al. 2003a, 2003b). The same reviews identified that anti beta-2 glycoprotein 1 showed association with arterial thrombosis in 3 of 10 studies and therefore there was insufficient evidence to support its role. Lupus anticoagulant dependent on beta-2-GPI, however, has been associated with arterial thrombosis again emphasising the importance of standardisation of laboratory methodology (De Laat et al. 2004). Highest recurrence risk of overall thrombosis (both arterial and venous) is seen in the relatively small cohort of APS with 'triple positive' APAs (Pengo et al. 2010). In the same cohort of young patients admitted to the hyperacute stroke unit, we examined antiphospholipid antibody screening in young stroke and TIA, and whether practice adhered to guidelines.

8.3 Young stroke full blood count and thrombophilia: methods

i) The full blood count and II) Thrombophilia screening in young stroke

In a regional hyperacute stroke unit, we retrospectively reviewed consecutive clinical and laboratory records for all patients aged <60 years presenting to the service and discharged with a final diagnosis of stroke or TIA. Since this was review of practice rather than instituting change, there was no institutional review board approval. Patients were either ward in-patients or attendees of the daily TIA clinic, inclusive from January 1st 2015 to August 7th 2016.

Firstly, the full blood count was examined. Abnormal test results, specifically pertaining to either the platelet count or haematocrit, were followed up to see if they were repeated, and whether there was resolution. Case notes were reviewed to confirm whether there was a secondary cause in all those with thrombocytosis (defined as platelet count >400 x 10^9/L) and/ or raised haematocrit (defined as Hct >0.45) and if not, whether further genetic testing, such as for the JAK II mutation, was considered. Case notes were similarly examined in those patients presenting with thrombocytopenia (defined as platelet count <150 x 10^9/L), and if no cause determined, whether ADAMTS13 testing was conducted.

Secondly, antiphospholipid antibody screening was examined: whether abnormal results were documented, whether repeated, and whether taken forward with haematology referrals and subsequent diagnosis of antiphospholipid syndrome. Looking at antiphospholipid antibody screening specifically, each type of test was examined individually.

8.4 Lab methodology

8.4.1 Lupus anticoagulant

Both dilute Russell Viper venom time (DRVVT) and taipan venom time (TVT) methodologies were utilised by the laboratory. TVT methodology was used as per lab protocol if the sample came from a warfarinised patient.

The DRVVT test uses Russells viper venom, present in the 'LA1 screening reagent'. LA1 initiates plasma clotting by directly activating factor X. Lupus anticoagulant antibodies prolong the LA1 clotting time. 'LA2 screening reagent' is of the same composition as LA1, but with a high phospholipid concentration. The extra phospholipid counteracts the LA antibody and largely corrects the clot time.

The DRVVT test bypasses factor VII of the extrinsic pathway, and the contact and 'anti-haemophilic' factors of the intrinsic pathway. Therefore LA1 sccreening reagent is more specific for lupus anticoagulants than the APTT since not affected by contact factor abnormalities or by factor VIII deficiencies or antibodies.

Normal ranges were batch specific and extablished prior to using each reagent batch by running at least 20 healthy individuals aged 18 to 55 years for LA1 and LA2.

LA1 and LA2 results are expressed as a ratio of normal plasma ie:

LA1 ratio= LA1 seconds patient/ LA1 mean pooled normal seconds

LA2 ratio= LA2 second patient/ LA2 mean pooled normal seconds

The correction between the 2 ratios is determined to see if sufficient correction has occurred.

Equipment:

1) Sysmex Corporation CS Automated Coagulation Analyser

2) Sysmex specimen racks

3) HP Officejet printer

4) Zebra barcode label printer

Reagents:

1) LA1 (reference OQGP), 10 x 2ml – simplified dilute Russell's Viper Venom Time test: to determine lupus anticoagulants in patient plasma.
2) LA2 (reference OQGR), 10 x 1ml - confirmatory step described above.
3) Control plasma N- control plasma for normal range.
4) LA control high, 6 x 1ml- control plasma for the pathological/ abnormal range.
5) Biophen normal plasma 2 (reference 223602), 10 x 2ml- platelet poor plasma (PPP) used for 50/50 mixing studies.

Process:

1) Reagents reconstituted with the volume of distilled water stated on the vial. Left at room temperature for 15 minutes, mixed by inversion to ensure complete re-suspension of lyophilized material.
2) Calibration: for each lot of LA1 and LA2 reagent, the mean normal value entered into the calibration setting of the analyser.
3) Pre-determined by running at least 20 normal healthy individuals aged 16-56 years, and calculating the mean values of LA and LA2 in seconds.
4) Calibration value used to divide the LA seconds with to obtain the LA1 ratio.

5) Reconstituted LA1 and LA2 reagents, control plasma N and LA control high loaded onto analyser in labelled 4ml cups. Placed into barcoded holders, placed in to reagent section of the CS 2100 instrument.
6) Biophen normal plasma 2 loaded into 4ml cup and inserted into buffer table insert holder with preassigned barcode (Lup Ctln). Placed into buffer section of the analyser.
7) Quality control run by selecting LA1 and LA2 for LA high and CtlN respectively.
8) Internal quality control- run and reviewed prior to running patient samples.
9) Re-run at interval of 6 hours and when replenishing reagents.
10) Following quality control, patient plasma smaples defrosted at 37 degrees for 10 minutes, then mixed after thawing.
11) Patient sample aliquots placed on Sysmex CS2500 racks.
12) Analyser works on bidirectional interface, so barcode fully exposed.
13) Analysis run: performs LA1, result given in seconds, LA ratio calculated by using mean normal LA value.
14) LA ratio of <1.2- no futher testing requested.
15) LA ratios >1.2- ananlyser performs confirmation studies LA2 and LA1 50/50 by reflex rule.
16) All seconds and ratio results sent to Laboratory Information System for authorization once complete.

For patients on anticoagulation:

1) Sample dluted out using normal plasma by performing 50:50 mixing studies on LA1 and LA2 assays.
2) Taipan venom time used if INR >3.0.

Reporting results:

i) If DRVVT test ratio is within normal range (<1.2), test is therefore negative for LA.
ii) If DRVVT test ratio >1.2, then confirmation and mixing studies required.
iii) DRVVT 50/50 results should show signs of inhibitor by resisting correction. If DRVVT 50/50 corrects to a rato of <1.1. result suggests a common pathway factor deficiency or oral anticoagulant interference- but potential co-exisitng lupus anticoagulant. A weak lupus anticoagulant can be diluted out in a 50/50 mix.
iv) Positive lupus ratio:
v) Raised DRVVT ratio (LA1) with >10% correction with DRVVT confirm ratio (LA2) and/ or DRVVT confirm ratio correcting back to <1.2 is consistnet with lupus anticoagulant.
vi) If DRVVT confirm ratio (LA2) and/or DRVVT confirm ratio demonstrate sufficient correction, then result does not support lupus anticoagulant.

8.4.2 Anti-cardiolipin antibody IgG/ IgM:

The laboratory uses the Quanta Lite ACA IgM, an enzyme-lined immunosorbent assay (ELISA) for the semi-quantitative detection of IgM cardiolipin antibodies in human serum.

Principles: purified cardiolipin antigen is bound to the wells of a polystyrene microwell plate under conditions that will preserve antigen in its native state. Pre-diluted controls and diluted patient sera are added to separate wells, allowing any cardiolipin antibodies present to bind to the immbolised antigen. Unbound sample is washed away and an enzyme labelled anti- human IgM conjugate is added to each well. A second incubation allows the enzyme labelled anti-human IgM to bind to any patient antibodies, which have become attached to the microwells. After washing away any unbound enzyme labelled anti-human IgM, the remaining enzyme activity is measured by adding a chromogenic substrate and measuring the intensity of the colour that develops. After stopping the enzymatic production of coloured product, the presence or absence of cardiolipin antibody is determined by comparing the sample optical density with that of a five-point calibration curve. Results are reported out semi-quantitatively in standard IgM anti-cardiolipin units (MPL).

Reagents:

1) Polystyrene microwell ELISA platelet coated with a purified cardiolipin antigen and bovine beta-2 GPI (12-1 x 8 wells)
2) ACA negative control, 1 vial of buffer containing preservative and human serum with no human antibodies to cardiolipin, prediluted, 1.2ml
3) ACA IgM III control, 1 vial of buffer containing preservative and human serum antibodies to cardiolipin, predilulted, 1.2ml.
4) ACA IgM III calibrator A, 1 vial buffer
5) ACA IgM III calibrator B, 1 vial buffer
6) ACA IgM calibrator C, 1 vial buffer
7) ACA IgM III calibrator D, 1 vial buffer
8) ACA IgM calibrator E, 1 vial buffer
9) ACA III sample diluent, 1 vial, containing PBS- buffered saline, protein stabilisers and preservative, 50ml.
10) ACA III PBS concentrate, 1 vial of 20x concentrate, containing PBS- buffered saline, 50ml.
11) HRP IgM conjugate (goat), anti-human IgM, 1 vial containing buffer, protein stabilizers and preservative, 10ml.
12) TMB chromogen, 1 vial containing stabilisers, 10ml.
13) HRP stop solution, 0.344M sulphuric acid, 1 vial, 10ml.

Procedure:

1) All reagents were brought to room temperature (20-26 degrees Celsius) prior to beginning the assay. The required number of microwells were added to the holder.
2) Added to the wells: 100 microlitres of each five calibrators, diluted patient samples, the ACA negative control and ACA IgM III control.
   Both the ACA IgM III control and ACA negative control were pre-diluted and ready to use. The acceptable range of the ACA IgM III control was printed on the vial label. If the control failed to fall into the acceptable range, the run was repeated.
3) Wells covered and incubated for 30 minutes at room temperature.
4) Wash step: contents of each well thoroughly aspirated. 200-300microlitres of the diluted ACA III PBS buffer added to all wells and then aspirated. The sequence was repeated twice more for a total of three washes. The plate was inverted and tapped onto absorbent material to remove any residual fluid after the last wash. Every well was completely emptied after each washing step. The same sequence was maintained for the aspiration as was used for the sample addition.
5) Addition of 100 microlitres of the HRP IgM conjugate to each well. Conjugate was removed from the bottles using standard aspetic conditions. No unused conjugate was returned to the bottle to avoid potential microbial or chemical contamination. The wells were incubated for 30 minutes.
6) Wash step repeated.
7) TMB chromagen: 100 microlitres to each well and incubated in the dark for 30 minutes at room temperature.
8) HRP stop solution: 100 microlitres added to each well. The same sequence and timing of HRP stop solution was maintained as for the TMB chromagen. The plate was gently tapped to thoroughly mix the wells.
9) The absorbance of each well was read at 450nm within one hour of stopping the reaction. A reference wavelength of 620nm was used.

Quality control:

1) The ACA IgM III control, ACA IgM III calibrators and the ACA negative control were run with every batch of samples to ensure that all reagents performed properly.
2) The following criteria were met for quality control assurances:
   i) The absorbance of prediluted ACA IgM III calibrator A had to be greater than the absorbance the prediluted ACA IgM III control, which had to be greater than the absorbance of the prediluted ACA negative control.

ii) The prediluted ACA IgM III calibrator control had an absorbance of greater than 1.0, while the prediluated ACA negative control absorbance was not over 0.2.

iii) The ACA IgM III control absorbance was more than twice the ACA negative control, or over 0.25.

Expected values: normal range. 489 random normal donor samples were assayed for ACA IgM III. Of this number, 14 samples were within the indeterminate range of 12.5- 20 MPL. Nine positive samples were also found during the random normal testing. These samples were determined to be true positives based on additional testing with other 510 cleared kits. 95.3% of the normals were found to be less than 12.5 MPL.

Precision and reproducibility: the between run precision and reproducibility of the assay was measured by running two replicates each of a positive and negative in 4 separate assays on 4 days. The within run precision and reproducibility of the assay was measured by running 16 replicates each of a positive and negative in a single assay. The mean value, standard deviation and coefficient of variation for each sample are summarised below:

Table 46: Precision and reproducibility of anticardiolipin antibody assay adjustment

Table 46: the within run precision and reproducibility of the assay was measured by running replicates of a positive and negative in a single assay. The mean value, standard deviation and coefficient of variation are displayed.

	Positive			Negative		
	Mean M-phospholipids	SD	%CV	Mean M-phospholipids	SD	%CV
Overall	66.0	5.4	8.2	4.1	0.8	19.1
Within run	65.0	4.4	6.8	4.1	0.9	0.2
Between run	67.8	7.5	11.0	3.9	0.6	15.9

ACL was considered positive if in medium or high titre: >40G-phospholipid or M- phospholipid units or >99th centile. Exactly the same methodology was used for testing IgG anticardiolipin antibody.

8.4.3 Anti-beta-2-glycoprotein 1 IgG/ IgM:

The same ELISA technique was used as outlined in detail above for testing for anticardiolipin antibodies. Anti-beta-2 glycoprotein was considered positive if titre >99th centile.

8.5 Results: haematological investigation of young stroke

4.12.1 The full blood count in young stroke

In this retrospective review, 609 patients aged under 60 years were included: 379 ischaemic stroke (62.2%), 193 TIA (31.7%) and 38 haemorrhagic stroke (6.2%).

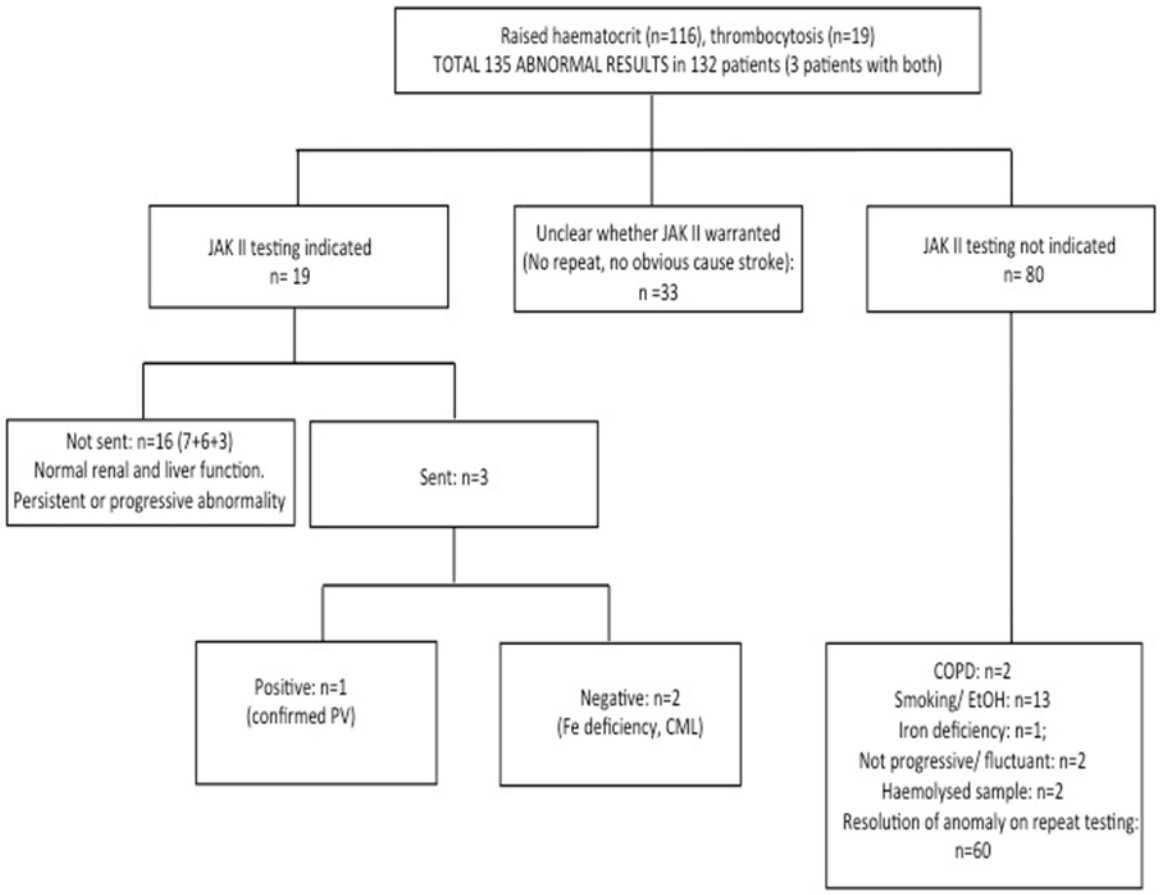
A total of 161 abnormal results with respect to haematocrit or platelet count were found in 153 patients (26.4%): 116 (19%) had a raised haematocrit (>0.45), 19 (3.1%) thrombocytosis (platelet >400), and 26 (4.2%) thrombocytopenia (platelet <150). Eight patients demonstrated abnormalities of both cell lines.

Of these initial 161 abnormal results, 118 *(*73.3%) were repeated in a total of 111 patients. There was no further follow up in 43 *(*26.7%) of the abnormal results seen in 42 patients. One patient was not included since he died shortly after initial presentation.

In those repeated tests, there was resolution of the discrepancy in 70 tests (59.3%). In the repeated tests showing no resolution; other aetiologies were taken into account (figures 15 and 16 below). However, even in those with repeated testing there were a number of patients with a persistent or progressive abnormality, which was not further investigated (n=22, 14.4 % of patients with abnormal results).

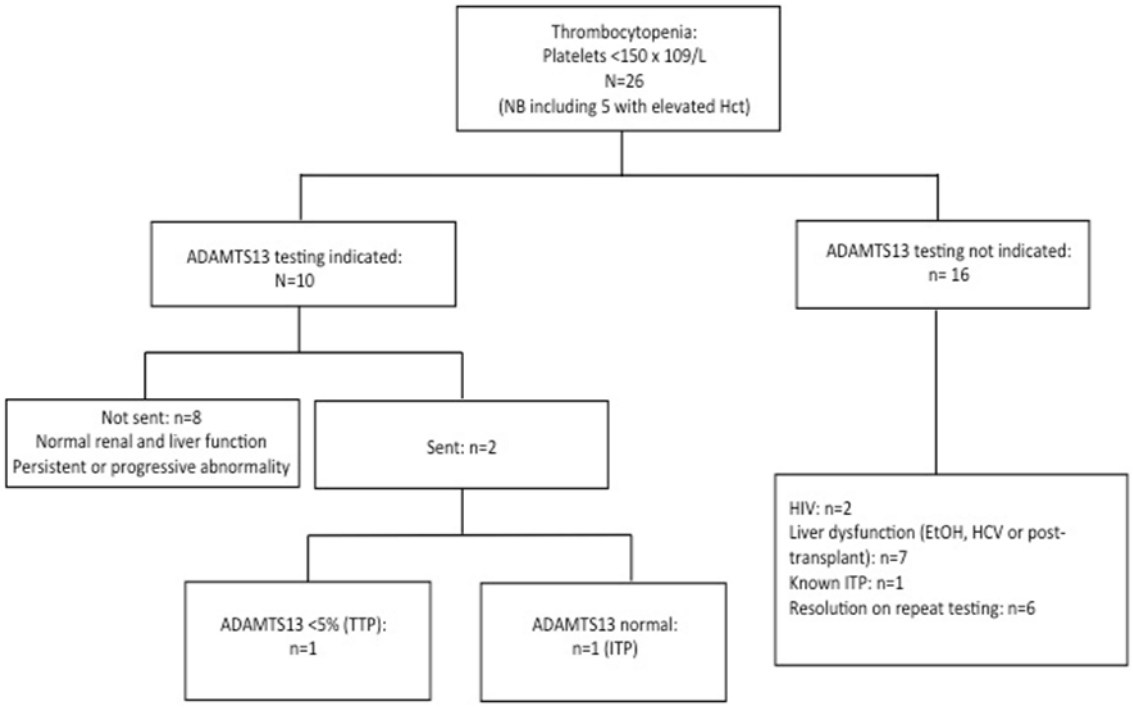
The British Society of Haematology guidelines include JAK II testing in the algorithms for investigation of a raised haematocrit; or in thrombocytosis, once iron deficiency and acute phase response have been ruled out. (Harrison et al. 2014; McMullin et al. 2019). Overall, JAK II testing was deemed warranted in 19 (2.8%): a persistently raised or progressively raised haematocrit or platelet count respectively, with normal liver and renal function and no other explicable cause. JAK II mutational analysis was only performed in 3 patients (0.5%). One was proven positive for the *V617F* mutation, hence diagnosed with polycythaemia vera. Of the 2 negative JAK II results, one patient was subsequently diagnosed with chronic myeloid leukaemia. Fourteen patients had no further testing or monitoring (8% of all abnormal results, 74% of all those patients who warranted JAK II testing).

Figure 20: Outcomes of raised haematocrit (>0.45) and/or thrombocytosis (platelet count >400 x 10^9/L)



26(4.3%) patients had thrombocytopenia. ADAMTS13 testing was not warranted in 16 of these since there was either resolution of thrombocytopenia on repeat testing, or a clear alternative aetiology (subsequent resolution of platelet count n=6, HIV n=2, liver derangement n=7, known ITP with no MAHA n=1). ADAMTS13 testing was indicated in 10 of these patients (38.5% of thrombocytopenic patients), defined as a persistent thrombocytopenia with no clear cause, normal liver and renal function and negative HIV status. In these 10 patients, the median platelet count was 117 x 10^9/L (range 34-150, mean 102.8). It is arguable whether ADAMTS13 screening is warranted in mild thrombocytopenia, but anecdotal experience suggests that congenital TTP can be increasingly diagnosed in cases of presumed immune thrombocytopenia (ITP). Eight of these patients did not have ADAMTS13 considered, according to the clinical documentation. Of the 2 tested for ADAMTS13, one result was normal, helping to resolve the clinical diagnosis of ITP. In the other patient, ADAMTS13 was <5%, confirming TTP and facilitating life-saving plasma exchange to take place.

Figure 21: Outcomes of thrombocytopenia (platelet count <150 x 10^9 /L)



4.13 Results: thrombophilia screening

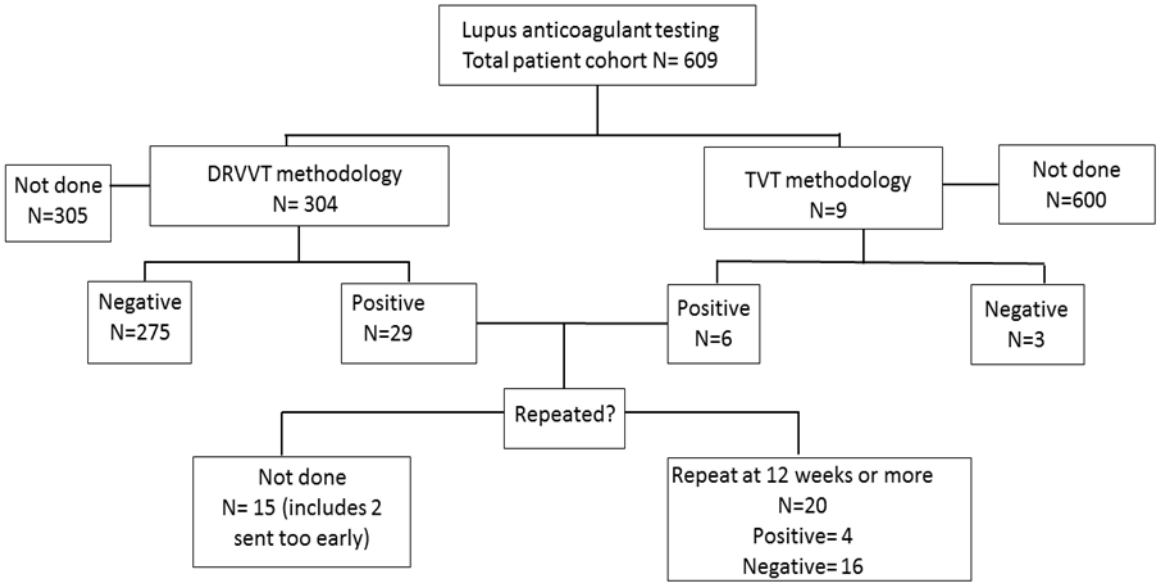
The same cohort of 609 patients less than 60 years were included: 378 ischaemic stroke (62.1%), 193 TIA (31.6%) and 38 haemorrhagic stroke (6.2%). Overall 235 patients had thrombophilia screening sent (39% of the cohort), and 374 patients did not (61% of the cohort).

4.13.1 Lupus anticoagulant testing

Examining the DRVVT testing first, 304 patients were tested and 305 were not. Of those whom were tested, 29 proved positive for a lupus anticoagulant and 275 were negative. With taipan methodology, 9 patients were tested for a lupus anticoagulant. The result was positive in 6 and negative in 3.

Therefore in sum there were 35 patients testing positively for a lupus anticoagulant. Of these, 15 were not repeated in the Trust, nor arrangements made for them to be repeated at local hospitals, according to the recommended timescale of at least 12 weeks later. The remaining 20 patients were tested again, and a lupus anticoagulant was persistently positive in 4 patients and negative in 16.

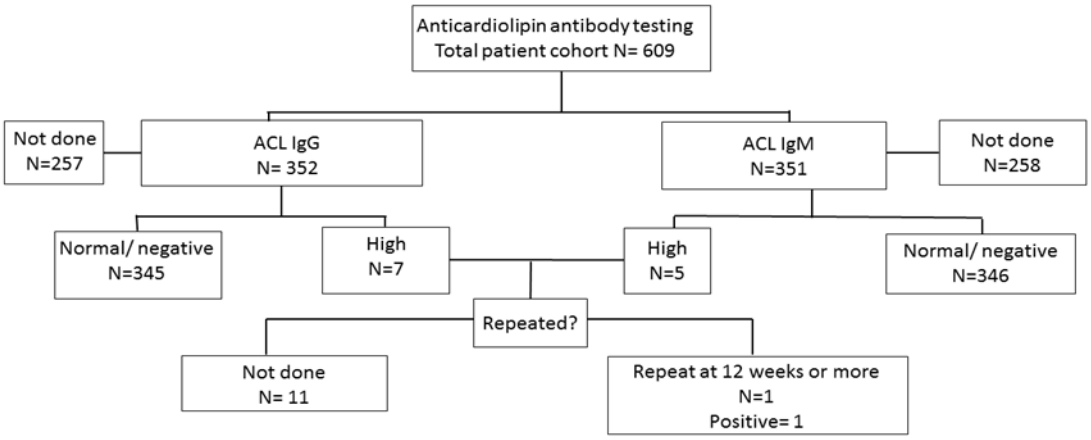
Figure 22: Lupus anticoagulant antibody testing



4.13.2 Anticardiolipin antibody testing

Anticardiolipin IgG antibody testing was performed in 352 patients, and not in 257. Of these, 345 had a normal/ negative result, with 7 patients with a high/ positive result. Anticardiolipin IgM antibody testing was performed in 351 patients, and not in 258. Of these, 346 had a normal/ negative result, with 5 patients with a high/ positive result. Of these 12 patients with a positive result, 11 were not tested again. The one patient who was tested again showed a persistently positive result.

Figure 23: Anticardiolipin antibody testing

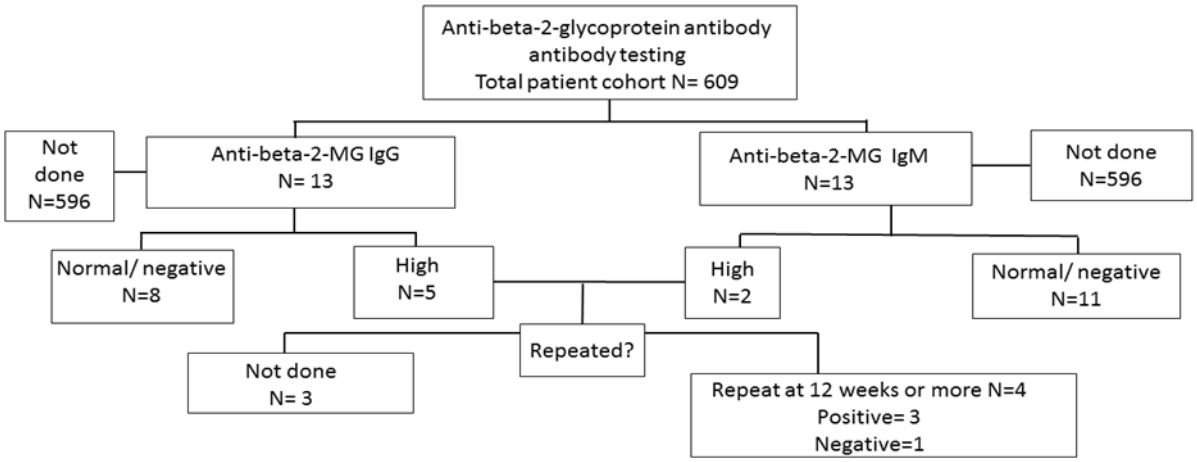


4.13.3 Anti-beta-2 glycoprotein 1 antibody testing

Anti-beta-2 glycoprotein 1 IgG antibody testing was performed in 13 patients and not done in 596. Of the patients tested, 8 had a normal/ negative result while 5 had a high/ positive result. Anti-beta-2 glycoprotein 1 IgM antibody testing was performed in 13 patients and not done in 596. Of the patients tested, 11 had a normal/ negative result while 2 had a high/positive result.

Of the total 7 patients with a positive result, 3 were not repeated. In the 4 patients with repeat testing as per the criteria, 3 showed persistent positivity, while 1 was normal/ negative.

Figure 24: Anti beta-2 glycoprotein 1 antibody testing



In sum, this means there were 54 (5.2%) positive results out of a total of 1042 individual tests performed. Less than half of these positive tests were repeated in our Trust (n=25), with no suggestion in correspondence to GPs and local hospitals for repeat tests to be performed in the others. A persistently positive result was seen in 8 of those repeated (32%), potentially indicating a diagnosis of antiphospholipid syndrome if the clinical criteria were in keeping. Of initially positive results, 29 were not repeated. One of these patients was 'triple APA' positive, already with a known diagnosis of APLS on rivaroxaban, testing positive for LA via TVT, anti-beta-2GP IgG antibody and ACL IgG antibody.

Examining practice, there were clearly far more patients tested for lupus anticoagulant (DRVVT and TVT methods combined: 313/ 609= 51.4%) and anticardiolipin antibody status (IgG 352/609 = 57.8%, IgM 351/609= 57.6%) compared to anti-beta-2 glycoprotein antibody testing (both IgG and IgM tested in 13/609= 2.1%). This was a protocol issue, since the standard thrombophilia screen at that time included factor V Leiden, prothrombin gene mutation, anticardiolipin antibodies, lupus anticoagulant, functional assays for protein C and antithrombin and free protein S quantification. Anti –beta-2 glycoprotein antibody was included as a later confirmatory step on request only.

4.14 Discussion: haematological investigation of young stroke

Cryptogenic stroke is seen most in the younger age group and acknowledged in the literature to have a more heterogeneous aetiology before cardiovascular risk factors have yet to yield effect (Nacu et al. 2016; Wolf et al. 2015). Although modifiable vascular risk factors may be increasingly prevalent, overall 'young stroke' is still considered a different phenomenon to the same aetiology and effect witnessed in an older patient (Simonetti et al. 2015; Maaijwee et al. 2014). Work up is correspondingly detailed and comprehensive.

Literature reviews based on the full blood count (or 'complete blood count') in the aetiology of young stroke yield little, often with the baseline full or complete blood count omitted altogether from comprehensive lists of investigations considered. Current models of stroke care in the UK mean that patients are often rapidly transferred to an acute stroke unit after a few days on the hyperacute stroke unit (HASU) so that any discrepancy in baseline tests may not be acknowledged and complex thrombophilia testing protocols are either poorly applied or if applied, poorly followed up.

We have specifically looked at the outcome of a basic first-line test- the full blood count, performed in almost every patient attending the hyperacute stroke unit. We focused on the haematocrit and the platelet count in the young stroke patients in our cohort. We considered a haematocrit of >0.45 to be of note since our experience has shown younger patients presenting with myeloproliferative disease may have a more subtle discrepancy than the normal range dictated by the laboratory. The platelet count was deemed abnormal if either $<150 \times 10^9$/L or $>400 \times 10^9$/L. We examined results outwith these ranges on a case-by-case basis.

We chose to study this population since there is controversy about the complexity of investigation and its relative worth, while the relevance of baseline testing may be missed. Although a carotid arterial dissection might be thought adequate reason for a stroke, there is little uniformity about whether more exhaustive investigations are still worthwhile. Our study included all patients under 60 years of age presenting to the service with confirmed stroke or TIA. A limitation is that our results may well represent an underestimate since this was a retrospective cohort and we lacked long term follow up on patients, particularly those repatriated to local hospital stroke services or from out of region.

Overall 26.7% of results outside our defined ranges were not repeated- this may have been because a haematocrit was borderline, or there was a clear explicable cause, or due to the patient's care being transferred either to another hospital or to primary care. In those patients who had repeated testing and resolution of the abnormality, transient anomalies in haematocrit or platelet count could be related to an acute phase response (thrombocytosis) or a relative polycythaemia due to dehydration (raised haematocrit).

Examining each case, molecular testing for MPD or TTP was indicated in at least 27 and not considered or performed in 22 of those patients (81.5%).

From a haematology perspective, we deemed that genetic testing for MPDs such as JAK II should be considered if there was a persistently raised haematocrit or platelet count respectively, with normal liver and renal function and no other obvious cause. In a similar fashion, we examined whether patients presenting with thrombocytopenia were considered for ADAMTS13 testing. Our results suggest that at least 22 further patients of those whom had repeat testing should have been considered for more specialist testing, or at least to have the anomaly documented and/ or discussed with haematology (14.4% of patients with abnormal haematocrit and/ or platelet count). It is arguable that such secondary investigation may not have been warranted if there was a clear and more obvious other aetiology such as cervical artery dissection. However, considering the complexity of tertiary investigations for young stroke patients, a simple full blood count seems worthy of consideration.

In stroke patients <60 years, one quarter had abnormalities in their routine full blood count, specifically the haematocrit level or platelet count. Myeloproliferative disease or TTP was present in 3 patients of 5 specifically investigated in the cohort. From a haematological perspective, 22 further patients merited further investigation. However, this number may be higher since a quarter of those patients with initial discrepancies of haematocrit and/ or platelet count did not have repeated testing. Although primary haematological disorders are rare as a cause of stroke, a basic full blood count result should not be ignored in considering the aetiology of arterial thrombosis in a younger cohort. Further validation is needed in a larger prospective observational study.

Beyond the full blood count, we examined the same young stroke cohort to look further at how antiphospholipid antibody screening features in the more comprehensive work-up. All 609 patients were examined on a case-by-case basis for whether antiphospholipid antibody screening was performed, which tests were included and whether repeat testing was arranged for those who proved positive. The same limitations apply as for our full blood count study: this was a retrospective cohort study, potentially affected by patients being repatriated elsewhere and hence lost to our follow up. Clinical documentation was examined for every patient. We fully expected approximately two-thirds of patients to be repatriated elsewhere. A key point of interest was whether discharge documentation flagged up abnormal results or requested repeated screening to be organised in either primary or secondary care. If not, then there would be no reason to expect it to be done.

Firstly examining the protocol for thrombophilia testing itself, there is limited evidence linking venous thrombosis risk factors such as protein S deficiency to arterial thrombosis (Gomez-Aranda et al. 1992; Martinez

et al. 1993). In young stroke patients with a patent foramen ovale, we acknowledge that venous risks could be directly relevant to an arterial event. Acquired deficiency of antithrombin, protein C or S may be seen in anticoagulation, liver dysfunction and infection, rather than a genuine heritable deficiency of a natural anticoagulant. Antiphospholipid syndrome demonstrates a more established risk for arterial thrombosis (De Laat et al. 2004; Galli et al. 2003a; Gómez-Puerta and Cervera 2014; Urbanus et al. 2009). Overall our results suggest that half of the cohort were not tested for antiphospholipid antibody status without clear clinical reasoning documented. The other half was tested for lupus anticoagulant and anticardiolipin antibody, but only 2.1% were tested for anti-beta-2 glycoprotein antibody status. Clinical reasoning for testing or not testing was not generally documented. This meant the antiphospholipid screen was incomplete while testing focused on less evidence-based venous thrombosis risk markers, as we have written up as a clinical cost-utility exercise (Alakbarzade et al. 2018).

Secondly, there may be careful attention paid to investigative algorithms in work-up of the young patient presenting with stroke, but thrombophilia testing will be a slower process, requiring confirmatory steps. As discussed earlier with regard to full blood count testing, current models of stroke care in the UK mean that patients are often rapidly transferred to an acute stroke unit after a few days on the hyperacute stroke unit (HASU). Discharge to another stroke service with thrombophilia results still pending can mean that results are missed. If positive results are documented and referred to the responsible clinician, timing of repeated testing needs to be coordinated to be in keeping with protocol. This would seem apparent with our population, with less than half of positive results repeat tested. In those whom were tested, 5.4% (n=54) were positive of the 1042 individual tests performed in 609 patients. Repeat testing was done in 25 of the initially positive 54 tests, with eight persistently positive. Of the initially positive tests, 29 were not repeated, potentially missing further diagnoses of APLS as a cause of seemingly cryptogenic stroke.

This is of particular pertinence with regard to APLS, since the diagnosis cannot be made based on a transient antiphospholipid antibody, but one that is persistent on repeat testing with a minimum of 12 weeks in between. The utility of positive results in influencing management is another question, with one recent publication suggesting hypercoagulability screening in young patients with cryptogenic stroke changed management in approximately one in every 20 tested (Omran et al. 2017).

There is no clear consensus regarding management of young patients presenting with stroke, including those whom may have a seemingly clear-cut precipitant such as a carotid arterial dissection and how far to proceed with more extensive investigation. In thrombophilia testing, however, the role of antiphospholipid antibody screening appears more robust than a generic ‘thrombophilia’ approach including molecular markers for venous thromboembolism risk and examining for deficiencies of natural anticoagulants that would be an unusual cause of adult stroke. Confirmation of APLS would have therapeutic implications, with a general

preference for anticoagulant as opposed to antiplatelet treatment for patients with APLS and stroke (Levine 2004; Ruiz-Irastorza et al. 2011). This has subsequently led to a change in our local practice, with cessation of front-line venous thromboembolism risk testing and uptake of triple antiphospholipid antibody testing in young patients presenting with stroke.

5 Final conclusions

This thesis has incorporated alliances between clinical haematology, stroke and cardiovascular medicine, and laboratory investigation of haemostasis. Our main focus has been the role of the VWF-ADAMTS13 axis in acute ischaemic brain injury. We have demonstrated the relevance of this haemostatic axis at both presentation and at later follow up. Furthermore, we have demonstrated its link with clinical severity, as predictive of later functional recovery and even mortality. Recovery of ADAMTS13 was seen to be specific for ischaemic stroke in convalescence. This potential role as a biomarker for clinical severity of acute ischaemic brain injury merits further investigation, both in a laboratory and clinical setting.

In conjunction with this, we investigated congenital TTP, defined by absence of ADAMTS13. Clinical practice has until now been empirically driven, based on symptom avoidance long before biochemical suggestion of TTP relapse. Our pharmacokinetic analysis of 6 patients with congenital TTP has shown enormous variability in half-life, suggesting that investigation of interindividual clearance of ADAMTS13 is necessary for future optimisation of treatment, to enable rationale for dose and frequency of prophylaxis. As recombinant ADAMTS13 is rolled out in clinical trials for treating TTP, extending into investigation and potential treatment of the disordered VWF-ADAMTS13 balance in arterial thrombosis and ischaemic stroke is critical.

With the application of haematological principles to stroke medicine, we also investigated protocols in place on the hyperacute stroke unit. A higher proportion of strokes in the younger population are classified as crytogenic. We performed a retrospective review of all stroke and TIA patients presenting to the HASU under the age of 60 years over a 31-month period. Separate studies firstly examined the initial full blood count test and whether discrepancies in the haematocrit and/ or platelet count were investigated further in consideration of primary haematological cause of stroke such as myeloproliferative disease or TTP. A second study examined thrombophilia testing in this young stroke cohort. Review of practice and available evidence, has now altered local practice so that triple antiphospholipid antibody testing is performed in young stroke rather than molecular markers for venous thromboembolism risk.

Understanding the basic science of the pathophysiology of arterial thrombus formation has been pivotal to direct clinical application, from antiplatelets to anticoagulation to thrombolytic therapy. Although the VWF-ADAMTS13 axis and congenital TTP may be in the domain of haematologists, whereas thrombolysis and protocols for investigation of young stroke falls to neurologists and stroke physicians; there is a clear overlap and common interest. Developing and establishing links between haematologists, neurologists, stroke physicians and laboratory scientists, and sharing expertise to the benefit of all, has been integral to this work. Further deepening of our understanding of arterial thrombosis, and ability to use emerging therapies effectively, merits ongoing collaboration to allow union of specialty knowledge across the field.

6 References

Adams, H.P. et al. 1993. “Classification of Subtype of Acute Ischemic Stroke.” *Stroke: a journal of cerebral circulation*.

Adams, Harold P. et al. 2008. “Emergency Administration of Abciximab for Treatment of Patients with Acute Ischemic Stroke: Results of an International Phase III Trial: Abciximab in Emergency Treatment of Stroke Trial (AbESTT-II).” *Stroke* 39(1): 87–99.

Adams Jr., H.P. et al. 2007. “Guidelines for the Early Management of Adults With Ischemic Stroke: A Guideline From the American Heart Association/ American Stroke Association Stroke Council, Clinical Cardiology Council, Cardiovascular Radiology and Intervention Council, and the Athero.” *Circulation* 115: e478–534. http://stroke.ahajournals.org/cgi/content/abstract/38/5/1655.

Agoram, Balaji M. 2009. “Use of Pharmacokinetic/ Pharmacodynamic Modelling for Starting Dose Selection in First-in-Human Trials of High-Risk Biologics.” *British Journal of Clinical Pharmacology*: 67 (2), 153–60.

Aguilar, MI, R Hart, and LA Pearce. 2007. “Oral Anticoagulants versus Antiplatelet Therapy for Preventing Stroke in Patients with Non-Valvular Atrial Fibrillation and No History of Stroke or Transient Ischemic Attacks.” *Cochrane Database Syst Rev* (3): 2–4.

Ahmad, S S, R Rawala-Sheikh, and P N Walsh. 1992. “Components and Assembly of the Factor X Activating Complex.” *Seminars in thrombosis and hemostasis* 18(3): 311–23. http://www.ncbi.nlm.nih.gov/pubmed/1455249.

Alakbarzade, Vafa et al. 2018. “Utility of Current Thrombophilia Screening in Young Patients with Stroke and TIA.” *Stroke and Vascular Neurology*.

Allford, S. L. et al. 2000. “Von Willebrand Factor-Cleaving Protease Activity in Congenital Thrombotic Thrombocytopenic Purpura.” *British Journal of Haematology* 111(4): 1215–22.

Alpoim, Patrícia N. et al. 2011. “ADAMTS13, FVIII, von Willebrand Factor, ABO Blood Group Assessment in Preeclampsia.” *Clinica Chimica Acta*.

Alwan, Ferras et al. 2017. “Presenting ADAMTS13 Antibody and Antigen Levels Predict Prognosis in Immune-Mediated Thrombotic Thrombocytopenic Purpura.” *Blood* 130(4): 466–71.

Ammash, Naser et al. 2011. “Left Atrial Blood Stasis and von Willebrand Factor-Adamts13 Homeostasis in Atrial Fibrillation.” *Arteriosclerosis, Thrombosis, and Vascular Biology* 31(11): 2760–66.

Anand, Sonia et al. 2007. “Oral Anticoagulant and Antiplatelet Therapy and Peripheral Arterial Disease.” *New England Journal of Medicine* 357: 217–27.

Andersen, Klaus Kaae, and T. S. Olsen. 2007. “Reduced Poststroke Mortality in Patients with Stroke and Atrial Fibrillation Treated with Anticoagulants: Results from a Danish Quality-Control Registry of 22 179 Patients with Ischemic Stroke.” *Stroke* 38(2): 259–63.

Andersson, HM et al. 2012. “High VWF, Low ADAMTS13, and Oral Contraceptives Increase the Risk of Ischemic Stroke and Myocardial Infarction in Young Women.” *Blood* 119(6): 1555 LP – 1560. http://www.bloodjournal.org/content/119/6/1555.abstract.

Andersson, John, Peter Libby, and Göran K. Hansson. 2010. “Adaptive Immunity and Atherosclerosis.” *Clinical Immunology* 134(1): 33–46.

Arslan, Yildiz, Tahir Kurtulus Yoldas, and Yasar Zorlu. 2013. “Interaction Between VWF Levels and Aspirin Resistance in Ischemic Stroke Patients.” *Translational Stroke Research* 4(5): 484–87.

Balogun, Ibrahim O et al. 2016. “Thrombin Generation in Acute Ischaemic Stroke.” *Stroke Research & Treatment* 2016: 1–9. http://myaccess.library.utoronto.ca/login?url=http://search.ebscohost.com/login.aspx?direct=true&db=rzh&AN=120387022&site=ehost-live.

Barber, Mark et al. 2004. “Hemostatic Function and Progressing Ischemic Stroke: D-Dimer Predicts Early Clinical Progression.” *Stroke* 35(6): 1421–25.

Bas De Laat, H. et al. 2004. “B2-Glycoprotein I-Dependent Lupus Anticoagulant Highly Correlates with Thrombosis in the Antiphospholipid Syndrome.” *Blood*.

Bath, P M, a Blann, N Smith, and R J Butterworth. 1998. “Von Willebrand Factor, P-Selectin and Fibrinogen Levels in Patients with Acute Ischaemic and Haemorrhagic Stroke, and Their Relationship with Stroke Sub-Type and Functional Outcome.” *Platelets* 9(3–4): 155–59. http://www.ncbi.nlm.nih.gov/pubmed/16793694.

Becker, RC et al. 1995. “Dynamic Nature of Thrombin Generation, Fibrin Formation, and Platelet Activation in Unstable Angina and Non-Q-Wave Myocardial Infarction.” *J Thromb Thrombolysis* 2(1): 57–64.

Bentler, P. M., and Douglas G. Bonett. 1980. “Significance Tests and Goodness of Fit in the Analysis of Covariance Structures.” *Psychological Bulletin* 88(3): 588–606.

Bergmeier, Wolfgang et al. 2006. “The Role of Platelet Adhesion Receptor GPIbalpha Far Exceeds That of Its Main Ligand, von Willebrand Factor, in Arterial Thrombosis.” *Proceedings of the National Academy of Sciences of the United States of America* 103(45): 16900–905. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1636551&tool=pmcentrez&rendertype=abstract.

Bernardo, A. et al. 2005. “Platelets Adhered to Endothelial Cell-Bound Ultra-Large von Willebrand Factor Strings Support Leukocyte Tethering and Rolling under High Shear Stress.” *Journal of Thrombosis and Haemostasis* 3(3): 562–70.

Berndt, M. C. et al. 2001. “The Vascular Biology of the Glycoprotein Ib-IX-V Complex.” *Thrombosis and Haemostasis* 86(1): 178–88.

Berntorp, Erik. 2009. “Differential Response to Bypassing Agents Complicates Treatment in Patients with Haemophilia and Inhibitors.” *Haemophilia* 15(1): 3–10.

Bhatia, Rohit et al. 2010. “Low Rates of Acute Recanalization with Intravenous Recombinant Tissue Plasminogen Activator in Ischemic Stroke: Real-World Experience and a Call for Action.” *Stroke*.

Bhatt, Deepak L; Topol, Eric J. 2000. “Current Role of Platelet Glycoprotein IIb / IIIa Inhibitors in Acute Coronary Syndromes.” 284(12): 1549–58.

Bhatt, Deepak L. et al. 2012. “The Relationship between CYP2C19 Polymorphisms and Ischaemic and Bleeding Outcomes in Stable Outpatients: The CHARISMA Genetics Study.” *European Heart Journal* 33(17): 2143–50.

Blombery, Piers, and Marie Scully. 2014. “Management of Thrombotic Thrombocytopenic Purpura: Current Perspectives.” *Journal of Blood medicine* 5: 15–23.

http://www.ncbi.nlm.nih.gov/pubmed/24523598%5Cnhttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC3921093.

Boekholdt, S. Matthijs, and Mark H H Kramer. 2007. “Arterial Thrombosis and the Role of Thrombophilia.” *Seminars in Thrombosis and Hemostasis*.

Bongers, Tamara N. et al. 2006. “High von Willebrand Factor Levels Increase the Risk of First Ischemic Stroke: Influence of ADAMTS13, Inflammation, and Genetic Variability.” *Stroke* 37(11): 2672–77.

Bonini, Pierangelo, Mario Plebani, Ferruccio Ceriotti, and Francesca Rubboli. 2002. “Errors in Laboratory Medicine.” *Clinical Chemistry*.

Borissoff, Julian I. et al. 2012. “Accelerated in Vivo Thrombin Formation Independently Predicts the Presence and Severity of CT Angiographic Coronary Atherosclerosis.” *JACC: Cardiovascular Imaging* 5(12): 1201–10. http://dx.doi.org/10.1016/j.jcmg.2012.01.023.

Brandt, John T. et al. 2007. “Common Polymorphisms of CYP2C19 and CYP2C9 Affect the Pharmacokinetic and Pharmacodynamic Response to Clopidogrel but Not Prasugrel.” *Journal of Thrombosis and Haemostasis* 5(12): 2429–36.

Bray, Benjamin D. et al. 2016. “Weekly Variation in Health-Care Quality by Day and Time of Admission: A Nationwide, Registry-Based, Prospective Cohort Study of Acute Stroke Care.” *Lancet (London, England)* 388(10040): 170–77.

Brey, R L et al. 2002. “Antiphospholipid Antibodies and Stroke in Young Women.” *Stroke* 33(10): 2396–2400. http://ovidsp.ovid.com/ovidweb.cgi?T=JS&CSC=Y&NEWS=N&PAGE=fulltext&D=emed5&AN=2002362475%5Cnhttp://openurl.ac.uk/athens:lee/?sid=OVID:embase&id=pmid:&id=doi:10.1161%2F01.STR.0000031927.25510.D1&issn=0039-2499&isbn=&volume=33&issue=10&spage=2396&pages=2396.

Brown, Martin M. 2012. “New National Guideline for Stroke Management: Where Do We Go from Here?” *Clinical Medicine* 12(5): 407–9.

Bustamante, A et al. 2015. “ADAMTS13 Activity Predicts Response to Thrombolysis in the Acute Stroke Setting.” *Stroke* 46. http://www.embase.com/search/results?subaction=viewrecord&from=export&id=L71818321.

Butenas, S., and Kenneth G. Mann. 2007. “Caution in the Interpretation of Continuous Thrombin Generation Assays [10].” *Journal of Thrombosis and Haemostasis*.

Cai, Ping et al. 2015. “Recombinant ADAMTS 13 Attenuates Brain Injury after Intracerebral Hemorrhage.” *Stroke* 46(9): 2647–53.

CAPRIE Steering committee. 1996. “A Randomized Blinded Trial of Clopidogrel versus Aspirin in Patients at Risk of Ischemic Events (CAPRIE).” *Lancet* 348: 1329–39.

Carcaillon, Laure et al. 2011. “Increased Thrombin Generation Is Associated with Acute Ischemic Stroke but Not with Coronary Heart Disease in the Elderly: The Three-City Cohort Study.” *Arteriosclerosis, Thrombosis, and Vascular Biology* 31(6): 1445–51.

Carobbio, Alessandra et al. 2011. “Risk Factors for Arterial and Venous Thrombosis in WHO-Defined Essential Thrombocythemia : An International Study of 891 Patients Brief Report Risk Factors for Arterial and Venous Thrombosis in WHO-Defined Essential Thrombocythemia : An International Stud.” *Blood* 117(22): 5857–59.

Cate, Hugo ten. 2012. “Thrombin Generation in Clinical Conditions.” *Thrombosis Research* 129(3): 367–70. http://dx.doi.org/10.1016/j.thromres.2011.10.017.

ten Cate, Hugo, and H. Coenraad Hemker. 2016. “Thrombin Generation and Atherothrombosis: What Does the Evidence Indicate?” *Journal of the American Heart Association* 5(8): e003553. http://jaha.ahajournals.org/lookup/doi/10.1161/JAHA.116.003553.

Cattaneo, Marco. 2007. “Laboratory Detection of ‘Aspirin Resistance’: What Test Should We Use (If Any)?” *European Heart Journal* 28: 2482–94.

Cervera, Ricard et al. 2002. “Antiphospholipid Syndrome: Clinical and Immunologic Manifestations and Patterns of Disease Expression in a Cohort of 1,000 Patients.” *Arthritis and Rheumatism* 46(4): 1019–27.

Chakroun, Tahar et al. 2004. “In Vitro Aspirin Resistance Detected by PFA-100^{TM} Closure Time: Pivotal Role of Plasma von Willebrand Factor.” *British Journal of Haematology* 124(1): 80–85.

Chauhan, A K et al. 2008. “ADAMTS13: A New Link between Thrombosis and Inflammation.” *J Exp Med* 205(9): 2065–74. http://www.ncbi.nlm.nih.gov/pubmed/18695007.

Chauhan, Anil K et al. 2006. “Systemic Antithrombotic Effects of ADAMTS13.” *The Journal of experimental medicine* 203(3): 767–76.

Chen, Zheng-Ming. 1997. “CAST: Randomised Placebo-Controlled Trial of Early Aspirin Use in 20 000 Patients with Acute Ischaemic Stroke.” *The Lancet* 349(9066): 1641–49. http://www.sciencedirect.com/science/article/pii/S0140673697040105.

Collins, P.W. et al. 2011. “Implications of Coagulation Factor VIII and IX Pharmacokinetics in the Prophylactic Treatment of Haemophilia.” *Haemophilia* 17: 2–10.

Conway, Dwayne S G et al. 2003. “Prognostic Value of Plasma von Willebrand Factor and Soluble P-Selectin as Indices of Endothelial Damage and Platelet Activation in 994 Patients with Nonvalvular Atrial Fibrillation.” *Circulation* 107(25): 3141–45.

Crawley, James T B et al. 2005. “Proteolytic Inactivation of ADAMTS13 by Thrombin and Plasmin.” *Blood* 105(3): 1085–93.

Crawley, James T B et al. 2011. “Unraveling the Scissile Bond: How ADAMTS13 Recognizes and Cleaves von Willebrand Factor.” *Blood* 118(12): 3212–21.

Danesh, John et al, and The Authors and Writing Committee and other members of the Fibrinogen Collaboration. 2005. “Plasma Fibrinogen Level and the Risk of Major Cardiovascular Diseases and Nonvascular Mortality.” *JAMA* 294(14): 1799–1810.

Davi, Giovanni, and Carlo Patrono. 2007. “Platelet Activation and Atherothrombosis.” *The New England journal of medicine* 357: 2482–94.

Dawson, Jesse et al. 2011. “Aspirin Resistance and Compliance with Therapy.” *Cardiovascular Therapeutics* 29(5): 301–7.

Deb, Prabal, Suash Sharma, and K. M. Hassan. 2010. “Pathophysiologic Mechanisms of Acute Ischemic Stroke: An Overview with Emphasis on Therapeutic Significance beyond Thrombolysis.” *Pathophysiology* 17(3): 197–218.

Demaerschalk, Bart M. et al. 2016. 47 Stroke *Scientific Rationale for the Inclusion and Exclusion Criteria for Intravenous Alteplase in Acute Ischemic Stroke A Statement for Healthcare Professionals from the*

American Heart Association/American Stroke Association.

Denorme, F et al. 2016. "ADAMTS13-Mediated Thrombolysis of t-PA Resistant Occlusions in Ischemic Stroke in Mice." *Blood* 127(19): 2337–46. http://www.ncbi.nlm.nih.gov/pubmed/26929275.

Denorme, Frederik, and Simon F. De Meyer. 2016. "The VWF-GPib Axis in Ischaemic Stroke: Lessons from Animal Models." *Thrombosis and Haemostasis* 116(4): 597–604.

Denorme, Frederik, and Matthew T. Rondina. 2019. "Targeting Glycoprotein VI for Thromboembolic Disorders: All Gain with No Pain?" *Arteriosclerosis, Thrombosis, and Vascular Biology*.

Department of Health. 2007. "National Stroke Strategy." *Policy*: 1. http://www.dh.gov.uk/prod_consum_dh/groups/dh_digitalassets/documents/digitalasset/dh_081059.

Department of Health National Audit Office. 2005. *Reducing Brain Damage: Faster Access to Better Stroke Care.*

Devaraj, S., DY. Xu, and I. Jialal. 2003. "C-Reactive Protein Increases Plasminogen Activator Inhibitor-1 Expression and Activity in Human Aortic Endothelial Cells: Implications for the Metabolic Syndrome and Atherothrombosis." *Circulation* 107(3): 398–404.

Diener, Hans Christoph et al. 2008. "Effects of Aspirin plus Extended-Release Dipyridamole versus Clopidogrel and Telmisartan on Disability and Cognitive Function after Recurrent Stroke in Patients with Ischaemic Stroke in the Prevention Regimen for Effectively Avoiding Second Strokes (PRoFE." *The Lancet Neurology* 7(10): 875–84.

Dirnagl, U, C Iadecola, and M a Moskowitz. 1999. "Pathobiology of Ischaemic Stroke: An Integrated View. 4441." *Trends in Neurosciences* 22(9): 391–97.

Dong, Jing fei et al. 2002. "ADAMTS-13 Rapidly Cleaves Newly Secreted Ultralarge von Willebrand Factor Multimers on the Endothelial Surface under Flowing Conditions." *Blood* 100(12): 4033–39.

Eikelboom, J. W. et al. 2017. "Rivaroxaban with or without Aspirin in Stable Cardiovascular Disease." *New England Journal of Medicine* 377: 1319–30.

Elliott, Michelle A., and A. Tefferi. 2005. "Thrombosis and Haemorrhage in Polycythaemia Vera and Essential Thrombocythaemia." *British Journal of Haematology* 128(3): 275–90.

Evans, Matthew R.B., Phil White, Peter Cowley, and David J. Werring. 2017. "Revolution in Acute Ischaemic Stroke Care: A Practical Guide to Mechanical Thrombectomy." *Practical Neurology*.

Farrell, B., J. Godwin, S. Richards, and C. Warlow. 1951. "The United Kingdom Transient Ischaemic Attack (UK-TIA) Aspirin Trial: Final Results." *Journal of Neurology, Neurosurgery and Psychiatry*.

Feher, Gergely et al. 2010. "Clinical Importance of Aspirin and Clopidogrel Resistance." *World journal of cardiology* 2(7): 171–86. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2998916&tool=pmcentrez&rendertype=abstract.

Feigin, Valery L., Carlene M M Lawes, Derrick A. Bennett, and Craig S. Anderson. 2003. "Stroke Epidemiology: A Review of Population-Based Studies of Incidence, Prevalence, and Case-Fatality in the Late 20th Century." *Lancet Neurology* 2(1): 43–53.

Feng, Yun et al. 2016. "ADAMTS13: More than a Regulator of Thrombosis." *International Journal of Hematology*: 104: 534-539.

Feys, H. B. et al. 2009. “Multi-Step Binding of ADAMTS-13 to von Willebrand Factor.” *Journal of Thrombosis and Haemostasis* 7(12): 2088–95.

Fischer, Urs et al. 2005. “NIHSS Score and Arteriographic Findings in Acute Ischemic Stroke.” *Stroke*.

Folsom, A R et al. 1999. “Prospective Study of Markers of Hemostatic Function with Risk of Ischemic Stroke.” *Circulation* 100(7): 736–42. http://www.embase.com/search/results?subaction=viewrecord&from=export&id=L29383644%5Cnhttp://mgetit.lib.umich.edu/sfx_locater?sid=EMBASE&issn=00097322&id=doi:&atitle=Prospective+study+of+markers+of+hemostatic+function+with+risk+of+ischemic+stroke&stitle=C.

Food and Drug Administration. 2010. “Reduced Effectiveness of Plavix (Clopidogrel) in Patients Who Are Poor Metabolizers of the Drug.” *FDA Drug Safety Communication*.

Fujimura Y, Kokame K, Yagi H, Isonishi A, Matsumoto M, Miyata T.Rodgers, George M. 2015. “Hereditary Deficiency of ADAMTS13 Activity: Upshaw−Schulman Syndrome.” In *ADAMTS13: Biology and Disease*, , 73–90.

Fujioka, Masayuki et al. 2010. “ADAMTS13 Gene Deletion Aggravates Ischemic Brain Damage: A Possible Neuroprotective Role of ADAMTS13 by Ameliorating Postischemic Hypoperfusion.” *Blood* 115(8): 1650–53.

Furie, Bruce, and Barbara C Furie. 2008. “Mechanisms of Thrombus Formation.” *The New England journal of medicine* 359(9): 938–49.

Furie, Karen L et al. 2011. “Guidelines for the Prevention of Stroke in Patients with Stroke or Transient Ischemic Attack: A Guideline for Healthcare Professionals from the American Heart Association/American Stroke Association.” *Stroke; a journal of cerebral circulation* 42(1): 227–76. http://stroke.ahajournals.org/content/42/1/227.abstract.

Furlan, Miha et al. 1999. “Recovery and Half-Life of von Willebrand Factor-Cleaving Protease after Plasma Therapy in Patients with Thrombotic Thrombocytopenic Purpura.” *Thrombosis and Haemostasis* 81(1): 13.

Furlan, Miha, and Bernhard Lämmle. 2002. “Assays of von Willebrand Factor-Cleaving Protease: A Test for Diagnosis of Familial and Acquired Thrombotic Thrombocytopenic Purpura.” *Seminars in Thrombosis and Hemostasis* 28(2): 167–72.

Galli, Monica, Davide Luciani, Guido Bertolini, and Tiziano Barbui. 2003a. “Anti-B2-Glycoprotein I, Antiprothrombin Antibodies, and the Risk of Thrombosis in the Antiphospholipid Syndrome.” *Blood* 102(8): 2717–23.

Galli, Monica, Davide Luciani, Guido Bertolini, and Tiziano Barbui. 2003b. “Lupus Anticoagulants Are Stronger Risk Factors for Thrombosis than Anticardiolipin Antibodies in the Antiphospholipid Syndrome: A Systematic Review of the Literature.” *Blood* 101(5): 1827–32.

Gao, Weiqiang, Patricia J. Anderson, and J. Evan Sadler. 2008. “Extensive Contacts between ADAMTS13 Exosites and von Willebrand Factor Domain A2 Contribute to Substrate Specificity.” *Blood* 112(5): 1713–19.

Garlichs, Christoph D. et al. 2003. “Upregulation of CD40-CD40 Ligand (CD154) in Patients with Acute Cerebral Ischemia.” *Stroke* 34(6): 1412–17.

Ghabaee, Mojdeh et al. 2014. “Predictive Ability of C-Reactive Protein for Early Mortality after Ischemic Stroke: Comparison with NIHSS Score.” *Acta Neurologica Belgica* 114(1): 41–45.

Goeggel Simonetti, B et al. 2015. “Risk Factors, Aetiology and Outcome of Ischaemic Stroke in Young Adults: The Swiss Young Stroke Study (SYSS).” *J Neurol* 262(9): 2025–32.

Gomez-Aranda, F, JM Lopez Dominquez, V Rivera Fernandez, and E Martin Garcia. 1992. “Stroke and Familial Protein S Deficiency.” *Stroke* 23(2): 299.

Gómez-Puerta, Jose A., and Ricard Cervera. 2014. “Diagnosis and Classification of the Antiphospholipid Syndrome.” *Journal of Autoimmunity*.

Granger, Christopher B. et al. 1998. “Thrombin Generation, Inhibition and Clinical Outcomes in Patients with Acute Myocardial Infarction Treated with Thrombolytic Therapy and Heparin: Results from the GUSTO-I Trial.” *Journal of the American College of Cardiology* 31(3): 497–505. http://dx.doi.org/10.1016/S0735-1097(97)00539-1.

Green, David et al. 2016. “Association of the von Willebrand Factor–ADAMTS13 Ratio With Incident Cardiovascular Events in Patients With Peripheral Arterial Disease.” *Clinical and Applied Thrombosis/Hemostasis*: 107602961665561. http://journals.sagepub.com/doi/10.1177/1076029616655615.

Gregory W. Albers, , Pierre Amarenco, , J. Donald Easton, , Ralph L. Sacco, , Philip Teal. 2004. “Hemorrhagic Complications of Anticoagulant Treatment.” *Chest* 126(3): 287S-310S. http://dx.doi.org/10.1378/chest.126.3_suppl.287S.

Groot, E. et al. 2006. “FRETS-VWF73: A Rapid and Predictive Tool for Thrombotic Thrombocytopenic Purpura [8].” *Journal of Thrombosis and Haemostasis* 4(3): 698–99.

Gubitz, G, Sandercock P, and Counsell C. 2008. “Anticoagulants for Acute Ischaemic Stroke.” *Cochrane database of systematic reviews (Online)* (4): CD000024.

Hacke, Werner et al. 2008. “Thrombolysis with Alteplase 3 to 4.5 Hours after Acute Ischaemic Stroke.” *New England Journal of Medicine* 359(13): 1317–29.

Halawani, Saeed H M et al. 2011. “Aspirin Failure in Patients Presenting with Acute Cerebrovascular Ischaemia.” *Thrombosis and haemostasis* 106(2): 240–47. http://www.ncbi.nlm.nih.gov/pubmed/21544317.

Halkes, P H a et al. 2008. “Dipyridamole plus Aspirin versus Aspirin Alone in Secondary Prevention after TIA or Stroke: A Meta-Analysis by Risk.” *Journal of neurology, neurosurgery, and psychiatry* 79: 1218–23.

Hanson, E. et al. 2009. “Association between Genetic Variation at the ADAMTS13 Locus and Ischemic Stroke.” *Journal of Thrombosis and Haemostasis* 7(12): 2147–48.

Harrison, Claire N. et al. 2014. “Modification of British Committee for Standards in Haematology Diagnostic Criteria for Essential Thrombocythaemia.” *British Journal of Haematology*: 418–38.

Harrison, P et al. 2008. “Lack of Reproducibility of Assessment of Aspirin Responsiveness by Optical Aggregometry and Two Platelet Function Tests.” *Platelets.* 19(2): 119–24.

Harrison, Paul et al. 2005. “Screening for Aspirin Responsiveness after Transient Ischemic Attack and Stroke: Comparison of 2 Point-of-Care Platelet Function Tests with Optical Aggregometry.” *Stroke* 36(5): 1001–5.

Hart, R. G. et al. 2018. “Rivaroxaban for Stroke Prevention after Embolic Stroke of Undetermined Source.” *New*

England Journal of Medicine 378: 2191–2201.

Hemker, H. C. et al. 2002. "The Calibrated Automated Thrombogram (CAT): A Universal Routine Test for Hyper- and Hypocoagulability." *Pathophysiology of Haemostasis and Thrombosis* 32(5–6): 249–53.

Hemker, H. C. et al. 2015. "The Application of Thrombin Generation in Real Life Clinical Situations." *Thrombosis Research* 136(1): 3–4. http://dx.doi.org/10.1016/j.thromres.2015.04.010.

Hemker, H. Coenraad et al. 2003. "Calibrated Automated Thrombin Generation Measurement in Clotting Plasma." *Pathophysiology of Haemostasis and Thrombosis* 33(1): 4–15.

Henn, V et al. 1998. "CD40 Ligand on Activated Platelets Triggers an Inflammatory Reaction of Endothelial Cells." *Nature* 391(6667): 591–94. http://www.nature.com/nature/journal/v391/n6667/abs/391591a0.html%5Cnhttp://www.ncbi.nlm.nih.gov/pubmed/9468137.

Heo, Ji Hoe, Kyung Yul Lee, Seo Hyun Kim, and Dong Ik Kim. 2003. "Immediate Reocclusion Following a Successful Thrombolysis in Acute Stroke: A Pilot Study." *Neurology* 60(10): 1684–87. http://www.ncbi.nlm.nih.gov/pubmed/12771267.

Hermann, A et al. 2001. "Platelet CD40 Ligand (CD40L): Subcellular Localisation, Regulation of Expression and Inhibition by Clopidogrel." *Platelets* 12(2): 74–82.

Hochholzer, Willibald et al. 2010. "Impact of Cytochrome P450 2C19 Loss-of-Function Polymorphism and of Major Demographic Characteristics on Residual Platelet Function After Loading and Maintenance Treatment With Clopidogrel in Patients Undergoing Elective Coronary Stent Placement." *Journal of the American College of Cardiology* 55(22): 2427–34.

Hofman, Albert et al. 2015. "The Rotterdam Study: 2016 Objectives and Design Update." *European Journal of Epidemiology* 30(8): 661–708.

Holmes, David R. et al. 2010. "ACCF/AHA Clopidogrel Clinical Alert: Approaches to the FDA 'Boxed Warning': A Report of the American College of Cardiology Foundation Task Force on Clinical Expert Consensus Documents and the American Heart Association." *Journal of the American College of Cardiology*.

Holmes, M.V., Perel P., et al. 2011. "CYP2C19 Genotype, Clopidogrel Metabolism, Platelet Function, and Cardiovascular Events. A Systematic Review and Meta-Analysis." *JAMA - Journal of the American Medical Association*.306(24): 2704–14.

Hovinga, Johanna A.Kremer et al. 2007. "ADAMTS-13, von Willebrand Factor and Related Parameters in Severe Sepsis and Septic Shock." *Journal of Thrombosis and Haemostasis*.

Hu, Hu et al. 2010. "Platelets Enhance Lymphocyte Adhesion and Infiltration into Arterial Thrombus." Thrombosis(1): 1184–92.

Hughes, M, G Y Lip, and National Clinical Guideline for Management of Atrial Fibrillation in Primary and Secondary Care Guideline Development Group National Institute for Health and Clinical Excellence. 2008. "Stroke and Thromboembolism in Atrial Fibrillation: A Systematic Review of Stroke Risk Factors, Risk Stratification Schema and Cost Effectiveness Data. [Review] [53 Refs]." *Thrombosis & Haemostasis* 99(2): 295–304.

Hulot, Jean Sébastien et al. 2006. "Cytochrome P450 2C19 Loss-of-Function Polymorphism Is a Major Determinant of Clopidogrel Responsiveness in Healthy Subjects." *Blood* 108(7): 2244–47.

van Hylckama Vlieg, A. et al. 2015. "The Risk of a First and a Recurrent Venous Thrombosis Associated with an Elevated D-Dimer Level and an Elevated Thrombin Potential: Results of the THE-VTE Study." *Journal of Thrombosis and Haemostasis* 13(9): 1642–52.

Ingelman-Sundberg, Magnus, Sarah C. Sim, Alvin Gomez, and Cristina Rodriguez-Antona. 2007. "Influence of Cytochrome P450 Polymorphisms on Drug Therapies: Pharmacogenetic, Pharmacoepigenetic and Clinical Aspects." *Pharmacology and Therapeutics* 116(3): 496–526.

Ishikawa, Mami et al. 2005. "CD40/CD40 Ligand Signaling in Mouse Cerebral Microvasculature after Focal Ischemia/Reperfusion." *Circulation* 111(13): 1690–96.

ISIS study group. 1988. "Randomized Trial of Intravenous Streptokinase, Oral Aspirin, Both, or Neither among 17,187 Cases of Suspected Acute Myocardial Infarction: ISIS-2.ISIS-2 (Second International Study of Infarct Survival) Collaborative Group." *Journal of the American College of Cardiology* 12(August): 3A-13A.

Jaffre, A. et al. 2017. "Non-Obstructive Carotid Atherosclerosis and Patent Foramen Ovale in Young Adults with Cryptogenic Stroke." *European Journal of Neurology*: 663–66. http://doi.wiley.com/10.1111/ene.13275.

Jimenez, Alfredo H. et al. 1992. "Rapidity and Duration of Platelet Suppression by Enteric-Coated Aspirin in Healthy Young Men." *The American Journal of Cardiology* 69(3): 258–62.

Joutel, Anne et al. 1996. "Notch3 Mutations in CADASIL, a Hereditary Adult-Onset Condition Causing Stroke and Dementia." *Nature* 383(6602): 707–10. http://www.nature.com/doifinder/10.1038/383707a0.

Juul, Klaus et al. 2002. "Plenary Paper Factor V Leiden : The Copenhagen City Heart Study and 2 Meta-Analyses." *Blood* 100(1): 3–10.

Kaikita, K et al. 2006. "Reduced von Willebrand Factor-Cleaving Protease (ADAMTS13)Activity in Acute Myocardial Infarction.Pdf." *Journal of Thrombosis and Haemostasis* 4: 2490–93.

Kamalian, Shervin et al. 2013. "Clot Length Distribution and Predictors in Anterior Circulation Stroke: Implications for Intra-Arterial Therapy." *Stroke*.

Kang, Dong Wha et al. 2009. "Inflammatory and Hemostatic Biomarkers Associated with Early Recurrent Ischemic Lesions in Acute Ischemic Stroke." *Stroke* 40(5): 1653–58.

Karpman, Diana, Lars Holmberg, Lena Jirgård, and Stefan Lethagen. 1996. "Increased Platelet Retention in Familial Recurrent Thrombotic Thrombocytopenic Purpura." *Kidney International* 49(1): 190–99.

Keeling, David et al. 2012. "Guidelines on the Investigation and Management of Antiphospholipid Syndrome." *British Journal of Haematology* 157(1): 47–58.

Keeling, David M. et al. 2011. "Guidelines on Oral Anticoagulation with Warfarin - Fourth Edition." *British Journal of Haematology* 154(3): 311–24.

Keizer, Ron J., Alwin D R Huitema, Jan H M Schellens, and Jos H. Beijnen. 2010. "Clinical Pharmacokinetics of Therapeutic Monoclonal Antibodies." *Clinical Pharmacokinetics* 49(8): 493–507.

Kennedy R Lees, Erich Bluhmki, Rüdiger von Kummer, Thomas G Brott, Danilo Toni, James C Grotta, Gregory W Albers, Markku Kaste, John R Marler, Scott A Hamilton, Barbara C Tilley, Stephen M Davis, Geoff rey A Donnan, Werner Hacke, for the ECASS, ATLANTIS, and EPITHET rt-PA Study Group Investigators. 2010. "Time to Treatment with Intravenous Alteplase and Outcome in Stroke: An Updated Pooled Analysis of ECASS, ATLANTIS, NINDS, AND EPITHET Trials." *Lancet* 375: 1695–1703.

Khan, MM, DG Motto, SR Lentz, and AK Chauhan. 2012. "ADAMTS13 Reduces VWF-Mediated Acute

Inflammation Following Focal Cerebral Ischemia in Mice." *Journal of Thrombosis and Haemostasis* 10(8): 1665–71. http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3419774/pdf/nihms-387375.pdf.

Kim, K a, P W Park, S J Hong, and J-Y Park. 2008. "The Effect of CYP2C19 Polymorphism on the Pharmacokinetics and Pharmacodynamics of Clopidogrel: A Possible Mechanism for Clopidogrel Resistance." *Clinical pharmacology and therapeutics* 84(2): 236–42.

Kinsella, J A et al. 2015. "Increased Thrombin Generation Potential in Symptomatic versus Asymptomatic Moderate or Severe Carotid Stenosis and Relationship with Cerebral Microemboli." *Journal of Neurology, Neurosurgery & Psychiatry* 86(4): 460–67. http://jnnp.bmj.com/lookup/doi/10.1136/jnnp-2013-307556.

Kleindorfer, Dawn et al. 2004. "Eligibility for Recombinant Tissue Plasminogen Activator in Acute Ischemic Stroke: A Population-Based Study." *Stroke; a journal of cerebral circulation* 35(2): e27-9. http://stroke.ahajournals.org/content/35/2/e27.abstract.

Kleinschnitz, Christoph et al. 2007. "Targeting Platelets in Acute Experimental Stroke: Impact of Glycoprotein Ib, VI, and IIb/IIIa Blockade on Infarct Size, Functional Outcome, and Intracranial Bleeding." *Circulation* 115(17): 2323–30.

Kleinschnitz, Christoph et al. 2009. "Deficiency of von Willebrand Factor Protects Mice from Ischemic Stroke." *Blood* 113(15): 3600–3603.

Koga, Masatoshi et al. 2013. "Factors Associated with Early Recanalization Failure Following Intravenous Rt-PA Therapy for Ischemic Stroke." *Cerebrovascular diseases (Basel, Switzerland)* 36(4): 299–305. http://www.ncbi.nlm.nih.gov/pubmed/24135558.

Kokame, Koichi et al. 2005. "FRETS-VWF73, a First Fluorogenic Substrate for ADAMTS13 Assay." *British Journal of Haematology* 129(1): 93–100.

Koleba, Tamar, and Mary H. H Ensom. 2006. "Pharmacokinetics of Intravenous Immunoglobulin: A Systematic Review." *Pharmacotherapy* 26(6): 813–27. http://doi.wiley.com/10.1592/phco.26.6.813.

Kuwashiro, Takahiro et al. 2013. "Predictive Role of C Reactive Protein in Stroke Recurrence after Cardioembolic Stroke: The Fukuoka Stroke Registry." *BMJ open* 3(11): e003678. http://bmjopen.bmj.com/content/3/11/e003678.full.

Labarthe, Benoît, Pierre Théroux, Michaël Angioï, and Marta Ghitescu. 2005. "Matching the Evaluation of the Clinical Efficacy of Clopidogrel to Platelet Function Tests Relevant to the Biological Properties of the Drug." *Journal of the American College of Cardiology*.

Lambers, Moritz et al. 2013. "Role of Reduced ADAMTS13 in Arterial Ischemic Stroke: A Pediatric Cohort Study." *Annals of Neurology* 73(1): 58–64.

Landersdorfer, Cornelia B. et al. 2013. "Pharmacokinetic Modeling and Simulation of Biweekly Subcutaneous Immunoglobulin Dosing in Primary Immunodeficiency." *Postgraduate medicine* 125(6): 53–61.

Larrue, V et al. 2011. "Etiologic Investigation of Ischemic Stroke in Young Adults." *Neurology* 76(23): 1983–88.

Lawrie, A. S. et al. 2013. "A Comparative Evaluation of a New Automated Assay for von Willebrand Factor Activity." *Haemophilia* 19(2): 338–42.

Lawrie, Andrew et al. 2010. "The Effect of Prion Reduction in Solvent/Detergent-treated Plasma on Haemostatic Variables." *Vox Sanguinis* 99(3): 232–38.

Lawson, Jeffrey H., and Kenneth G. Mann. 1991. "Cooperative Activation of Human Factor IX by the Human

Extrinsic Pathway of Blood Coagulation." *Journal of Biological Chemistry* 266(17): 11317–27.

Lenting, Peter J., Olivier D. Christophe, and Cécile V. Denis. 2015. "Von Willebrand Factor Biosynthesis, Secretion, and Clearance: Connecting the Far Ends." *Blood* 125(13): 2019–28.

Lester, Will A. et al. 2002. "Successful Treatment of Congenital Thrombotic Thrombocytopenic Purpura Using the Intermediate Purity Factor VIII Concentrate BPL 8Y." *British Journal of Haematology* 119(1): 176–79.

Levine, Steven R. 2004. "Antiphospholipid Antibodies and Subsequent Thrombo-Occlusive Events in Patients with Ischemic Stroke." *Journal of the American Medical Association* 291(5): 576–84.

Li, N. 2008. "Platelet-Lymphocyte Cross-Talk." *Journal of Leukocyte Biology* 83(5): 1069–78.

Lip, Gregory Y.H., and Andrew Blann. 1997. "Von Willebrand Factor: A Marker of Endothelial Dysfunction in Vascular Disorders?" *Cardiovascular Research*: 34, 2,255-265.

Lippi, Giuseppe, Massimo Franchini, and Giovanni Targher. 2011. "Arterial Thrombus Formation in Cardiovascular Disease." *Nature reviews. Cardiology* 8(9): 502–12. http://dx.doi.org/10.1038/nrcardio.2011.91.

Loeffen, R et al. 2014. "Associations Between Thrombin Generation and the Risk of Cardiovascular Disease in Elderly Patients: Results From the PROSPER Study." *J.Gerontol.A Biol.Sci.Med.Sci.* (1758-535X (Electronic)): 982–88.

López-López, José A. et al. 2017. "Oral Anticoagulants for Prevention of Stroke in Atrial Fibrillation: Systematic Review, Network Meta-Analysis, and Cost Effectiveness Analysis." *BMJ (Clinical research ed.)*.

Lotta, Luca A. et al. 2010. "ADAMTS13 Mutations and Polymorphisms in Congenital Thrombotic Thrombocytopenic Purpura." *Human Mutation* 31(1): 11–19.

Lozano, Rafael et al. 2012. "Global and Regional Mortality from 235 Causes of Death for 20 Age Groups in 1990 and 2010: A Systematic Analysis for the Global Burden of Disease Study 2010." *The Lancet* 380(9859): 2095–2128.

Lukasik, Maria et al. 2011. "Aspirin Treatment Influences Platelet-Related Inflammatory Biomarkers in Healthy Individuals but Not in Acute Stroke Patients." *Thrombosis Research* 128(5): e73–80. http://dx.doi.org/10.1016/j.thromres.2011.06.016.

Lundberg, Anna M., and Göran K. Hansson. 2010. "Innate Immune Signals in Atherosclerosis." *Clinical Immunology* 134(1): 5–24.

Maaijwee, N A et al. 2014. "Ischaemic Stroke in Young Adults: Risk Factors and Long-Term Consequences." *Nat Rev Neurol* 10(6): 315–25.

Maguire, Jane M. et al. 2008. "Polymorphisms in Platelet Glycoprotein 1b Alpha and Factor VII and Risk of Ischemic Stroke: A Meta-Analysis." *Stroke* 39(6): 1710–16.

Mann, Kenneth G., Saulius Butenas, and Kathleen Brummel. 2003. "The Dynamics of Thrombin Formation." *Arteriosclerosis, Thrombosis, and Vascular Biology* 23(1): 17–25.

Mant, J, DT Wade, and S Winner. 2004. *Health Care Needs Assessment: Stroke Health Needs Assessment: The Epidemiologically Based Assessment Reviews.* 2nd editio. eds. A Stevens, M Raftery, and J Mant. Oxford Radcliffe Medical Press.

Markus, Hugh S. 2010. "Unravelling the Genetics of Ischaemic Stroke." *PLoS Medicine* 7(3): 1–5.

Markus, Hugh S. 2012. “Stroke Genetics: Prospects for Personalized Medicine.” *BMC medicine* 10(1): 113. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3521189&tool=pmcentrez&rendertype=abstract.

Martinez, Hector R., Ricardo A. Rangel-Guerra, and Luis J. Marfil. 1993. “Ischemic Stroke Due to Deficiency of Coagulation Inhibitors Report of 10 Young Adults.” *Stroke*.

Matsuda, S. et al. 2011. “Human C-Reactive Protein Enhances Thrombus Formation after Neointimal Balloon Injury in Transgenic Rabbits.” *Journal of Thrombosis and Haemostasis* 9(1): 201–8.

McCabe, Dominick J H et al. 2004. “Platelet Degranulation and Monocyte-Platelet Complex Formation Are Increased in the Acute and Convalescent Phases after Ischaemic Stroke or Transient Ischaemic Attack.” *British Journal of Haematology* 125(6): 777–87.

McCabe, Dominick J H et al. 2005. “Assessment of the Antiplatelet Effects of Low to Medium Dose Aspirin in the Early and Late Phases after Ischaemic Stroke and TIA.” *Platelets* 16(5): 269–80. http://www.ncbi.nlm.nih.gov/pubmed/16011977.

McCabe, Dominick J H et al. 2015. “Relationship between ADAMTS13 Activity, von Willebrand Factor Antigen Levels and Platelet Function in the Early and Late Phases after TIA or Ischaemic Stroke.” *Journal of the Neurological Sciences* 348(1–2): 35–40. http://dx.doi.org/10.1016/j.jns.2014.10.035.

McMullin, Mary Frances et al. 2019. “A Guideline for the Diagnosis and Management of Polycythaemia Vera. A British Society for Haematology Guideline.” *British Journal of Haematology* 184: 176–91.

Mega, Jessica L et al. 2008. “Cytochrome P-450 Polymorphisms and Response to Clopidogrel.” *Society* 360(4): 1–9. http://www.ncbi.nlm.nih.gov/pubmed/19106084.

Menon, Bijoy K., and Jukka Putaala. 2018. “Search for a Panacea Continues.” *Stroke* 49: 3118–19.

Meschia, James F. et al. 2014. 45 Stroke *Guidelines for the Primary Prevention of Stroke: A Statement for Healthcare Professionals from the American Heart Association/American Stroke Association*.

Methia, Nassia et al. 2001. “Localized Reduction of Atherosclerosis in von Willebrand Factor-Deficient Mice.” *Blood* 98(5): 1424–28.

De Meyer, Simon F., Guido Stoll, Denisa D. Wagner, and Christoph Kleinschnitz. 2012. “Von Willebrand Factor: An Emerging Target in Stroke Therapy.” *Stroke*.

Miyakis, S et al. 2006. “International Consensus Statement on Preliminary Classification Criteria for Definite Antiphospholipid Syndrome.” *Journal of Thrombosis and Haemostasis* 4: 295–306.

Miyata, Toshiyuki. 2015. “GWA Study for ADAMTS13 Activity.” *Blood* 125(25): 3833–34.

Mohan, Keerthi M. et al. 2011. “Risk and Cumulative Risk of Stroke Recurrence: A Systematic Review and Meta-Analysis.” *Stroke* 42(5): 1489–94.

Momi, Stefania et al. 2013. “Reperfusion of Cerebral Artery Thrombosis by the GPIb – VWF Blockade with the Nanobody ALX-0081 Reduces Brain Infarct Size in Guinea Pigs.” 121(25): 5088–97.

Montaner, Joan. 2015. “The Post-Stroke Clotting Battle: ADAMTS13 Falls and Puts out of Control VWF into Brain Arteries.” *Journal of the Neurological Sciences* 348(1–2): 1–2. http://dx.doi.org/10.1016/j.jns.2014.10.038.

Morris, Jane G., Swaraj Singh, and Marc Fisher. 2010a. “Testing for Inherited Thrombophilias in Arterial Stroke:

Can It Cause More Harm than Good?" *Stroke* 41(12): 2985–90.

Mould, D. R., and R. N. Upton. 2012. "Basic Concepts in Population Modeling, Simulation, and Model-Based Drug Development." *CPT: Pharmacometrics and Systems Pharmacology*.

Muroi, C. et al. 2014. "Effect of ADAMTS-13 on Cerebrovascular Microthrombosis and Neuronal Injury after Experimental Subarachnoid Hemorrhage." *Journal of Thrombosis and Haemostasis* 12(4): 505–14.

Murray, V. et al. 2010. "The Molecular Basis of Thrombolysis and Its Clinical Application in Stroke." *Journal of Internal Medicine* 267(2): 191–208.

Nacu, A et al. 2016. "Age Dependency of Ischaemic Stroke Subtypes and Vascular Risk Factors in Western Norway: The Bergen Norwegian Stroke Cooperation Study." *Acta neurologica Scandinavica* 133(3): 202–7.

Naik, Swati, and Donald H. Mahoney. 2013. "Successful Treatment of Congenital Ttp with a Novel Approach Using Plasma-Derived Factor VIII." *Journal of Pediatric Hematology/Oncology* 35(7): 551–53.

Nakano, Takafumi et al. 2015. "Delayed Treatment with ADAMTS13 Ameliorates Cerebral Ischemic Injury without Hemorrhagic Complication." *Brain Research* 1624: 330–35. http://dx.doi.org/10.1016/j.brainres.2015.07.027.

National Institute for Health and Clinical Excellence (NICE). 2002. Technology *Guidance on the Use of Glycoprotein IIb/IIIa Inhibitors in the Treatment of Acute Coronary Syndromes*.

National Institute for Health and Clinical Excellence (NICE). 2008. "Stroke and Transient Ischaemic Attack in over 16s: Diagnosis and Initial Management Clinical Guideline." (July). http://www.nice.org.uk/guidance/CG68.

Ni, Heyu et al. 2000. "Persistence of Platelet Thrombus Formation in Arterioles of Mice Lacking Both von Willebrand Factor and Fibrinogen." *Journal of Clinical Investigation* 106(3): 385–92.

Nieswandt, Bernhard, Guido Stoll, and Washington Dc. 2013. "(Dis) Solving the Stroke Problem By." *Blood* 121(25): 4972–74.

Nightingale, T., and D. Cutler. 2013. "The Secretion of von Willebrand Factor from Endothelial Cells; an Increasingly Complicated Story." *Journal of Thrombosis and Haemostasis*: 11 (Suppl 1) 192-201.

NINDS Study Group. 1995. "Tissue Plasminogen Activator for Acute Ischemic Stroke. The National Institute of Neurological Disorders and Stroke Rt-PA Stroke Study Group." *The New England journal of medicine* 333(24): 1581–87.

Nishio, K, P J Anderson, X L Zheng, and J E Sadler. 2004. "Binding of Platelet Glycoprotein Ibalpha to von Willebrand Factor Domain A1 Stimulates the Cleavage of the Adjacent Domain A2 by ADAMTS13." *Proc Natl Acad Sci U S A* 101(29): 10578–83. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=15249683.

Nomura, E et al. 2001. "Sequential Changes in von Willebrand Factor and Soluble Thrombomodulin in Acute Ischemic Stroke." *Journal of stroke and cerebrovascular diseases : the official journal of National Stroke Association* 10(6): 257–64. http://www.ncbi.nlm.nih.gov/pubmed/17903836.

Ofosu, Frederick A. 2003. "Protease Activated Receptors 1 and 4 Govern the Responses of Human Platelets to Thrombin." *Transfusion and Apheresis Science* 28(3): 265–68.

Omari, K M, and K Dorovini-Zis. 2003. “CD40 Expressed by Human Brain Endothelial Cells Regulates CD4+ T Cell Adhesion to Endothelium.” *J Neuroimmunol* 134(1–2): 166–78. http://ac.els-cdn.com/S016557280200423X/1-s2.0-S016557280200423X-main.pdf?_tid=2dcc6d6a-dfd5-11e5-ab86-00000aab0f27&acdnat=1456854419_e9dbe819f94122ff684102cd7f5ff246.

Omran S.S. et al. 2017. “Clinical Utility of Hypercoagulability Screening in Young Adults with Ischemic Stroke.” *Stroke*.

Orbe, Josune et al. 2008. “Increased Thrombin Generation after Acute versus Chronic Coronary Disease as Assessed by the Thrombin Generation Test.” *Thrombosis and Haemostasis* 99(2): 382–87.

Pandey, Soumya et al. 2015. “Rituximab and Intermediate-Purity Plasma-Derived Factor VIII Concentrate (Koate®) as Adjuncts to Therapeutic Plasma Exchange for Thrombotic Thrombocytopenic Purpura in Patients with an ADAMTS13 Inhibitor.” *Journal of Clinical Apheresis* 30(1): 50–54.

Pare, G., and J. W. Eikelboom. 2011. “CYP2C19 Genetic Testing Should Not Be Done in All Patients Treated With Clopidogrel Who Are Undergoing Percutaneous Coronary Intervention.” *Circulation: Cardiovascular Interventions* 4(5): 514–21.

Passamonti, Francesco et al. 2004. “Life Expectancy and Prognostic Factors for Survival in Patients with Polycythemia Vera and Essential Thrombocythemia.” *Am J Med* 117(10): 755–61.

Pendu, Ronan et al. 2006. “P-Selectin Glycoprotein Ligand 1 and B2-Integrins Cooperate in the Adhesion of Leukocytes to von Willebrand Factor.” *Blood* 108(12): 3746–52.

Pengo, V. et al. 2010. “Clinical Course of High-Risk Patients Diagnosed with Antiphospholipid Syndrome.” *Journal of Thrombosis and Haemostasis*.

Pengo, Vittorio et al. 2007. “Survey of Lupus Anticoagulant Diagnosis by Central Evaluation of Positive Plasma Samples.” *Journal of Thrombosis and Haemostasis*.

Penz, S M et al. 2007. “Glycoprotein Iba Inhibition and ADP Receptor Antagonists, but Not Aspirin, Reduce Platelet Thrombus Formation in Flowing Blood Exposed to Atherosclerotic Plaques.” *Thrombosis and Haemostasis* 97(3): 435–43. http://dx.doi.org/10.1160/TH06-07-0415.

Penz, Sandra et al. 2005. “Human Atheromatous Plaques Stimulate Thrombus Formation by Activating Platelet Glycoprotein VI.” *The FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 19(8): 898–909.

Pham, Mirko et al. 2011. “Sustained Reperfusion after Blockade of Glycoprotein-Receptor-IB in Focal Cerebral Ischemia: An MRI Study at 17.6 Tesla.” *PLoS ONE*.

Pinsky, David J. et al. 1996. “Hypoxia-Induced Exocytosis of Endothelial Cell Weibel-Palade Bodies: A Mechanism for Rapid Neutrophil Recruitment after Cardiac Preservation.” *Journal of Clinical Investigation* 97(2): 493–500.

Putaala, Jukka et al. 2009. “Analysis of 1008 Consecutive Patients Aged 15 to 49 with First-Ever Ischemic Stroke the Helsinki Young Stroke Registry.” *Stroke* 40(4): 1195–1203.

Qu, Le et al. 2016. “Assessment of the Diagnostic Value of Plasma Levels, Activities, and Their Ratios of von Willebrand Factor and ADAMTS13 in Patients with Cerebral Infarction.” *Clinical and applied thrombosis/hemostasis : official journal of the International Academy of Clinical and Applied*

Thrombosis/Hemostasis 22(3): 252–59. http://www.ncbi.nlm.nih.gov/pubmed/25916953.

Quinn, Terence J., J. Dawson, and M. Walters. 2008. "Dr John Rankin; His Life, Legacy and the 50th Anniversary of the Rankin Stroke Scale." *Scottish Medical Journal*.

Reininger, A J et al. 2010. "A 2-Step Mechanism of Arterial Thrombus Formation Induced by Human Atherosclerotic Plaques." *J Am Coll Cardiol* 55(11): 1147–58. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=20223370.

Reiter, Rosemarie A., Paul Knöbl, Katalin Varadi, and Peter L. Turecek. 2003. "Changes in von Willebrand Factor-Cleaving Protease (ADAMTS13) Activity after Infusion of Desmopressin." *Blood* 101(3): 946–48.

Rho, R et al. 1995. "Plasma Markers of Procoagulant Activity Among Individuals with Coronary Artery Disease." *J Thromb Thrombolysis* 2(3): 239–43.

Riedel, Christian H. et al. 2011. "The Importance of Size: Successful Recanalization by Intravenous Thrombolysis in Acute Anterior Stroke Depends on Thrombus Length." *Stroke*.

Rios, Danyelle R.A. et al. 2012. "Relationship between ABO Blood Groups and von Willebrand Factor, ADAMTS13 and Factor VIII in Patients Undergoing Hemodialysis." *Journal of Thrombosis and Thrombolysis*.

Roberts, Jason D. et al. 2012. "Point-of-Care Genetic Testing for Personalisation of Antiplatelet Treatment (RAPID GENE): A Prospective, Randomised, Proof-of-Concept Trial." *The Lancet*.

Robson, S C, E G Shephard, and R E Kirsch. 1994. "Fibrin Degradation Product D-Dimer Induces the Synthesis and Release of Biologically Active IL-1 Beta, IL-6 and Plasminogen Activator Inhibitors from Monocytes in Vitro." *British journal of haematology* 86(2): 322–26. http://www.ncbi.nlm.nih.gov/pubmed/8199021.

Rolfs, Arndt et al. 2013. "Acute Cerebrovascular Disease in the Young: The Stroke in Young Fabry Patients Study." *Stroke* 44(2): 340–49.

Rooth, Elisabeth et al. 2013. "Thrombin Generation in Acute Cardioembolic and Non-Cardioembolic Ischemic Stroke." *Scandinavian journal of clinical and laboratory investigation* 73(7): 576–84. http://www.ncbi.nlm.nih.gov/pubmed/24063505.

Rosing, Jan et al. 1985. "The Role of Activated Human Platelets in Prothrombin and Factor X Activation." *Blood* 65(2): 319–33.

Rowland, Malcolm, and Thomas N. Tozer. 2011. *Clinical Pharmacokinetics and Pharmacodynamics: Concepts and Applications*. Philadelphia : Wolters Kluwer Health/Lippincott William & Wilkins.

Royal College of Physicians. 2012. Intercollegiate Stroke Working Party *National Clinical Guideline for Stroke*.

Royal College of Physicians. 2016. "National Clinical Guideline for Stroke." (5th Edition): 151.

Ruiz-Irastorza, G. et al. 2011. "Evidence-Based Recommendations for the Prevention and Long-Term Management of Thrombosis in Antiphospholipid Antibody-Positive Patients: Report of a Task Force at the 13th International Congress on Antiphospholipid Antibodies." In *Lupus*,.

Sahan, M et al. 2013. "Ischemic Stroke : Do They Have Any Relationship With Short-Term Mortality ?" *European Review for Medical and Pharmacological Sciences* 17: 2773–77.

Sambrano, Gilberto R et al. 2001. "Role of Thrombin Signalling in Platelets in Haemostasis and Thrombosis."

Nature 413(6851): 74–78. http://www.ncbi.nlm.nih.gov/pubmed/11544528.

Sandercock, Peter et al. 2012. "The Benefits and Harms of Intravenous Thrombolysis with Recombinant Tissue Plasminogen Activator within 6 h of Acute Ischaemic Stroke (the Third International Stroke Trial [IST-3]): A Randomised Controlled Trial." *The Lancet* 379(9834): 2352–63. http://dx.doi.org/10.1016/S0140-6736(12)60768-5.

van Schie, M C, J E van Loon, M P M de Maat, and F W G Leebeek. 2011. "Genetic Determinants of von Willebrand Factor Levels and Activity in Relation to the Risk of Cardiovascular Disease. A Review." *Journal of thrombosis and haemostasis : JTH* 9(November 2010): 899–908. http://www.ncbi.nlm.nih.gov/pubmed/21342431.

Scully, M. et al. 2017. "Consensus on the Standardization of Terminology in Thrombotic Thrombocytopenic Purpura and Related Thrombotic Microangiopathies." *Journal of Thrombosis and Haemostasis* 15(2): 312–22.

Scully, M et al. 2008. "ADAMTS 13 in Non-Thrombotic Thrombocytopaenic Purpura Conditions." *British journal of Haematology* 141(2): 262–65.

Scully, Marie et al. 2008. "Regional UK TTP Registry: Correlation with Laboratory ADAMTS 13 Analysis and Clinical Features." *British Journal of Haematology* 142(5): 819–26.

Scully, Marie et al. 2012. "Guidelines on the Diagnosis and Management of Thrombotic Thrombocytopenic Purpura and Other Thrombotic Microangiopathies." *British Journal of Haematology* 158(3): 323–35.

Scully, Marie et al. 2014. "Thrombotic Thrombocytopenic Purpura and Pregnancy: Presentation, Management, and Subsequent Pregnancy Outcomes." *Blood* 124(2): 211–19.

Scully, Marie et al. 2016. "Thrombotic Thrombocytopenic Purpura and Atypical Hemolytic Uremic Syndrome Microangiopathy in Pregnancy." *Seminars in Thrombosis and Hemostasis* 42(7): 774–79.

Scully, Marie et al. 2017. "Recombinant ADAMTS-13: First-in-Human Pharmacokinetics and Safety in Congenital Thrombotic Thrombocytopenic Purpura." *Blood* 130(19): 2055–63.

Scully, Marie, Michael Gattens, Kate Khair, and Ri Liesner. 2006. "The Use of Intermediate Purity Factor VIII Concentrate BPL 8Y as Prophylaxis and Treatment in Congenital Thrombotic Thrombocytopenic Purpura." *British Journal of Haematology* 135(1): 101–4.

Scully, Marie, and Tim Goodship. 2014. "How I Treat Thrombotic Thrombocytopenic Purpura and Atypical Haemolytic Uraemic Syndrome." *British Journal of Haematology* 164(6): 759–66.

Seo, Woo Keun et al. 2012. "C-Reactive Protein Is a Predictor of Early Neurologic Deterioration in Acute Ischemic Stroke." *Journal of Stroke and Cerebrovascular Diseases* 21(3): 181–86.

Shelat, Suresh G., Jihui Ai, and X. Long Zheng. 2005. "Molecular Biology of ADAMTS13 and Diagnostic Utility of ADAMTS13 Proteolytic Activity and Inhibitor Assays." *Seminars in Thrombosis and Hemostasis* 31(6): 659–72.

Shida, Yasuaki et al. 2008. "Functional Imaging of Shear-Dependent Activity of ADAMTS13 in Regulating Mural Thrombus Growth under Whole Blood Flow Conditions." *Blood* 111(3): 1295–98.

Shim, Kyuhwan et al. 2008. "Platelet-VWF Complexes Are Preferred Substrates of ADAMTS13 under Fluid Shear Stress Platelet-VWF Complexes Are Preferred Substrates of ADAMTS13 under Fluid Shear Stress." *Platelets* 111(2): 651–57.

Shuldiner, Alan R. et al. 2009. “Association of Cytochrome P450 2C19 Genotype with the Antiplatelet Effect and Clinical Efficacy of Clopidogrel Therapy.” *Jama* 302(8): 849–57. http://jama.jamanetwork.com/article.aspx?doi=10.1001/jama.2009.1232%5Cnhttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3641569&tool=pmcentrez&rendertype=abstract.

Sibbing, D. et al. 2011. “Current Evidence for Genetic Testing in Clopidogrel-Treated Patients Undergoing Coronary Stenting.” *Circulation: Cardiovascular Interventions* 4(5): 505–13. http://circinterventions.ahajournals.org/cgi/doi/10.1161/CIRCINTERVENTIONS.111.962142%5Cnhttp://circinterventions.ahajournals.org/cgi/doi/10.1161/CIRCINTERVENTIONS.111.962183.

Siedlecki, Christopher A. et al. 1996. “Shear-Dependent Changes in the Three-Dimensional Structure of Human von Willebrand Factor.” *Blood* 88(8): 2939–50. http://www.ncbi.nlm.nih.gov/pubmed/8874190.

Singh, Indrajeet et al. 2006. “Solution Structure of Human von Willebrand Factor Studied Using Small Angle Neutron Scattering.” *Journal of Biological Chemistry* 281(50): 38266–75.

Singh, Indrajeet, Efrosyni Themistou, Lionel Porcar, and Sriram Neelamegham. 2009. “Fluid Shear Induces Conformation Change in Human Blood Protein von Willebrand Factor in Solution.” *Biophysical Journal* 96(6): 2313–20. http://dx.doi.org/10.1016/j.bpj.2008.12.3900.

Singh, Uma, Sridevi Devaraj, and Ishwarlal Jialal. 2005. “C-Reactive Protein Decreases Tissue Plasminogen Activator Activity in Human Aortic Endothelial Cells: Evidence That C-Reactive Protein Is a Procoagulant.” *Arteriosclerosis, Thrombosis, and Vascular Biology* 25(10): 2216–21.

Smid, M. et al. 2011. “Thrombin Generation in Patients with a First Acute Myocardial Infarction.” *Journal of Thrombosis and Haemostasis* 9(3): 450–56.

Sonneveld, Michelle A.H. et al. 2016. “Von Willebrand Factor, ADAMTS13, and the Risk of Mortality.” *Arteriosclerosis, Thrombosis, and Vascular Biology* 36(12): 2446–51.

Sonneveld, Michelle A H et al. 2015. “Low ADAMTS13 Activity Is Associated with an Increased Risk of Ischemic Stroke.” *Blood* 126(25): 2739–46.

Spronk, Henri M.H. et al. 2009. “Monitoring Thrombin Generation: Is Addition of Corn Trypsin Inhibitor Needed?” *Thrombosis and Haemostasis*.

De Stefano, Valerio et al. 2008. “Recurrent Thrombosis in Patients with Polycythemia Vera and Essential Thrombocythemia: Incidence, Risk Factors, and Effect of Treatments.” *Haematologica* 93(3): 372–80.

Sternberg, Zohara et al. 2013. “Clopidogrel Responsiveness in Stroke Patients on a Chronic Aspirin Regimen.” *Journal of Stroke and Cerebrovascular Diseases* 22(6): 725–32. http://dx.doi.org/10.1016/j.jstrokecerebrovasdis.2011.11.009.

Stoll, G, C Kleinschnitz, and B Nieswandt. 2008. “Molecular Mechanisms of Thrombus Formation in Ischemic Stroke: Novel Insights and Targets for Treatment.” *Blood* 112(9): 3555–62. http://bloodjournal.hematologylibrary.org/content/112/9/3555.full.pdf.

Stoll, Guido, and Martin Bendszus. 2006. “Inflammation and Atherosclerosis: Novel Insights into Plaque Formation and Destabilization.” *Stroke* 37(7): 1923–32.

Szczeklik, A, J Dropinski, J Radwan, and M Krzanowski. 1992. “Persistent Generation of Thrombin after Acute Myocardial Infarction.” *Arteriosclerosis and thrombosis : a journal of vascular biology / American Heart Association* 12(5): 548–53.

Teasdale, Graham, and Bryan Jennett. 1974. “Assessment of Coma and Impaired Consciousness. A Practical Scale.” *The Lancet* 304(7872): 81–84.

Tefferi, Ayalew, and Tiziano Barbui. 2015. “Polycythemia Vera and Essential Thrombocythemia: 2015 Update on Diagnosis, Risk-Stratification and Management.” *American Journal of Hematology* 90(2): 162–73.

Ternant, David et al. 2018. “Model-Based Therapeutic Drug Monitoring of Infliximab Using a Single Serum Trough Concentration.” *Clinical Pharmacokinetics* 57(9): 1173–84.

The ESPIRIT study group. 2006. “Aspirin plus Dipyridamole versus Aspirin Alone after Cerebral Ischaemia of Arterial Origin (ESPRIT): Randomised Controlled Trial.” *Lancet* 367(9523): 1665–73.

Tsai, Han-Mou. 2010. “Pathophysiology of Thrombotic Thrombocytopenic Purpura.” *Int J Hematol* 91(1): 1–19.

United Kingdom government. 2013. “Clopidogrel: Risk of Acquired Haemophilia.” *UK government Medicines and Healthcare Products regulatory agency. Drug Safety Update* 5(5): A2.

Urbanus, Rolf T. et al. 2009. “Antiphospholipid Antibodies and Risk of Myocardial Infarction and Ischaemic Stroke in Young Women in the RATIO Study: A Case-Control Study.” *The Lancet Neurology* 8(11): 998–1005. http://dx.doi.org/10.1016/S1474-4422(09)70239-X.

Van Veen, Joost J. et al. 2008. “Corn Trypsin Inhibitor in Fluorogenic Thrombin-Generation Measurements Is Only Necessary at Low Tissue Factor Concentrations and Influences the Relationship between Factor VIII Coagulant Activity and Thrombogram Parameters.” *Blood Coagulation and Fibrinolysis*.

Van ’t Veer, C, and K G Mann. 1997. “Regulation of Tissue Factor Initiated Thrombin Generation by the Stoichiometric Inhibitors Tissue Factor Pathway Inhibitor, Antithrombin- III, and Heparin Cofactor-II.” *J Biol Chem* 272(7): 4367–77. http://www.ncbi.nlm.nih.gov/htbin-post/Entrez/query?db=m&form=6&dopt=r&uid=0009020158%5Cnpapers2://publication/uuid/084095B3-0C2E-4BD5-8D66-1453E0EF963F.

Vergouwen, M. D I et al. 2014. “Effect of Recombinant ADAMTS-13 on Microthrombosis and Brain Injury after Experimental Subarachnoid Hemorrhage.” *Journal of Thrombosis and Haemostasis* 12(6): 943–47.

Verro, Piero, Phillip B. Gorelick, and Danh Nguyen. 2008. “Aspirin plus Dipyridamole versus Aspirin for Prevention of Vascular Events after Stroke or Tia: A Meta-Analysis.” *Stroke* 39(4): 1358–63.

Vischer, U M. 2006. “Von Willebrand Factor, Endothelial Dysfunction, and Cardiovascular Disease.” *Journal of thrombosis and haemostasis : JTH* 4(6): 1186–93. http://www.ncbi.nlm.nih.gov/pubmed/16706957.

Völler, Swantje et al. 2017. “Model-Based Clinical Dose Optimization for Phenobarbital in Neonates: An Illustration of the Importance of Data Sharing and External Validation.” *European Journal of Pharmaceutical Sciences* 109(Supplement): S90–97.

De Vries, Paul S. et al. 2015. “Genetic Variants in the ADAMTS13 and SUPT3H Genes Are Associated with ADAMTS13 Activity.” *Blood* 125(25): 3949–55.

Wang, Lixiang et al. 2013. “Recombinant ADAMTS13 Reduces Tissue Plasminogen Activator-Induced Hemorrhage after Stroke in Mice.” *Annals of Neurology* 73(2): 189–98.

Wang, Yilong et al. 2016. “Association Between CYP2C19 Loss-of-Function Allele Status and Efficacy of Clopidogrel for Risk Reduction Among Patients With Minor Stroke or Transient Ischemic Attack.” *JAMA* 316(1): 70. http://jama.jamanetwork.com/article.aspx?doi=10.1001/jama.2016.8662.

Wannamethee, S. Goya et al. 2012. “Fibrin D-Dimer, Tissue-Type Plasminogen Activator, von Willebrand

Factor, and Risk of Incident Stroke in Older Men." *Stroke* 43(5): 1206–11.

Wardlaw JM, Murray V, Berge E, del Zoppo G. 2009. "Thrombolysis for Acute Ischaemic Stroke." *Cochrane Database of Systematic Reviews* (4).

Wardlaw, Joanna M. et al. 2012. "Recombinant Tissue Plasminogen Activator for Acute Ischaemic Stroke: An Updated Systematic Review and Meta-Analysis." *The Lancet* 379(9834): 2364–72. http://dx.doi.org/10.1016/S0140-6736(12)60738-7.

WAVE Investigators. 2006. "The Effects of Oral Anticoagulants in Patients with Peripheral Arterial Disease: Rationale, Design, and Baseline Characteristics of the Warfarin and Antiplatelet Vascular Evaluation (WAVE) Trial, Including a Meta-Analysis of Trials." *American Heart Journal* 151(1): 1–9.

Welsh, Paul et al. 2008. "Associations of Proinflammatory Cytokines with the Risk of Recurrent Stroke." *Stroke* 39(8): 2226–30.

Weston-Smith, S, P Revell, and GF Savidge. 1989. "Thrombophilia." *British Journal of Hospital Medicine* 41(4): 368–71.

Wieberdink, Renske G. et al. 2010. "High von Willebrand Factor Levels Increase the Risk of Stroke: The Rotterdam Study." *Stroke* 41(10): 2151–56.

Wolf, M E et al. 2015. "Phenotypic ASCO Characterisation of Young Patients with Ischemic Stroke in the Prospective Multicentre Observational Sifap1 Study." *Cerebrovasc Dis* 40(3–4): 129–35.

Wu, T et al. 2010. "Force-Induced Cleavage of Single VWF A1A2A3-Tridomains by ADAMTS-13." *Blood* 115(2): 370–78. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=19897584.

Xu, Haochen et al. 2017. "ADAMTS13 Controls Vascular Remodeling by Modifying VWF Reactivity during Stroke Recovery." *Blood* 130(1): 11–22.

Yilmaz, Gokhan, Thiruma V. Arumugam, Karen Y. Stokes, and D. Neil Granger. 2006. "Role of T Lymphocytes and Interferon-Gamma in Ischemic Stroke." *Circulation* 113(17): 2105–12.

Yilmaz, Gokhan, and DN Granger. 2010. "Leukocyte Recruitment and Ischemic Brain Injury." *Neuromolecular medicine* 12(2): 193–204. http://link.springer.com/article/10.1007/s12017-009-8074-1.

Zhang, Jing et al. 2006. "C-Reactive Protein Induced Expression of Adhesion Molecules in Cultured Cerebral Microvascular Endothelial Cells." *Life sciences* 78(26): 2983–88. http://www.ncbi.nlm.nih.gov/pubmed/16412476.

Zhang, Li et al. 2005. "Multitargeted Effects of Statin-Enhanced Thrombolytic Therapy for Stroke with Recombinant Human Tissue-Type Plasminogen Activator in the Rat." *Circulation* 112(22): 3486–94.

Zhang, Xiaohui et al. 2009. "Mechanoenzymatic Cleavage of the Ultralarge Vascular Protein, von Willebrand Factor." *Science* 324(5932): 1330–34.

Zhao, Bing-qiao et al. 2009. "Von Willebrand Factor – Cleaving Protease ADAMTS13 Reduces Ischemic Brain Injury in Experimental Stroke." *Blood* 114(15): 3329–34. http://www.bloodjournal.org.ezp-prod1.hul.harvard.edu/content/114/15/3329.abstract.

Zheng, X. L. 2013. "Structure-Function and Regulation of ADAMTS-13 Protease." *Journal of Thrombosis and Haemostasis* 11(SUPPL.1): 11–23.

Zheng, X Long. 2015. “ADAMTS13 and von Willebrand Factor in Thrombotic Thrombocytopenic Purpura.” *Annual review of medicine* 66(5): 211–25. http://www.ncbi.nlm.nih.gov/pubmed/25587650.