CHARACTERISATION OF THE FIBRINOLYTIC SYSTEM AND THE VON WILLEBRAND FACTOR-ADAMTS13 AXIS IN THE CHACMA BABOON

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Abstract

Background: The Chacma baboon (*Papio ursinus*) model of acquired thrombotic thrombocytopenic purpura (aTTP) is ideally suited to investigate novel treatments with potential application in this lethal thrombotic disorder, which hinges on the dysfunction of the VWF–ADAMTS13 axis. One such modality is the thrombolytic drugs, which activate the fibrinolytic system. Our recently published pilot study demonstrated the thrombolytic drug streptokinase as ineffective in resolving aTTP in this model and highlighted the deficits in our knowledge of the Chacma baboon's fibrinolytic system and VWF-ADAMTS13 axis. The present study aimed to characterise these components of the Chacma baboon's haemostatic system to better understand the aTTP model, the effects of thrombolytics in this model, and the implications for other haemostatic disease models in this species.

Materials and Methods: Forty baboons were tested using observational and experimental assays. The VWF–ADAMTS13 axis was investigated by determining ADAMTS13 antigen and activity levels, VWF:Ag, VWF:RCo, and VWF:CB levels and VWF multimer patterns. The fibrinolytic system was explored by measuring the concentrations of fibrinogen, plasminogen, tPA, PAI-1, PAP complexes, TAFI, and α2-antiplasmin, and by assessing its in vitro clot lysis ability in a modified clot lysis time assay. The plasminogen activation potentials of streptokinase and tPA were determined using concentration escalation experiments. The effect of tPA-induced plasmin activity on VWF multimer patterns when in the non-globular state was assessed in the presence of the anti-ADAMTS13 mAb 3H9. Thrombin generation, which can influence the aTTP model and its response to thrombolytics, was also evaluated, as well as the effects of sex and ABO blood group. Reference intervals and interindividual variation were calculated and compared with human values.

Results and Discussion: ADAMTS13 activities were generally below (but still comparable to) human ranges. VWF:Ag and VWF:CB values tended towards the lower limit of the human reference interval, whereas VWF:RCo activities were higher. All VWF multimer patterns were essentially equivalent to normal human patterns. Fibrinogen

concentrations were similar to human values, but tPA, PAP complex, PAI-1 and α2-antiplasmin concentrations all tended toward the lower human ranges. Meaningful results could not be generated for ADAMTS13 antigen, plasminogen, or TAFI, possibly due to structural differences with the human protein. Streptokinase resulted in minimal plasmin activity, but tPA led to concentration-dependent increases. All baboon samples exceeded 100% of human plasmin activity at baseline and had clot lysis times shorter than pooled normal human plasma when activated by tPA. In the absence of ADAMTS13 activity, tPA reduced the non-globular high molecular weight VWF multimers in both human and baboon samples. Baboons had greater overall endogenous thrombin potentials than humans, which was more prominent in females ($p=0.0238$). Except for lower fibrinogen concentrations ($p=0.0134$) in male baboons, and PAP complex concentrations which were higher ($p=0.0188$), no other sex-related differences were apparent. No baboons were typed as ABO group B or AB, and none were Rh(D) negative. Group O baboons had higher fibrinogen concentrations ($p=0.0355$), but all other parameters were unaffected. Fibrinogen and especially α2-antiplasmin were subject to considerable interindividual biological variation.

Conclusion: The central thesis of this research is that the components of the Chacma baboon's haemostatic system pertinent to the pathogenesis of aTTP and its treatment with thrombolytics are similar enough to their human counterparts to enable continued use of this species as a model of human haemostasis, provided quantitative results are interpreted within the context of the novel reference intervals, and the identified limitations and interspecies differences are considered. Finally, tPA should be explored further in the Chacma baboon aTTP model in vivo to provide proof-of-concept for the use of thrombolytic drugs in the treatment of aTTP in humans.

Key terms: Thrombotic thrombocytopenic purpura, Chacma baboon, *Papio ursinus*, Von Willebrand factor–ADAMTS13 axis, Fibrinolytic system, Animal model, Streptokinase, Tissue-type plasminogen activator.

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Leana, thank you for always being available for valued academic inputs. You remain my angel, my joy, and the love of my life. This accomplishment would not have been possible without you. Thank you.

Declaration

I, Jaco Joubert, declare that the thesis that I herewith submit for the Doctoral Degree PhD (Haematology) at the University of the Free State, is my independent work, and that I have not previously submitted it for a qualification at another institution of higher education.

Candidate:

Jaco Joubert
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Signed: _____ Date: _____

Jaco Joubert

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List of Abbreviations

°C	Degrees Celsius
µg	Microgram
µL	Microliter
ADAMTS13	A disintegrin and metalloproteinase with a thrombospondin type I motif, member 13
Arg	Arginine
Asn	Asparagine
Asp	Aspartate
aTTP	Acquired thrombotic thrombocytopenic purpura
Ca^{2+}	Calcium
CLSI	Clinical and Laboratory Standards Institute
CLT	Clot lysis time
CUB	Complement 1r/s, Uegf, Bone morphogenic protein 1
CV	Coefficient of variation
Cys domain	Cysteine-rich domain
Cys	Cysteine
DIC	Disseminated intravascular coagulation
Dis	Disintegrin-like
DNA	Deoxyribonucleic acid
DSPA	Desmodus salivary plasminogen activator
EC	Endothelial cell
ELISA	Enzyme-linked immunosorbent assay
ETP	Endogenous thrombin potential

FDPs	Fibrinogen degradation products
FRET	Fluorescence resonance energy transfer
FVIII	Factor VIII
FXIa	Activated factor XI
g/L	Gram per litre
Gln	Glutamine
Glu	Glutamic acid
Gly	Glycine
GP	Glycoprotein
His	Histidine
HIV	Human immune deficiency virus
HRP	Horseradish peroxidase
HUVECs	Human umbilical vein endothelial cells
IgG	Immunoglobulin gamma
IgM	Immunoglobulin mu
INR	International Normalised Ratio
ISO	International Organization for Standardization
ISTH	International Society on Thrombosis and Haemostasis
IU/mL	International units per millilitre
KR	Kringle
L	Litre
Leu	Leucine
Lys	Lysine
M	Molar

mAb	Monoclonal antibody
Met	Methionine
mg	Milligram
mL	Millilitre
MP	Metalloprotease
ms	Millisecond
NAC	*N*-acetylcysteine
NAP c2	Nematode anticoagulant protein c2
NETs	Neutrophil extracellular traps
ng	Nanogram
NHLS	National Health Laboratory Service
NIBSC	National Institute for Biological Standards and Control
NIH	National Institute of Health
nm	Nanometre
nM/L	Nanomolar per litre
OD	Optical density
PAI-1	Plasminogen activator inhibitor-1
PAI-2	Plasminogen activator inhibitor-2
Pap	Pan-apple
PAP	Plasmin-α2-antiplasmin
PBS	Phosphate buffered saline
PE	Pulmonary embolism
PEX	Plasma exchange
PFA-100	Platelet Function Analyser-100

pg	Picogram
Phe	Phenylalanine
POX	Peroxidase
Pro	Proline
PT	Prothrombin time
PTCI	Potato tuber carboxypeptidase inhibitor
SA	South Africa
SAVC	South African Veterinary Council
SD	Standard deviation
Ser	Serine
SOP	Standard operating procedure
SP	Serine protease
SSC	Scientific and Standardization Committee
STEMI	ST-segment elevation acute myocardial infarction
TAFI	Thrombin activatable fibrinolysis inhibitor
TAFIa	Activated thrombin activatable fibrinolysis inhibitor
TB	Tuberculosis
TEG	Thromboelastogram
TFPI	Tissue factor pathway inhibitor
TMA	Thrombotic microangiopathy
TMB	Tetramethylbenzidine
TOS-Gly-Pro-Lys-4-NA	*N*-Tosylglycyl-L-prolyl-L-lysine 4-nitroanilide
tPA	Tissue-type plasminogen activator
Trp	Tryptophan

TSP1	Thrombospondin type-1
TTP	Thrombotic thrombocytopenic purpura
Tyr	Tyrosine
U/mL	Units per millilitre
UFS	University of the Free State
ULVWF	Ultra-large Von Willebrand factor
uPA	Urokinase-type plasminogen activator
USA	United States of America
UTR	Untranslated region
VWF	Von Willebrand factor
VWF:Ag	Von Willebrand factor antigen level
VWF:CB	Von Willebrand factor collagen binding activity
VWF:RCo	Von Willebrand factor ristocetin cofactor activity
x g	G-force

Chapter 1 - **Introduction**

Thrombotic thrombocytopenic purpura (TTP) is a severe thrombotic disorder, caused by a quantitative or qualitative deficiency of the plasma proteolytic enzyme ADAMTS13 (a disintegrin and metalloproteinase with a thrombospondin type I motif, member 13); (Kremer Hovinga and Lämmle, 2012) which cleaves large Von Willebrand factor (VWF) multimers into smaller, less adhesive forms (Scully et al., 2012). As a consequence of this deficiency, ultra-large VWF (ULVWF) multimers accumulate, resulting in abnormal platelet aggregation, micro-thrombus formation and microvascular occlusion (Moake, 2002), with a concomitant thrombocytopenia and fragmentation haemolysis (Scully et al., 2012). The classical diagnostic pentad consists of thrombocytopenia, micro-angiopathic haemolytic anaemia, neurological signs, renal impairment and fever (Amorosi and Ultmann, 1966), but it can also present without the full pentad (Scully et al., 2012).

Hereditary (congenital) TTP, also known as Upshaw-Schulman syndrome, is a rare disorder caused by mutations of the ADAMTS13 gene on chromosome 9q34, which results in an inherited deficiency of the enzyme (Kremer Hovinga and Lämmle, 2012). Acute idiopathic TTP is the most common variant of TTP and is characterised by the presence of autoantibodies, usually immunoglobulin G (IgG), directed against ADAMTS13 (Scully et al., 2012). It is considered an autoimmune disease (Malak et al., 2008), although the underlying process leading to its manifestation remains incompletely understood. Other subtypes of TTP have also been identified and include drug-associated TTP, pregnancy-associated TTP, transplant-associated microangiopathy, malignancy-associated thrombotic microangiopathy (TMA), pancreatitis-associated TTP, human immune deficiency virus (HIV)-associated TTP and the related condition, haemolytic uraemic syndrome (Scully et al., 2012).

HIV-associated TTP is of particular importance in South Africa (Opie, 2012), accounting for more than 80% of all cases in the academic hospitals associated with the University of the Witwatersrand in Johannesburg, South Africa (Gunther et al., 2006). It differs from acquired idiopathic TTP in that anti-ADAMTS13 autoantibodies are frequently absent (Meiring et al., 2012). Even the role of ADAMTS13 deficiency in its pathogenesis is

unclear. Although ADAMTS13 activity is severely decreased in some HIV-positive TTP patients, it has been found to be within the normal range in others (Malak et al., 2008).

These and other clinical observations suggest that the pathophysiology of TTP is not fully elucidated. Patients with hereditary TTP are only intermittently symptomatic, and patients with acquired ADAMTS13 deficiency can achieve partial or even complete remission of TTP without recovery of ADAMTS13 levels. These observations suggest the presence of additional factors affecting the pathophysiology of TTP (Chauhan, 2014), some of which may be responsive to therapeutic manipulation.

The current standard-of-care is plasma exchange (PEX) therapy (Scully et al., 2012), which aims to replace the deficient ADAMTS13 enzyme, while removing/diluting pathogenic ULVWF multimers as well as any potentially inhibitory autoantibodies (Yarranton et al., 2004, Brunskill et al., 2007). Although effective, global TTP mortality is still approximately 15% (Veyradier, 2016), as PEX is not readily available at all facilities. It is expensive and cumbersome, and requires high levels of expertise to perform. It also involves exposure to blood products, and since it has numerous potential complications (Sutton et al., 1989), the search is continuing for simpler, more targeted therapies.

Recent human in vitro and murine in vivo research has identified plasmin, a plasma serine protease and the fibrinolytic system's major enzyme, as a possible physiological backup mechanism for ADAMTS13, and therefore, a potential role-player in the development (and possibly treatment) of TTP (Tersteeg et al., 2014). Moreover, the thrombolytic drug streptokinase has been suggested as a possible candidate for the treatment of acquired TTP (aTTP) (Tersteeg et al., 2014, Chauhan, 2014). There is a large body of clinical experience with streptokinase in the treatment of myocardial infarction (Bryan, 2014), but not in TTP treatment. Therefore, before human trials can be conducted data from non-human primates are preferred as pre-clinical proof-of-concept due to the greater homology between non-human primates and man (Vanhoorelbeke and De Meyer, 2013).

A Chacma baboon (*Papio ursinus*) model of aTTP has previously been developed, established, and refined locally at our institution (Feys et al., 2010a). Although this model has been used fruitfully to investigate various drugs with potential therapeutic application in TTP (Feys et al., 2012, Callewaert et al., 2012, Tersteeg et al., 2017b), many

haemostatic aspects of this animal model (including aspects relating to aTTP) remain unexplored.

A pilot study (attached as appendix A) investigating the potential utility of streptokinase in the treatment of TTP was subsequently conducted in this model by the candidate and his promoters, in a set of in vitro and in vivo experiments (Joubert et al., 2021). The results of this pilot study, while encouraging in terms of the continued use of the model to investigate aTTP (and its treatment options), highlighted various gaps in our knowledge of Chacma baboon haemostasis. Moreover, this study suggested possible differences between the Chacma baboon and human haemostatic systems, with potential implications for the translational value of results.

To contextualise the results of this pilot study (and the various other previous pre-clinical drug testing studies conducted in this model), a more detailed haematological characterisation of the Chacma baboon's fibrinolytic system, VWF and ADAMTS13 was required to better define and elucidate the relevant aspects of this model as they pertain to TTP. Not only does this research broaden our understanding of the pathophysiology of aTTP and inform decisions on the continued validity of this model as a pre-clinical testing platform for drugs with potential application in TTP, but it also has more comprehensive utility for the future use of this species in other pre-clinical testing models, in which the haemostatic system is also affected.

Chapter 2 - **Literature Review**

The literature review of this thesis describes the pathophysiology and treatment of TTP, as well as the non-human primate model of this condition, which provide the broader framework within which the additional research opportunities outlined below, were identified. In particular, the recent advances and remaining research opportunities regarding the exploration of the potential utility of thrombolytic drugs within the *Papio ursinus* aTTP model are reviewed below.

2.1 Thrombotic thrombocytopenic purpura

2.1.1 Introduction

TTP is a life-threatening disorder, part of a diverse group of conditions collectively known as the TMA syndromes (George and Nester, 2014). Although heterogeneous, these disorders all share widespread microvascular occlusion in several organs as a central pathogenic event (George and Nester, 2014). In TTP, this is due to a deficiency of the enzyme ADAMTS13, the plasma protease that cleaves large VWF multimers into smaller, less adhesive forms (Scully et al., 2012). As a consequence of this deficiency, unusually large VWF multimers accumulate, resulting in abnormal platelet aggregation and micro-thrombus formation, with subsequent thrombocytopenia and associated fragmentation haemolysis (Scully et al., 2012).

As summarized by Kremer Hovinga and Lämmle (2012), TTP became recognised as a unique entity in 1947, when Singer et al. (1947) reviewed the 12 cases known at the time – the first clinical case (described by Moschcowitz in 1924), together with 11 subsequent, related cases (Kremer Hovinga and Lämmle, 2012, Singer et al., 1947).

2.1.2 Clinical presentation

In 1966, in a report of 16 cases and a review of the literature, Amorosi and Ultmann further defined TTP as a syndrome consisting of a pentad of clinical findings, namely thrombocytopenia, micro-angiopathic haemolytic anaemia, neurological signs, renal

impairment and fever (Amorosi and Ultmann, 1966). However, TTP can present without the full pentad, with up to 35% of patients not having neurological signs at presentation, and patients may be afebrile and without significant renal dysfunction (Scully et al., 2012). In contrast to the other primary TMA syndromes, TTP rarely causes acute renal injury, presumably because TTP primarily leads to vascular thrombi and not direct renal cell injury, as seen with the other TMA syndromes (George and Nester, 2014). A diagnosis of TTP should, therefore, also be considered in the presence of thrombocytopenia and micro-angiopathic haemolytic anaemia alone (Galbusera et al., 2006) – a recommendation also stipulated in the 2012 British Committee for Standards in Haematology (BCSH) diagnostic guidelines, despite the possibility of over-diagnosis of TTP with this approach (Scully et al., 2012).

TTP is a rare disease, with an incidence of 4 to 11 cases per million people per year reported in the United States (George, 2006) and 6 cases per million per year in the United Kingdom (Scully et al., 2012). It is more common in women, with a female-to-male ratio of 3:2 (Amorosi and Ultmann, 1966). Although TTP cases have been described in patients ranging from age 1 to 90 years (Kennedy et al., 1980), the peak incidence occurs in the third decade of life (Galbusera et al., 2006). In children, the estimated incidence is only 0.1 cases per million per year (George and Nester, 2014). Unfortunately, published data on the incidence of TTP in South Africa was not available at the time of writing.

2.1.3 Von Willebrand factor

VWF is a glycoprotein (GP), organised in a large, multimeric structure. It is synthesised by endothelial cells and megakaryocytes and stored as ULVWF multimers within the Weibel-Pallade bodies (storage granules) of endothelial cells, and the α-granules of platelets (Chauhan, 2014).

The haemostatic functions of VWF are to stabilise factor VIII (FVIII) and enhance platelet adhesion and aggregation at sites of vascular injury (Chauhan, 2014). It is promoted by the unique structure of VWF (Figure 1) that allows for platelet-, collagen- and FVIII-binding. VWF molecules are arranged in multimers of increasing size, ranging from the

smallest homodimers to large multimers. The largest multimers are the most adhesive (Huck et al., 2014) and, therefore, of particular importance in TTP.

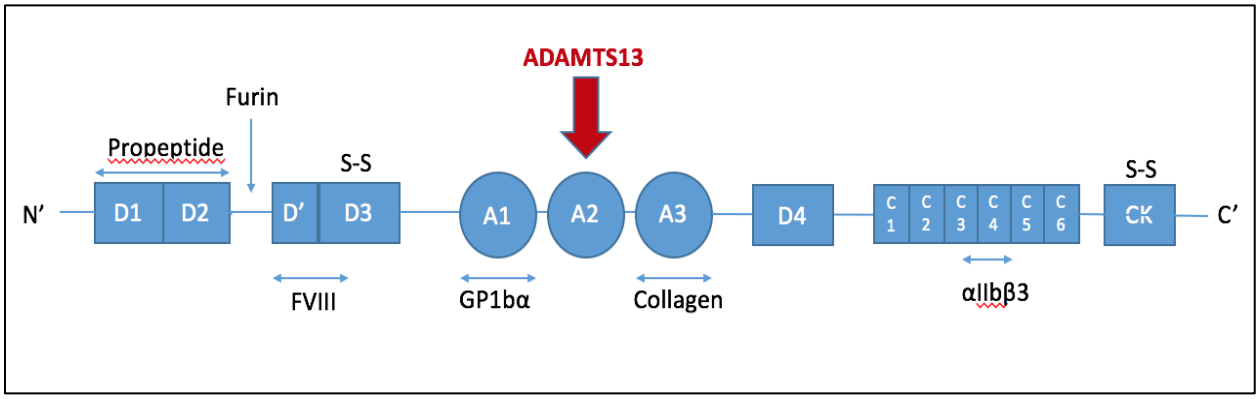


Figure 1: The domain structure of Von Willebrand factor. Adapted from Crawley and Scully (Crawley and Scully, 2013).

The VWF A1 domain-GPIbα interaction is the only effective initiator of platelet adhesion under high shear stress conditions (Savage et al., 1996), allowing VWF-mediated platelet adhesion at sites of vascular injury to act as the first step in thrombus formation (Mannucci, 1998). High shear flow also exposes the ADAMTS13 cleavage site on the elongated multimers and exposes various binding sites. By constantly cleaving the multimers at the A2 domain into smaller, less thrombotic forms, ADAMTS13 regulates the function of VWF (Chauhan, 2014). The unfolded A2 domain is involved in multiple ADAMTS13 exosite interactions (please see detail in section 2.1.5). Firstly, upon binding VWF, the ADAMTS13 Cysteine-rich (Cys) and Spacer domain exosites bring the enzyme and its substrate into close proximity. Subsequent binding of the ADAMTS13 disintegrin-like (Dis) domain exosite to VWF leads to allosteric activation of the adjacent metalloprotease (MP) domain to initiate proteolysis. Importantly, since the MP domain primarily exists in a latent conformation where the active-site cleft is occluded, allosteric activation is mandatory to enable proteolysis. VWF, therefore, functions as both the substrate and activating cofactor for ADAMTS13 (Petri et al., 2019) – the VWF-ADAMTS13 axis.

2.1.4 ADAMTS13

ADAMTS13, so called as it is the 13th member of the ADAMTS family of metalloproteases, cleaves VWF at the Tyr_{1605}-Met_{1606} peptide bond in its A2 domain (Kremer Hovinga and Lämmle, 2012). It is the primary enzyme responsible for the physiological processing of VWF (Scully et al., 2012).

As also depicted in Figure 2 below, from its N-terminus, ADAMTS13 consists of a propeptide, a metalloprotease (MP) domain, a disintegrin (Dis) domain, the first thrombospondin type-1 (TSP1) motif, a cysteine-rich (Cys) domain, a Spacer domain, seven additional TSP1 repeats, and two CUB (Complement 1r/s, Uegf, Bone morphogenic protein 1) domains at the C-terminal (Zheng et al., 2001). Its existence was first proposed in 1982 by Moake et al. (Moake et al., 1982) after observing unusually large VWF multimers in patients with chronic relapsing TTP (George and Nester, 2014). In 2001, ADAMTS13 deficiency was identified as the molecular mechanism of TTP (Levy et al., 2001).

2.1.5 The Von Willebrand Factor-ADAMTS13 axis

As also noted and reviewed by others (Petri et al., 2019), ADAMTS13 is an interesting enzyme in that proteolysis is directed by shear-dependent conformational changes in the substrate (VWF) and not by on-demand activation of the enzyme (Crawley et al., 2011). In contrast to other plasma proteases, such as those of the complement or fibrinolytic system, or the coagulation cascade, where zymogens are proteolytically activated when needed, ADAMTS13 is secreted in a seemingly constitutively active form, in that cleavage of the propeptide is not required for proteolytic activity (Majerus et al., 2003). Regardless of its apparent constitutive activity and lack of a dedicated circulating inhibitor (in contrast to other plasma proteases), ADAMTS13 appears to specifically target only a single site on only one physiological substrate, which is the Tyr_{1605}-Met_{1606} peptide bond on VWF. Thus, leading to the hypothesis that ADAMTS13 circulates in a latent form that requires allosteric activation by VWF (Muia et al., 2014), mediated by one or more of its exosite interactions with VWF (Petri et al., 2019).

After Muia et al. (2014) were able to show that the C-terminal domains of ADAMTS13 inhibit its activity, and that binding to VWF relieves this auto-inhibition, Petri et al. (2019) performed a range of kinetic analyses and investigated the crystal structure of ADAMTS13. They subsequently put forth a model whereby VWF functions as both the substrate for ADAMTS13 and its activating cofactor. In this model outlined in Figure 2, it is clear that VWF and ADAMTS13 are dependent on each other for regulation and activation (Petri et al., 2019). As depicted in Figure 2a, multimeric VWF usually circulates in the plasma in a globular conformation under normal circumstances, with its A1 domains concealed so that it does not bind to platelets. ADAMTS13 also circulates in a stabilised "closed" conformation, rendered through the interaction of its C-terminal CUB domains with the central Spacer domain. The MP domain is also in a latent configuration under normal circumstances, in that the active-site cleft is obscured by the Ca^{2+}-binding loop, which prevents proteolysis of off-target substrates and confers resistance to plasma inhibitors. As shown in Figure 2b, with endothelial cell (EC) disruption and consequent exposure of subendothelial collagen, globular VWF binds to the subendothelium through its A3 domain, unfurling into an elongated conformation, brought on by the shear forces exerted by rapidly flowing blood. The A1 domain is now exposed, enabling it to capture passing platelets via their GPIbα surface receptors. The unfurling of VWF also exposes the VWF A2 domain, revealing the binding sites for ADAMTS13 and the Tyr_{1605}-Met_{1606} cleavage site, only now rendering it susceptible to proteolysis by ADAMTS13.

As demonstrated in Figure 2c, ADAMTS13 recognizes the unfurled VWF through multiple interactions. In step (1), the CUB domains bind the VWF D4-CK domains, which induces their dissociation from the Spacer domain: step (2). The Spacer, step (3), and Cys, step (4), domain exosites recognize the C-terminal region of the unfurled A2 domain to bring the enzyme and its substrate into close proximity. Figure 2d shows that, once bound, step (5), the Dis domain exosite binds VWF residues Asp_{1614}-Asp_{1622}. This interaction induces an allosteric change in the MP domain: step (6). The resulting conformational change disrupts a "gatekeeper triad" that otherwise obscures the active-site cleft to reveal the S1′ pocket. Once allosterically activated, step (7), the MP domain cleaves the scissile bond. Should the cleavage site in the VWF-A2 domain be inaccessible (as found in VWF's globular configuration or in low-shear states), ADAMTS13 would dissociate from VWF

and revert to the auto-inhibited state (Chung, 2019). From these interactions, it is clear that VWF and ADAMTS13 can be said to function as an axis, that is, they are interrelated in their purpose and function.

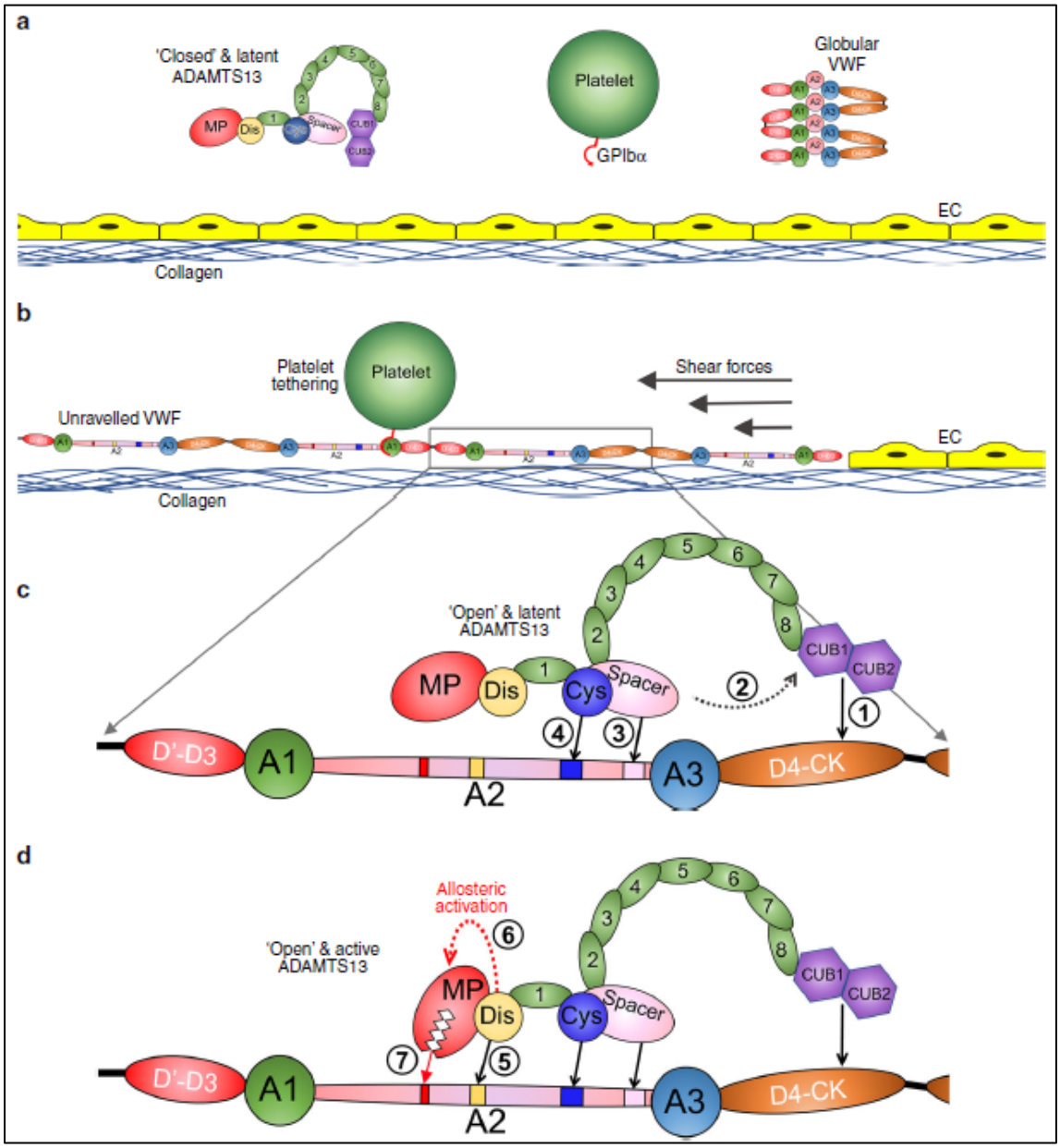


Figure 2: The Von Willebrand Factor-ADAMTS13 axis, as expounded by Petri et al. (Petri et al., 2019). See text for elucidation. *Reproduced unaltered from an open access article, under the terms of a Creative Commons Attribution 4.0 International License: http://creativecommons.org/licenses/by/4.0/.*

2.1.6 Pathophysiology of thrombotic thrombocytopenic purpura

With ADAMTS13 deficiency, the unfurled ULVWF multimers remain attached to the endothelial cell's luminal surface. Passing platelets adhere to the exposed A1 domains on the long multimers through their GPIbα receptors. This initial platelet adhesion leads to platelet activation and degranulation, which is rapidly followed by the aggregation of many additional platelets by means of their GPIIb/IIIa receptor complexes. It results in the formation of large, potentially vaso-occlusive, platelet-rich thrombi (Moake, 2002). Intravascular mechanical destruction (fragmentation) of erythrocytes follows as they pass through these platelet-rich thrombi in the microcirculation (Opie, 2012), resulting in microangiopathic haemolytic anaemia.

2.1.7 Aetiology

Congenital TTP, or Upshaw-Schulman syndrome, is a rare disorder caused by mutations of the ADAMTS13 gene on chromosome 9q34, which results in an inherited deficiency of the enzyme. As recently reviewed by Kremer Hovinga and George (2019), in excess of 200 different ADAMTS13 mutations have been identified (Kremer Hovinga and George, 2019). ADAMTS13 deficiency without a demonstrable autoantibody is required for the diagnosis of congenital TTP (George and Nester, 2014). Molecular techniques have been used in recent years to confirm the diagnosis, with the presence of either homozygous or compound heterozygous defects in the ADAMTS13 gene (Scully et al., 2012).

Acute idiopathic TTP is the most common form of TTP and is characterised by autoantibodies (usually IgG) directed against ADAMTS13 (Scully et al., 2012). Other subtypes of TTP have also been identified and include drug-associated TTP, pregnancy-associated TTP, transplant-associated microangiopathy, malignancy-associated TMA, pancreatitis-associated TTP, HIV-associated TTP and the related condition, haemolytic uraemic syndrome (Scully et al., 2012).

Anti-ADAMTS13 antibodies may be inhibitory, with direct neutralisation of enzyme activity, or non-inhibitory, in which case opsonisation (amongst other unconfirmed mechanisms) is thought to reduce enzyme activity (Malak et al., 2008). It should be

considered an autoimmune disease (Malak et al., 2008), and although the underlying process leading to its manifestation ultimately remains obscure, a possible genetic predisposition to the development of ADAMTS13 autoantibodies is suggested by the overrepresentation in acquired idiopathic TTP of the major histocompatibility complex Class II allele, human leukocyte antigen DRB1*11, in some studies (Kremer Hovinga and Lämmle, 2012).

Patients with congenital TTP are only intermittently symptomatic, and patients with acquired ADAMTS13 deficiency can achieve remission of TTP without recovery of ADAMTS13 levels. These observations suggest the presence of additional factors affecting the pathophysiology of TTP (Chauhan, 2014). An additional trigger, the so-called "second hit", that precipitates an acute attack of TTP, has been the subject of much research (Tersteeg et al., 2016). Infection has long been suspected, with the presence of increased levels of DNA-histone complexes and neutrophil-derived components in the circulation during an acute TTP episode, put forward as presumptive evidence (Tersteeg et al., 2016). Whether these features of infection are a cause or a consequence of TTP remains to be elucidated (Tersteeg et al., 2016).

2.1.8 The effects of ABO blood group on the main determinants in the pathophysiology of TTP

The ABO blood group antigens are comprised of complex carbohydrate structures. They are not only present on erythrocytes but also on various glycolipids and GPs, including the *N*-linked oligosaccharide chains of circulating plasma VWF (Jenkins and O'Donnell, 2006).

There is a well-described relationship between the ABO blood group and VWF level, which has been the subject of multiple reviews (Franchini et al., 2007, Franchini et al., 2014, O'Donnell and Laffan, 2001). Lower VWF levels and activities are a consistent finding in blood group O individuals (Franchini et al., 2007). Although the mechanism by which ABO antigens influence plasma VWF levels remains somewhat unclear, it is likely multifactorial.

Since the ABO blood group antigens, A, B and H, are found on VWF (Sodetz et al., 1979), and the mature VWF subunits are glycosylated with ABO *N*-linked oligosaccharides (Matsui et al., 1992), it is conceivable that these *N*-linked oligosaccharides decrease the rate of VWF clearance from the plasma by protecting circulating VWF from proteolysis, which is a postulate supported by multiple studies (O'Donnell et al., 2002, Federici et al., 1984, Gallinaro et al., 2008, Eikenboom et al., 2013).

Another hypothesis involves increased VWF cleavage by ADAMTS13 (Franchini et al., 2007). Whether this is solely due to the elevated ADAMTS13 activity levels associated with blood group O individuals, as reported by Mannucci et al. (Mannucci et al., 2004), or as a consequence of augmented ADAMTS13-mediated proteolysis of blood group O VWF, as documented by Bowen and Collins (Bowen and Collins, 2004), is still to be confirmed, but would suggest that measured levels and activities of both VWF and ADAMTS13, should be interpreted in the context of the ABO blood group.

Recently, it was also shown immunohistochemically that the ABO blood group is a determinant of VWF protein content in human pulmonary endothelial cells, which suggests that ABO antigens also influence endothelial VWF synthesis and/or secretion, in addition to plasma clearance (Murray et al., 2020).

2.2 Advances and research opportunities in the understanding of the pathophysiology of TTP

2.2.1 Introduction

Despite commendable advances, such as identifying ADAMTS13 as a key role player in TTP, many aspects surrounding its pathophysiology remain unclear. It seems especially true in HIV-associated TTP, where Meiring et al. (2012) reported normal ADAMTS13 activities in a third of adult cases, with only 50% of patients presenting with ADAMTS13 autoantibodies (Meiring et al., 2012).

Moreover, patients treated for aTTP can have undetectable ADAMTS13 activities or levels and remain in remission. At the same time, patients with congenital ADAMTS13 deficiency also develop sporadic attacks of TTP instead of a continuous TMA (Tersteeg

et al., 2016). These observations have been duplicated in murine models, where congenitally ADAMTS13-deficient mice also do not spontaneously develop TTP (nor experience a continuous TMA) but only exhibit features of TTP after a Shiga toxin (Motto et al., 2005) or recombinant human VWF (Tersteeg et al., 2014) challenge, casting doubt on ADAMTS13 deficiency as the only factor of importance.

2.2.2 A second hit as the precipitating factor in acute TTP

It is becoming increasingly clear that ADAMTS13 deficiency alone cannot explain all acute episodes of TTP, and that a triggering event, the so-called "second hit", is possibly required (Tersteeg et al., 2016). Irrespective of the exact nature of the second hit, VWF multimer size remains central to the pathophysiology of TTP. Therefore, it is logical that any process affecting VWF multimer size (whether directly or indirectly) could influence the pathophysiology of TTP.

2.2.3 Proposed backup mechanisms for ADAMTS13

Since the presence of ULVWF multimers is a predictable feature of TTP, the search for the elusive second hit has stimulated much research into potential backup systems for ADAMTS13 and was also the subject of a recent review (Tersteeg et al., 2016). Should these systems be neutralised or overwhelmed, it could potentially contribute to the development of TTP or precipitate an acute attack, thus, providing the second hit. A better understanding of these putative backup mechanisms may ultimately lead to the development of novel therapeutic options.

2.2.3.1 The complement system

The recognition of complement-mediated TMA due to mutations in the complement-regulatory factors H and I (Maga et al., 2010, Zipfel et al., 2007) has implicated the complement system as a possible role player in the second hit (Tersteeg et al., 2016). The complement system interacts with VWF in multiple ways. Indiscriminate membrane-

attack complex formation on endothelial cell surfaces leads to increased VWF release, which will exacerbate microthrombosis (Turner et al., 2014). Conversely, platelet-VWF complexes tethered to endothelial cell surfaces can initiate the alternative complement pathway (Turner et al., 2014) to possibly function as an additional clearance mechanism. Factor H may be an important co-factor for ADAMTS13, since its presence enhances cleavage of soluble VWF by ADAMTS13 (Feng et al., 2013). However, it has also been found to inhibit VWF cleavage by ADAMTS13 on the surface of endothelial cells (Rayes et al., 2014).

More direct evidence for the function of the complement system as a backup mechanism for ADAMTS13 can be found in the observation that factor H directly modifies the multimeric composition of VWF in a non-enzymatic manner through a reduction in the number of large soluble multimers, via a mechanism that is different and independent from ADAMTS13 (Nolasco et al., 2013). Dysregulation of the complement system can therefore constitute (or at least contribute to) the second hit, but direct therapeutic manipulation of this system in TTP is yet to be attempted.

2.2.3.2 Enzymes

As recently reviewed (Tersteeg et al., 2016), several enzymes can cleave VWF in vitro. Their molecular mechanisms differ with some targeting the Tyr_{1605}-Met_{1606} bond in the VWF A2 domain, which is also targeted by ADAMTS13, while others target different sites. Their effects may contribute to in vivo VWF proteolysis, which could precipitate an acute attack of TTP if negated. Since most of them can be secreted by various cells upon stimulation or activated during clot formation/breakdown (Tersteeg et al., 2016), they may be amenable to therapeutic manipulation.

2.2.3.2.1 Neutrophil enzymes

The enzymes released during neutrophil activation, such as elastase, can cleave VWF close to or at the same position in the A2 domain as ADAMTS13 (Tersteeg et al., 2016, Wohner et al., 2012, Raife et al., 2009). However, their role in TTP is unclear since

neutrophil depletion in *Adamts13-/-* mice was not found to affect VWF multimer composition (Budde and Schneppenheim, 2014). As summarised in Figure 3, the overall contribution of neutrophil activation to acute exacerbations of TTP appears ambivalent. In response to (especially bacterial) infection, neutrophils release chromatin fibres known as neutrophil extracellular traps (NETs) (Brinkmann et al., 2004), which have been shown to contribute to thrombosis (Brill et al., 2012), and may well constitute the second hit. Conversely, neutrophil enzymes released during activation may assist ADAMTS13 in clearing ULVWF multimers during microangiopathy (Tersteeg et al., 2016). Direct blockade or supplementation of these responses for the treatment of acute TTP has not been attempted in vivo yet, probably because of the various possible non-specific effects such an approach would have.

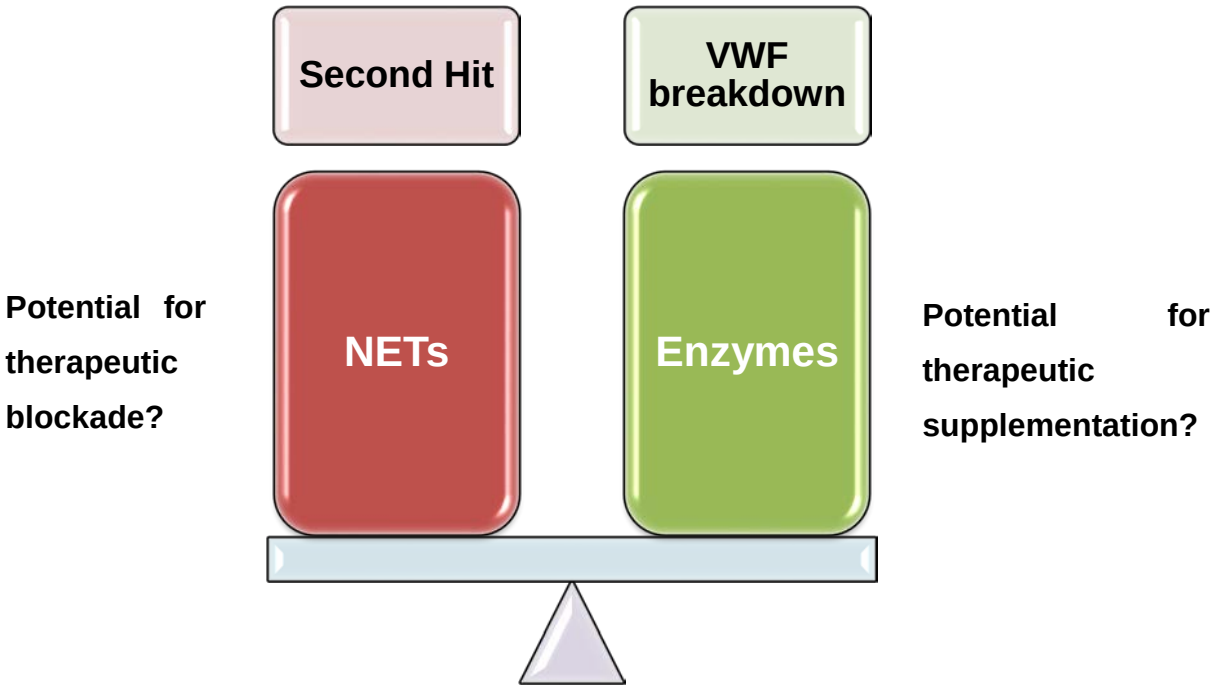


Figure 3: The dichotomous roles of neutrophils in acute thrombotic thrombocytopenic purpura. Both offer possibilities for therapeutic intervention that remain to be explored. NETs; Neutrophil extracellular traps, VWF; Von Willebrand factor

2.2.3.2.2 Granzymes

The granzymes are usually secreted by cytotoxic lymphocytes to initiate apoptosis in a highly regulated process, but may also be secreted indiscriminately by other cell types, such as mast cells, into the extracellular compartment following degranulation (Tersteeg et al., 2016). Granzyme B can cleave extracellular matrix proteins, as well as fibrinogen and VWF, although its effect on VWF is only exerted when VWF is unfolded, destroying its platelet binding capacity (Buzza et al., 2008). In contrast, granzyme M abrogates VWF's ability to carry FVIII but does not affect its platelet-binding capacity, as it only cleaves VWF in its globular conformation (Hollestelle et al., 2011). Granzyme B may, therefore, have potential therapeutic application in TTP, although its exact role in the pathophysiology of TTP has not been fully explored. Furthermore, multiple off-target effects may also limit its ultimate clinical utility.

2.2.3.2.3 Thrombin

Thrombin, a key enzyme in the coagulation cascade, has procoagulant and anticoagulant functions during both primary and secondary haemostasis (Tersteeg et al., 2016). Although it cleaves VWF less efficiently than neutrophil elastase (Wohner et al., 2012), it is able to detach platelets anchored to a collagen matrix under high shear conditions via VWF cleavage (Wohner et al., 2012). Its role in the pathophysiology of TTP is, however, unclear. Although elevated plasma markers for thrombin activity are described in several older case reports of acute TTP (Monteagudo et al., 1991, Wada et al., 1998, Sagripanti et al., 1996, Takahashi et al., 1989), this does not appear to lead to fibrin-rich thrombi, as the microthrombi found in TTP are generally platelet and VWF-rich, but fibrin-poor (Asada et al., 1985, Hosler et al., 2003), suggesting limited/absent thrombin activity in TTP (Tersteeg et al., 2016). Whether the enhancement of thrombin generation will have therapeutic utility in TTP is open to speculation.

2.2.3.2.4 Plasmin and the fibrinolytic system as a critical physiological backup for ADAMTS13

Plasmin, a plasma serine protease, is the fibrinolytic system's major enzyme; its primary substrates are fibrin and fibrinogen. Breakdown of fibrin by plasmin leads to the generation of a distinct set of soluble degradation products (Pizzo et al., 1973), of which D-dimers (formed when fibrin, cross-linked by factor XIII, is degraded by plasmin) are used diagnostically (Cesarman-Maus and Hajjar, 2005). Other fibrin breakdown products may inhibit platelet function, influence immune modulation and chemotaxis, or potentiate the hypotensive effects of bradykinin (Cesarman-Maus and Hajjar, 2005, Hajjar, 2003), although some of their actual in vivo effects have been disputed (Gaffney, 2001). The soluble fibrinogen degradation products (FDPs), known as fragments D, E, X, and Y, may inhibit the spontaneous polymerisation of fibrinogen and are produced when circulating fibrinogen is degraded by plasmin (Hajjar, 2003, Cesarman-Maus and Hajjar, 2005).

Plasmin's circulating inactive precursor form, plasminogen, is produced by the liver and converted to plasmin by several plasminogen activators (Medcalf, 2015). Plasminogen, which consists of a Pan-apple (PAp) domain, five kringle domains (KR1-5), and a serine protease (SP) domain, exists in a closed, activation-resistant state in the circulation. After the kringle domains have initiated interactions with fibrin clots or cell-surface receptors, plasminogen adopts an open configuration that can be readily cleaved by tissue-type plasminogen activator (tPA) or urokinase-type plasminogen activator (uPA), resulting in its conversion to plasmin (Law et al., 2012, Law et al., 2013).

tPA, first described by Astrup and Permin in 1947 (Astrup and Permin, 1947), generally only becomes effective as an activator when bound to fibrin (Hoylaerts et al., 1982). Plasminogen and tPA both bind to fibrin in a lysine-dependent manner, which essentially limits its activation of plasminogen to areas of fibrin deposition, since fibrin-bound plasminogen is activated into plasmin orders of magnitude faster in the presence of fibrin than free in the circulation (Medcalf, 2015).

uPA, first identified in urine by Sobel et al. in 1952 (Sobel et al., 1952), primarily activates plasminogen on the surfaces of cells via a receptor-mediated mechanism (Ellis et al., 1991). It does not require the presence of fibrin (Tersteeg et al., 2016) and can activate

plasminogen whether bound to fibrin or in solution (Cesarman-Maus and Hajjar, 2005, Medcalf, 2015). The uPA receptor (uPAR) is present on monocytes, macrophages, and fibroblasts (Hajjar, 1995), but pertinently to TTP, also on platelets and endothelial cells (Tersteeg et al., 2016). Cellular receptors for plasminogen have also been identified (Miles and Parmer, 2013), which help to diversify plasmin's functions beyond fibrinolysis (Tersteeg et al., 2016, Medcalf, 2015).

As recently reviewed (Medcalf, 2015), plasmin formation and activity is regulated (either directly or indirectly) at three different levels. Two of these levels involve three different serine protease inhibitors known as serpins. All serpins function in essentially the same manner by forming irreversible complexes with the active site serine of the target protease after proteolytic cleavage of the inhibitor by the target protease. Both the protease and the inhibitor are permanently inactivated in the process (Cesarman-Maus and Hajjar, 2005).

At the first level, plasmin is directly inhibited by α_2-antiplasmin. It is the most important inhibitor of fibrinolysis and a highly efficient serpin, with the association rate between α_2-antiplasmin and plasmin being the fastest described among all the serine proteases and serpins (Medcalf, 2015). Very high plasma levels (~1 µmol/L) of α_2-antiplasmin (Medcalf, 2015), together with its long half-life of ~2.6 days (Collen and Wiman, 1979), ensures that the half-life of plasmin activity is less than 10 ms (Medcalf, 2015). On the other hand, the half-life of plasminogen is approximately 2.2 days (Medcalf, 2015). However, when bound to fibrin or a cell surface receptor, plasmin is temporarily shielded from inactivation, allowing it more prolonged activity on cell surfaces than in solution (Tersteeg et al., 2016).

At the second level, plasmin activity is regulated indirectly by the serpins plasminogen activator inhibitor-1 (PAI-1) (described in the early 1980s) (Loskutoff et al., 1983, Medcalf, 2015, Coleman et al., 1982) and PAI-2 (described in 1970) (Kawano et al., 1970, Medcalf, 2015), both of which rapidly bind to and inactivate both of plasminogen's primary activators; uPA and tPA (Medcalf, 2015, Tersteeg et al., 2016).

The third level of plasmin regulation does not involve protease inhibition but rather the stabilisation of fibrin clots, rendering them resistant to plasmin degradation (Medcalf, 2015). After activation by thrombin, the carboxypeptidase Thrombin Activatable

Fibrinolysis Inhibitor (TAFI) enzymatically removes C-terminal lysine residues from fibrin, decreasing the capacity of plasminogen and tPA to bind to the fibrin surface, thereby indirectly inhibiting fibrin breakdown (Bajzar et al., 1995, Nesheim and Bajzar, 2005).

While the importance of plasminogen activation during fibrinolysis is well-recognised, plasmin was also proposed in 1985 as a substantial role player in various other processes (Dano et al., 1985). Several studies have demonstrated plasmin's ability to selectively cleave particular peptide bonds in other proteins, including FVIII (Guisasola et al., 1978, Rick et al., 1985), VWF (Hamilton et al., 1985, Federici et al., 1992), ADAMTS13 (Feys et al., 2010b, Crawley et al., 2005), the platelet receptor GPIb (Adelman et al., 1985) through plasmin's lysine binding regions (Adelman et al., 1986), and, notably, also in platelet-VWF complexes (Tersteeg et al., 2014, Wohner et al., 2012) which is of particular importance in TTP.

The role of plasmin as a possible ADAMTS13 backup mechanism for proteolytic degradation of the culprit VWF multimers leading to aTTP was consequently investigated by Tersteeg et al. (Tersteeg et al., 2014), and reported in a 2014 paper that also received an editorial comment (Chauhan, 2014). They demonstrated that plasminogen does indeed bind to the VWF A1 domain in a lysine-dependent manner, similar to its mechanism of binding to fibrin (Medcalf, 2015), but that binding occurs at a site that remains inaccessible when VWF is in a globular state (Tersteeg et al., 2014). In addition, plasminogen activation (mediated through a uPAR-dependent mechanism) resulted in rapid cleavage of platelet-VWF complexes on activated human umbilical vein endothelial cells (HUVECs) in the absence of ADAMTS13, which was also lysine-dependent (Tersteeg et al., 2014). Another group confirmed that globular VWF is resistant to plasmin degradation under static conditions but is promptly cleaved by plasmin under shear conditions (Brophy et al., 2017), and that this occurs at the Lys_{1491}-Arg_{1492} peptide bond within the VWF A1-A2 linker region in a shear- and glycan-dependent manner, and not at the Tyr_{1605}-Met_{1606} ADAMTS13 proteolytic site in the A2 domain (Brophy et al., 2017).

During acute TTP episodes, plasmin-α_2-antiplasmin (PAP) complexes, a marker of plasminogen activation, were elevated in proportion to the degree of thrombocytopenia and, thus, the extent of the disease (Tersteeg et al., 2014). In addition, hypoxia has been

shown to accelerate endothelium-mediated plasminogen activation through the upregulation of uPAR (Kroon et al., 2000, Graham et al., 1998). Tersteeg et al. (2014) ultimately hypothesised that the ischaemia following microvascular thrombosis evokes plasminogen activation with ensuing plasmin-mediated degradation of platelet-VWF complexes and proposed that plasmin independently aids in the local resolution of the microvascular obstruction seen in aTTP (Tersteeg et al., 2014).

In a later murine model of aTTP, Tersteeg et al. (2017a) also subsequently demonstrated that by amplifying endogenous plasmin activity and plasminogen activation through the inhibition of PAI-1 in *α2-antiplasmin*$^{-/-}$ mice, acute TTP could be resolved (Tersteeg et al., 2017a).

After investigating the effects of sex, age, and blood group on plasminogen, antithrombin and protein C levels in a cross-sectional study involving a cohort of healthy plasma donors, Dolan et al. (1994) found no association between plasminogen concentration and ABO blood group (Dolan et al., 1994).

2.3 Advances and research opportunities in the treatment of TTP

2.3.1 Manipulation of the fibrinolytic system

2.3.1.1 Thrombolytic (fibrinolytic) drugs

The thrombolytic drugs exert their therapeutic effect through the exogenous activation of plasminogen (Collen and Lijnen, 2005), thereby therapeutically manipulating the fibrinolytic system for the treatment of thrombotic conditions such as acute ischaemic stroke (Bivard et al., 2013), ST-segment elevation acute myocardial infarction (STEMI) (O'Gara et al., 2013), arterial thromboembolism (Alonso-Coello et al., 2012), haemodynamically unstable pulmonary embolism (PE) (Kearon et al., 2012), and certain cases of upper extremity deep venous thrombosis (Kearon et al., 2012).

They can be divided into fibrin-selective and non-fibrin-selective compounds. The latter include two-chain uPA and the bacterial product streptokinase; both activate plasminogen in circulating blood as well as fibrin-bound plasminogen (Collen and Lijnen, 2005). Streptokinase, described as early as 1933 (Tillett and Garner, 1933), is a single chain

polypeptide derived from β-haemolytic streptococcus cultures (Anderson and Willerson, 1993). It binds to plasminogen, forming a 1:1 stoichiometric complex that becomes an active enzyme that cleaves peptide bonds on other plasminogen molecules, leading to plasmin activation (McClintock and Bell, 1971, Anderson and Willerson, 1993). By binding to the plasminogen SP domain in a way that elegantly avoids the kringle array (Law et al., 2012), streptokinase is able to readily activate plasminogen while it is still in the closed conformation (Wang et al., 1998, Parry et al., 2000), effectively circumventing the need for prior fibrin binding.

The generated plasmin is rapidly inactivated by α_2-antiplasmin but will also degrade other plasma proteins after the inhibitor's saturation, leading to the so-called "lytic state" (Collen and Lijnen, 2005). Although streptokinase is antigenic, resulting in the production of antibodies that preclude repeat doses (Gurman et al., 2015), it is relatively inexpensive and has been proposed as the thrombolytic of choice for developing countries (Kunamneni et al., 2007).

In contrast, fibrin-selective drugs preferentially activate fibrin-bound plasminogen. As reviewed by Collen and Lijnen (Collen and Lijnen, 2005), these include wild-type recombinant tPA (alteplase) as well as several recombinant tPA variants with differing half-lives, fibrin specificity and PAI-1 resistance (reteplase, tenecteplase, lanoteplase, monteplase, pamiteplase), the Desmodus salivary plasminogen activator (DSPA) desmoteplase, single-chain uPA, and the bacterial product staphylokinase, secreted by certain strains of *Staphylococcus aureus*.

In light of their findings that plasminogen is activated during bouts of microangiopathy and that plasmin could substitute for ADAMTS13 to degrade platelet–VWF complexes, Tersteeg et al. (2014) investigated the induction of plasminogen activation by non-fibrin-selective thrombolytic agents in vitro and in a murine model in vivo (Tersteeg et al., 2014). They explored the hypothesis that thrombolytic drugs can effectively treat aTTP, by administering streptokinase and human plasminogen to *Adamts13*$^{-/-}$ mice after inducing TTP through the injection of high-dose recombinant human VWF. A single dose of streptokinase, roughly equivalent to 20% of the loading dose used to treat human PE and stroke, was able to attenuate TTP symptoms and correct any thrombocytopenia (Tersteeg

et al., 2014). Moreover, histochemistry indicated that thrombolytic treatment had prevented the appearance of microvascular thrombi in the livers of challenged mice (Tersteeg et al., 2014), suggesting a possible role for drugs such as uPA, streptokinase, or even tPA, as adjuvant or alternative therapies for aTTP (Chauhan, 2014).

An obvious limitation of such an approach is the potential risk of bleeding, brought on by the use of thrombolytic therapy in a severely thrombocytopenic patient (Chauhan, 2014). However, it was reassuring that Tersteeg et al. (2016) found no macroscopic evidence of bleeding or disrupted secondary haemostasis post-streptokinase (Tersteeg et al., 2016). Moreover, normal platelet aggregate formation on immobilized collagen in the presence of plasminogen and streptokinase under arterial shear conditions suggested that plasminogen activation does not impair physiological platelet aggregation (Chauhan, 2014). Their results also suggest that the amount of streptokinase required to successfully dissolve pathogenic platelet-VWF complexes is much lower than the dose required for fibrinolysis. A single dose at ~20% of the loading dose given for PE successfully attenuated the TTP phenotype (Tersteeg et al., 2014) without the need for continuous infusion; an essential consideration since thrombolytic treatment intensity correlates with bleeding risk (Sinnaeve et al., 2014).

Although non-fibrin-selective manipulation of the fibrinolytic system with streptokinase appears safe and effective in mice, higher animal model data are desirable before this drug's utility can be investigated in human clinical trials. Data from non-human primates would be preferred due to the more significant homology between non-human primates and humans (Vanhoorelbeke and De Meyer, 2013).

2.4 Non-human primate model of aTTP

Although useful results can be obtained from in vitro studies of human haemostasis, and human haemostatic genes and proteins have been well characterised, animal models still have great utility in the investigation of *fatal* haemostatic diseases, where in vitro studies may be found wanting and human in vivo studies of novel treatment approaches may simply not be possible. It is in this context that non-human primate models of aTTP are

particularly relevant and useful, due to their proven utility in the development (and testing) of new treatment strategies for this rare disease (Vanhoorelbeke and De Meyer, 2013).

A Chacma baboon (*Papio ursinus*) model of aTTP was developed and established locally at our institution in collaboration with the Laboratory for Thrombosis Research at KU Leuven in Kortrijk, Belgium (Feys et al., 2010a). This model has been used successfully to investigate various drugs with therapeutic potential in TTP (Feys et al., 2012, Callewaert et al., 2012, Tersteeg et al., 2017b). It has also been useful to elucidate many haemostatic aspects of this condition's basic pathophysiology.

Like all other baboons, the Chacma baboon or *Papio ursinus* is classified as an Old World monkey in the Cercopithecidae family. Along with guenons, macaques and mangabeys, it is further sub-classified in the sub-family Cercopithecinae (Fortman et al., 2002). Currently, five or six *Papio* species are generally recognized, although there is some disagreement on whether they are indeed separate species or merely subspecies of each other (Newman et al., 2004, Frost et al., 2003, Wildman et al., 2004), and their taxonomic status is consequently debated (Kopp et al., 2014). They are the Chacma baboon (*Papio ursinus*; found in southern Africa), Western, Red, or Guinea baboon (*Papio papio;* found in far western Africa), Hamadryas baboon (*Papio hamadryas*; found in the Horn of Africa and southwestern Arabia), Olive baboon (*Papio anubi*s; found in the north-central African savannah), Kinda baboon (*Papio kindae;* found in south-central and eastern Africa), and Yellow baboon (*Papio cynocephalus*; found in south-central and eastern Africa) (Kopp et al., 2014).

Papio spp. are commonly used in scientific research. Their relatively large size (adult females range on average from 11 kg to 15 kg, and adult males from 22 kg to 30 kg) (Fortman et al., 2002) makes venepuncture reasonably easy. Their haemodynamic systems are also tolerant to repeat blood sampling without cardiovascular compromise (Leadley et al., 2000). At 50-70 mL/kg, the blood volumes of baboons make them particularly suitable for studies that require frequent sampling or the analysis of large amounts of blood (Fortman et al., 2002).

2.4.1 Available animal data – the *Papio ursinus* model of aTTP

The development of the *Papio ursinus* model of aTTP started with the generation of a monoclonal antibody (mAb) able to inhibit human recombinant ADAMTS13 (Vanhoorelbeke and De Meyer, 2013). This antibody, named 3H9, binds to an epitope in the MP domain of the enzyme, which contains the active site. Crucially, it is able to cross-react with *Papio ursinus* ADAMTS13 and induce acute TTP in vivo (Feys et al., 2010a). Differences between this model and murine models already became apparent early in its development. In contrast to established congenital *Adamts13*$^{-/-}$ murine models, in *Papio ursinus,* mere 3H9-mediated ADAMTS13 inhibition is sufficient to predictably and reproducibly provoke immediate clinical TTP in all treated animals (Feys et al., 2010a, Vanhoorelbeke and De Meyer, 2013). It seems to be a characteristic feature of the *Papio ursinus* model, as even in an aTTP murine model, complete ADAMTS13 inhibition by anti-mADAMTS13 mAbs is not sufficient to induce TTP, with a second hit such as the addition of recombinant human VWF required (Deforche et al., 2016).

With 48-hourly injections of this antibody, TTP is generally well established by approximately day 4; a feature confirmed in multiple experiments using this model (Feys et al., 2012, Callewaert et al., 2012, Tersteeg et al., 2017b, Feys et al., 2010a). Inhibition of ADAMTS13 function results in severe haemolytic anaemia, as evidenced by rapid decreases in haemoglobin and haptoglobin levels, and elevated serum lactate dehydrogenase levels. Schistocytes (red cell fragments) consistently appear at approximately day 2 (Feys et al., 2010a), reaching levels of over 40% in some experiments (Callewaert et al., 2012), on about day 9. Thrombocytopenia typically develops within 24 hours, reaching a nadir of roughly $12 \pm 7 \times 10^9$/L after 48 hours (Feys et al., 2010a). As expected, mild mucocutaneous bleeding manifests in the form of bruising at injection sites and mild gingival bleeding (Callewaert et al., 2012, Feys et al., 2010a), but more severe bleeding such as intracranial haemorrhages was not evident in any of the previous experiments, including those investigating drugs with potential bleeding effects (Feys et al., 2012, Callewaert et al., 2012, Tersteeg et al., 2017b).

Post-mortem immunohistochemistry confirmed the presence of platelet and VWF-rich (but fibrin-poor) microthrombi in the brain, heart, kidneys, and spleen, but not in the lungs

(Feys et al., 2010a). Although these results confirm that inhibition of ADAMTS13 function is sufficient to cause TTP in baboons, even in the absence of a second hit, none of the animals developed renal impairment, and only one baboon had evidence of myocardial ischaemia (Feys et al., 2010a). Moreover, to date, no other important clinical manifestations of TTP, such as neurologic symptoms or overt renal failure, have been reported in this model, and no animals have died (Feys et al., 2012, Callewaert et al., 2012, Tersteeg et al., 2017b, Feys et al., 2010a).

Consequently, this model probably represents mild, early-stage TTP (Feys et al., 2010a, Callewaert et al., 2012), and a second hit could be needed to establish end-stage disease with overt organ failure, in order to more closely mimic the situation in clinical practice, where TTP may only become clinically apparent after the onset of organ damage (George, 2006). Nevertheless, since not all patients with TTP present with neurological deficit or renal failure (Vesely et al., 2003), valuable inferences can still be made from this model, and since it provides consistency with minimal animal discomfort, it remains a valuable tool for the investigation of novel therapies (Feys et al., 2010a).

Perhaps more importantly, the *Papio ursinus* model provides an opportunity to study the basic pathophysiology of TTP as well as other conditions where haemostasis is disturbed, and has already contributed significantly to our understanding of the fundamental mechanisms of TTP. It demonstrated that ADAMTS13 activity is essential for microvascular patency in primates as TTP spontaneously and rapidly occurs after ADAMTS13 inhibition even in the absence of a second hit (Feys et al., 2010a). It has suggested the existence of "early-stage" and "advanced-stage" TTP, where early-stage TTP may be mild or even unnoticed, as measurable organ failure (i.e. advanced-stage TTP) only manifests after 96 hours of ADAMTS13 inhibition (Feys et al., 2010a) or after aggravation by additional triggers (Vanhoorelbeke and De Meyer, 2013).

Genetic, physiological and even environmental differences between humans, baboons and mice, can unfortunately impede direct comparison and extrapolation of results obtained in different species (Vanhoorelbeke and De Meyer, 2013). Recently, direct platelet receptor comparisons found *Papio ursinus* to be a suitable animal model for the evaluation of human-targeted anti-platelet agents formulated against the P2Y12,

GPIIb/IIIa and GPIbα receptors (Janse van Rensburg, 2016), but not for the assessment of human-targeted anti-GPVI agents (Janse van Rensburg et al., 2015). Aggregation responses to various platelet agonists are generally lower in Chacma baboons than in humans (Ponschab et al., 2016). Although thromboelastometric assays indicate broad similarity between humans and Chacma baboons, blood clots formed by Chacma baboons are more fibrinolysis-resistant than human clots (Schöchl et al., 2012), implying possible fundamental differences that require further investigation.

2.4.2 Remaining research opportunities in *Papio ursinus* in general, and the *Papio ursinus* model of aTTP in particular

Although data is available on basic haematological parameters (such as haemoglobin concentration, packed cell volume, white blood cell count, platelet count, red blood cell count, mean cell volume, mean cell haemoglobin, and mean cell haemoglobin concentration), for both wild (Melton and Melton, 1982) and laboratory (Steyn et al., 1975, Schöchl et al., 2012) *Papio ursinus* populations, there is a paucity of published data on basic haemostatic components, with only one study, conducted by Schöchl et al., reporting on coagulation (Schöchl et al., 2012). In addition to fibrinolysis resistance, they found significantly shorter prothrombin times (PTs), lower International Normalised Ratios (INRs), and lower fibrinogen levels in *Papio ursinus* compared to humans, despite generally comparable thromboelastometric profiles. Hampton and Matthews, however, found no difference between human and baboon fibrinogen levels, although the exact species of baboon studied is not mentioned in their 1966 publication (Hampton and Matthews, 1966). Prior to our pilot study (Joubert et al., 2021) (see section 2.6 below, Appendix A), which reported a large difference in the baseline fibrinogen concentrations of the two test animals (1.12 g/L vs. 5.30 g/L), baseline fibrinogen level data is only available for the initial description of the aTTP model (Feys et al., 2010a) and not for any of the later studies. In this seminal study, baseline fibrinogen levels were found to be comparable to human ranges (Feys et al., 2010a).

In addition to these conflicting reports, which represent potential areas for future research, there is no detailed information on the thrombin generation capabilities of this species'

haemostatic system. Although thrombin generation was assessed as part of a preclinical Chacma baboon study of a synthetic heparin mimetic (Herbert et al., 2001), thorough characterisation of Chacma baboon thrombin generation was not the primary aim of the study. Similarly, preclinical Olive baboon (*Papio anubis)* studies of protein S (Heeb et al., 2012) and the factor IXa inhibitor, pegnivacogin, and its reversal agent, anivamersen (Bel et al., 2015), as well as a Hamadryas baboon (*Papio hamadryas)* heat-stroke model study of nematode anticoagulant protein (NAP) c2 (Bouchama et al., 2012), included some assessment of thrombin generation, but none of these studies primarily aimed to specifically characterise these species' thrombin generation capabilities in detail. It was, however, explicitly investigated in the Olive baboon, as part of a study comparing thrombin and plasmin generation in humans, Olive baboons, Rhesus monkeys, Yorkshire pigs, Sprague-Dawley rats, New Zealand White rabbits and Hartley guinea pigs (Tarandovskiy et al., 2020), which showed that Olive baboon and Rhesus macaque thrombin generation are both comparable to that of humans. Whether Chacma baboon thrombin generation is similar to that of other non-human primate species remains to be investigated, but cannot be simply assumed, since interspecies variation has not only been described in both the endogenous thrombin potentials (of humans, rats, pigs and rabbits) and the thrombin generation lag phase times (of humans, rats, pigs, sheep and rabbits), as measured by the thrombin generation assay (Siller-Matula et al., 2008), but also in the endogenous thrombin potentials of different non-human primates, such as Cynomolgus and Rhesus monkeys (Poitout-Belissent et al., 2020).

The *Papio ursinus* model offers an opportunity for basic and translational research on TTP by providing a matrix in which alternative therapies and novel theories can be investigated (Feys et al., 2010a). However, despite the wealth of information generated by previous experiments, many haemostatic aspects of this model (including aspects relating to TTP) remain unexplored. There are no or very limited published data on essential elements of this model, such as the general reference ranges in these animals for the levels and activities of the key role-players in TTP (ADAMTS13 and VWF), the effects of TTP on the *Papio ursinus* fibrinolytic system, and how these differ from humans. Although the distribution and genetics of ABO blood groups in *Papio ursinus* were studied in the 1970s (Downing et al., 1973, Downing et al., 1975), there are no published data on

the correlation of ADAMTS13 or VWF levels & activities with ABO blood group in these animals.

Evaluations of certain compounds (such as recombinant glycosylated human interleukin-6, both in normal baboons (Mestries et al., 1994) and in a disseminated intravascular coagulation (DIC) model (Kruithof et al., 1997), soluble-fibre concentrate in obese baboons (Venter et al., 1997), and puff adder venom (Brink and Steytler, 1974) in normal baboons) have included assessments of their effects on *Papio ursinus* fibrinolysis, with varying depth and levels of sophistication. However, apart perhaps from a 1959 study which assessed the effects of the menstrual cycle on the euglobulin clot lysis time in three baboons (Gillman et al., 1959), and the study by Schöchl et al. (2012), which assessed similarities in thromboelastometric findings between humans and baboons (Schöchl et al., 2012), there are no publications focusing specifically on the *Papio ursinus* fibrinolytic system in general, its level of homology with human fibrinolysis, or its interplay with other components of the haemostatic system, under physiological conditions. The reason for this is unclear, but may simply be a consequence of the high priority awarded to other haemostatic components (such as platelets, ADAMTS13 and VWF, in the context of aTTP) in previous *Papio ursinus* animal model research (Vanhoorelbeke and De Meyer, 2013) which may have come at the expense of studies focusing purely on fibrinolysis.

Notably, although the in vivo effects of various systemic thrombolytic drugs (recombinant single-chain uPA, recombinant tPA, and a hybrid recombinant plasminogen activator consisting of an anti-fibrin antibody combined with low-molecular-weight single-chain uPA) have been investigated previously in an unspecified baboon species (Runge et al., 1996), prior to the research efforts of the candidate and his promoters (please see section 2.6 below) only two studies have investigated the in vivo responses of the *Papio ursinus* fibrinolytic system to systemic thrombolytic drugs: Van Zyl et al. investigated the utility of the chimeric protein PLATSAK (a recombinantly produced antithrombin-antiplatelet-staphylokinase conjugate) in a *Papio ursinus* arterial and venous thrombosis model (van Zyl et al., 2000), and Franz et al. investigated the effect of tPA (Franz et al., 2004), as part of a more extensive study assessing the utility of rotational thromboelastography in the quantification of the resultant lytic state during thrombolytic therapy (Franz, 2009). At the time of the candidate's pilot study outlined in section 2.6 below, no publications were

reporting on the in vitro or systemic in vivo effects of streptokinase in the normal Chacma baboon, nor in the *Papio ursinus* model of aTTP, which presented a unique research opportunity.

Streptokinase is the only non-fibrin-selective thrombolytic drug currently registered for clinical use in South Africa (Rossiter, 2012). It is listed as a primary medication in the South African Essential Medicines List for primary health care hospitals (National Department of Health, 2019) and could therefore be a simple, low-cost, easily obtainable treatment alternative/supplement for patients with HIV-associated TTP in South Africa. It may theoretically offer a solution to at least some of the challenges posed by PEX or may at the very least be used as emergency management, while the patient is transferred to a tertiary-level centre for definitive therapy.

The *Papio ursinus* model is ideal for investigating this treatment approach, which could be of particular benefit in the South African setting, where access to PEX is often limited.

2.5 Use of streptokinase in baboon models

Although there is a dearth of practical, published experience with the thrombolytic drugs (especially streptokinase) in *Papio ursinus* in vivo, other baboon models (most notably *Papio hamadryas*) have been used successfully to investigate the effects of many of the thrombolytic drugs (including streptokinase). Most of these studies were performed during the pre-clinical development phases of these drugs for the treatment of thrombotic conditions such as acute myocardial infarction.

In 1966, Hampton and Matthews studied the in vitro effect of streptokinase on fibrinolysis in the plasma of an unnamed baboon species. They found that although high-dose urokinase was able to activate baboon fibrinolysis to an extent comparable with humans, streptokinase was not (Hampton and Matthews, 1966).

Collen et al. investigated the comparative thrombolytic properties and immunogenicity of streptokinase and the (at the time) relatively poorly studied thrombolytic drug recombinant staphylokinase in a *Papio hamadryas* arterial and venous thrombosis model (Collen et al., 1993a). Streptokinase was found to be relatively inefficient at dissolving platelet-rich

thrombi in their femoral arterial eversion graft model, and this was not due to anti-streptokinase antibodies or other inhibitors. The shortest time to reflow was over an hour (63±33 minutes), and persistent patency was only achieved in one of the twelve test animals, at a dose of 0.50 mg/kg. In comparison, staphylokinase resulted in reflow in 24±12 minutes at a dose of 0.25 mg/kg, and persistent patency was achieved in four of the twelve test baboons.

However, systemic infusion of streptokinase led to progressive dose-dependent clot lysis in baboons with jugular vein thrombosis (comparable to the lysis obtained with staphylokinase), indicating meaningful fibrinolytic activation and that the relative potencies of these two drugs for activation of the *Papio hamadryas* fibrinolytic system in vivo may be comparable. The authors caution that in vitro clot lysis systems may not be representative of in vivo thrombolytic performance and that results obtained in different animal models cannot always be extrapolated to human clinical conditions (Collen et al., 1993a).

Different experimental animal models can also not be extrapolated to one another. Variability in the reactivity of human, rabbit and hamster (Lijnen et al., 1991), as well as human, equine, feline, canine, bovine, and leporine (Wohl et al., 1983) fibrinolytic systems, has been reported for streptokinase and was also investigated *inter alia* in *Papio hamadryas* baboons by Lijnen et al. (Lijnen et al., 1992). In their study, streptokinase was a relatively poor plasminogen activator in dogs, rabbits, hamsters, rats and *Papio hamadryas* when compared to humans, with significant interspecies variation.

Collen et al. (1993b) investigated this interspecies variability in the human, baboon, leporine, canine and bovine fibrinolytic systems to streptokinase and recombinant staphylokinase activation. They concluded that this variability is determined by the ability of these compounds to form complexes with plasminogen, by the catalytic efficiencies of the resultant complexes for the activation of autologous plasminogen, and by the rate at which these complexes are inhibited by α_2-antiplasmin (Collen et al., 1993b). Notably, the plasminogen molecule of the (unspecified) baboon species tested in their experiment was unable to form stable stoichiometric complexes with streptokinase, but did react

stoichiometrically and quantitatively with staphylokinase, although these complexes had low catalytic efficiencies.

Since there are demonstrable differences between the propensities of the human, *Papio hamadryas* and other unnamed baboon fibrinolytic systems for activation by streptokinase, it is conceivable that there may also be differences across the various *Papio spp*., which creates a potential research opportunity.

2.6 Use of streptokinase in the *Papio ursinus* model of aTTP

As part of a larger research effort investigating the pathogenesis and treatment of acute aTTP in humans, the candidate and his promoters performed a pilot study to determine the in vitro and in vivo effects of low, intermediate, and high dose streptokinase in the established Chacma baboon model of aTTP (Joubert et al., 2021). This study was performed to ascertain whether a more extensive scale baboon study of streptokinase would be feasible. It is attached as Appendix A and is briefly reviewed below.

The pilot study consisted of an in vitro and an in vivo component. In vitro, the effects of different spiking concentrations of streptokinase (which correlated broadly with the intravenous doses used in the in vivo studies) on routine coagulation screening tests, VWF activities and multimer patterns, Platelet Function Analyser-100 (PFA-100) closure times, thromboelastograms (TEGs) and plasmin activities, were assessed at two incubation time-points.

In the in vivo experiments, after the induction of TTP with the anti-ADAMTS13 mAb 3H9, the effects of escalating intravenous streptokinase doses (ranging from 50 000 to 3 000 000 IU) on peripheral blood counts, schistocytes, routine coagulation screening tests, VWF activities and multimer patterns, PFA-100 closure times, ADAMTS13 antigen levels and activities, fibrinolysis, and TEGs, were assessed.

In the in vitro study, streptokinase spiking of baboon plasma had no effect on the routine coagulation screening tests, PFA-100, VWF antigen, or VWF collagen binding activity (VWF:CB), compared with baseline, although, at 8%, the baseline VWF:CB activity was well below the human reference interval (51% to 143%)(Meiring et al., 2007). VWF

ristocetin cofactor activity (VWF:RCo) was reduced at the 3-hour incubation time point, with a moderate loss of large VWF multimers at high streptokinase concentrations. TEGs showed evidence of primary fibrinolysis at the intermediate and high concentrations, and a slight (but discernible) concentration-dependent escalation in plasmin activity was evident, all of which indicated at least some effect that could potentially be therapeutically harnessed in vivo.

In the in vivo part of the study, which involved the use of two baboons (a low & intermediate-dose test animal, and a separate, high-dose test animal) administration of escalating intravenous streptokinase doses was not effective in resolving the clinical features of aTTP in either animal (mostly mild mucocutaneous haemorrhages), and also had no (or only minimal) effects on the main laboratory parameters. Actual plasmin activity was undetectable (0%) throughout the low & intermediate-dose experiment, and reached a maximum level of only 12% in the high-dose test animal, with minimal effects on the VWF antigen levels (VWF:Ag) and multimer patterns. Plasminogen levels generally declined immediately post-streptokinase infusion, possibly suggesting consumption during fibrinolysis activation. However, this effect was temporary, with a generally upward trend as TTP was progressively established. At low & intermediate streptokinase doses, PAP complex levels showed a very similar pattern suggesting they may be formed by endogenous plasminogen activation consequent on the rising plasminogen levels (which is possibly secondary to TTP), rather than on meaningful plasminogen activation induced by streptokinase. In contrast, in the high-dose test animal, PAP complex levels remained essentially unchanged from baseline (or even decreased slightly) after streptokinase infusion, indicating negligible free plasmin activity. Although fibrinogen concentrations did decrease significantly during streptokinase treatment suggesting possible fibrinogenolysis, concomitant increases in FDPs were not observed.

The pilot study demonstrated that streptokinase was not able to resolve TTP in the Chacma baboon aTTP model, possibly due to its limited activation of the *Papio ursinus* fibrinolytic system, in contrast to its potent effects in humans (Lijnen et al., 1992). Other possibly significant differences between the human and *Papio ursinus* haemostatic systems also emerged, such as a marked discrepancy between the baseline in vitro (8%)

and low & intermediate dose in vivo experiment (7%) VWF:CB activity results, and the human reference interval (51% to 143%) (Meiring et al., 2007). Furthermore, the baseline ADAMTS13 antigen and activity levels of 8% and 33%, respectively, in the low & intermediate-dose test animal, were well below the human reference intervals of 70%–160% and 40%–130% for these assays (Peyvandi et al., 2010).

There was also variation between the fibrinolytic system data obtained from the two in vivo studies, with baseline PAP complex levels and plasminogen concentrations differing dramatically between the two (otherwise identical) test animals. The lack of published reference intervals for these parameters in *Papio ursinus* limits researchers' ability to correctly interpret data from this model. Inherent haemostatic system (including fibrinolytic system) differences across the various *Papio spp*. is also conceivable and pose an additional potential research opportunity.

The candidate and his promoters concluded that alternative thrombolytic drugs (such as uPA or tPA) should first be explored in this model to establish proof-of-concept before streptokinase can be tested in human clinical trials, or discarded outright as a potential future treatment option. Ultimately, this study highlighted critical hiatuses in our knowledge of Chacma baboon haemostasis and emphasised the need for a more comprehensive characterisation of this model and its limitations. Moreover, it signalled the presence of possibly significant differences between the *Papio ursinus* and human haemostatic systems, which may also have implications for the aTTP model's broader applicability and translational value, which should be explored in future studies. Specifically, large interindividual variation needs to be excluded as a cause for the limited streptokinase-induced plasminogen activation for instance, by repeating the pilot study assays in a larger number of animals. A deeper exploration of streptokinase's maximum plasminogen activation potential in vitro may also be useful, to decide whether a repeat of the pilot study using even higher streptokinase doses is justified.

2.7 Summary of literature review

TTP remains a partially understood condition for which additional treatment options are actively sought, resulting in numerous research opportunities. The validated *Papio*

ursinus model of aTTP is ideally placed to investigate novel theories and treatment strategies, such as the thrombolytic drugs like streptokinase. Although the effects of streptokinase have been studied in other baboon models and in the *Papio ursinus* model of aTTP, they have not been investigated in *Papio ursinus* under physiological conditions, whether in vitro or in vivo. There are also many other haemostatic aspects of this model (including factors pertaining to TTP) that remain to be explored.

Chapter 3 - **Motivation, Aim and Objectives**

3.1 Motivation

As discussed in section 2.4.2 of the Literature Review above, many key aspects of Chacma baboon haemostasis (including factors of direct importance in TTP) remain to be explored. There is no or very limited published data on reference intervals in this species for the concentrations and activities of the main role-players in TTP (ADAMTS13 and VWF), and how these differ from humans. There is a shortage of information on the *Papio ursinus* fibrinolytic system in general, its level of homology with human fibrinolysis, and its interplay with other components of the haemostatic system, both under physiological conditions and when stimulated by plasminogen activators such as the thrombolytic drugs. In addition, there is also no data on the thrombin generation capabilities of this species' haemostatic system.

The recently published pilot study reviewed above (Section 2.6 of the Literature Review, Appendix A) has highlighted the importance of these gaps in our knowledge and reiterated the need for a more thorough characterisation of this model. Moreover, it has warned of possible fundamental differences between *Papio ursinus* and human haemostasis.

All these factors have bearing on:

- Our understanding of TTP
- The continued use of this animal model to investigate TTP
- The use of *Papio ursinus* in other experiments involving the haemostatic system
- The translational value of results generated in this species, especially regarding the use of streptokinase in the treatment of human TTP

It is clear from the pilot study results that streptokinase was ineffective in treating aTTP in the *Papio ursinus* model. More than merely investigating *why* it was ineffective in this model, one needs to take a step even further back first to *define* this model's parameters before additional studies can be performed rationally.

The first step in this process, to define the context of this and future studies, is a more complete haematological characterisation of the *Papio ursinus* fibrinolytic system, ADAMTS13, VWF and thrombin generation capability.

3.2 Aim

To characterise the components of the Chacma baboon (*Papio ursinus*) haemostatic system relevant to the pathophysiology of aTTP (and its possible treatment with streptokinase or other thrombolytic agents).

3.3 Objectives

3.3.1 Primary objectives

1. To determine normal reference intervals/patterns in the Chacma baboon for the following, and compare it with known human data and other animal model data, where available/applicable:
    - VWF:
        - VWF antigen level (VWF:Ag)
        - VWF Ristocetin cofactor activity (VWF:RCo)
        - VWF Collagen binding activity (VWF:CB)
        - VWF multimer pattern
    - ADAMTS13
        - ADAMTS13 antigen level
        - ADAMTS13 activity
    - Fibrinogen level
    - Fibrinolytic system component levels:
        - tPA

    - PAI-1
    - Plasminogen
    - PAP complex
    - TAFI
    - α2-antiplasmin
- Clot lysis ability:
    - Modified clot lysis time
- Thrombin generation ability:
    - Lag phase
    - Peak thrombin
    - Time to peak thrombin
    - Velocity index
    - Endogenous thrombin potential

2. To determine and compare the maximum plasmin activities generated in Chacma baboon plasma when activated by streptokinase and tPA in vitro, in comparison with human plasma.
3. To determine and compare the in vitro streptokinase and tPA concentrations at which maximum plasmin activity is obtained in Chacma baboon plasma compared to human plasma.
4. To determine the effect of thrombolytic-induced plasmin activity on Chacma baboon VWF multimer patterns when VWF is in the non-globular (unfurled) state, compared with the baseline (globular) state.

3.3.2 Secondary objective

To determine whether any of the parameters outlined above are influenced by ABO or Rh blood group in the Chacma baboon, should a sufficient number of test animals be obtained with enough ABO and Rh blood group variation.

Chapter 4 - **Methodology**

4.1 Study location

This study was conducted at the University of the Free State (UFS):

- All animal work was performed at the UFS Animal Research Centre's Primate Research Facility on the West Campus of the UFS Main Campus.
- Laboratory preparation and analysis was done at the Special Haemostasis Laboratory of the Department of Haematology and Cell Biology, UFS, and the Universitas Academic Laboratories of the National Health Laboratory Service (NHLS) at Universitas Academic Hospital.

4.2 Study design

The study was conducted primarily as a cross-sectional observational study with an in vitro experimental component.

4.3 Experimental animals

As part of the routine care of Chacma baboons at the Animal Research Centre of the UFS, all baboons are screened for tuberculosis (TB). Venous blood specimens are collected for TB culture from all baboons in the unit annually. Additional venous blood specimens were collected for the current study at this event in order to subject animals to only one phlebotomy. The environmental factors were identical for all animals in the breeding colony. Blood was collected from 51 baboons, from which a subgroup of 40 baboons was selected based on their ABO blood groups and sex, to have as broad a representation of the different ABO blood groups and sexes as possible. Only 10 baboons were of non-O ABO blood group and were all included. The rest were all of group O, from which the 30 animals with the largest available number of plasma aliquots (that would be able to accommodate all the planned tests) were selected. Only 14 animals were male

and were all included. All 11 baboons not selected for further testing were therefore female and of group O. All 51 animals screened were either adults or young adults.

The exact genetic heterogeneity of the animals is unknown, but it is unlikely that they were all derived from the same original breeding pair (or pairs) since various different baboons were used for breeding purposes in the colony in the past.

4.4 Drugs

Animals were sedated using ketamine hydrochloride (Anaket V®, Bayer Pharmaceuticals, Isando, South Africa) at a dosage of 1 mg/kg intramuscularly every 30 minutes or as needed. No study drug or other compound was administered. No animal was euthanized after sampling.

4.5 Adverse effects

No serious adverse effects were encountered, as only a single routine phlebotomy was performed. Mild, self-limiting nausea or vomiting due to the anaesthetic can occur, but was not encountered. Superficial bruising at the site of phlebotomy may also be experienced, but it was not evident, nor was any excessive bleeding.

4.6 Post-procedure care

Animals were temporarily housed in single-animal holding cages at the Primate Research Facility on the West Campus of the UFS during and after sampling, for as long as deemed necessary by the staff of the facility, who also monitored the animals during and after sampling.

Routine care during and after sampling continued as per Primate Research Facility protocols, consisting of personnel directly observing the animal's condition (including on weekends and public holidays), with assessments also being documented by staff.

Monitoring included direct interaction with the animal, noting its level of interaction with staff, its ability to vocalise and track movement, its tendency to spontaneously take food and water, as well as any possible bleeding from venepuncture or other sites. Monitoring frequency was dependent on the clinical progression of the animal.

In the unlikely event that an animal required euthanasia, Sodium Pentobarbitone (Euthanaze®, 220 mg/mL, Bayer Pharmaceuticals, Isando, South Africa) was available on site, to be administered at a dosage of 1 mg/kg intravenously if needed.

4.7 Methods

4.7.1 Sample acquisition and preparation

All blood sampling and administration of sedation was performed by a South African Veterinary Council (SAVC) registered veterinarian of the Animal Research Centre, with assistance from the candidate. Blood sampling was performed under sedation, with ketamine-hydrochloride given intramuscularly every 30 minutes at 1 mg/kg body weight, as needed. All doses were based on the measured body weight of each animal. Animals' eyelid and toe pinch reflexes were monitored during sedation. Animals were never left alone while sedated. To prevent hypothermia animals were kept under a blanket and their extremities covered with sleeves/socks during periods of sedation.

All blood sampling procedures were performed with strict aseptic technique: the insertion area was cleaned with povidone-iodine (Betadine®, Mundipharma, Cape Town, South Africa) in a circular pattern beginning at the insertion site and gradually moving outward in concentric circles.

Blood specimens were collected into BD Vacutainer® tubes (BD Biosciences, Franklin Lakes, New Jersey, USA) and were drawn from a 20 gauge IV catheter inserted in the femoral/cephalic/great saphenous vein by an experienced veterinarian, with assistance from the candidate. A maximum of fifteen separate 5 mL specimen tubes, each containing 0.5 mL 3.2% sodium citrate, were filled with 4.5 mL blood per tube, for a maximum total of 67.5 mL citrated whole blood per baboon. Sampling volumes, therefore, did not exceed 10% of the total blood volume, based on a total blood volume of 70 mL/kg measured body weight (Fortman et al., 2002). As approximately 2 mL of blood was initially drawn for TB

culture, the maximum volume of blood collected from any given baboon during this event was 69.5 mL, which fell within the 10% (+/- 70 mL) limit.

Platelet poor plasma was prepared primarily by the candidate, with assistance from technical personnel of the Special Haemostasis Laboratory of the Department of Haematology and Cell Biology. Whole blood was centrifuged at 1500 x *g* for 15 minutes at room temperature, then aliquoted and stored at -80°C as soon as possible in the Special Haemostasis Laboratory, until testing. The aim was to have all aliquots frozen within 90 minutes of specimen collection.

4.7.2 Observational assays

Where available, assays were performed according to the standard operating procedures (SOPs) of the Special Haemostasis Laboratory of the Department of Haematology and Cell Biology, UFS, and the NHLS. Where no SOPs were available, assays were performed according to the kit manufacturer's instructions or as outlined below. Unless otherwise specified, the reference sample/standard for each assay was pooled normal human plasma, or a manufacturer-supplied standard of human origin.

Many of these assays, especially those employing monoclonal antibodies directed against human antigens, might not be optimal to assess the haemostatic parameters of the Chacma baboon. Due to cost-constraints, the choice of these assays was based on local availability and routine practice, and since this study was a first, preliminary attempt at characterising these haemostatic parameters in the Chacma baboon, multiple independent assays of the same parameter (but with a different methodology, such as molecular studies for instance) were not used. The rationale was that should marked discrepancies be identified with any of the assays outlined below, alternative approaches would be employed in future studies to investigate these discrepancies.

4.7.2.1 ADAMTS13 antigen

ADAMTS13 antigen levels were measured using an enzyme-linked immunosorbent assay (ELISA) kit (TECHNOZYM® ADAMTS13 Antigen ELISA kit, Technoclone GmbH,

Vienna, Austria) according to the manufacturer's instructions. The ADAMTS13 Antigen ELISA kit is a chromogenic ELISA to determine ADAMTS13 antigen concentration in human plasma. The kit contains ELISA test strips pre-coated with a monoclonal anti-ADAMTS13 capture antibody, directed against the CUB domains of the protein.

To measure the ADAMTS13 antigen concentration, plasma incubation was followed by the addition of a polyclonal anti-ADAMTS13-peroxidase (POX) conjugate. The chromogenic substrate tetramethylbenzidine (TMB) was added after the excess sample and conjugate had been washed off in separate washing steps. After a 15-minute incubation period was stopped using a sulphuric acid stopping solution, the colour change was measured within 10 minutes, using an ELISA reader, at 450 nm. The ADAMTS13 antigen concentration was read from a human calibration reference curve.

4.7.2.2 ADAMTS13 activity

ADAMTS13 activity levels were measured according to the manufacturer's instructions, using a chromogenic ELISA kit (TECHNOZYM® ADAMTS13 Activity ELISA Kit, Technoclone GmbH, Vienna, Austria) designed for the determination of ADAMTS13 activity in human plasma. The kit's ELISA test strips are pre-coated with a monoclonal anti-glutathione-S-transferase (GST) antibody. A recombinant VWF fragment (VWF73) that contains the ADAMTS13 cleavage site, is tagged with GST to form the GST-VWF73 substrate solution, which was then added to the anti-GST-coated test strips and incubated. Subsequent incubation with the test plasma allowed cleavage of the VWF73 fragment by any ADAMTS13 present in the test sample. This step was followed by the conjugation reaction, with a horseradish peroxidase (HRP)-conjugated monoclonal anti-N10 antibody that recognises only the cleaved VWF fragment. TMB colour reagent was used to detect the reaction.

Measurements were performed using an ELISA reader with a 450 nm filter, and the concentrations were read from the calibration reference curve.

4.7.2.3 VWF antigen (VWF:Ag)

An ELISA-based assay that measures the amount of VWF in test plasma was used. An ELISA plate (high binding, Nunc, Denmark) was pre-coated with a polyclonal rabbit anti-human VWF antibody (Dako, USA) diluted at 1:6000 in phosphate buffered saline (PBS) that captures the VWF to be measured, and incubated overnight at 4°C in a humid container. After the incubation period, the plate was washed once with PBS/0.1% Tween-20 (Merck, USA) using a microplate washer (VACUTEC, SA) to remove any unbound antibody. The 6th international standard (National Institute for Biological Standards and Control; NIBSC, United Kingdom) was used as a calibrator and added in the following concentrations: 100%, 50%, 25%, 12.5%, and 6.25%. Normal (N) and abnormal (P) controls (Siemens, Tarrytown, USA) were also evaluated to ensure accuracy and reproducibility of results. Test samples and controls were double diluted at 1:50 and 1:100 in PBS/0.1% Tween. PBS/Tween was used as a blocking solution to cover all the remaining binding surfaces of the plate that were not occupied by the coated protein. Standard, plasma and control samples (100 µL) were added to coated wells in duplicate, covered with Parafilm (Lasec, USA), and incubated for 2 hours at 36°C. After 2 hours, the plates were washed four times with PBS/0.1% Tween-20 wash buffer. A polyclonal rabbit anti-human VWF antibody conjugated to HRP (Dako, USA, 1:8000 dilution in PBS/Tween) was then added and incubated for 1 hour at room temperature. The plate was rewashed four times, followed by the addition of 90 µL/well colour developer solution (0.1 M citric acid, 0.2 M Na_2HPO_4, OPD, H_2O_2) for colour development. After 3 minutes, 30 µL/well stop solution (sulphuric acid; 4 M H_2SO_4) was added to stop the reaction. The absorbance was read at 490 nm for colour intensity using a microplate reader (BioTek Synergy HT reader, USA). The colour intensity produced is directly proportional to the VWF concentration initially present in the test sample. The BioTek Synergy HT software calculates the results automatically.

The final results were read from the calibration reference curves, and the actual VWF:Ag concentrations were calculated using GraphPad Prism® (GraphPad Software, USA). The values are expressed as percentages (Meiring, 2011a).

4.7.2.4 VWF ristocetin cofactor activity assay (VWF:RCo)

This assay measures platelet binding to VWF in test plasma. Formalin-fixed washed platelets require normal plasma, as a source of VWF, to agglutinate in the presence of ristocetin. The resultant agglutination then follows a dose-response curve that is dependent on the quantity (and quality) of the added plasma VWF. The ristocetin cofactor assay was done using a PAP-8E whole-blood light transmission aggregometer (Bio/Data Corporation, Horsham, Pennsylvania, USA). The test plasma activity was measured against a standard curve of calibrated human plasma and expressed as percentages (Meiring, 2011c).

4.7.2.5 VWF collagen binding activity assay (VWF:CB)

This is an ELISA-based functional assay that measures the ability of large VWF multimers to bind to collagen. The method for this assay was almost identical to the method described above for the VWF:Ag assay, except that the ELISA plate was pre-coated with human type III umbilical cord collagen (50 µg/mL) instead of an anti-human VWF antibody. Dilutions of test plasma were then added to the collagen-coated ELISA plate, and the amount of bound VWF was assessed using an anti-HRP-conjugated VWF antibody (1:3000 dilution in PBS/Tween). The values are expressed as percentages (Meiring, 2011b).

4.7.2.6 VWF multimer analysis

This procedure enables visualisation of VWF's multimeric structure in plasma. Multimer patterns were determined by employing the rapid visualization method described by Krizek and Rick (Krizek and Rick, 2000), which involves electrophoretic separation of the multimers on an agarose gel, followed by the transfer (or "blotting") of the VWF multimers onto a polyvinylidene fluoride membrane. Localisation is facilitated by the binding of a polyclonal rabbit anti-human VWF/HRP, which is followed by luminographic visualisation, aided by x-ray film and cassettes.

Using this method, after the submerged horizontal agarose gel electrophoresis had been completed, VWF was manually transferred onto a polyvinylidine fluoride membrane for immune-localisation and subsequent luminographic visualisation of the multimer pattern. In addition, the individual multimer densities were determined using a gel-documentation system, and the individual VWF multimer patterns were compared with normal human plasma (Kelderman and Meiring, 2011).

Since multimers are distributed on a continuum using this method, the distinction between low, intermediate and high molecular weight multimers was made semi-arbitrarily, based on their location on the gel. High molecular weight multimers are visible at the top of all figures in this thesis, intermediate weight multimers in the middle, and low weight multimers towards the bottom. The lowest bands in each pattern (separated from the rest of the full multimer pattern by an empty space) represent collections of VWF dimers, which do not migrate with the low molecular weight multimers.

4.7.2.7 Fibrinogen

Fibrinogen was measured using the Clauss fibrinogen method, in an ISO15189 accredited laboratory, on a Sysmex CS-2100i® fully automated coagulation analyser (Sysmex, Kobe, Japan, supported locally by Siemens), according to the manufacturer's instructions and NHLS SOPs. Briefly, in principle, an excess of the enzyme thrombin is added to test plasma, which converts the soluble plasma protein fibrinogen into its insoluble polymer, fibrin. The clotting time for the diluted plasma is inversely proportional to the fibrinogen concentration in the test plasma (Laffan, 2006). This assay was performed by technical personnel of the coagulation section of NHLS Universitas under the candidate's direction.

4.7.2.8 Plasminogen

A Glu-Plasminogen ELISA kit (TECHNOZYM® Glu-Plasminogen ELISA kit, Technoclone GmbH, Vienna, Austria) was used to measure Glu-plasminogen levels. Glu-plasminogen

is the predominant native plasminogen that is converted to active plasmin through two consecutive cleavage actions by specific plasma proteases (Israels and Israels, 2013). Plasmin itself also modifies Glu-plasminogen and exposes lysine binding sites to form the Lys-plasminogen variant that, through its superior fibrin binding and activation capacity, mediates a positive feedback mechanism leading to the enhanced conversion of plasminogen to plasmin (Israels and Israels, 2013). By assessing Glu-plasminogen instead of Lys-plasminogen or plasmin, the total potential initial pool available for plasmin formation is therefore considered.

The Glu-Plasminogen ELISA test is a chromogenic solid-phase enzyme immunoassay, and the mAbs employed in this test system recognise only uncleaved Glu-plasminogen. The results are, therefore, not affected by the presence of PAP complexes or plasmin-modified Lys-plasminogen.

The ELISA test strips, which had been pre-coated with a monoclonal anti-plasminogen antibody and blocked with 1% bovine serum albumin, were incubated with the test samples and controls. A POX-conjugated, monoclonal anti-plasminogen antibody was added, followed by the TMB substrate. Measurement was performed using an ELISA reader with a 450 nm filter, and concentrations read from the calibration reference curve.

4.7.2.9 tPA

tPA levels were measured using a tPA antigen ELISA kit (TECHNOZYM® t-PA Antigen ELISA kit, Technoclone GmbH, Vienna, Austria) according to the manufacturer's instructions. It consisted of ELISA test strips pre-coated with an anti-tPA monoclonal capture antibody. After incubation of test plasma, a POX-conjugated anti-tPA antibody was added, followed by the chromogen TMB as substrate. Measurement was performed using an ELISA reader at 450 nm, and concentrations were read from the calibration reference curve.

4.7.2.10 PAI-1

PAI-1 levels were also measured using an ELISA method, according to the manufacturer's instructions. The PAI-1 antigen ELISA kit (TECHNOZYM® PAI-1 Antigen ELISA kit, Technoclone GmbH, Vienna, Austria) consisted of ELISA test strips coated with anti-PAI-1 monoclonal capture antibody, to which test plasma was added, followed by a POX-conjugated anti-PAI-1 antibody. Upon addition of the TMB chromogenic substrate, a colour change ensued, which is directly proportional to the amount of PAI-1 in the test plasma. This colour change was measured at 450 nm using an ELISA reader, and compared with a calibration reference curve.

4.7.2.11 PAP complex

PAP complex levels were also measured using an ELISA kit (TECHNOZYM® PAP Complex ELISA Kit, Technoclone GmbH, Vienna, Austria) according to the manufacturer's instructions. The PAP complex ELISA kit consisted of ELISA test strips pre-coated with a monoclonal capture antibody directed against the neoantigen in the PAP complex, in which test plasma and controls were incubated. A conjugated, polyclonal anti-plasminogen antibody (POX-conjugated antibody) was added, followed by its TMB substrate. An ELISA reader with a 450 nm filter was used to perform the measurement, and concentrations were read from the calibration reference curve.

4.7.2.12 TAFI

TAFI concentrations were determined using a high-sensitivity ELISA kit (Human TAFI ELISA Kit, Elabscience, Houston, Texas, USA) which uses the Sandwich-ELISA principle, according to the manufacturer's instructions. The micro ELISA plate provided in the kit had been pre-coated with an anti-human TAFI-specific capture antibody. Standards or samples were added to the micro ELISA plate wells and combined with the specific antibody. Then a biotinylated detection antibody specific for human TAFI and Avidin-HRP conjugate were added successively to each micro plate well and incubated. Free

components were washed away, and the substrate solution was added to each well. The enzyme-substrate reaction was terminated by adding a stop solution, and the optical density (OD) was measured spectrophotometrically at a wavelength of 450 nm ± 2 nm. The OD value is proportional to the concentration of TAFI in the sample, which was then calculated by comparing the OD of the sample to the standard reference curve.

ELISA kits with differing sensitivities and quantification ranges are available for the measurement of TAFI concentrations. We selected a high-sensitivity kit, with a detection range of 5.00 to 320 ng/mL and a sensitivity of 3.00 ng/mL. Although human reference intervals for TAFI are usually in the µg/mL range, this kit was selected in order to be certain that low concentrations of TAFI would still be detected and accurately quantified, even if present at exceedingly low levels. In the event that this kit demonstrated baboon sample TAFI values to be >320 ng/mL, a second kit with a higher detection range would have been used.

4.7.2.13 α2-antiplasmin

α2-antiplasmin levels were determined using the SimpleStep ELISA® kit (Abcam, Cambridge, United Kingdom), which employs an affinity tag labelled capture antibody and a reporter conjugated detector antibody, for immunocapture of the sample analyte of interest. This complex (capture antibody/analyte/detector antibody) is consequently immobilised by an anti-tag antibody, coating micro ELISA plate wells. Samples or standards were added to the wells, followed by the antibody mixture. After incubation at room temperature for 1 hour, the wells were washed to remove any unbound substances. TMB development solution was added and catalysed by HRP during incubation, leading to blue colouration. The reaction was then stopped by adding the stop solution, which completes the colour change from blue to yellow. The resultant OD is proportional to the amount of bound α2-antiplasmin and was measured spectrophotometrically at 450 nm. The final concentration of α2-antiplasmin in each sample was then calculated by comparing the OD of the sample to the standard reference curve.

4.7.2.14 Thrombin generation

Thrombin generation was measured on the Technoclone Ceveron Alpha instrument (Technoclone GmbH, Vienna, Austria) according to the manufacturer's instructions. The TECHNOTHROMBIN TGA assay is based on monitoring the fluorescence generated by the cleavage of a fluorogenic substrate by thrombin over time, upon activation of the coagulation cascade by a low concentration of tissue factor (1 picomolar) and negatively charged phospholipids in plasma (RC Low reagent, Technoclone GmbH, Vienna, Austria). From the changes in fluorescence over time, the concentration of thrombin in the sample can be calculated using the reference thrombin calibration curve. The increase in thrombin concentration with time then allows calculation of total thrombin generation in the sample. The *Papio ursinus* total endogenous thrombin generation potential and thrombin generation curves were then compared with known data for humans and other animal models.

4.7.2.15 ABO and Rh blood group

ABO and Rh blood grouping were performed according to NHLS SOP (Venter et al., 2018). The test procedure is based on the principle of antibody-induced erythrocyte clumping. Erythrocytes with a given ABO or Rh blood group antigen on their surface will agglutinate when exposed to immunoglobulin M (IgM) antibodies directed against the given antigen. Briefly, 2-5% erythrocyte suspensions were prepared and mixed with anti-A, anti-B, anti-AB and anti-D (Rh) antibodies to determine the red cell ABO and Rh phenotype (forward grouping). Additionally, in the ABO blood group system, as an individual's serum will naturally contain antibodies directed against the particular antigen(s) lacking on their erythrocytes, reverse grouping was also performed as a confirmatory step, whereby test serum was added to reagent red cells of known ABO blood group. These assays were performed by technical personnel of the immuno-haematology section of NHLS Universitas under the candidate's direction.

4.7.3 Assays with an experimental component

Those assays that involved some form of experimental manipulation are grouped below:

4.7.3.1 Plasmin activity

Baseline streptokinase-induced plasmin activity was measured chromogenically, using a method obtained from the IRF Life Sciences Laboratory for Thrombosis Research at the Kulak Kortrijk Campus of KU Leuven, Kortrijk, Belgium (C Tersteeg 2017, personal communication, 30 August). Briefly, a commercially available chromogenic plasmin substrate *N*-Tosylglycyl-L-prolyl-L-lysine 4-nitroanilide acetate salt (TOS-Gly-Pro-Lys-4-NA; Merck KGaA, Darmstadt, Germany) was added to 2 µL buffered test plasma, after which streptokinase (10 µL of a 400 IU/mL solution) was added, and plasmin activity measured kinetically at 405 nm for 2 hours with an ELISA reader set at >37°C. Readings were compared with a standard curve derived from commercially available pooled buffered normal human plasma and reported as a percentage. This procedure was also performed using a comparable amount of recombinant human tPA (alteplase; Actilyse®, Boehringer Ingelheim, Ingelheim am Rhein, Germany) as plasminogen activator (10 µL of a 2700 µg/mL solution). The total doses of streptokinase and alteplase used to treat STEMI in humans is 1 500 000 IU and 100 mg (in patients >67 kg), respectively (O'Gara et al., 2013). Therefore, the concentrations of these solutions represent approximately 0.03% of the total STEMI treatment dose per millilitre.

These procedures were repeated using escalating concentrations of streptokinase and tPA as activators, until the point of maximum streptokinase-generated plasmin activity and maximum tPA-generated plasmin activity was reached. It was done by adding 10 µL of a streptokinase or tPA solution of the following concentrations until a plateau in plasmin activity was observed:

- Streptokinase: 800 IU/mL, 3200 IU/mL, 25600 IU/mL
- tPA: 28.125 µg/mL, 56.25 µg/mL, 112.50 µg/mL, 225 µg/mL, 450 µg/mL, 900 µg/mL, 1800 µg/mL, 2700 µg/mL, 3600 µg/mL, 5400 µg/mL, 7200 µg/mL.

4.7.3.2 Modified clot lysis time

Plasma clot lysis was assessed based on a modification of the standardised method of clot lysis time (CLT) determination proposed by Pieters et al. for the Subcommittee on Factor XIII and Fibrinogen, and the Subcommittee on Fibrinolysis, of the Scientific and Standardization Committee (SSC) of the International Society on Thrombosis and Haemostasis (ISTH) (Pieters et al., 2018). Briefly, in the standardised method described by Pieters et al. (2018), buffered plasma with and without tPA is clotted at 37°C with an activation mix consisting of human α-thrombin, $CaCl_2$ and buffer, in the presence of an excess of phospholipids, in a 96-well ELISA plate. Changes in turbidity are then read with an ELISA reader, measuring the absorbance at 405 nm every 10 seconds up to 30 minutes (180 readings) and then every minute until the clots have broken down or up to 180 minutes (n=150 readings). The timepoint at which 50% clot lysis has occurred is defined as the CLT. This method was used as a basis for the modified CLT, reconfigured for baboon plasma (please see modifications below).

The 50% CLT was calculated from a graph on the Prism software program, using a Boltzmann sigmoidal curve fitting, as indicated in Figure 4 below. The 50% CLT was calculated as the time in minutes from the midpoint of the ascending curve (from clear to maximum turbidity), to the midpoint in the descending curve (the transition from maximum turbidity to the final baseline turbidity). These midpoints were identified by applying two separate sigmoidal curve fittings to the ascending and descending parts of the turbidity curves. The Prism software programme was used to calculate the midpoints from the fitted curves. The time of the midpoint of the ascending curve was then subtracted from the time of the midpoint of the descending curve to provide the 50% CLT.

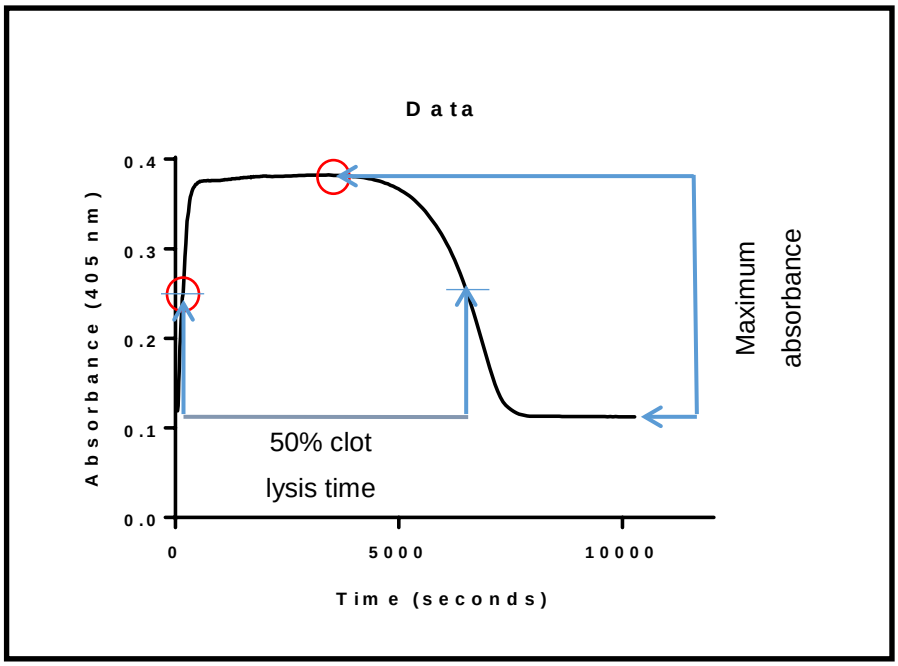


Figure 4: Positions on the turbidity curve to be used to determine the 50% clot lysis time. From Pieters et al. (Pieters et al., 2018). *Reproduced unaltered with author's permission.*

According to the original method, each laboratory should first determine the tPA concentration to be used locally, by establishing the concentration at which a CLT of 100 ± 5 minutes is obtained on lyophilized NIBSC plasma. It is suggested to start these experiments with tPA concentrations in the range of 20-40 ng/mL.

The procedure was modified as follows:

- To apply the assay to the individual baboon plasma samples, a tPA concentration of 225 ng/mL was selected, since this was the lowest concentration able to elicit complete clot lysis in both pooled normal human plasma and pooled baboon plasma samples, but resulted in a CLT of ±10 minutes on pooled baboon plasma and ±13 minutes on pooled normal human plasma. The pooled normal human plasma used for this experiment was prepared from 3.2% citrated plasma previously collected before 11:00 in the morning from 20 fasting, healthy individuals. Exclusion criteria were pregnancy, elevated temperature, known blood disorders, use of oral contraceptives, or use of acute or chronic medication. Whole

blood specimens were kept at room temperature after collection for no longer than 30 minutes and then centrifuged at 2000 x g for 20 minutes to prepare platelet-poor plasma. Plasma of all individuals was pooled, mixed and aliquoted before freezing at -70°C. Pooled baboon plasma was prepared by pooling and mixing previously prepared individual baboon plasma aliquots that had been thawed in a waterbath at 37°C just before use.

- All plasma samples were clotted with locally available bovine thrombin (Dade® Thrombin, Siemens Healthcare GmbH, Erlangen, Germany) at a concentration of 2.5 NIH U/mL in the final activation mixture instead of human α-thrombin, due to ease of availability and cost constraints.
- The commercial phospholipid preparation recommended by Pieters et al. (Rossix, Cat no: PL604T, 0.5 mmol/L) was not used due to cost constraints. Instead, phospholipids were prepared locally as follows: 50 mL human platelet-rich plasma from the South African National Blood Service was washed twice with Tris-buffered saline and frozen for 20 minutes at -80°C, thawed at 37°C and frozen again. This freeze-thaw cycle was repeated until the platelet count was $<20 \times 10^9$/L, confirming that virtually all platelets were lysed, yielding a platelet lysate suspension of phospholipids.

4.7.3.3 Urea-altered VWF multimer analysis

Exposure of plasma to mild denaturing urea concentrations leads to a conformational change in the structure of VWF, causing it to transform from the globular state to an unfurled/unfolded state; a process referred to by some as “urea-induced unfolding” (De Cristofaro et al., 2006). To assess the effect of fibrinolytic-induced plasmin activity on *Papio ursinus* VWF multimer patterns when VWF is in the unfurled/unfolded (non-globular) state in comparison with the baseline (globular) state, VWF multimer patterns were also assessed (using the method described in section 4.7.2.6 above) after incubation of pooled *Papio ursinus* plasma for 30 minutes and 3 hours, with escalating concentrations of tPA (1800 µg/mL, 7200 µg/mL, and 10000 µg/mL), after overnight

dialysis in 1.5 M urea, as previously described (De Cristofaro et al., 2006). The tPA concentrations were selected based on the results of the tPA plasminogen activation potential experiments (see section 6.2.2.2 of Chapter 6 below), where a concentration of 1800 µg/mL resulted in mean plasmin activity that was closest to baseline human plasmin activity, 7200 µg/mL led to a plateau in plasmin activity in the majority of animals (n=31; 78%) and 10000 µg/mL represented an excess of tPA. This procedure was performed in the presence of an excess of the monoclonal anti-ADAMTS13 antibody 3H9, added to neutralise any ADAMTS13 effects on the unfurled VWF multimers. The 3H9 dose given in vivo in the *Papio ursinus* TTP model is 600 µg/kg (Feys et al., 2010a). Assuming a total blood volume of 70 mL/kg (Fortman et al., 2002), the dose given in vivo is approximately 0.00857143 µg/µL whole blood, or about 1 µg/110 µL whole blood. Assuming a maximum possible haematocrit of 0.49 L/L (Melton and Melton, 1982), the in vivo dose is roughly 2 µg/110 µL plasma. An excess of 3H9 was added to the pooled plasma test mixtures so that the final 3H9 concentration in each test well was 5 µg/110 µL. Effective neutralisation of ADAMTS13 activity was confirmed by performing the ADAMTS13 activity ELISA outlined in section 4.7.2.2 above on all specimens.

4.8 Statistical analysis

Statistical analysis was performed using the EP Evaluator® (version 10.3.0.556, Data Innovations, 2013), Microsoft Excel®, and XLSTAT-Biomed® statistical analysis software systems. Quantitative (e.g. antigen and activity values) and qualitative (i.e. VWF multimer patterns) data were generated.

Quantitative data were summarised using simple descriptive statistics, namely frequencies and percentages for categorical data, and means, medians, standard deviations (SDs), coefficients of variation (CVs), and percentiles, for continuous data.

Biological reference intervals were calculated for all parameters where relevant, following the Clinical and Laboratory Standards Institute (CLSI) guidelines for non-parametric data (CLSI., 2008). The central 95% interval of the distribution, i.e. the interval from the 2.5th percentile to the 97.5th percentile (or upper limit of this interval if appropriate), was defined

as the biological reference interval/limit, as stipulated in ISO standard 15189 (ISO, 2012). The CVs calculated were also compared with available human biological variation database values.

An unpaired Student's t-test was performed to determine if there was a significant difference ($p<0.05$) between ABO blood group O and non-O baboons and between male and female baboons for all parameters. The paired Student's t-test was used to determine if there was a significant difference ($p<0.05$) in the baseline and maximum *Papio ursinus* plasmin activities between the two different thrombolytic drugs (streptokinase and tPA).

Qualitative data, i.e. VWF multimer patterns, were compared with human patterns. The large, intermediate and small multimers were classified as absent/decreased/similar/increased, when compared to human plasma. These proportions of absent/decreased/similar/increased multimers were then compared across the different baboon subgroups (i.e. male vs. female, ABO blood group O vs. non-O) by cross-tabulation, with the Fisher's exact p-value.

4.9 Animal fate

All animals were returned to the holding area at the Primate Research Facility after the completion of sampling for physical examination by the staff of the Animal Research Centre, under the direction of the designated veterinarian, Dr Lizanne Meiring. After recovery from the effects of sedation, all animals were reintegrated into the colony.

No animal was euthanized during or after this study.

4.10 Ethics considerations

Ethics approval was obtained from the UFS Interfaculty Animal Ethics Committee before the commencement of the study: UFS-AED2019/0054 (Appendix B). Most of the pertinent ethical aspects have already been addressed elsewhere in this thesis. Importantly, only

purpose-bred animals were sampled; wild-caught baboons were not tested. No specimens were collected from humans or other animal species.

The 3R (Replacement, Reduction and Refinement) framework for humane animal research was addressed as follows:

4.10.1 Replacement

Life-threatening conditions such as TTP cannot be simulated by in vitro studies with sufficient levels of confidence to directly allow human trials, although new techniques to replace animal experimentation and to reduce animal distress are always sought.

For justification of human in vivo studies of potentially dangerous drugs in already severe conditions such as TTP, non-human primate data are required. In vitro and murine in vivo studies (as a replacement for non-human primate experiments) can only enhance our understanding of the potential clinical utility of novel drugs up to a point before non-human primate data becomes imperative to justify any human trials. The use of rat or murine models also hinders data collection due to the limited volumes of blood that can be obtained from these species. The next logical step is to obtain non-human primate data.

As the *Papio ursinus* model of acute TTP is validated and well-established, it would be prudent to characterise this model as extensively as possible so that any future data generated in this very useful model can be appropriately interpreted. As the development of a new lower-animal model would only represent a complicated, costly, and time-consuming regression, the continued use (and deeper exploration) of the established non-human primate model to find better treatment approaches is probably justified.

4.10.2 Reduction

No animal was venepunctured solely for study purposes. By obtaining samples at the time of TB screening, the total number of Animal Research Centre animals exposed to sedation and phlebotomy was greatly reduced. Sample acquisition and processing, with aliquoting and storage of frozen plasma, was also set up in such a way that the maximum

amount of data could be gathered from this single phlebotomy event. Repeat sampling of animals to obtain additional plasma was not required. The results of this study will potentially also enable future studies to use much fewer animals.

4.10.3 Refinement

In essence, this entire study represented an attempt at refinement. By characterising the established baboon model of TTP even better, future studies using this species will be much more refined. Because of this, future studies can be planned and executed more rationally. The data generated in future studies will be much better understood, with more contextualised conclusions.

Refinement was also addressed by providing species-appropriate housing, performing all sampling under sedation, maintaining vigilance for hypothermia, regularly interacting with the animals, and (perhaps most importantly) by using the ideal species for this study; *Papio ursinus*, in which the utility of the aTTP model is well-established.

Chapter 5 - **Results**

The results of the purely observational assays and calculated biological intervals are presented in this chapter separately from the results of the assays with an experimental component in Chapter 6.

Part I – Observational assays and biological reference intervals

5.1 Introduction

The observational assay results are presented in the sections below for all forty baboons. Unless otherwise stipulated, all results are compared with the normal human reference ranges provided by the kit manufacturers or with the ranges in use by the UFS Special Haemostasis Laboratory when the assays were performed. These ranges were calculated previously, using normal human samples, in accordance with CLSI guidelines (CLSI., 2010, CLSI., 2008) and the relevant ISO standard (ISO, 2012). It is important to note that all the assay kits we used are designed for use with human samples and not for use with other animal species.

5.2 ADAMTS13 antigen and activity

The ADAMTS 13 antigen and activity statistical analyses are summarised in Table 1 below. The antigen values were well below the human reference range of 41 – 141% for the particular kit used, with the highest value (21%) still falling well below the lower limit of the human reference range. The activity values were more comparable to the human reference range of 40 – 130%, although 18 of the 40 baboons tested (45%) had values below the human reference range.

Table 1: The range, median, mean, standard deviation (SD), coefficient of variation (CV), and central 95% interval of ADAMTS13 antigen (Ag) and activity (Act).

Parameter, with human reference range for kit	Range	Median	Mean	SD	CV%	Central 95% interval
ADAMTS13 Ag (%) **41-141% (Technoclone, 2012a)**	2-21	7	8	3	46	3-14
ADAMTS13 Act (%) **40-130% (Technoclone, 2011)**	22-70	40	41	11	27	23-69

5.3 VWF level and function

VWF was assessed using multiple methods. The quantitative and qualitative data are grouped separately below.

5.3.1 VWF antigen, ristocetin cofactor activity, and collagen binding activity

The statistical analyses of the VWF antigen level, as well as the VWF functional activity assays, are summarised in Table 2 below. Although the VWF:Ag values tended towards the lower limit of the human reference range, only four baboons (10%) had values below the lower limit cut-off value of 50%. The VWF:CB values also tended towards the lower limit of the human reference range, but the majority of baboons (55%, n=22) had values below the lower limit cut-off value of 51%. The VWF:RCo values tended towards the higher limit of the human reference range, with values above the upper limit cut-off value of 150%, encountered in 24 animals (60%).

Table 2: The range, median, mean, standard deviation (SD), coefficient of variation (CV), and central 95% interval of Von Willebrand factor antigen (VWF:Ag), ristocetin cofactor activity (VWF:RCo) and collagen binding activity (VWF:CB).

Parameter, with human reference range	Range	Median	Mean	SD	CV%	Central 95% interval
VWF:Ag (%) **50-150% (Meiring et al., 2009, Meiring, 2011a)**	27-94	68	69	16	23	47-92
VWF:RCo (%) **50-150% (Meiring et al., 2009, Meiring, 2011c)**	52-280	178	174	57	33	76-275
VWF:CB (%) **51-143% (Meiring et al., 2007, Meiring, 2011b)**	11-86	49	50	17	34	26-76

5.3.2 VWF multimers

The multimer patterns of each of the individual baboons are shown in Figure 5 below, which is a composite of all the individual multimer patterns obtained throughout four separate runs. Figure 6 and Figure 7 show the individual multimer densitograms of baboons 1 to 20 and baboons 21 to 40, respectively. Although not always identical to human patterns, all animals were deemed to have multimer patterns that are more or less equivalent to a normal human pattern, showing broad similarity with human patterns and intact intermediate and high molecular weight multimers. None of the baboon patterns would be interpreted as abnormal had they been regarded as routine human diagnostic specimens (Kelderman and Meiring, 2011, Meiring et al., 2009).

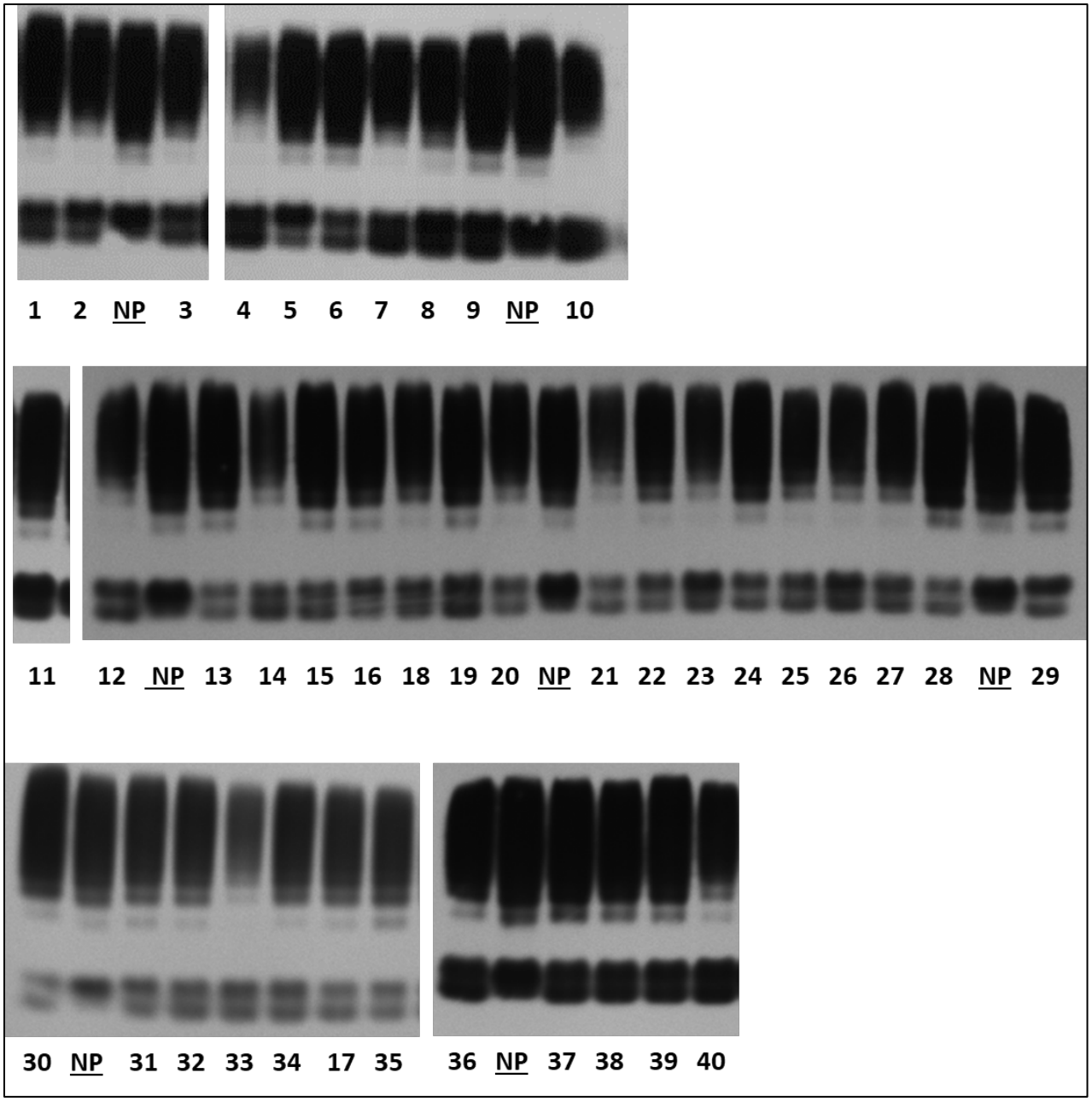


Figure 5: Von Willebrand factor multimer patterns of all baboons (n=40), compared with pooled normal human plasma (NP). Composite image of patterns obtained over four separate runs.

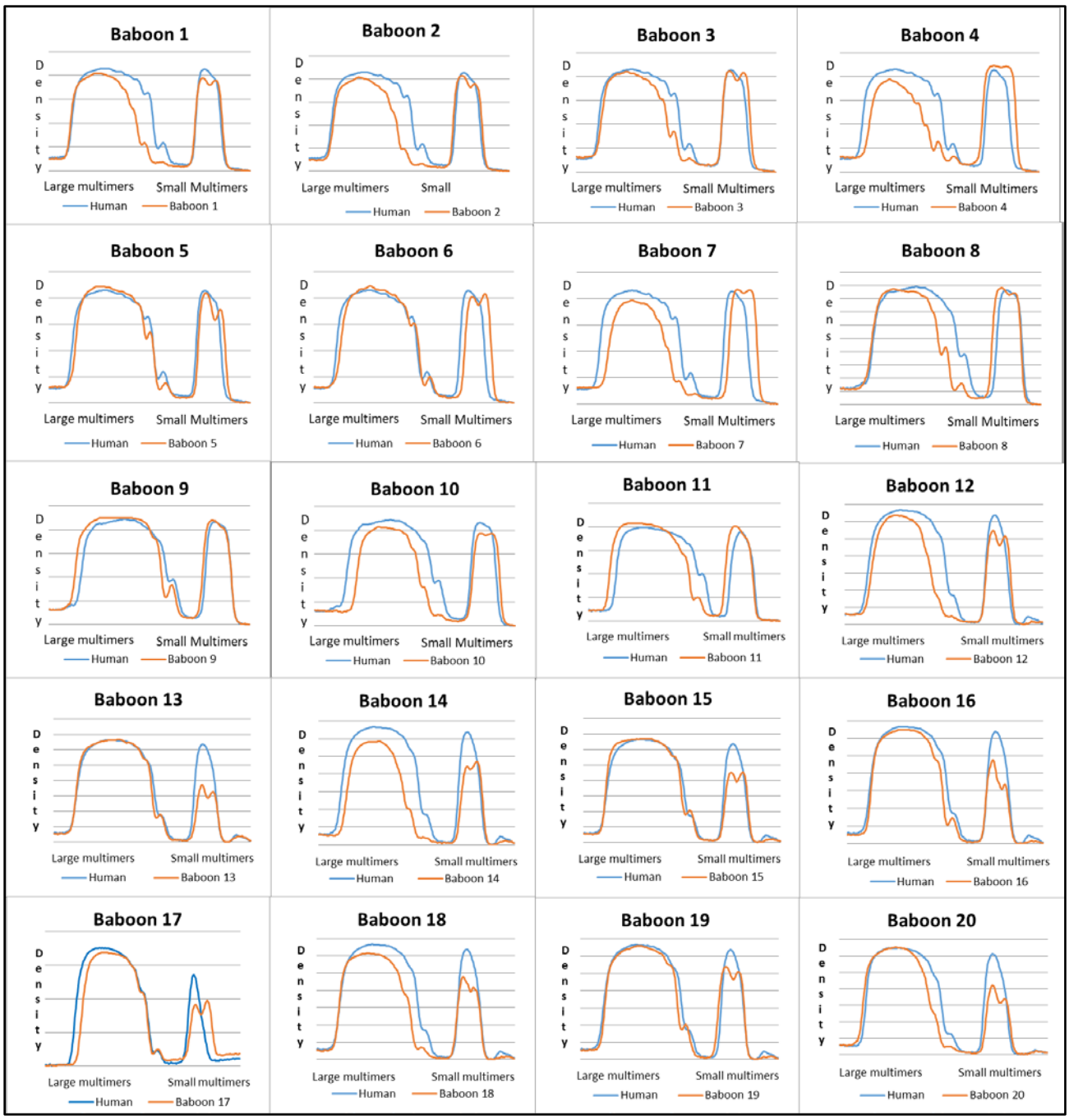


Figure 6: Individual Von Willebrand factor densitograms of baboons 1-20, compared with the pooled normal human plasma sample ran with each particular run (Human).

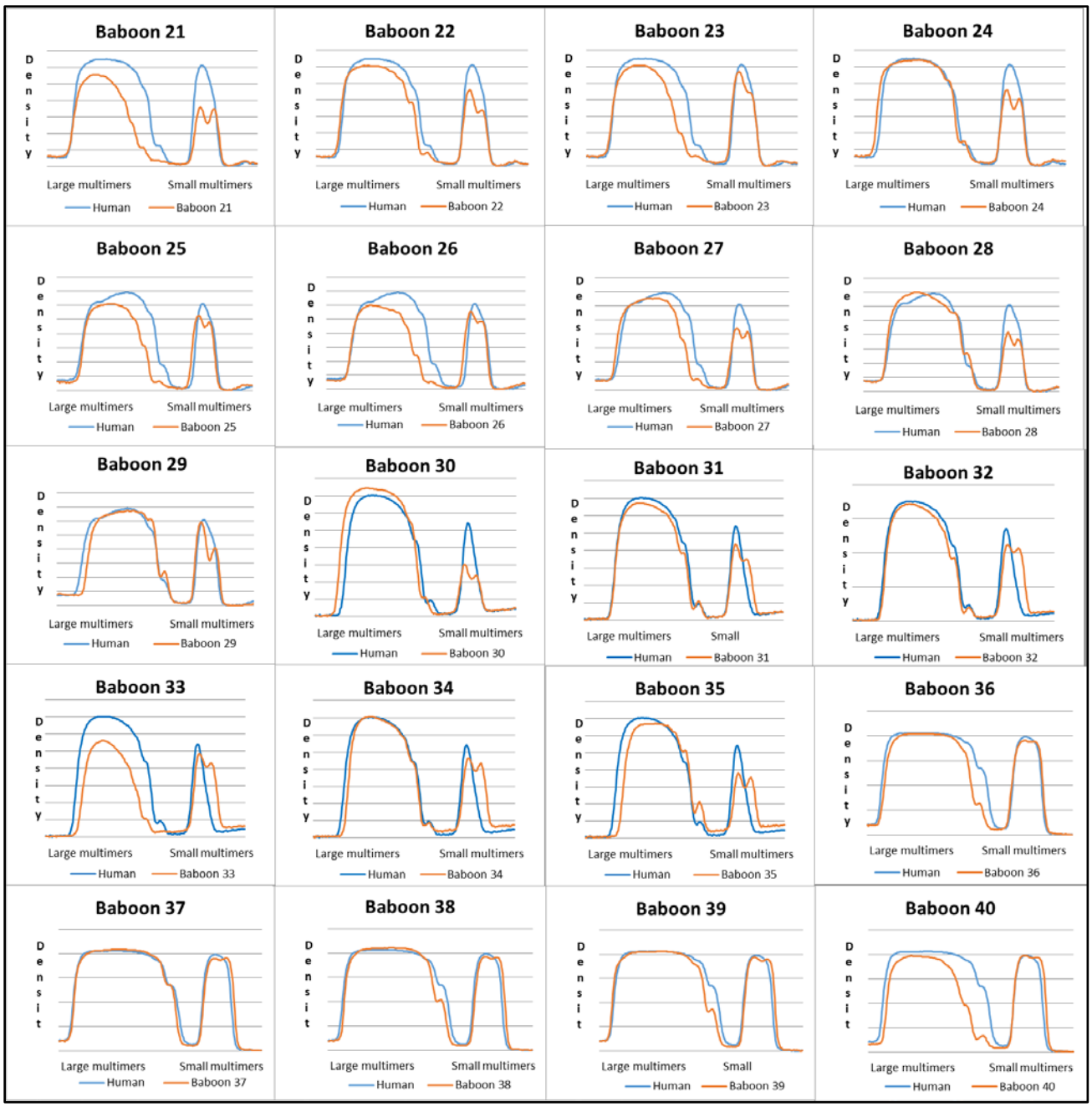


Figure 7: Individual Von Willebrand factor densitograms of baboons 21-40, compared with the pooled normal human plasma sample ran with each particular run (Human).

5.4 Fibrinogen and the fibrinolytic system

The statistical analyses of the fibrinogen level and the various components of the fibrinolytic system are summarised in Table 3 below. The fibrinogen levels were generally consistent with human values, with nine baboons (22.5%) falling below the human reference range cut-off value of 1.7 g/L. Plasminogen levels were virtually undetectable with the human ELISA kit used, with all values measuring far below the lower human reference range cut-off value for this kit of 60 µg/mL, even after the assay had been repeated on a second set of aliquots. tPA levels tended to the lower limit of the human reference range, with 21 baboons (52.5%) below the human reference range cut-off. The tPA central 95% interval was much narrower (1.4 ng/mL) than the range expected in humans (6 ng/mL). PAI-1 levels also tended towards the lower limit of the human reference range, and 11 baboons (27.5%) had values below the cut-off value. All animals had PAP complex levels within the human reference range, but unlike what may be seen in humans, no baboon had a PAP complex level of 0 ng/mL. TAFI was not detectable in 15 baboons (37.5%), with the rest of the values obtained also far below the human reference interval for this kit. Note that the Chacma baboon TAFI results are reported in ng/mL in Table 3, but that human reference intervals for this analyte (including the reference interval supplied by the manufacturer of the ELISA kit used in this experiment) are usually in the µg/mL range.

The very low, virtually undetectable levels of plasminogen and TAFI suggest that these two kits may not be suitable for quantifying these proteins in *Papio ursinus* plasma. Moreover, it suggests fundamental structural differences between these two proteins and their human equivalents, since ELISA kits designed for use in humans could not generate results with *Papio ursinus* plasma. It is likely due to structural differences that affect the binding of the antibodies used in these kits.

α2-antiplasmin concentrations varied dramatically between individual animals with a CV of 270.5%. Although α2-antiplasmin concentrations also tended to be well below the human reference interval for this kit (25 – 50 µg/mL), five animals (12.5%) did have values >10 µg/mL, with a value as high as 95.8 µg/mL obtained in one animal. In contrast to the plasminogen and TAFI data where these analytes were virtually undetectable in all

animals assayed, the α2-antiplasmin results suggest that this particular kit is suitable for use in *Papio ursinus* (although the kit does seem to be reasonably species-specific, with <3% reactivity for monkey, mouse, rat or cow α2-antiplasmin according to the manufacturer), and that Chacma baboon α2-antiplasmin concentrations are indeed lower than human concentrations.

Table 3: The range, median, mean, standard deviation (SD), coefficient of variation (CV), and central 95% interval of fibrinogen, plasminogen, tissue-type plasminogen activator (tPA), plasminogen activator inhibitor-1 (PAI-1), plasmin-α2-antiplasmin (PAP) complex, thrombin activatable fibrinolysis inhibitor (TAFI) and α2-antiplasmin values.

Parameter, with human reference range	Range	Median	Mean	SD	CV%	Central 95% interval
Fibrinogen 1.70-4.20 g/L (Conradie, 2015)	1.16-4.26	1.83	2.02	0.68	33.67	1.28-4.00
Plasminogen 60-250 µg/mL (Technoclone, 2014)	1.5-28.2	3.1	2.4	4.1	132.0	1.8-4.2
tPA 2-8 ng/mL (Technoclone, 2012d)	1.4-4.0	1.9	2.0	0.5	24.9	1.6-3.0
PAI-1 7-43 ng/mL (Technoclone, 2012b)	1.9-32.3	9.6	11.3	7.1	63.1	2.6-30.1
PAP complex 0-514 ng/mL (Technoclone, 2012c)	99.9-340.8	130.8	141.8	40.8	28.8	109.0-215.8
TAFI ng/mL 1700-20000 ng/mL (Elabscience, 2019)	0.0-66.3	1.8	9.7	15.4	158.8	0.0-59.3
α2-antiplasmin 25-50 µg/mL (Abcam, 2019)	1.2-95.8	1.5	5.7	15.3	270.5	1.2-21.8

5.5 Thrombin generation

The thrombin generation curves of all 40 test animals are shown below in Figure 8 (evaluated for 90 minutes) and Figure 9 (evaluated for 120 minutes), in comparison with

pooled normal human plasma and pooled normal human plasma as well as baboon plasma, respectively. Although there was wide interindividual variation in the thrombin peak, the time-to-peak and lag-time appeared less variable and similar to human plasma. However, the thrombin generated by baboon plasma did appear to persist for longer than with human plasma. After approximately 30 minutes, the gradual decrease in the available thrombin was less prominent in the baboon samples than in human plasma, with a protracted residual thrombin “tail” evident from the curves. On the extended run of 120 minutes shown in Figure 9 (a second run, performed additionally), it was apparent that thrombin generation persisted in *Papio ursinus* plasma long after it had ceased in the pooled normal human plasma; at approximately 90 minutes.

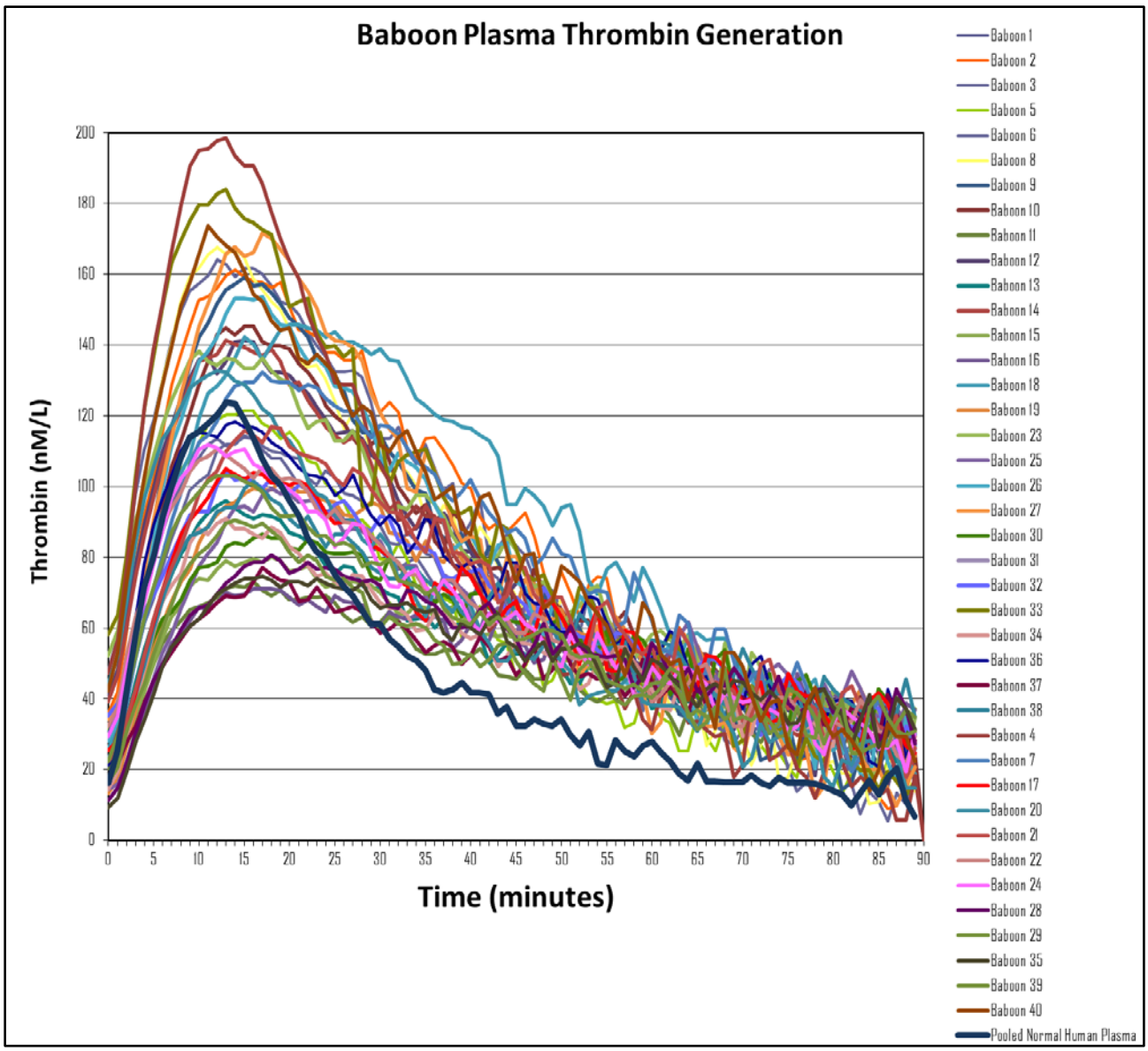


Figure 8: Thrombin generation curves of all 40 baboons, compared with pooled normal human plasma, ran for 90 minutes. nM/L; nanomolar per litre.

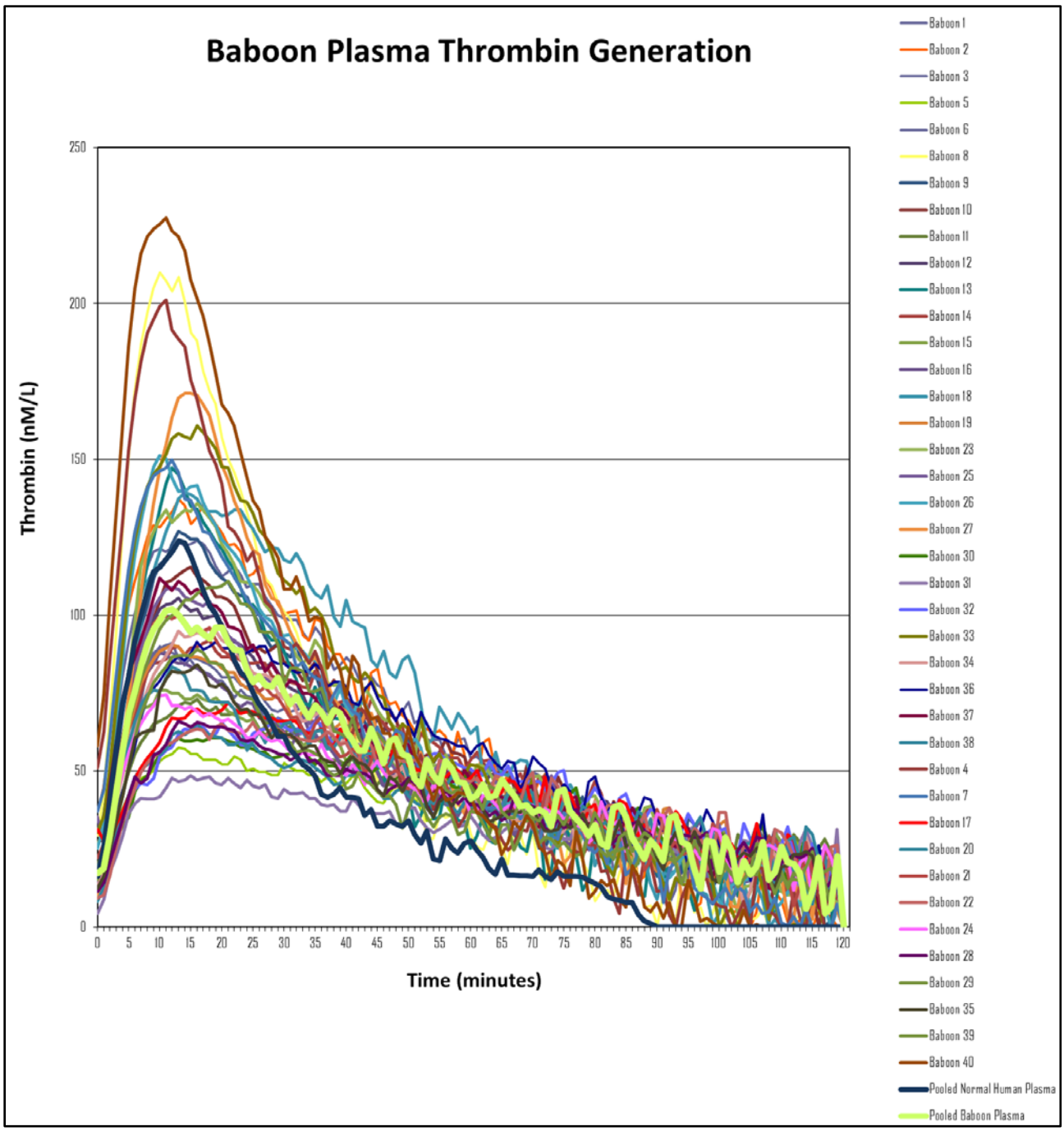


Figure 9: Thrombin generation curves of all 40 baboons, compared with pooled normal human plasma, as well as pooled *Papio ursinus* plasma, ran for 120 minutes in a separate additional run. nM/L; nanomolar per litre.

Thrombin generation assays also led to the production of a large amount of quantitative data. The statistics of the 90-minute run are summarised in Table 4 below. The 90-minute

run data were chosen for comparison with the human reference interval, since no thrombin generation was attained after 90 minutes in the pooled normal human plasma sample during the 120-minute run (Figure 9). The local SOP also stipulates a run of 90 minutes. The baboon data were compared with a normal human reference range previously derived locally (Meiring, 2017). As there is much variation in how thrombin generation is performed internationally, it is recommended that each laboratory establishes and validates its own in-house reference intervals (Bloemen et al., 2019). The local human reference ranges were also previously established using runs of 90 minutes.

Table 4: The range, median, mean, standard deviation (SD), coefficient of variation (CV), and central 95% interval of the thrombin generation lag time, peak height (maximum thrombin), time-to-peak (time to maximum thrombin), velocity index and endogenous thrombin potential (ETP; area under the curve).

Parameter, with human reference range (derived at local laboratory)	**Range**	**Median**	**Mean**	**SD**	**CV%**	**Central 95% interval**
Lag-time **1.8–6.3 minutes**	3.0-5.0	4.0	4.2	0.5	11.4	4.0-5.0
Peak-height **35.3–661.7 nM/L**	46.7-198.6	114.2	120.0	35.7	29.8	70.5-184.5
Time-to-peak **4.1–12.1 minutes**	10.0-20.0	15.0	14.8	2.5	16.6	11.0-19.0
Velocity index **24.6 – 270.7 nM/L/minute**	4.2-24.8	11.3	12.1	5.2	42.6	5.9-23.1
ETP **1565.4–3975.0 nM/L.minute**	3209.4-7850.7	5687.4	5942.0	1114.6	18.8	4252.7-7796.8

The lag-times and peak-height values for all baboons fell within the respective human reference ranges. However, the peak-height values tended toward the lower limit and had a much narrower range than would be locally accepted as normal in humans (114.0 nM/L vs 626.4 nM/L in humans). The majority (n=32; 80%) of test animals had times-to-peak above the upper limit of the human reference range. It resulted in velocity index values markedly below the human reference range, with only one baboon (2.5%) falling above the lower reference limit. All but one of the baboons tested (97.5%) had an ETP above the human reference range. Therefore, the calculated central 95% reference interval was much higher than the human reference range, indicating greater overall thrombin generation.

5.6 ABO and Rh blood group results and their influence on observational assay parameters

All baboons were Rh-positive. The majority of animals (n=30; 75%) were ABO blood group O, while the remainder (n=10; 25%) typed as subgroups of A. No baboons typed as group B or AB. The effects of ABO blood group on the various quantitative and qualitative parameters are summarised in the tables below. In only the ADAMTS13 antigen and fibrinogen levels, were significant differences seen between blood groups. Baboons with blood group O had lower ADAMTS13 antigen levels (p=0.0010) and higher fibrinogen levels (p=0.0355) than baboons typed as subgroups of A.

Table 5: The effects of ABO blood group on the quantitative parameters of the VWF-ADAMTS13 axis.

Parameter, with human reference range	Group O (n=30)			Subgroups of A (n=10)			p-value
	Range	Mean	SD	Range	Mean	SD	
ADAMTS13							
ADAMTS13 Ag (%) **41-141% (Technoclone, 2012a)**	2-14	6.5	2.4	5-21	10.5	4.5	***0.0010***
ADAMTS13 Act (%) **40-130% (Technoclone, 2011)**	22-68	39.4	9.6	23-70	46.4	14.7	0.0884
Von Willebrand factor							
VWF:Ag (%) **50-150% (Meiring et al., 2009, Meiring, 2011a)**	27-94	68.8	16.2	47-86	70.6	14.8	0.7539
VWF:RCo (%) **50-150% (Meiring et al., 2009, Meiring, 2011c)**	52-280	176.0	55.9	77-253	168.8	61.8	0.7329
VWF:CB (%) **51-143% (Meiring et al., 2007, Meiring, 2011b)**	11-86	49.9	17.4	26-71	48.2	15.2	0.7805

Table 6: The effects of ABO blood group on fibrinogen levels and the quantitative parameters of the fibrinolytic system, measured via observational assays.

Parameter, with human reference range	Group O (n=30)			Subgroups of A (n=10)			p-value
	Range	Mean	SD	Range	Mean	SD	
Fibrinogen **1.7-4.2 g/L (Conradie, 2015)**	1.16-4.26	2.1	0.7	1.28-1.83	1.6	0.2	***0.0355***
Plasminogen **60-250 µg/mL (Technoclone, 2014)**	1.5-28.2	3.3	4.7	2.0-3.3	2.4	0.5	0.5538
tPA **2-8 ng/mL (Technoclone, 2012d)**	1.6-4.0	2.1	0.5	1.4-2.7	1.8	0.4	0.1993
PAI-1 **7-43 ng/mL (Technoclone, 2012b)**	1.9-32.3	11.6	7.7	3.4-21.7	10.4	5.5	0.6409
PAP complex **0-514 ng/mL (Technoclone, 2012c)**	99.9-212.6	139.1	27.1	109.2-340.8	150.0	68.9	0.4716
TAFI ng/mL **1700-20000 ng/mL (Elabscience, 2019)**	0.0-66.3	10.2	16.4	0.0-30.5	8.0	12.1	0.6944
α2-antiplasmin **25-50 µg/mL (Abcam, 2019)**	1.2-17.5	3.1	4.3	1.3-95.8	13.3	29.5	0.0675

Table 7: The effects of ABO blood group on the quantitative parameters of thrombin generation.

Parameter, with human reference range	Group O (n=30)			Subgroups of A (n=10)			p-value
	Range	Mean	SD	Range	Mean	SD	
Lag time **1.8–6.3 minutes**	3.0-5.0	4.2	0.5	4.0-5.0	4.3	0.5	0.5748
Peak height **35.3–661.7 nM/L**	46.7-198.6	121.8	39.2	80-164.2	114.6	23.2	0.5911
Time-to-peak **4.1–12.1 minutes**	10.0.20.0	15.0	2.3	11.0-18.0	14.2	2.8	0.3804
Velocity index **24.6 – 270.7 nM/L/minute**	4.2-24.8	12.0	5.5	6.2-20.5	12.4	4.3	0.8533
ETP **1565.4–3975.0 nM/L.minute**	3209-7851	5976.8	1230.5	4908-7344	5837.5	699.3	0.7368

5.7 Influence of animals' sex on observational assay parameters

The majority of animals were female (n=26; 65%, males: n=14; 35%) and the effects of animals' sex on the various quantitative and qualitative parameters are summarised in the tables below. Males had less potent thrombin generation, evidenced by longer lag times (p=0.0475), lower peak thrombin concentrations (p=0.0203), and lower ETPs (p=0.0238), as well as lower fibrinogen levels (p=0.0134) than females, but higher PAP complex concentrations (p=0.0188).

Table 8: The effects of baboons' sex on the quantitative parameters of the VWF-ADAMTS13 axis.

Parameter, with human reference range	Females (n=26)			Males (n=14)			p-value
	Range	Mean	SD	Range	Mean	SD	
ADAMTS13							
ADAMTS13 Ag (%) **41-141% (Technoclone, 2012a)**	2-14	6.9	2.9	5-21	8.7	4.2	0.1387
ADAMTS13 Act (%) **40-130% (Technoclone, 2011)**	22-68	40.1	10.2	23-70	43.1	13.3	0.4188
Von Willebrand factor							
VWF:Ag (%) **50-150% (Meiring et al., 2009, Meiring, 2011a)**	27-92	66.9	16.9	52-94	73.5	12.8	0.2109
VWF:RCo (%) **50-150% (Meiring et al., 2009, Meiring, 2011c)**	52-280	170.8	60.5	113-258	180.5	50.4	0.6125
VWF:CB (%) **51-143% (Meiring et al., 2007, Meiring, 2011b)**	11-76	47.8	17.0	31-86	52.6	16.3	0.3897

Table 9: The effects of baboons' sex on fibrinogen levels and the quantitative parameters of the fibrinolytic system, measured via observational assays.

Parameter, with human reference range	Females (n=26)			Males (n=14)			p-value
	Range	Mean	SD	Range	Mean	SD	
Fibrinogen **1.7-4.2 g/L (Conradie, 2015)**	1.16-4.26	2.2	0.8	1.43-1.91	1.7	0.1	***0.0134***
Plasminogen **60-250 µg/mL (Technoclone, 2014)**	1.5-28.2	3.4	5.1	2.0-3.3	2.5	0.5	1.0000
tPA **2-8 ng/mL (Technoclone, 2012d)**	1.6-4.0	2.1	0.6	1.4-2.7	1.8	0.3	0.1156
PAI-1 **7-43 ng/mL (Technoclone, 2012b)**	1.9-32.3	11.5	7.2	2.6-30.0	11.0	7.3	0.8316
PAP complex **0-514 ng/mL (Technoclone, 2012c)**	99.9-184.9	130.9	18.2	110.8-340.8	162.1	60.6	***0.0188***
TAFI ng/mL **1700-20000 ng/mL (Elabscience, 2019)**	0.0-66.3	11.7	17.4	0.0-28.4	5.9	10.0	0.2651
α2-antiplasmin **25-50 µg/mL (Abcam, 2019)**	1.2-20.0	4.0	5.7	1.2-95.8	8.7	25.1	0.3628

Table 10: The effects of baboons' sex on the quantitative parameters of thrombin generation.

Parameter, with human reference range	Females (n=26)			Males (n=14)			p-value
	Range	Mean	SD	Range	Mean	SD	
Lag time **1.8–6.3 minutes**	3.0-5.0	4.1	0.4	4.0-5.0	4.4	0.5	***0.0475***
Peak height **35.3–661.7 nM/L**	46.7-198.6	129.5	37.1	71.1-164.2	102.4	25.8	***0.0203***
Time-to-peak **4.1–12.1 minutes**	10.0-20.0	14.7	2.4	11.0-18.0	15.0	2.7	0.7114
Velocity index **24.6 – 270.7 nM/L/minute**	4.2-24.8	13.0	5.3	5.9-20.5	10.5	4.7	0.1554
ETP **1565.4–3975.0 nM/L.minute**	3209-7851	6230.2	1180.1	4429-7344	5406.8	758.1	***0.0238***

Chapter 6 - **Results**

Part II – Experimental assays

6.1 Introduction

The results of assays with an experimental component (or some external specimen manipulation) are summarised in the tables and figures below (n=40 throughout).

6.2 Plasmin activity

Plasmin activity was assessed after induction with the thrombolytic drugs streptokinase and tPA at baseline and at escalating concentrations. The baseline and thrombolytic drug-induced activities obtained during the concentration escalation studies are presented separately below. Unless otherwise stipulated, differences between activators were assessed using paired Student's t-tests.

6.2.1 Baseline plasmin activity

The baseline plasmin activities induced by streptokinase and tPA are compared in Table 11 and Figure 10 below, using the concentrations defined in section 4.7.3.1 of Chapter 4 – 400 IU/mL for streptokinase and 2700 µg/mL for tPA. Compared with tPA, very little activity could be demonstrated with streptokinase as activator ($p<0.0001$). tPA was able to induce plasmin activity in *Papio ursinus* plasma to a greater extent than possible in pooled normal human plasma, with the activity in all baboon samples exceeding 100% of human activity.

Table 11: Baseline plasmin activity, expressed as a percentage of the activity induced in pooled normal human plasma, using streptokinase and tPA (tissue-type plasminogen activator) as activators (n=40).

Thrombolytic drug, concentration	Plasmin activity (%)						Two-tailed p-value
	Range	Median	Mean	SD	CV%	Central 95% interval	
Streptokinase, 400 IU/mL	1-14	4	4	3	73	1-12	***<0.0001***
tPA, 2700 µg/mL	114-294	160	178	46	26	124-261	

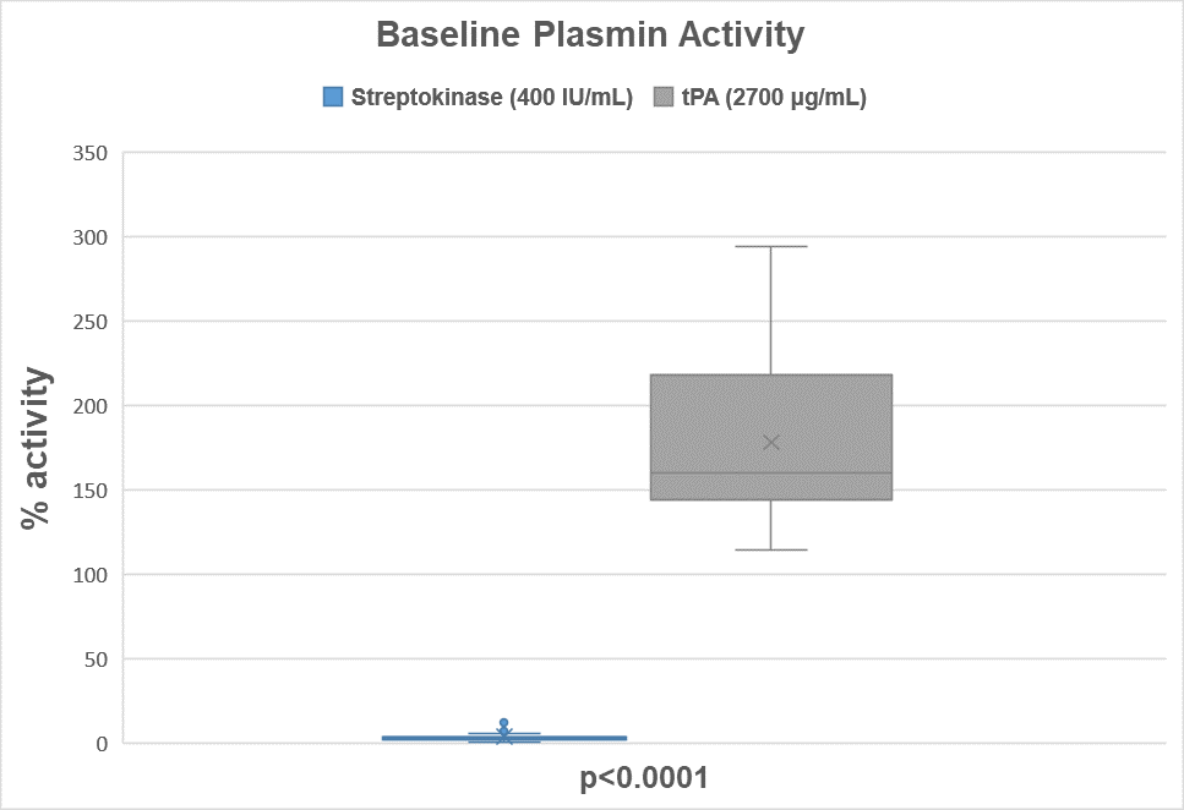


Figure 10: Box & Whisker plot of baseline plasmin activity, expressed as a percentage of the activity induced in pooled normal human plasma, using streptokinase and tPA (tissue-type Plasminogen Activator) as activators (n=40).

6.2.2 Thrombolytic drug-induced plasmin activity – concentration escalation studies

The plasminogen activation potentials of streptokinase and tPA, using escalating concentrations of activator, are shown in the tables and figures below as percentages of the activities obtained in pooled normal human plasma, using the baseline concentrations defined above, i.e. 400 IU/mL for streptokinase and 2700 µg/mL for tPA.

6.2.2.1 Streptokinase plasminogen activation potential

The plasmin activities obtained with escalating streptokinase concentrations are shown in Table 12 and Figure 11 below. Virtually no activity could be demonstrated at any concentration, even when the streptokinase stock solution [300 000 IU/mL] was used neat on a pooled Chacma baboon plasma sample for confirmation. A maximum concentration was thus not identified.

Table 12: Plasmin activity elicited by increasing concentrations of streptokinase, expressed as a percentage of the baseline activity induced in pooled normal human plasma (n=40).

[Streptokinase]	**Plasmin activity** (%)					
	Range	**Median**	**Mean**	**SD**	**CV%**	**Central 95% interval**
800 IU/mL	1.0-15.8	1.7	2.1	2.3	111.0	1.0-3.5
3200 IU/mL	0.9-9.6	1.2	1.7	1.6	93.0	0.8-5.0
25 600 IU/mL	1.1-1.5	1.2	1.6	1.2	71.2	0.9-5.0

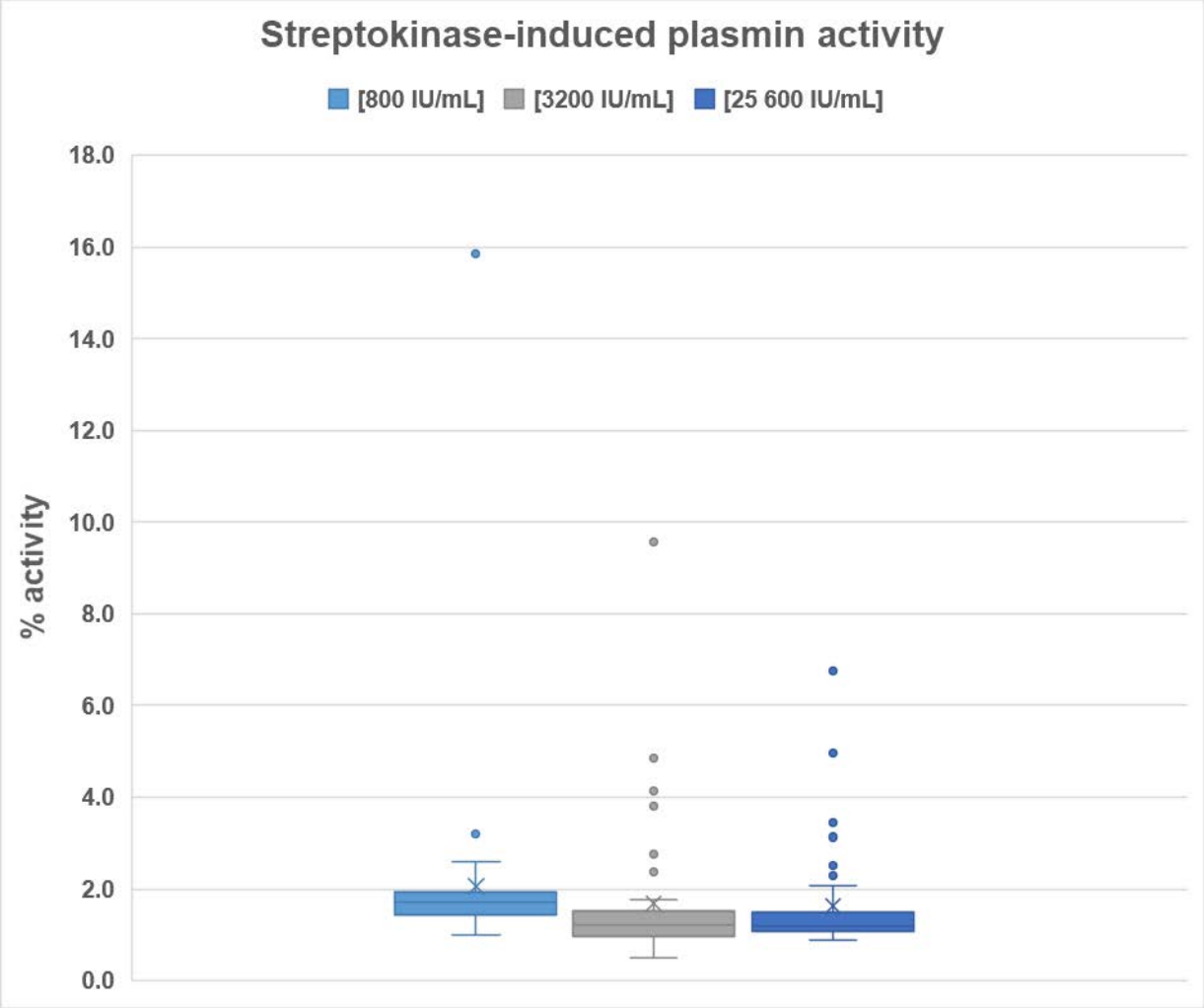


Figure 11: Box & Whisker plot of plasmin activity elicited by increasing concentrations of streptokinase, expressed as a percentage of the baseline activity induced in pooled normal human plasma (n=40).

6.2.2.2 tPA plasminogen activation potential

The table and figures below demonstrate the concentration-dependent increases in plasmin activity encountered when Chacma baboon plasma samples were exposed to escalating concentrations of tPA. Plateaus in activity were encountered in all samples at concentrations of either 5400 µg/mL (n=9) or 7200 µg/mL (n=31).

Table 13: Plasmin activity elicited by escalating concentrations of tissue-type plasminogen activator (tPA), expressed as a percentage of the baseline activity induced in pooled normal human plasma (n=40).

[tPA]	Plasmin activity (%)						Number with maximum activity
	Range	Median	Mean	SD	CV%	Central 95% interval	
28.125 µg/mL	5-8	5.8	6.3	0.9	14.8	5.1-7.7	0
56.25 µg/mL	6-10	7.3	7.4	0.6	8.7	6.5-8.7	0
112.5 µg/mL	8-18	9.5	9.8	2.1	21.2	7.9-15.2	0
225 µg/mL	9-20	12.2	12.2	2.0	16.1	9.5-15.3	0
450 µg/mL	13-32	19.7	20.1	3.5	17.5	15.2-25.4	0
900 µg/mL	34-130	46.0	55.2	22.3	40.4	34.6-103.7	0
1800 µg/mL	59-200	99.6	112.6	37.6	33.4	60.1-181.3	0
2700 µg/mL	114-294	160.0	178.0	45.9	25.8	124.4-260.9	0
3600 µg/mL	146-301	202.5	212.3	43.2	20.3	145.7-298.5	0
5400 µg/mL	186-399	264.2	267.0	51.1	19.1	207.5-378.7	9
7200 µg/mL	225-400	287.1	288.3	48.3	16.8	227.1-394.1	31
Maximum activity	227-400	289.5	299.8	45.6	15.2	230.9-394.2	40

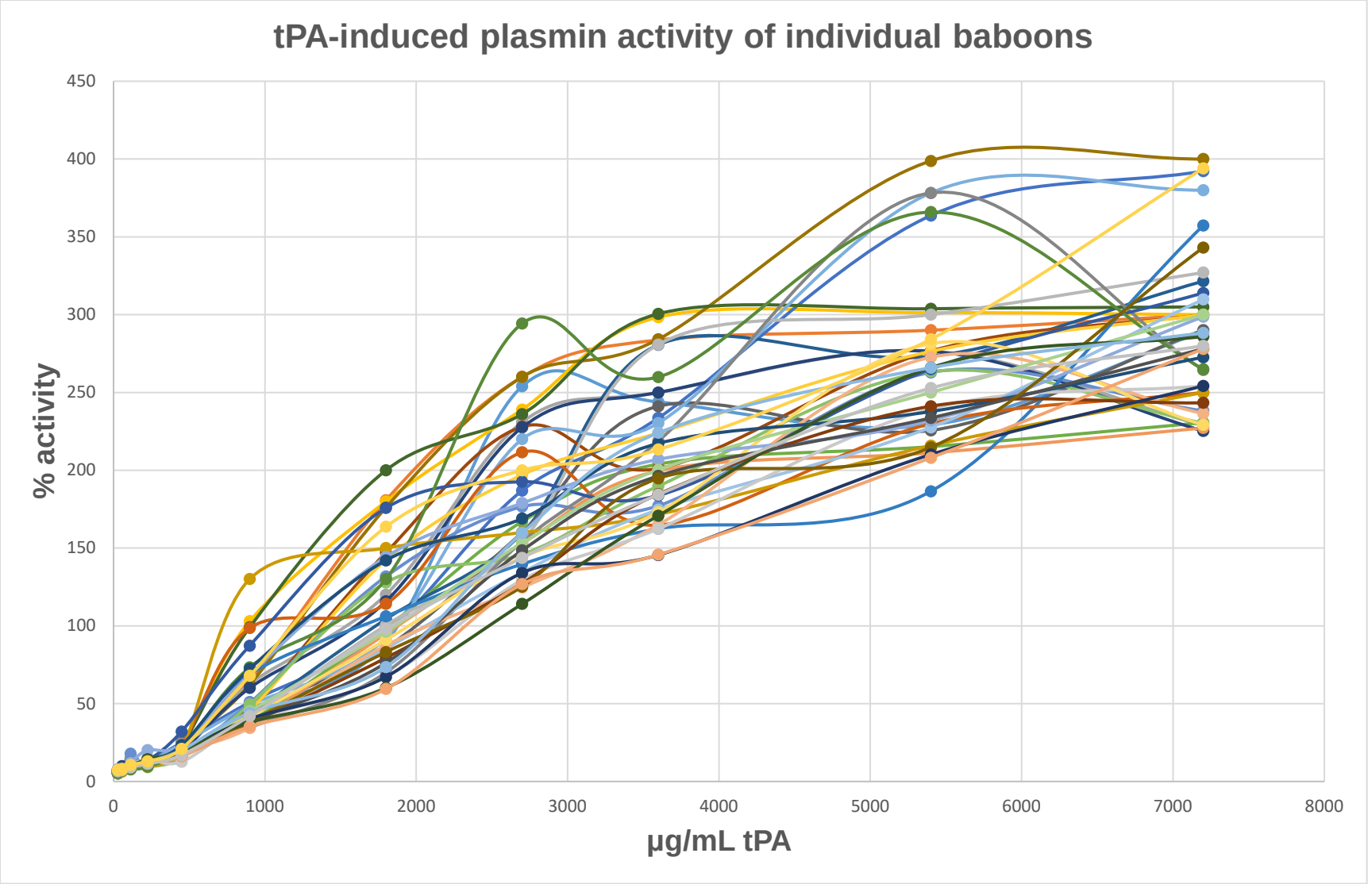


Figure 12: Plasmin activity of each individual baboon, elicited by increasing concentrations of tPA, expressed as a percentage of the baseline activity induced in pooled normal human plasma (n=40).

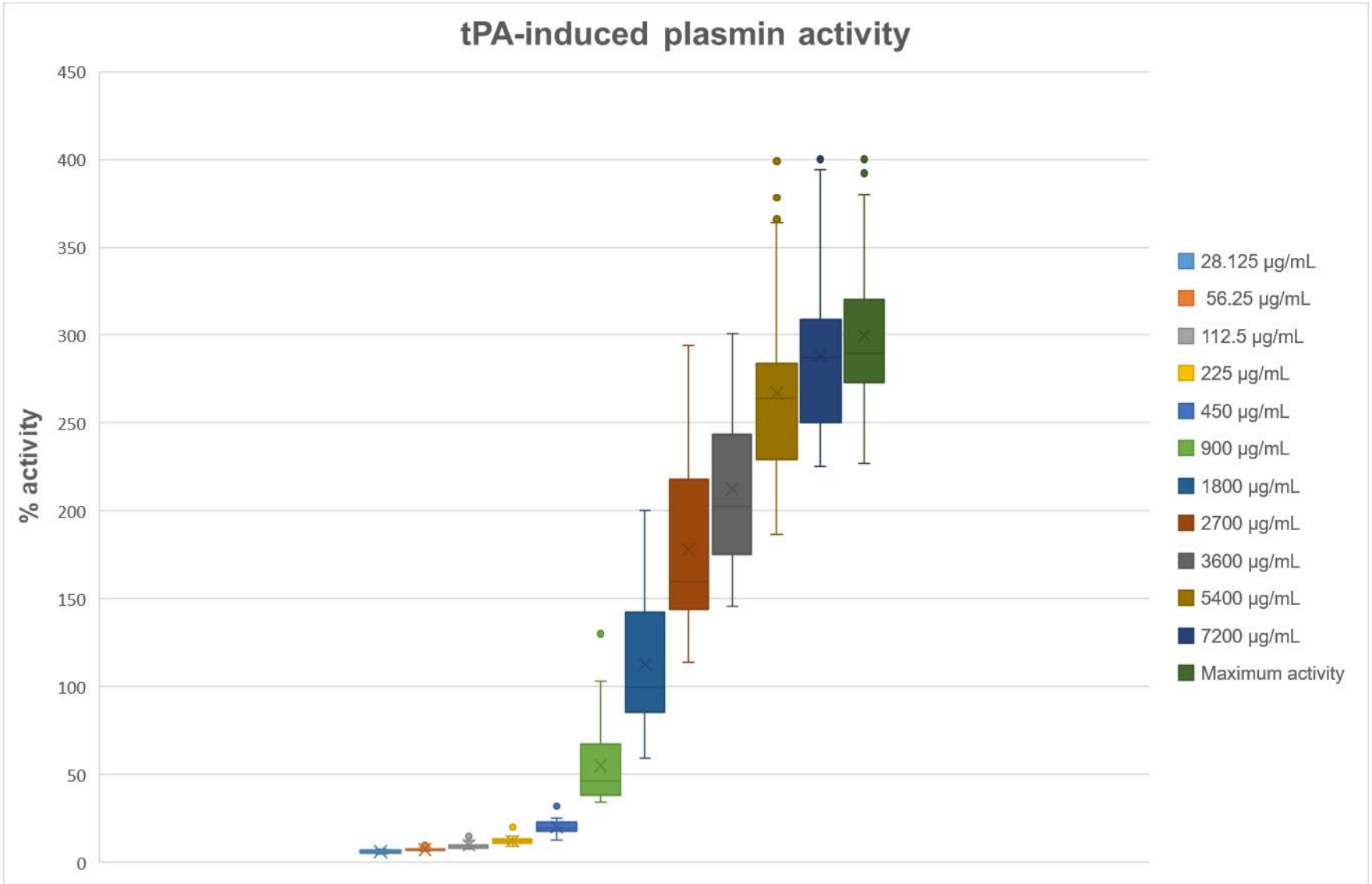


Figure 13: Box & Whisker plot of plasmin activity elicited by increasing concentrations of tPA, expressed as a percentage of the baseline activity induced in pooled normal human plasma (n=40).

6.3 Modified clot lysis time

The results of the modified clot lysis time experiment are summarised in Table 14 and Figure 14 below. All baboon samples had modified clot lysis times less than the pooled normal human plasma sample, indicating relatively brisk in vitro clot lysis when activated by tPA at a concentration of 225 ng/mL, which was the lowest concentration at which complete clot lysis could be elicited in both baboon and human plasma. A modified clot lysis assay using streptokinase as activator was not attempted, based on the findings in sections 6.2.1 and 6.2.2 above. A normal human reference range is not available for this modified assay. No lysis was observed in either of the two negative controls (pooled baboon plasma sample and pooled normal human plasma sample, with no added tPA).

Table 14: Modified clot lysis times compared with pooled normal human plasma and pooled baboon plasma when activated with tissue-type plasminogen activator (tPA) at a concentration of 225 ng/mL.

Modified clot lysis time (minutes)								
[tPA]	**Individual baboon samples (n=40)**						**Pooled plasma samples**	
	Range	**Median**	**Mean**	**SD**	**CV%**	**Central 95% interval**	**Human**	**Baboon**
225 ng /mL	7.9-13.2	10.2	10.4	1.2	11.6	8.5-12.6	13.4	10.8

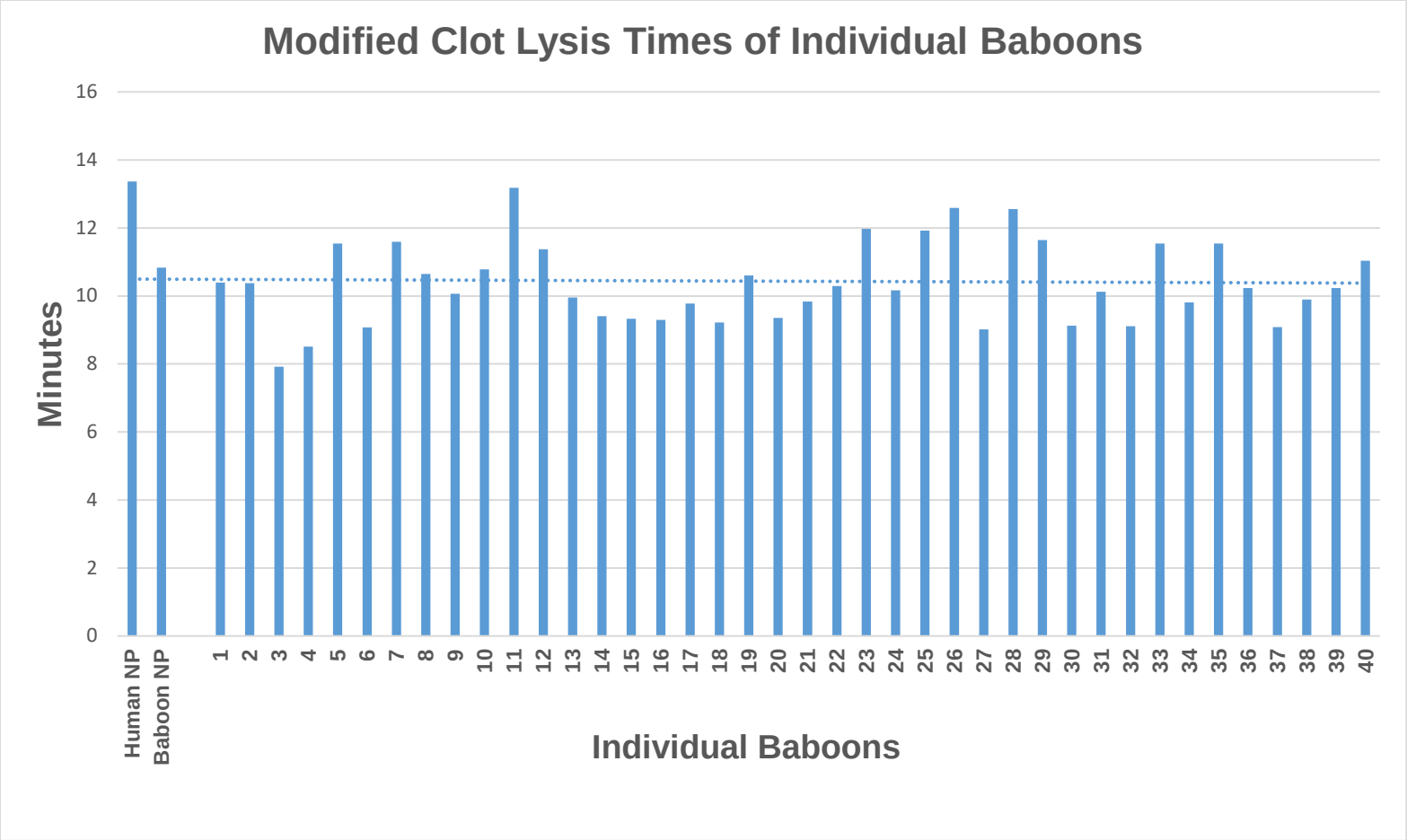


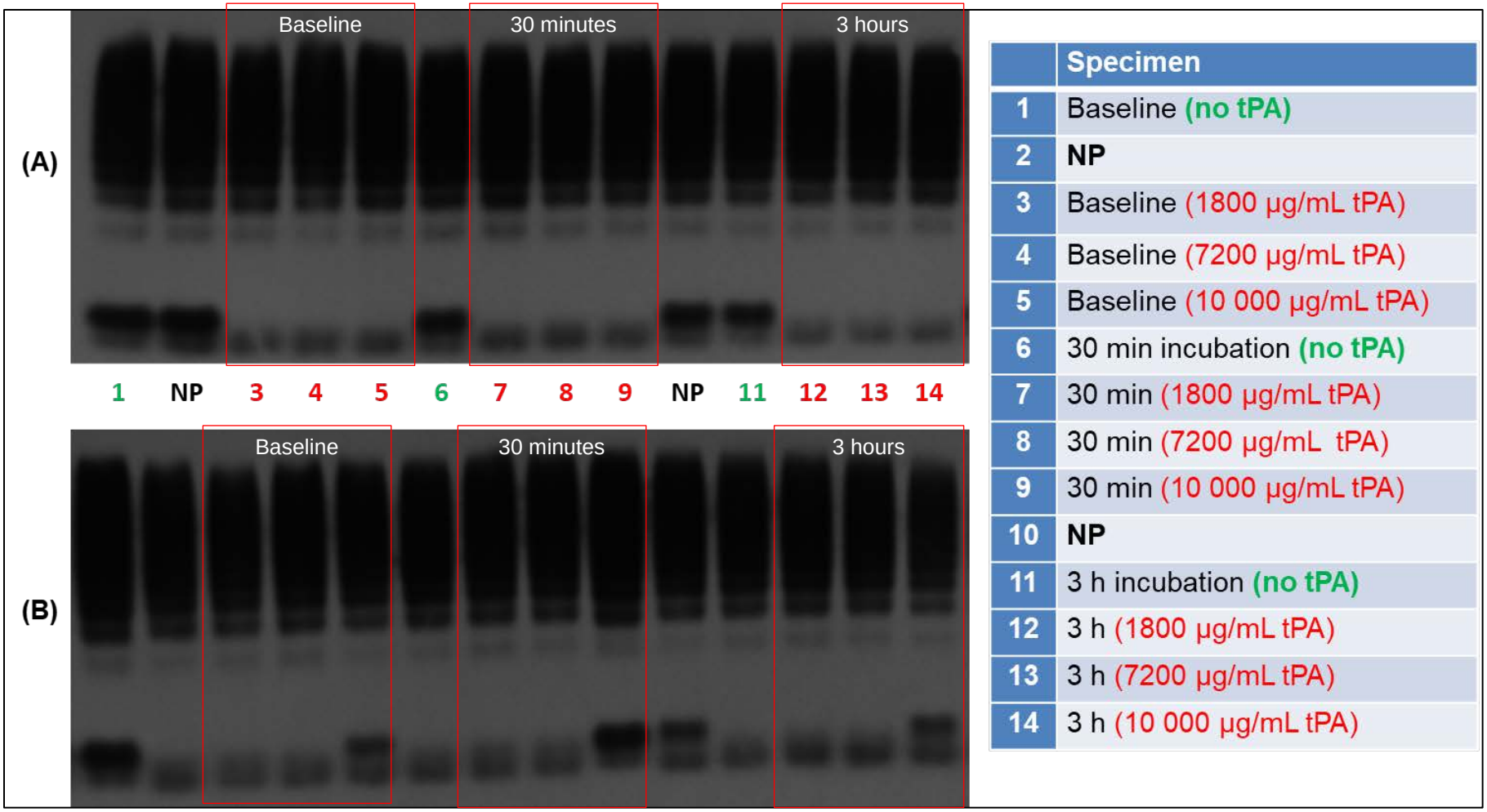
Figure 14: Modified clot lysis times of all individual baboon samples (n=40), compared with pooled normal human plasma (Human NP) and pooled baboon plasma (Baboon NP), when activated with tissue-type plasminogen activator (tPA) at a concentration of 225 ng/mL. The mean of all individual baboon samples is indicated with a dotted line.

6.4 Effects of tPA on VWF in the unfurled (non-globular) state

The effects of tPA on pooled *Papio ursinus* plasma and pooled normal human plasma, with and without urea dialysis (Figures 15 and 16), and after urea dialysis in the presence of excess 3H9 (Figure 17), are shown below. In Figure 17, baseline patterns and the pattern obtained at 30 minutes incubation without tPA are shown in duplicate due to the importance of these patterns. The addition of tPA had no effect, at any concentration, on either human or *Papio ursinus* multimer patterns, at any of the incubation time points, in the native (globular) state (Figure 15).

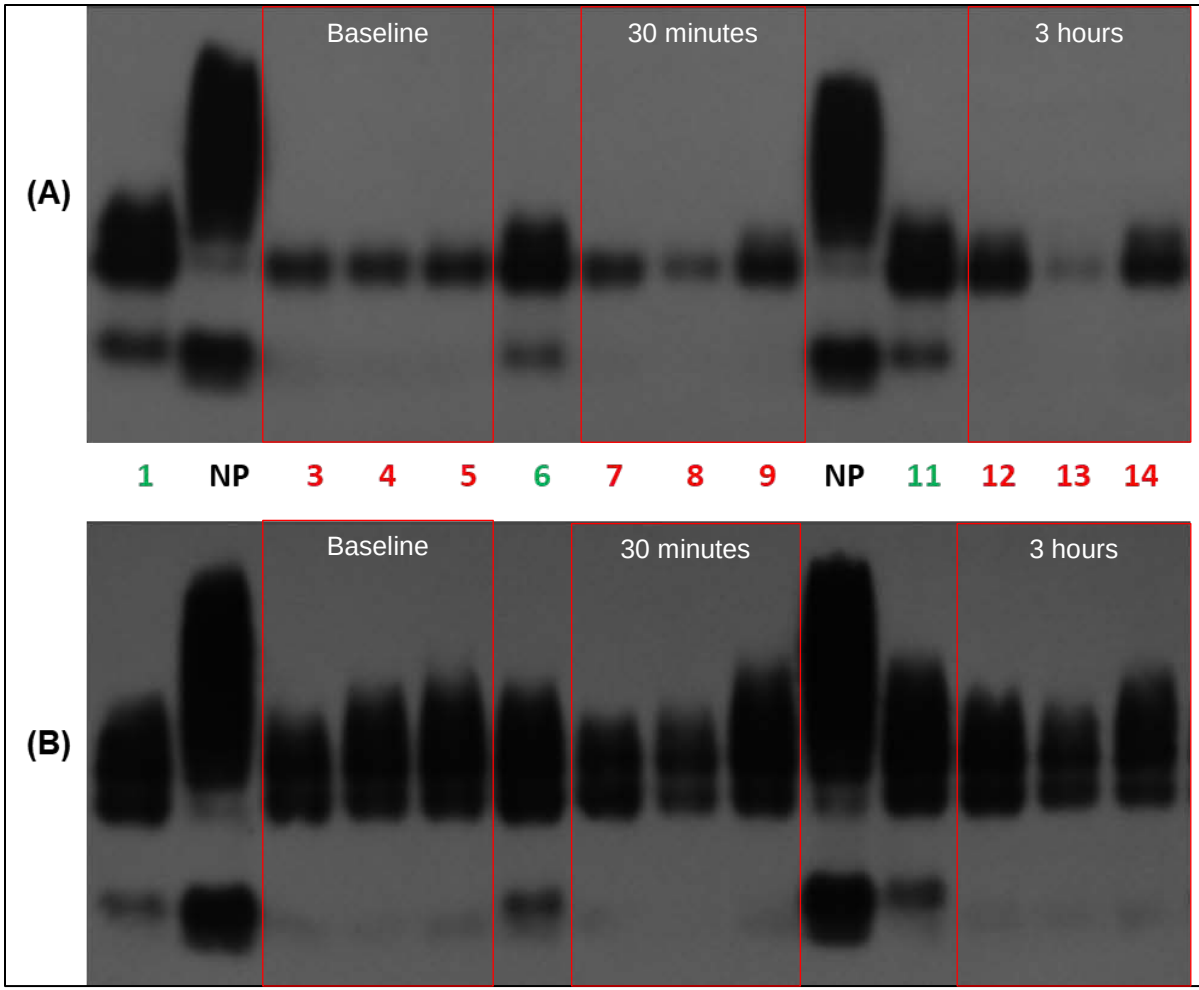
After urea dialysis (in the absence of 3H9), the high molecular weight multimers were reduced in *Papio ursinus* plasma exposed to tPA, compared with the patterns obtained without added tPA (Figure 16B). It was more prominent at the lower tPA concentrations. The effect was even more pronounced in pooled normal human plasma (Figure 16A). Although the reduction in high molecular weight multimers (and intermediate weight multimers in pooled normal human plasma) observed here may have solely been due to the activity of ADAMTS13 (as evidenced by a reduction also in the high molecular weight multimers seen in the absence of tPA, compared with untreated pooled normal human plasma) the reduction in multimers was still more prominent in those specimens exposed to tPA, than in those that were not (Figure 16B).

With urea dialysis after the addition of 3H9 (Figure 17), all ADAMTS13 activity had effectively been neutralised, as ADAMTS13 activity levels were confirmed to be 0% in all specimens after urea dialysis using the activity ELISA outlined in section 4.7.2.2 above. Human and *Papio ursinus* multimer patterns obtained in this experiment were remarkably similar, again showing a reduction in the high molecular weight multimers in specimens exposed to tPA compared with the patterns obtained without tPA, which was more prominent at lower tPA concentrations. The effect was also more pronounced at baseline and 30 minutes incubation than after 3 hours.



	Specimen
1	Baseline (no tPA)
2	NP
3	Baseline (1800 µg/mL tPA)
4	Baseline (7200 µg/mL tPA)
5	Baseline (10 000 µg/mL tPA)
6	30 min incubation (no tPA)
7	30 min (1800 µg/mL tPA)
8	30 min (7200 µg/mL tPA)
9	30 min (10 000 µg/mL tPA)
10	NP
11	3 h incubation (no tPA)
12	3 h (1800 µg/mL tPA)
13	3 h (7200 µg/mL tPA)
14	3 h (10 000 µg/mL tPA)

Figure 15: The effects of escalating tPA concentrations at baseline, 30 minutes, and 3 hours incubation, on pooled normal human plasma (A) and pooled *Papio ursinus* plasma (B) in the absence of 3H9, without urea dialysis. NP; (Human) Normal Pool, tPA; tissue-type plasminogen activator.



	Specimen
1	Baseline (no tPA)
2	NP
3	Baseline (1800 µg/mL tPA)
4	Baseline (7200 µg/mL tPA)
5	Baseline (10 000 µg/mL tPA)
6	30 min incubation (no tPA)
7	30 min (1800 µg/mL tPA)
8	30 min (7200 µg/mL tPA)
9	30 min (10 000 µg/mL tPA)
10	NP
11	3 h incubation (no tPA)
12	3 h (1800 µg/mL tPA)
13	3 h (7200 µg/mL tPA)
14	3 h (10 000 µg/mL tPA)

Figure 16: The effects of escalating tPA concentrations at baseline, 30 minutes, and 3 hours incubation, on pooled normal human plasma (A) and pooled *Papio ursinus* plasma (B) in the absence of 3H9, after urea dialysis. NP; (Human) Normal Pool (not subjected to urea dialysis), tPA; tissue-type plasminogen activator.

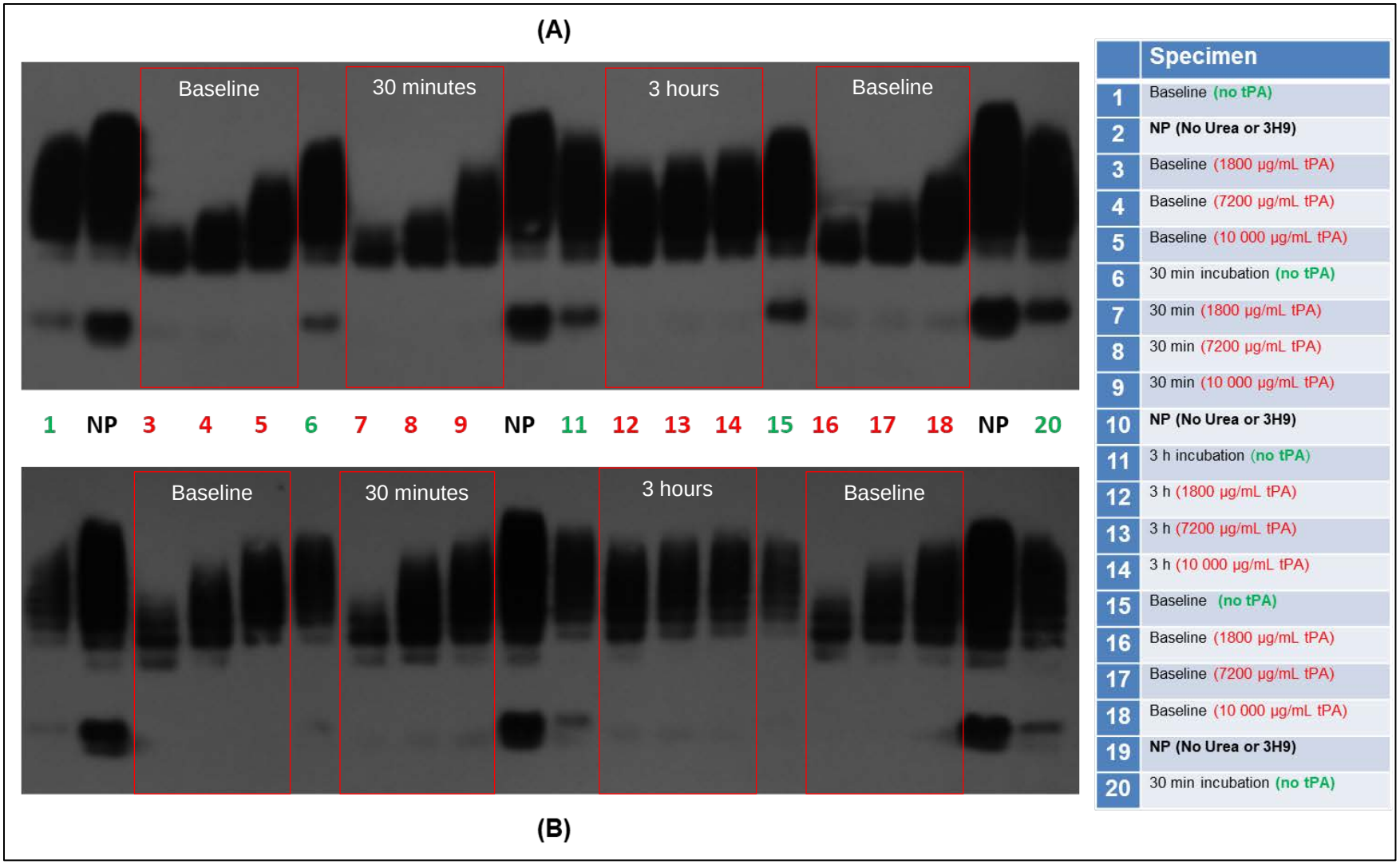


Figure 17: The effects of escalating tPA concentrations at baseline, 30 minutes, and 3 hours incubation, on pooled normal human plasma (A) and pooled *Papio ursinus* plasma (B) in the presence of an excess of 3H9, after urea dialysis. NP; (Human) Normal Pool (not subjected to urea dialysis, not exposed to 3H9), tPA; tissue-type plasminogen activator.

6.5 Effects of ABO and Rh blood group on experimental assay results

The effects of ABO blood group on the various quantitative and qualitative parameters assessed using assays with an experimental component (or some form of additional external specimen manipulation) are summarised in the tables below. ABO blood group did not affect streptokinase-induced plasminogen activation, tPA-induced plasminogen activation at baseline, the tPA concentration at which maximal activation occurred, or the modified clot lysis time. Baboons typing as subgroups of A had statistically significantly lower tPA-induced plasmin activities at concentrations of 56.25 µg/mL and 225 µg/mL, but higher activities at 112.5 µg/mL. However, these differences were slight and not consistent across all concentrations, and therefore unlikely to be of practical importance. All baboons were Rh-positive; therefore, the effect of the Rh-group could not be determined.

Table 15: The effects of ABO blood group on the quantitative parameters of the fibrinolytic system, measured via experimental assays.

Parameter	Group O (n=30)			Subgroups of A (n=10)			p-value
	Range	Mean	SD	Range	Mean	SD	
Plasmin activity (as % of human baseline activity)							
Baseline							
Streptokinase (400 IU/mL)	1-14	3.9	3.1	1-6	3.4	1.4	0.5826
tPA (2700 µg/mL)	114-260	177.7	44.6	134-294	179.2	52.2	0.9295
Escalation studies							
[Streptokinase]							
800 IU/mL	1.0-15.8	2.2	2.6	1.0-2.6	1.7	0.4	0.6131
3200 IU/mL	0.5-9.6	1.6	1.7	0.8-4.1	1.9	1.2	0.6633
25600 IU/mL	0.9-6.8	1.6	1.1	0.9-5.0	1.8	1.3	0.7090
[tPA]							
28.125 µg/mL	5-8	6.4	1.0	5-8	5.9	0.7	0.1929
56.25 µg/mL	7-10	7.5	0.7	6-8	7.1	0.4	***0.0387***
112.5 µg/mL	8-12	9.4	1.1	8-18	10.9	3.6	***0.0434***
225 µg/mL	9-20	12.6	1.9	9-15	11.1	1.7	***0.0268***
450 µg/mL	13-32	20.6	3.9	17-21	18.7	1.2	0.1526
900 µg/mL	34-103	55.9	20.0	35-130	53.0	29.3	0.7266
1800 µg/mL	59-200	115.2	41.1	70-150	104.9	24.6	0.4600
2700 µg/mL	114-260	177.7	44.6	134-294	179.2	52.2	0.9295
3600 µg/mL	146-301	212.6	45.3	172-281	211.5	38.5	0.9433
5400 µg/mL	186-399	267.4	48.1	211-378	265.9	62.2	0.9352
7200 µg/mL	225-400	295.0	51.0	227-327	268.1	33.5	0.1287
Maximum activity	243-400	302.4	43.6	227-378	292.1	52.8	0.5440
Modified clot lysis time in minutes (Human NP = 13.4 minutes)							
tPA (225 ng /mL)	7.9-13.2	10.5	1.3	9.1-11.9	10.0	0.8	0.3526

tPA: tissue-type plasminogen activator; Human NP: pooled normal human plasma

Table 16: The effect of ABO blood group on the tissue-type plasminogen activator (tPA) concentration at which maximum plasmin activity was attained.

[tPA] at maximum activity	ABO blood group	
	Group O (n=30)	Subgroups of A (n=10)
5400 µg/mL (n=9)	6	3
7200 µg/mL (n=31)	24	7
Fisher's exact test two-tailed p-value = 0.6650		

6.6 Influence of animals' sex on experimental assay results

The influence of animals' sex on the various quantitative and qualitative parameters assessed using assays with an experimental component (or some form of additional external specimen manipulation) are summarised in the tables below. Animals' sex did not influence any of the parameters assayed.

Table 17: The effects of animals' sex on the quantitative parameters of the fibrinolytic system, measured via experimental assays.

Parameter	Female (n=26)			Male (n=14)			p-value
	Range	Mean	SD	Range	Mean	SD	
Plasmin activity (as % of human baseline activity)							
Baseline							
Streptokinase (400 IU/mL)	1-14	3.8	3.2	1-7	3.8	1.8	0.9952
tPA (2700 µg/mL)	114-260	177.3	44.8	127-294	179.4	49.6	0.8953
Escalation studies							
[Streptokinase]							
800 IU/mL	1.0-15.8	2.2	2.8	1.0-3.2	1.8	0.6	0.6232
3200 IU/mL	0.5-9.6	1.7	1.8	0.9-4.1	1.7	1.1	0.9447
25600 IU/mL	0.9-6.8	1.7	1.2	1.0-5.0	1.6	1.0	0.7761
[tPA]							
28.125 µg/mL	5.1-7.7	6.3	0.9	5.1-7.9	6.2	1.0	0.7506
56.25 µg/mL	6.5-9.9	7.5	0.7	6.3-8.3	7.2	0.5	0.0651
112.5 µg/mL	7.9-17.9	9.9	2.3	7.8-15.2	9.5	1.7	0.6331
225 µg/mL	9.3-20.2	12.5	2.1	9.5-15.2	11.8	1.6	0.2657
450 µg/mL	12.7-32.1	20.3	4.0	16.5-24.2	19.6	2.3	0.6146
900 µg/mL	34.4-103.0	55.8	19.2	34.6-130.1	54.1	27.9	0.8289
1800 µg/mL	59.5-200.0	120.0	41.0	67.2-150.0	98.9	26.2	0.0891
2700 µg/mL	114.1-260.0	177.3	44.8	127.3-294.3	179.4	49.6	0.8953
3600 µg/mL	145.7-300.6	215.1	45.5	145.5-280.8	207.2	39.6	0.5911
5400 µg/mL	186.5-398.8	264.1	45.7	210.1-378.2	272.5	61.4	0.6300
7200 µg/mL	225.2-400.0	294.3	51.2	229.2-380.0	277.2	41.9	0.2921
Maximum activity	227.0-400.0	301.7	43.6	231.0-380.0	296.2	50.6	0.7199
Modified clot lysis time in minutes (Human NP = 13.4 minutes)							
tPA (225 ng /mL)	7.9-13.2	10.5	1.2	9.1-12.6	10.1	1.1	0.4319

tPA: tissue-type plasminogen activator; Human NP: pooled normal human plasma

Table 18: The effect of animals' sex on the tissue-type plasminogen activator (tPA) concentration at which maximum plasmin activity was attained.

[tPA] at maximum activity	**Sex**	
	Female (n=26)	**Male (n=14)**
5400 µg/mL (n=9)	6	3
7200 µg/mL (n=31)	20	11
Fisher's exact test two-tailed p-value = 1.0000		

Chapter 7 - **Discussion**

7.1 Introduction

This study aimed to characterise and quantify those components of the Chacma baboon haemostatic system that are relevant to the pathophysiology of aTTP (and its possible treatment with streptokinase or other thrombolytic drugs) to gain a deeper understanding of the Chacma baboon model of aTTP, and its response to pharmacological manipulation of the fibrinolytic system as a possible treatment of TTP. The findings of this study, virtually all of which are novel, may also apply to any experimental model in this species that involves or affects the haemostatic system.

7.2 The VWF-ADAMTS13 axis is generally similar (but not identical) to its human equivalent

7.2.1 ADAMTS13 – functionally similar but structurally different?

The lower ADAMTS13 activities and markedly lower ADAMTS13 antigen levels obtained in the forty baboons tested (Table 1) may be due to truly lower activities and levels in *Papio ursinus* plasma, but is possibly also an indication that the ELISA kits used for these assays may not be suitable for quantification and investigation of ADAMTS13 in *Papio ursinus* plasma, as they are designed for use in humans. Ideally, when embarking upon ADAMTS13 testing in this model, methods should be employed that have been developed explicitly for use in Chacma baboons. Nevertheless, the discrepant results suggest possible fundamental structural differences between the baboon ADAMTS13 protein and its human equivalent.

Firstly, structural differences would affect the binding of the antibodies used in these kits: the monoclonal capture antibody of the Technozym® ADAMTS13 antigen ELISA binds to the CUB domains of the protein (Technoclone, 2012a), implying a different structure (or complete absence) of these domains in the Chacma baboon. It is conceivable that the structures of *Papio ursinus* and human ADAMTS13 are fundamentally different. Muia et al. (2019) concluded that ADAMTS13 structure is exceptionally variable among

vertebrates after conducting a phylogenetic analysis of ADAMTS13 in 264 vertebrates, as well as functional assays of plasma ADAMTS13 from 20 birds, amphibians, and placental mammals, which included an analysis of the Olive baboon (*Papio anubis*) (Muia et al., 2019), but not of the Chacma baboon. Interestingly, they found that the proximal (N-terminal) MDTCS section (consisting of the MP domain, Dis domain, first TSP1 motif, Cys domain, and Spacer domain), and distal (C-terminal) seventh TSP1 (T7) and eighth TSP1 (T8) domains are invariably present, but that a minority of species had partially lost the distal CUB domains. Armadillo (*Dasypus novemcinctus*) and Upper Galilee mountains blind mole rat (*Nannospalax galili)* ADAMTS13 are truncated after the first disulphide loop of CUB1, leaving half a CUB domain. Ord's kangaroo rat (*Dipodomys ordii)* has no CUB domains at all. Therefore, it is possible that *Papio ursinus* ADAMTS13 may also exhibit complete or partial CUB domain loss, rendering the capture antibody of the antigen ELISA unable to bind properly.

However, both human and *Papio anubis* ADAMTS13 only lost the long propeptide and T6a domain when compared with ancestral ADAMTS13, which consists of a long propeptide, MDTCS section, eight additional T domains (numbered T3, T4, T5, T6a, T6, T7, T8), and two CUB domains (Muia et al., 2019). Baboon phylogenetic studies have confirmed *Papio ursinus* (found in southern Africa) and *Papio anubis* (found in the north-central African savannah) to belong to distinct southern and northern clades, respectively, mirroring their sharply demarcated geographical distribution (Fischer et al., 2019, Rogers et al., 2019, Newman et al., 2004).

Since they are also morphologically and behaviourally distinct (Rogers et al., 2019), differences in haemostasis physiology (such as ADAMTS13 and VWF structure) are possible, so that *Papio ursinus* may have lost other areas of ADAMTS13 (such as the CUB domains) that are still present in *Papio anubis* and human ADAMTS13. Nonetheless, a total loss of both CUB domains is probably unlikely since it has been shown that the CUB domains, primarily CUB1, contribute to auto-inhibition and to the VWF D4 domain binding affinity of ADAMTS13 (Zhu et al., 2019, South et al., 2017). A partial loss of one or more CUB domains (or other structural differences of the CUB domain area) that would decrease antibody binding, may however, explain our results.

Secondly, structural differences may affect the protein's relative enzymatic potency to cleave substrate. The nearer-to-human values obtained for ADAMTS13 activity than for the antigen levels suggest the Chacma baboon ADAMTS13 active site is perhaps more similar (although not identical in potency) to the corresponding human active site, than other areas of the protein are. With the ADAMTS13 activity ELISA, the substrate (a human VWF73 fragment) is captured, and free ADAMTS13 cleaves the VWF73 (Technoclone, 2011). The addition of an HRP-conjugated, monoclonal, anti-N10 antibody (that now recognises only the cleaved VWF fragment), and a TMB chromogenic substrate, completes the reaction. In this assay, no reagent antibodies ever bind to ADAMTS13. Instead, ADAMTS13 only interacts with the captured human VWF73 fragment. This fragment of 73 amino acids spans the region from Asp_{1596} to Arg_{1668} in the A2 domain of VWF, and is deemed the minimal substrate for ADAMTS13 (Kokame et al., 2004).

When investigating the enzymatic properties of plasma ADAMTS13 from 19 nonhuman species, Muia et al. (2019) found that Olive baboon plasma cleaved human VWF71 more slowly than human plasma did. Olive baboon plasma VWF71 cleavage activity was 71% ± 8.03 (mean ± SD) that of human plasma, although it is unclear exactly how many animals were included, since the authors only state that at least three individual animals were tested. VWF71 is a recombinant VWF fragment, spanning the Gln_{1599}-Arg_{1668} region in the A2 domain, with mutations N1610C and K1617R and an N-terminal Gly, designed for use in undiluted plasma (Muia et al., 2013). It has been shown to give equivalent results to VWF73-based assays (R^2 = 0.95) (Muia et al., 2013). Our Chacma baboon ADAMTS13 activity results of 41% ± 11 (mean ± SD; human reference range for kit: 40-130%) obtained using the (equivalent) VWF73-based ELISA, although comparable to Olive baboon activities, are still much lower than human and Olive baboon levels, again reiterating the potential differences not only between baboon and human ADAMTS13, but between different baboon species as well.

The addition of human VWF D4 by Muia et al. (2019) accelerated Olive baboon ADAMTS13 activity by a factor of 1.9 ± 0.1, again emphasising the importance of allostery in the VWF-ADAMTS13 axis, even across different species. One must bear in mind that the allosteric effect of baboon VWF D4 could potentially be quite important for complete baboon ADAMTS13 activity (perhaps even more critical than human VWF D4 is for

human ADAMTS13 activity) and could be another explanation for the low baboon ADAMTS13 activities seen in our experiments and in the results of Muia et al. (2019). Since, although baboon VWF would be present in the baboon plasma tested, it would be in the globular configuration, without exposure of the D4 domain, which is required for allosteric ADAMTS13 activation. It would be interesting to investigate the effects of human (and Chacma, as well as Olive baboon) VWF D4 on Chacma (and Olive) baboon ADAMTS13 activity, as this is possibly closer to actual physiological conditions, and one can speculate that the activity will be enhanced. Chacma baboon VWF D4 may have a more considerable allosteric activation effect on Chacma baboon ADAMTS13 than human VWF D4 has on human, Chacma, or Olive baboon ADAMTS13.

Another indication that the Chacma baboon ADAMTS13 active site is relatively similar to the human active site is the observation by Muia et al. (2019) that the mAb 3H9, which recognizes the metalloprotease domain of human ADAMTS13, prevented the cleavage of human VWF71 by plasma from all primates tested (human, marmoset, macaque, Olive baboon, and chimpanzee), which is consistent with the Chacma baboon model of aTTP, where 3H9 leads to profound and reproducible ADAMTS13 inhibition (Feys et al., 2010a).

Although consistent with the results obtained in the pilot study (Joubert et al., 2021), direct comparison of our ADAMTS13 antigen and activity quantification results with other preclinical studies performed previously in this model is difficult for several reasons. Firstly, with both the original description of the model (Feys et al., 2010a) and a subsequent study investigating the utility of the anti-VWF mAb GBR600 (Feys et al., 2012), the reported ADAMTS13 activity levels for all animals, assessed using a modified (Anderson et al., 2006) FRETS-VWF73 activity assay (Kokame et al., 2005), were not derived after comparison with a human calibration reference curve as in our study, but after comparison with individual baboons' baseline activities (before 3H9 injection), which was set as the 100% activity mark. However, in another 2012 study investigating the utility of the anti-VWF nanobody ALX-0681, Callewaert et al. used the same ADAMTS13 activity ELISA kit as in our present (and pilot) study (Technozym® ADAMTS-13 Activity ELISA kit, Technoclone GmbH, Vienna, Austria) and reported values comparable to ours, with a mean ADAMTS13 activity of approximately 40% (range: approximately 30% to 50%) in the control and test animals (n=16) at baseline. Tersteeg et al. (2017b) did not evaluate

ADAMTS13 activities as part of their study investigating the utility of *N*-acetylcysteine (NAC) in this model.

Secondly, ADAMTS13 antigen level results obtained in previous studies were either not shown (Feys et al., 2012), reported in µg/mL and not as a percentage (Callewaert et al., 2012), or generated using an in-house ELISA that was probably somewhat novel at the time (Feys et al., 2010a). With the establishment of the model in 2010, Feys et al. used an in-house antigen ELISA first described in 2006 (Feys et al., 2006) based on high-affinity murine anti-ADAMTS13 mAbs, which were generated using genetic immunization of female Balb/c mice. This sandwich ELISA is composed of a murine anti-ADAMTS13 monoclonal capture antibody termed 2G3, used in combination with a mixture of two biotinylated anti-ADAMTS-13 detection antibodies named 8C10 and 13F7. It is unclear to which ADAMTS13 domains these antibodies specifically bind. However, since the development of this assay involved the use of rabbit polyclonal antibodies raised against a recombinant ADAMTS13 C-terminal CUB-domain (used to capture rADAMTS13, to which any murine anti-ADAMTS13 antibodies would then bind, enabling their detection as part of a sandwich ELISA), it is unlikely (although not impossible) that either the 2G3 capture antibody or the 8C10 and 13F7 detection antibodies would be directed against the C-terminal CUB domain (as is the case with the capture antibody of the Technozym® ADAMTS-13 Antigen ELISA kit we used), since binding sites on this domain would have been occupied by the rabbit polyclonal capture antibodies used to originally identify antibody-producing mice. This ELISA, which is likely fundamentally different from the ELISA kit we used, resulted in ADAMTS13 antigen level values dramatically different from our results. When this ELISA was used during the initial development of the model (Feys et al., 2010a), pooled normal human plasma from 20 normal donors was apparently used as the reference point, set at 100%, yielding results ranging from approximately 85% to 105% in the control animals (n=5) for the experiment, which is much higher than our range of 2% to 21%. When looking at Figure 2 (and its accompanying caption) of the publication by Feys et al. (2012), it does, however, appear as if the ADAMTS13 antigen results were indeed ultimately also reported as a percentage of individual animals' baseline levels (and not derived from the pooled normal human plasma reference as stated in the text), which

further clouds direct comparison to our results. In addition, antigen testing was not done in the recent evaluation of NAC in this model (Tersteeg et al., 2017b).

In terms of the implications of our results for the Chacma baboon model of aTTP, it is clear that, although there appear to be significant structural and functional differences between human and Chacma baboon ADAMTS13, none of these differences would affect either the total inhibition of Chacma baboon ADAMTS13 by 3H9 or the resultant TTP phenotype. Although human ADAMTS13 is cleavable by plasmin (Feys et al., 2010b), the potential structural and functional differences highlighted here are also unlikely to affect the treatment of aTTP with fibrinolytic agents in this model, since ADAMTS13 is kept constantly inhibited by 3H9 anyway, regardless of any other possible lytic effects on ADAMTS13. Whether Chacma baboon plasmin can also cleave Chacma baboon ADAMTS13 as in humans, remains to be explored. When selecting/designing assays for ADAMTS13 activity measurement in future experiments using this model, it may be prudent to instead use individual animals' baseline (pre-injection of 3H9) values as the 100% reference point, especially if the activity trend (and not the absolute value) is the compelling factor. Information on the intra-individual trend in response to TTP induction, or (more importantly) the individual response to any novel therapeutic agent, will likely generally be more useful than an absolute value derived from comparison with human plasma. Should an absolute value be required, though, it may be more suitable to use pooled normal baboon plasma for the calibration reference curve in any ELISA kit, rather than human plasma reference curves. Sufficient amounts of pooled normal Chacma baboon plasma (n=40) are fortunately available after this study's conclusion.

It does, however, seem that the Technozym® ADAMTS-13 Antigen ELISA kit (Technoclone GmbH, Vienna, Austria) is not suitable for use in this model, and that other kits or the in-house assay previously described (Feys et al., 2006) should be considered for use in future experiments. Alternatively, mAbs against Chacma baboon ADAMTS13 should be developed for use in species-specific assays.

7.2.2 VWF – generally similar to human VWF

VWF:Ag levels were generally comparable to human levels, although the reference range obtained was slightly lower and markedly narrower (47-92% vs 50-150%), which must be considered when interpreting results from previous and future experiments. It also likely indicates that the Chacma baboon VWF molecule is at least similar enough in structure to human VWF, for the polyclonal rabbit anti-human VWF capture antibody used in the assay to bind sufficiently to Chacma baboon VWF. This is probably a consequence of the polyclonal antibody's ability to bind to multiple different epitopes, which may circumvent possible occasional structural differences and contribute to the comparable VWF:Ag concentrations.

VWF:RCo activity was also generally comparable to human activity, although the reference range obtained was higher and broader (76-275% vs 50-150%). Values above the human upper limit cut-off were encountered in 24 animals (60%), resulting in a mean of 174%, which is well above the human reference range. It is an interesting finding (which also has to be considered when interpreting results from previous and future experiments), indicating that Chacma baboon VWF is possibly more conducive to ristocetin-induced agglutination of human platelets than human VWF is. The elevated VWF:RCo activity could also (at least partly) explain the curious previous observations that ADAMTS13 inhibition alone is sufficient to induce (at least early-stage) TTP in Chacma baboons (Feys et al., 2010a), whereas a second hit is required in mice (Deforche et al., 2016) and humans (Vanhoorelbeke and De Meyer, 2013).

Ristocetin binds at two sites within human VWF that flank the platelet-binding areas of the A1 domain: Cys_{1237}–Pro_{1251} within the D3 domain, and Glu_{1463}–Asp_{1472} towards the C-terminal end of the A1 domain (Girma et al., 1990, Berndt et al., 1992, Azuma et al., 1993). Both sites contain numerous proline residues and are brought into proximity by a disulphide bond between Cys_{1272} and Cys_{1458}. This raises the possibility of structural differences between human and Chacma baboon VWF in the D3 and/or A1 domains, which also contain the binding sites for GPIbα and plasminogen, or perhaps even in the A2 domain, as it has been shown that ristocetin also changes the conformation of the A2

domain, in particular making the Tyr_{1605}–Met_{1606} peptide bond available for ADAMTS13 cleavage or oxidation by hypochlorous acid (Chen et al., 2012).

However, sequencing of the Chacma baboon *VWF* exon 28, which encodes the whole A1 and A2 domains, a C-terminal part of the D3 domain, and an N-terminal region of the A3 domain, reveals only 18 amino acid changes of which only four are radical, with none of the radical changes in the A1 domain (Janse van Rensburg, 2019). As outlined in Figure 18, there is a conservative amino acid substitution (Lys for Gln) at position 1238 in the Cys_{1237}–Pro_{1251} ristocetin binding site within the D3 domain. Whether this substitution can explain the enhanced VWF:RCo activity demonstrated by Chacma baboon plasma remains to be explored, but is unlikely. However, a possible effect cannot be excluded without performing in depth crystal structure modelling to ascertain the extent to which the substitution influences the protein's folding and ultimate structure, if at all. The Chacma baboon Glu_{1463}–Asp_{1472} ristocetin binding site in the A1 domain is identical to that of human VWF. Cys_{1272} and Cys_{1458}, which bring the two ristocetin binding sites into proximity through a disulphide bond between them, are also both conserved. The elevated VWF:RCo activity is likely also not an effect of enhanced binding to human platelets via altered GPIbα binding sites in the A1 domain, since all seven GPIbα binding amino acids, as well as Cys_{1272} and Cys_{1458} mentioned above (which are also critical for platelet GPIbα binding) (Cruz et al., 1993), are all preserved in the Chacma baboon (Janse van Rensburg, 2019). The possibility that some other region of the Chacma baboon VWF may exert a modulatory effect on ristocetin binding that is different from that seen with human VWF, i.e. augmenting its platelet-binding ability, cannot be excluded. The Lys_{1491}-Arg_{1492} peptide bond within the VWF A1-A2 linker region, identified as the cleavage site for plasmin (Brophy et al., 2017), is also conserved.

The central 95% interval for baboon VWF:CB values was 26-76% (mean=50%, median=49%), which, although tending towards the lower limit of the human reference range (51-143%), indicates that the very low baseline VWF:CB values found for the two test animals in our pilot study (8% and 7%) (Joubert et al., 2021) are not the norm, with 45% of the baboons (n=18) having VWF:CB levels >51%. These two test animals would in fact have been deemed to have subnormal values for this assay had the central 95% interval found in the current study been applied, leading to the possible exclusion of these

animals as test subjects. This reiterates the value of obtaining species-specific reference ranges for all assays used in this model.

The lower VWF:CB activities found with Chacma baboon plasma do, however, possibly point to structural differences in the baboon A3 domain, and that the specific type of collagen used in the VWF:CB assay is not ideal for assessing baboon VWF:CB activity. Collagen binds to VWF primarily via VWF's A3 domain (Lankhof et al., 1996), with a second site in the A1 domain (Bonnefoy et al., 2006) and additional putative binding sites in the Chacma baboon A2 domain (Janse van Rensburg, 2019). Although the Chacma baboon A1 domain has already been shown to contain no radical amino acid changes when compared to human VWF, and no amino acid changes at the putative collagen binding sites in the A2 domain, only a small portion (position 1673-1682, as part of exon 28) of the Chacma baboon A3 domain has been sequenced to date (Janse van Rensburg, 2019), so that structural differences in the A3 domain cannot be excluded as an explanation for our results. Such structural differences may lead to a decreased binding affinity for the human type III umbilical cord collagen used in our VWF:CB assay, to such an extent that alternative collagen types may yield substantially different results, as has been demonstrated with human VWF:CB experiments involving VWD patients (Favaloro, 2000). Several types of fibrillar collagen bind VWF, with types I and III, in particular, providing strong binding sites for VWF (Deckmyn and Vanhoorelbeke, 2006). It would be interesting to repeat the VWF:CB experiment using a variety of different collagen types, such as HORM® collagen (Takeda, Linz, Austria; derived from equine tendon, consists of a mixture of type I and type III collagen), in order to define the exact collagen type that will yield the most accurate and reliable assessment of Chacma baboon VWF:CB activity, in future experiments using this model.

VWF multimer patterns were generally comparable to human patterns, indicating that any observed differences in VWF:Ag, VWF:RCo or VWF:CB values from human ranges were likely not caused by differences in VWF multimeric composition, and that the VWF multimer pattern assay can be applied successfully in this model unaltered, with complete translatability. It also indicates that the binding of the polyclonal anti-human VWF antibody used for VWF localisation in this assay is not affected by any possible structural differences in the Chacma baboon A3 domain or any other domains. This is likely due to

the polyclonal antibody's ability to bind to multiple different epitopes, which would negate the effect of occasional mismatches and explain the comparable multimer patterns.

Similarly to our ADAMTS13 antigen and activity results, direct comparison of our VWF results with other preclinical studies performed in this model is difficult due to many of the same issues highlighted above, again reiterating the importance of devising standardised testing approaches and reference ranges for future use in this model. Generally, baseline mean VWF:Ag levels reported in previous preclinical studies appear to be higher than the mean of 69% (range: 27-94%, SD=16%) found in the forty baboons assayed in our present study. Although the details of the exact method used are not reported, with the original description of the aTTP baboon model Feys et al. (2010) determined VWF:Ag levels through comparison with pooled normal human plasma (n=20), similar to our approach. They, however, found mean values at baseline ranging from approximately 105% in the subject group (n=6, SD about 15%) to roughly 115% in the control group (n=5, SD approximately 20%). When investigating the potential utility of the anti-VWF nanobody ALX-0681, Callewaert et al. (2012) reported baseline stratification values ranging from 95% in the prevention group (n=4, SD=32%), to 97% in the therapeutic group (n=4, SD=26%), and 103% in the control group (n=4, SD=30%), using a "validated ELISA" not described in further detail. VWF:RCo and VWF:Ag results are expressed as a ratio by Feys et al. (2012) in their study investigating the effects of the anti-VWF mAb GBR600 in this model. However, similar to their approach for reporting ADAMTS13 activity results, the reported VWF:RCo and VWF:Ag results were recalculated from comparison with individual baboons' baseline activities (before 3H9 injection) which were set as 100%. Values derived from comparison with a human calibration reference curve (although obtained for the initial calculations) are not reported, so that baseline VWF:RCo and VWF:Ag results from their study are essentially unavailable for comparison. VWF:Ag levels were not evaluated in the 2017 preclinical study investigating the utility of NAC in this model (Tersteeg et al., 2017b).

In contrast to the VWF:Ag level findings, mean baseline VWF:RCo activities were lower in the single preclinical study with comparable results than in our study. Callewaert et al. (2012) employed a similar light transmission aggregometry-based VWF:RCo assay (using a PAP-8E aggregometer) to that used in our study, but reported mean baseline

VWF:RCo activities ranging from approximately 90% in the therapeutic group (n=4), to around 140% in both the control (n=4) and preventative (n=4) groups, which are all well below the mean of 174% (range: 52-280%, SD=57%) found in our group of 40 baboons. Although the upper limit of quantification in their study is stipulated as 145% for VWF:RCo (Callewaert et al., 2012), it would appear, based on our results, that normal baboon VWF:RCo activity is higher than may have been appreciated in this (Callewaert et al., 2012) and other previous preclinical studies (Feys et al., 2012) in this model, using this assay. This should be borne in mind when stratifying animals into different treatment, prevention, and control groups based on VWF, as was done by Callewaert et al. (2012). Although VWF:RCo activity was evaluated by Tersteeg et al. (2017b) as part of the assessment of the effects of escalating NAC concentrations on baboon VWF in vitro, baseline values were again set as 100%, and baboon VWF:RCo activities were not compared with a human reference curve as in our study. VWF:RCo activity assessment was not included in the initial description of the model (Feys et al., 2010a).

Except for our pilot study (Joubert et al., 2021) already mentioned above, VWF:CB activity was not assessed in any of the previously discussed preclinical studies performed in this model (Feys et al., 2010a, Feys et al., 2012, Callewaert et al., 2012, Tersteeg et al., 2017b). VWF multimer patterns, however, were evaluated as part of all the preclinical studies discussed above (Feys et al., 2010a, Feys et al., 2012, Tersteeg et al., 2017b, Joubert et al., 2021) except the ALX-0681 study by Callewaert et al. (2012). With the initial description of the model (Feys et al., 2010a), VWF multimers were determined using SDS agarose gel electrophoresis similar to our method, and although only one control animal and one subject animal's data are shown, the reported patterns appear essentially identical to normal human plasma, which also mirror our pilot study animals' baseline patterns (Joubert et al., 2021). Both Feys et al. (2012) and Tersteeg et al. (2017b) did not compare the baboon multimer patterns observed in their studies with human plasma, but all animals had baseline mean high molecular weight multimers comprising >30% of all multimers. This is consistent with our results, confirming firstly that the high molecular weight multimers are preserved in Chacma baboon plasma, secondly that Chacma baboon multimer patterns are similar to human patterns (with translatable results), and lastly that the VWF multimer pattern assay is suitable for use in this model.

Although translatability of the VWF results generated in the Chacma baboon model of aTTP appears to be generally retained, our study's VWF results have several implications for the future configuration of the model. Similar to our findings with ADAMTS13 activity, when selecting or designing assays for VWF:Ag, VWF:RCo and VWF:CB in future experiments using this model, it may be more useful to use individual animals' baseline (pre-injection of 3H9) values as the 100% reference point rather than a calibration reference curve based on human plasma, especially if intra-individual changes in response to the induction of TTP or the effects of novel treatment compounds, are to be assessed. Should an absolute value be required, it may be more suitable to use pooled normal baboon plasma for the calibration reference curve instead of human plasma. If human plasma is used as the 100% reference point, all VWF data should be interpreted in the context of our study's reference ranges (see section 7.7 below). Compared with human VWF, Chacma baboon VWF appears to be a more potent ristocetin-induced agglutinator of human platelets than previously appreciated, so that future experiments investigating novel drugs in this model should consider the possibility that Chacma baboon VWF may indeed be physiologically more active than human VWF. Future research (such as sequencing of the whole VWF gene, which is a rich avenue for potential further validation of the model) will be required to elucidate the possible underlying mechanism(s) of this observation.

This difference between human and Chacma baboon VWF activity will need to be considered when extrapolating results from the Chacma baboon model to humans: a more significant anti-VWF effect may potentially be encountered in humans in vivo than would have been expected from any novel drug's baboon model data, if these data were to be based on comparisons with normal human plasma, and assumptions of proof of efficacy were to be based on reductions of VWF:RCo activity to below the human reference range. Although this hypothesis was not explicitly explored with ALX-0681 (the only novel drug to have reached clinical use in humans, as caplacizumab, after successful use in this model), it is noteworthy that both the phase 2 (Peyvandi et al., 2016) and phase 3 (Scully et al., 2019) clinical trials of caplacizumab reported bleeding-related adverse events to be more common with caplacizumab than with placebo. This observation was

not consistent with the initial baboon model data, where complete neutralization of VWF by ALX-0681 was not associated with excessive bleeding (Callewaert et al., 2012).

The VWF:CB assay should possibly be reconfigured before future use in this model. As discussed above, the use of alternative collagen sources should be explored to ascertain the collagen formulation that will give the most accurate reflection of true Chacma baboon collagen binding activity together with specific anti-Chacma baboon VWF antibodies. All other VWF assays employed in this model generally appear suitable for use. VWF multimer patterns should indeed always be checked as a safety feature to confirm that the high molecular weight multimers are still present (if the novel drug's mechanism of action is based on neutralisation of VWF's platelet binding ability, such as with GBR600 or ALX-0681) or as a measure of efficacy to confirm that the high molecular weight multimers are properly degraded (if the novel drug's mechanism of action hinges on destruction/degradation of the VWF multimers, such as with NAC or the thrombolytic agents).

Future evaluation of human VWF-targeted drugs should consider possible structural differences between human and Chacma baboon VWF, as suggested by our VWF:CB results. Two human VWF-targeted molecules have been successfully evaluated in this model, namely GBR600 and ALX-0681. GBR600 is a humanised anti-human VWF mAb derived from the murine mAb NMC-4 (Feys et al., 2012), which recognises the GPIb binding domain of VWF (Fujimura et al., 1991) located in its A1 domain, and ALX-0681 is a bivalent humanised nanobody, also targeting the A1 domain of VWF (Callewaert et al., 2012), as first described for ALX-0081 (Ulrichts et al., 2011). The Chacma baboon A1 domain contains no radical amino acid changes compared with human VWF as shown in Figure 18 (Janse van Rensburg, 2019). However, should future drugs target different domains than GBR600 and ALX-0681, such as the A3 domain (major collagen binding site) or C4 domain (binding site for the platelet integrin αIIbβ3) (Crawley and Scully, 2013), binding may not occur to the same extent as seen with these compounds.

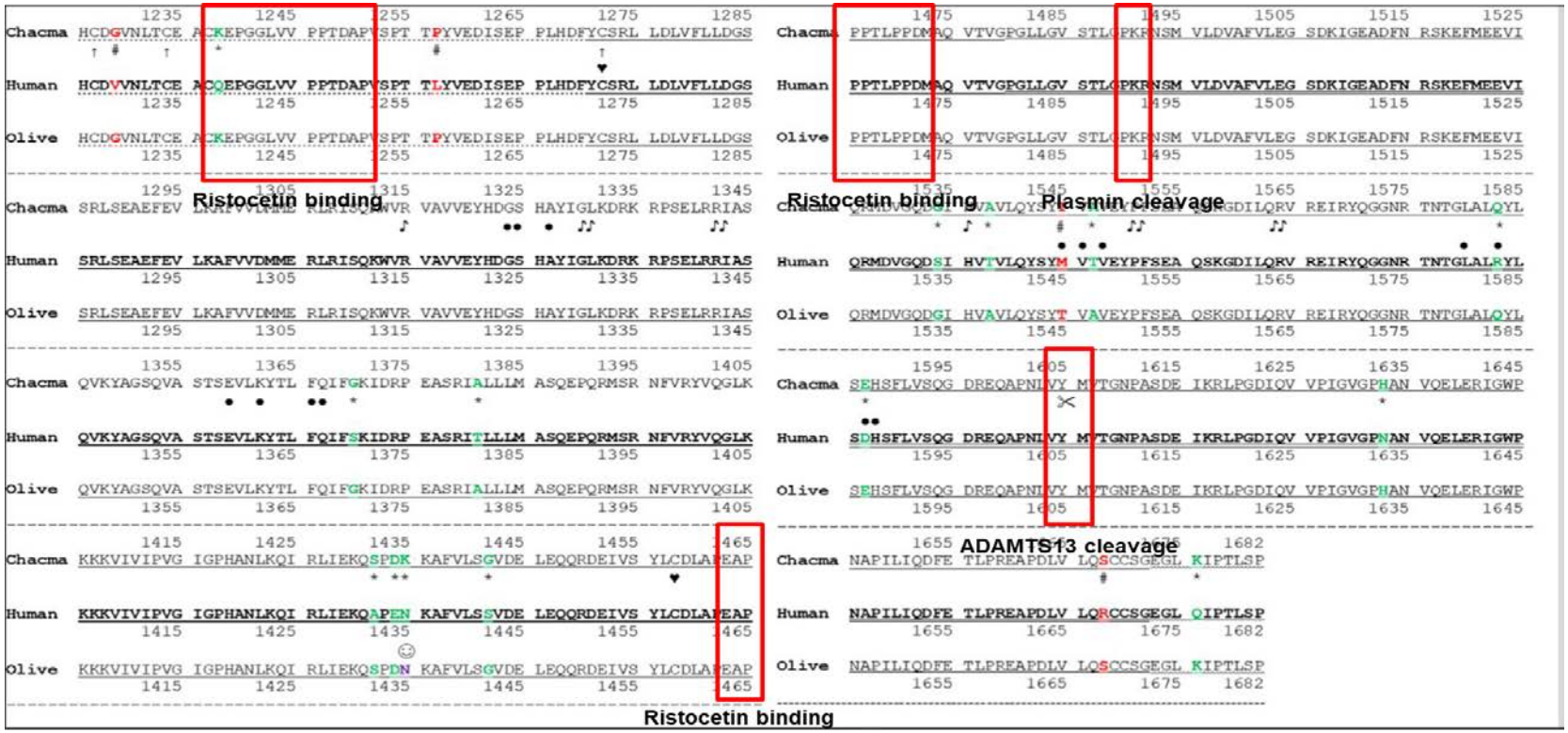


Figure 18: Von Willebrand factor amino acid sequence comparison between the Chacma baboon, Human and Olive baboon, highlighting the two ristocetin binding sites, as well as the cleavage sites for ADAMTS13 and plasmin (both of which are conserved). A portion of the E3 subdomain of the D3 domain is underlined with a dotted line (1226-1270). The A1 domain is underlined with a solid line (1271-1479), and the A2 domain is underlined with a double solid line (1480-1672). A portion of the A3 domain is underlined with a waved line (1673-1682). There is a conservative amino acid substitution in one of the ristocetin binding sites (1238), the significance of which is unclear but likely minimal. Adapted from Janse Van Rensburg (Janse van Rensburg, 2019).

7.3 Elevated thrombin generation ability

Thrombin generation lag times and peak heights of all baboons fell within human reference ranges, but most animals (n=32; 80%) had times-to-peak above (and thus velocity indexes markedly below) the human reference range. However, even when only considering the quantitative data generated by the 90-minute run (Table 4), 97.5% of baboons had an ETP above the human reference range (1565.4–3975.0 nM/L.minute), with a mean ETP of 5942.0 nM/L.minute, indicating a greater overall thrombin generation potential than humans, primarily due to the effect of a prominent thrombin generation "tail" which also persisted well after thrombin generation had ceased in the pooled normal human plasma sample (Figure 9; 120-minute run). This should be considered when designing future experiments involving the haemostatic system or evaluating novel therapies in these animals. Possible explanations for this observation include a less potent natural anticoagulant system in the Chacma baboon than in humans, with lower levels and/or activities of tissue factor pathway inhibitor (TFPI), antithrombin, protein C, protein S, protein Z, protein Z-dependent protease inhibitor, or soluble thrombomodulin, or alternatively, higher FVIII levels, since the most important determinants of the overall amount of thrombin generated in humans (as quantified by ETP) are FVIII, antithrombin, and free protein S (Calzavarini et al., 2019, Bloemen et al., 2019).

In humans, the procoagulant effect of FVIII appears to be particularly important when thrombin generation is initiated by low tissue factor concentrations (Bloemen et al., 2019), such as was used in our experiments. Baseline FVIII levels were determined in 15 male Chacma baboons by Jacquemin et al. (2009) as part of a study investigating the potential clinical utility of the human monoclonal antibody Mab-LE2E9Q (a partial FVIII inhibitor) and reported to generally be in excess of 200 ng/mL (Jacquemin et al., 2009). Since this is above the human reference interval of 100-200 ng/mL (Chavin, 1984), higher FVIII levels in the Chacma baboon may well explain (at least partially) their greater thrombin generation capability and could be examined further in a future study. The role of the natural anticoagulant system, apart from perhaps thrombomodulin which was assessed at baseline by Redl et al. (1995) in a Chacma baboon sepsis model (Redl et al., 1995),

has not been comprehensively explored in this species yet, creating an opportunity for future research.

Although thrombin generation assay methods similar to ours have been used in a preclinical Chacma baboon study of a synthetic heparin mimetic (Herbert et al., 2001) and in preclinical Olive baboon (*Papio anubis)* studies of protein S (Heeb et al., 2012) and the factor IXa inhibitor pegnivacogin and its reversal agent anivamersen (Bel et al., 2015), as well as in a *Papio hamadryas* heat-stroke model study of NAP c2 (Bouchama et al., 2012), the overall Chacma baboon thrombin generation potential has not been investigated in detail yet, using a formal thrombin generation assay. It has, however, been investigated in the Olive baboon, using a previously described simultaneous thrombin plasmin generation assay (Tarandovskiy et al., 2019), as part of a study comparing thrombin and plasmin generation in humans, Olive baboons, Rhesus monkeys, Yorkshire pigs, Sprague-Dawley rats, New Zealand White rabbits and Hartley guinea pigs (Tarandovskiy et al., 2020), which confirmed that the thrombin generation profiles of Olive baboon and Rhesus macaque plasma are broadly similar to that of humans, a finding which mirrored the baseline thrombin generation data of the experiments on pegnivacogin by Bel et al. (2015).

Interestingly, the mean peak thrombin concentrations obtained for Olive baboon (n=10) and human (n=28) plasma by Tarandovskiy et al. (2020) is almost four times greater than we obtained for Chacma baboon plasma, and the production rate is approximately ten times higher. Although ETP values are not reported in this study, it is noteworthy that our mean Chacma baboon ETP was well above the human reference range, despite low peak thrombin concentrations and production rates, again demonstrating the profound effect of the persistent thrombin generation tail on total ETP in Chacma baboon plasma. Whether results obtained in the Olive baboon can be directly compared to our Chacma baboon data remains to be explored, but should not be automatically assumed, due to probable interspecies differences already alluded to.

Regardless of the differences outlined above, our Chacma baboon ETPs are still more similar to human ETPs than pig, rabbit (Siller-Matula et al., 2008), Sprague-Dawley rat, and even Cynomolgus monkey (*Macaca fascicularis*) (Poitout-Belissent et al., 2020),

ETPs are. Siller-Matula et al. (2008), after performing TGAs on six members of each species using a method almost identical to ours, report median ETPs of 2043 nM/L.minute on pigs and 6295 nM/L.minute on rabbits, which represents differences of -52% and +49%, respectively, from the corresponding human median of 4235 nM/L.minute. In contrast, our Chacma baboon median ETP of 5687.4 nM/L.minute is only 34% higher than their human median.

Poitout-Belissent et al. (2020) investigated compound-related effects on coagulability in Sprague-Dawley rats, and Cynomolgus and Rhesus monkeys, using a TGA method similar to ours, and report baseline mean values for the three rats and three rhesus monkeys used in their experiments, which can be used for indirect comparison. The mean ETP of 664.3 nM/L.minute reported for rats is 84% lower than the median ETP reported for humans by Siller-Matula et al. (2008), who, interestingly, reported a much higher median ETP for the same rat species (3075 nM/L.minute, which represents a difference of only -27% from the human median), highlighting the large interindividual variation possibly present in rats, which may also complicate the use of this species in coagulation experiments. Although mean baseline values for Cynomolgus monkeys are not reported, Poitout-Belissent et al. (2020) do provide reference ranges for various TGA parameters for this species, which they calculated to investigate intra-and interassay variation. Cynomolgus monkey ETPs ranged from 1072.5 – 1827.5 nM/L.minute (n=13). The upper limit of this range is still 57% lower than the median human ETP reported by Siller-Matula et al. (2008), indicating that the Cynomolgus monkey is also not the ideal species to use as a model of human thrombin generation. Rhesus monkeys, however, had a mean baseline ETP of 2782.2 nM/L.minute, which, similar to our Chacma baboon ETPs, represents a difference of -34% from the median human ETP reported by Siller-Matula et al. (2008), indicating that this species may also be a suitable model. With a median ETP of 4092 nM/L.minute, which only differs by -3% from the median human ETP, sheep are also a possibly suitable species for coagulation studies (Siller-Matula et al., 2008).

Although formal thrombin generation assays were not performed in any of the preclinical studies conducted in the Chacma baboon model of aTTP (Feys et al., 2010a, Callewaert et al., 2012, Feys et al., 2012, Tersteeg et al., 2017b, Joubert et al., 2021), nor in any of the recent DIC/sepsis model experiments in this species (Schöchl et al., 2017, Silasi et

al., 2019, Puy et al., 2019, Redl et al., 2005), it has implications for these models, as well as for the potential investigation of thrombolytic drugs in the aTTP model. Firstly, since thrombin has also been shown to cleave platelet-bound VWF under flow so that excessive and sustained generation of thrombin would restrict the presence of VWF to its release point (Wohner et al., 2012), a greater overall ETP may explain why (in contrast to clinical experience with TTP in humans) no animal has ever demised or suffered from excessive bleeding, in any of the preclinical studies conducted in this model. Chacma baboons' apparently greater (longer) thrombin generation ability may have an ameliorative effect in the aTTP model, with thrombin acting as a backup mechanism for ADAMTS13.

Secondly, a greater ETP could possibly lead to increased activation of TAFI with a concomitant greater inhibition of fibrinolysis through TAFI's removal of C-terminal lysine residues from fibrin, thereby decreasing the capacity of plasminogen and tPA to bind to fibrin surfaces (Bajzar et al., 1995, Nesheim and Bajzar, 2005). The resultant indirect inhibition of fibrin breakdown could not only lessen TTP-associated bleeding in this model but could also redirect the actions of plasminogen and tPA away from fibrin clots, allowing more plasminogen and tPA to be available for platelet-VWF binding and degradation than would be expected in humans. This implies that should tPA be considered in a future experiment in this model, less fibrin binding may occur than would be expected in humans, with the presence of more free tPA for non-fibrin specific effects, such as VWF degradation via plasminogen.

Conversely, since increased thrombin generation has been reported as a *consequence* of thrombolytic therapy with tPA or uPA in humans (Owen et al., 1988, Wang et al., 2002), implicating a role for the fibrinolytic system in augmenting coagulation, the Chacma baboon's greater overall thrombin generation potential should be considered when designing future in vivo preclinical studies of thrombolytic drugs in the aTTP model. In addition, since this phenomenon may be due to tPA's ability to abrogate the capacity of PAI-1 to inhibit the activity of activated factor XI (FXIa), suggesting that thrombolytic therapy with tPA may increase thrombin generation by decreasing the inhibition of FXIa by PAI-1 (Puy et al., 2019), our finding that Chacma baboon PAI-1 (and tPA) concentrations are generally similar to human levels (see section 7.4.2.1 below), may be particularly important. Therefore, the use of therapeutic tPA in the Chacma baboon aTTP

model may result in significantly greater thrombin generation than expected from a comparable dose in humans. Thirdly, since excess thrombin generation is central to the pathogenesis of DIC (Gando et al., 1998, Toh and Dennis, 2003), Chacma baboons' more potent overall thrombin generation ability may influence DIC/sepsis models in this species, potentially leading to more severe DIC phenotypes than would be expected after a similar insult in humans.

7.4 Fibrinogen and the fibrinolytic system

7.4.1 Fibrinogen – the potential size of the fibrinolysis substrate pool

The fibrinogen concentration range was generally comparable to the human range, albeit with lower cut-off values at both ends of the reference interval. This is in contrast to the findings of Schöchl et al. (2012) that fibrinogen levels are significantly lower in Chacma baboons than in humans. They report a median fibrinogen concentration of 1.026 g/L (n=25, interquartile range: 0.785 – 1.236 g/L) which is 44% lower than our median of 1.83 g/L, obtained using the same (Clauss) method. It is also well below the human reference range for our assay (1.7-4.2 g/L). Although their study only included male baboons, which may explain this discrepancy, the 14 male baboons included in our study, still had a median fibrinogen concentration of 1.67 g/L, which is only 9% lower than the overall median. However, male baboons in our study did have significantly lower fibrinogen levels than the female animals (p=0.0134, see section 7.6 below).

Hampton and Matthews (1966), however, found no difference between human and baboon fibrinogen levels, which may be due to their study design, which included eight males and eight females. Although they used a (now out of favour) micro-Kjeldahl assay for fibrinogen measurement, and the exact species of baboon studied is not mentioned in their 1966 publication (Hampton and Matthews, 1966), they found a mean of 3.37 g/L (SD=0.69 g/L) which is within the human reference range for our assay (1.7-4.2 g/L) and is comparable to our mean of 2.02 g/L (SD=0.68 g/L, range: 1.16-4.26 g/L). Although Venter et al. (1997) report baseline mean fibrinogen concentrations of 2.1 g/L (n=20) in their Chacma baboon metabolic disease model using a similar assay to ours, results from

their study cannot be directly compared to our data since all the test animals used in their study were obese at baseline (Venter et al., 1997).

Data from the aTTP Chacma baboon model are limited to the baseline fibrinogen levels from our pilot study (Joubert et al., 2021) and the initial description of the model (Feys et al., 2010a). In the other preclinical studies, baseline fibrinogen concentrations were either not assessed (Callewaert et al., 2012, Tersteeg et al., 2017b) or assessed but not reported (Feys et al., 2012). Although only male baboons (n=6) were used for the initial description of the model, Feys et al. (2010) report a mean fibrinogen level of 3.0 g/L (SD=0.5 g/L) which is comparable to human ranges, using the same (Clauss) method as the present study. Our pilot study (Joubert et al., 2021), however, revealed a large difference between the two (male) in vivo test animals' baseline fibrinogen concentrations (1.12 g/L vs. 5.30 g/L, using the same method), both of which would have been deemed to fall outside the reference interval calculated from the present data.

Although fibrinogen levels in this species are therefore generally comparable to human ranges, viewed as a whole, these studies suggest that interindividual variation may be more considerable in Chacma baboons than previously anticipated (also see section 7.7 below), which should be considered when designing future experiments in this species. When assigning individual animals to different treatment and control groups, for instance, animals with very high fibrinogen levels should not be automatically assumed to have high levels secondary to an acute phase response, but should still be considered for inclusion (provided an acute phase response is excluded through other means), since the physiological range of fibrinogen may be broader than previously expected. Nevertheless, should a provisionally selected animal's fibrinogen concentration be above the upper limit calculated from our data (4.00 g/L) at initial screening, efforts should be made to ensure that the animal is not currently acutely ill which may confound results. Ultimately, there does appear to be an acceptable level of homology between Chacma baboon and human fibrinogen so that translatability of results is generally retained.

7.4.2 The fibrinolytic system and its potential therapeutic manipulation with thrombolytic drugs

7.4.2.1 tPA and its principal inhibitor, PAI-1

Similar to the fibrinogen data, tPA and PAI-1 levels were generally comparable to human ranges but also with lower cut-offs at both ends of the reference interval. Although this may reflect truly lower levels in *Papio ursinus* plasma, it also confirms at least some homology with the human fibrinolytic system, of at least one plasminogen activator and its inhibitor. This has important implications for the use of thrombolytic drugs in the aTTP model. Firstly, since Chacma baboon tPA was found to be similar to human tPA in number, and (since the assay was able to generate results) possibly in structure as well, the use of human tPA in this model may have clinical effects very similar to its use in humans – a hypothesis discussed in more detail below and probably worth exploring in vivo. Secondly, and perhaps more importantly, with similar PAI-1 levels (and possibly structure) to humans, similar levels of inhibition of any exogenously administered tPA can be expected. For instance if much higher PAI-1 levels had been encountered, tPA dosage adjustments may need to be considered.

Prior to our present study, tPA levels had not been specifically assessed in normal Chacma baboons, although tPA levels were measured by Franz et al. (2004), as part of their investigation of rotational thromboelastography as a tool to quantify the ensuing 'lytic state' during thrombolytic therapy with tPA in this species (Franz, 2009, Franz et al., 2004). Baseline tPA levels in their study (approximate mean = 16 ng/mL) are higher than our levels (mean=2.0 ng/mL, range: 1.4-4.0 ng/mL), again reiterating the value of establishing reference ranges in this species for contextualization of results.

Although one study, conducted in an Olive baboon (*Papio anubis*) sepsis model, reports baseline tPA concentrations of approximately 0 ng/mL (Keshari et al., 2017), most preclinical studies in other baboon species report higher levels than found in our present study. Higher baseline tPA levels are reported in a *Papio hamadryas* heatstroke model study of the effects of recombinant activated Protein C on endothelial injury and microparticle formation for instance (approximate mean = 15 ng/mL, n=14) (Bouchama et al., 2008), as well as in a study of the effects of hypovolaemia (induced by bleeding vs.

lower body negative pressure) on an unnamed baboon species (approximate mean = 25 ng/mL, n=13), which, interestingly, also revealed a subset of baboons with baseline hyperfibrinolysis, found to be strongly associated with tPA levels (Zaar et al., 2020). In this study, perhaps counterintuitively, hyperfibrinolytic baboons (defined using thromboelastography) had the lowest tPA levels at baseline, although the authors concede that correlation does not imply causation (Zaar et al., 2020). Whether these findings can be directly extrapolated to the Chacma baboon is open to speculation. The possibility of the existence of a hyperfibrinolytic subset of baboons (which can possibly be predicted based on tPA levels) is intriguing, however, and worth exploring (perhaps also using thromboelastography) in the Chacma baboon as this has definite implications for the use of thrombolytic drugs in the aTTP model.

Regardless, although it would appear that tPA levels may be physiologically lower in the Chacma baboon than in other baboon species, the mean Chacma baboon tPA concentrations obtained in our study did fall within the human reference range. In contrast, the approximate mean baseline concentrations reported by Bouchama et al. (2008), Keshari et al. (2017) and Zaar et al. (2020) do not, perhaps suggesting a higher level of homology of Chacma baboons to humans. This is encouraging in terms of the aTTP model's translatability and future use to investigate the potential therapeutic effects of thrombolytic drugs. Moreover, the baseline approximate mean PAI-1 concentration of 2 ng/mL (n=13) reported by Zaar et al. (2020), is below the human reference range of 7-43 ng/mL. Our mean of 11.3 ng/mL (range: 1.9-32.3 ng/mL; n=40) was within the human reference range, suggesting good comparability of Chacma baboon and human PAI-1 physiology. Therefore, the main inhibitor of any exogenously administered tPA appears to possibly have more similar levels to humans in the Chacma baboon than in other baboon species, reiterating the suitability of the Chacma baboon over other baboon species to preclinically investigate the in vivo effects of thrombolytic drugs in TTP.

Our results now also put the Chacma baboon results of Puy et al. (2019) into context. In a proof-of-concept pilot study investigating the inhibitory effects of PAI-1 on coagulation through its inactivation of FXIa in a Chacma baboon sepsis model using three baboons, Puy et al. (2019) report a mean baseline PAI-1 concentration of 0 ng/mL (which is below our range of 1.9-32.3 ng/mL; n=40), but also report sequential rises in PAI-1 levels after

bacterial challenge, ranging from approximately 100 ng/mL after 2 hours to concentrations over 600 ng/mL after 24 hours (Puy et al., 2019). Firstly, our data again highlight the importance of obtaining credible reference intervals in this species using a large number of individual animals. Our reference interval suggests that the test animals used by Puy et al. (2019) possibly had subnormal PAI-1 levels at baseline. This observation should also influence the selection or stratification of individual test animals in future experiments. Secondly, and perhaps more importantly, our data now also allow for the accurate interpretation of subsequent rises in PAI-1 values after any intervention in this species. We can for example now better appreciate just how extreme PAI-1 levels rose in the experiments by Puy et al. (2019). Knowing that baseline PAI-1 levels are comparable to human reference ranges with an upper limit that should generally not exceed 30.1 ng/mL in healthy baboons as calculated from our data, the magnitude of the mean PAI-1 level above 600 ng/mL observed 24 hours after *Staphylococcus aureus* injection in their study, can now be better appreciated. Similarly, any quantitative PAI-1 data will also be much better contextualised in future experiments following our present study.

PAI-1 activity (as opposed to concentration) was not assessed in our study but was evaluated in two older preclinical baboon studies: one investigating the association of PAI-1 and PAI-2 with pregnancy in the Olive baboon (Fazleabas et al., 1991) and a later study, comparing the effects of soluble fibre concentrate with those of bezafibrate, on, amongst others, PAI-1 activity in obese Chacma baboons (Venter et al., 1997). Since all test animals were obese at baseline, results from the latter study cannot be directly compared with normal human reference ranges. Therefore, it will be essential to confirm the conclusion discussed above (that human and Chacma baboon PAI-1 physiology may be similar, based on the finding of broadly similar concentrations) by also assessing PAI-1 activity as part of a future study.

7.4.2.2 PAP complexes – an indication of background plasminogen activation

PAP complex concentrations were comparable to human levels, although, unlike what may be seen in humans, no animal had a PAP complex level of 0 ng/mL (the lowest value

obtained was 99.9 ng/mL), possibly suggesting higher background plasminogen activation in Chacma baboons than in humans. It is consistent with the approximate mean baseline values of 30-40 ng/mL reported by Zaar et al. (2020) for an unspecified baboon species, but not with the mean baseline concentrations of approximately 0 ng/mL reported by Keshari et al. (2017) in a study involving Olive baboons (*Papio anubis*), suggesting possible interspecies differences in background plasmin activity, even across different baboon species. However, because PAP complexes are cleared approximately five times faster than α_2-antiplasmin (Collen and Wiman, 1979) or plasminogen (Collen and de Maeyer, 1975), and concentrations may, therefore, decrease within minutes (Levi et al., 1992), the PAP complex levels essentially provide a snapshot of current, ongoing plasminogen activation, so that non-physiological, transient plasminogen activation (related for example to the sedation and handling of the animals) cannot be excluded as a reason for the apparently higher background plasminogen activation evident from our data. Nevertheless, our data ultimately confirm that Chacma baboon PAP complex levels (and therefore background plasminogen activation) are generally comparable to human levels, reinforcing the suitability of the Chacma baboon for preclinical studies involving fibrinolysis.

PAP complex levels were only assessed in our pilot study (Joubert et al., 2021) and not in any of the other preclinical studies in the Chacma baboon aTTP model (Feys et al., 2010a, Feys et al., 2012, Callewaert et al., 2012, Tersteeg et al., 2017b). In our pilot study, the low & intermediate-dose test animal had a baseline PAP complex level (112 ng/mL) within the reference interval now derived from our current data (109.0-215.8 ng/mL), but the high-dose test animal did not. A baseline PAP complex level of 390 ng/mL suggests that this baboon was possibly already afflicted with either a thrombus or some other illness, since our current data would indicate that this animal demonstrated supra-physiological plasminogen activation already at baseline. Again, these observations demonstrate the value of credible reference intervals in this model for the common fibrinolytic system parameters.

7.4.2.3 Plasminogen – a dissimilar driver of fibrinolysis in the Chacma baboon?

Plasminogen was virtually undetectable using the Technozym® Glu-Plasminogen ELISA kit (Technoclone GmbH, Vienna, Austria), with the central 95% interval (1.8-4.2 µg/mL) far below the human reference range of 60-250 µg/mL for this kit (Technoclone, 2014) despite repeating the assay on a second (different) set of aliquots. It is unlikely that this reflects truly lower levels in the Chacma baboon since the discrepancy is so large. It is more plausible that the selected ELISA kit (in its current configuration at least – please also see below) may not be suitable for quantifying this protein in Chacma baboon plasma since it is designed for use in humans. Importantly, this finding suggests possible fundamental structural differences between this protein and its human equivalent. Several lines of evidence from our study indicate that Chacma baboon plasminogen is not only structurally different from human plasminogen but functionally dissimilar as well.

Our plasminogen concentration data indicate that either the monoclonal murine anti-human plasminogen capture antibodies or the POX-conjugated monoclonal murine anti-human plasminogen detection antibodies (or both) used in the Technozym® ELISA kit did not bind to Chacma baboon plasminogen, implying a structural difference in at least one domain. Our observations that streptokinase cannot induce any meaningful plasmin activity in Chacma baboon plasma, whereas comparable amounts of tPA led to greater plasmin activity (as well as a more remarkable clot lysis ability) in Chacma baboon plasma than found in humans, indicate significant functional differences. The implications of these activity differences for the future use of thrombolytic drugs in the Chacma baboon aTTP model are discussed in more detail in sections 7.4.2.6 and 7.4.2.7 below.

However, although plasminogen levels were not assessed in the other preclinical studies conducted in the Chacma baboon aTTP model (Feys et al., 2010a, Feys et al., 2012, Callewaert et al., 2012, Tersteeg et al., 2017b), our pilot study (Joubert et al., 2021) revealed baseline plasminogen levels that were either within (180 µg/mL, in the low & intermediate-dose test animal) the human reference range (60-250 µg/mL) or drastically above it (2562 µg/mL, in the high-dose test animal) using an ELISA kit from the same manufacturer (Technoclone GmbH, Vienna, Austria). Although the possibility exists that the manufacturer had changed the composition of the ELISA kit between the time the

pilot study was conducted in 2016-2017 and the time the current study's laboratory work was performed in 2020 (which would explain the discrepant results), it also raises the possibility that preanalytical factors may have influenced the current results. Since, under certain circumstances (such as during thrombolytic therapy or with endogenous hyperfibrinolysis), Glu-plasminogen may be partially degraded after blood collection (Technoclone, 2014), and since plasminogen can also be completely degraded by fairly ubiquitous proteinases such as elastase, procathepsin D, or metalloelastase (Aĭsina and Mukhametova, 2014), it is conceivable that Chacma baboon plasminogen may be more sensitive to post-collection degradation than previously anticipated. However, the preanalytical conditions of the present study were identical to those of the pilot study. The manufacturer advises that should an increased susceptibility to degradation by proteinases be a possibility, the addition of proteinase inhibitors (such as 2000 units aprotinin or 20 mM benzamidine/mL blood) be considered (Technoclone, 2014). It would be important to confirm our plasminogen concentration results in a repeat future study using the appropriate inhibitors before Chacma baboon plasminogen is conclusively deemed structurally non-homologous to its human counterpart.

Since human apolipoprotein(a), or apo(a), consists of structural domains that are also present in plasminogen (namely KR4, KR5, and the SP domain), and the gene encoding apo(a) (*LPA*) has been closely linked to plasminogen in humans with a high degree of plasminogen sequence homology (McLean et al., 1987, Eaton et al., 1987), the KR5 domain, SP domain and 3' Untranslated Region (UTR) of the Olive baboon (*Papio hamadryas*) plasminogen gene was sequenced (Genbank Accession Number: AF029692) as part of a study investigating the molecular basis of an apo(a) null allele in this species (Cox et al., 1998). Interestingly, the KR5 and SP domains of Olive baboons are respectively 97% and 98% similar to those of Rhesus monkeys, but only 94% and 95% similar, respectively, to those of humans. The Arg_{561}–Val_{562} bond cleaved by plasminogen activators (Aĭsina and Mukhametova, 2014) as well as the amino acid residues of the enzyme active site (His_{603}, Asp_{646}, and Ser_{741}), which form the catalytic triad (Aĭsina and Mukhametova, 2014), are however retained in the Olive baboon.

Whether it can be extrapolated to the Chacma baboon is open to speculation, but since streptokinase's activation of plasminogen is dependent on SP domain binding (Wang et

al., 1998, Parry et al., 2000), it would be interesting to investigate the level of homology of this domain in the Chacma baboon, Olive baboon, and man, by sequencing this gene in the Chacma baboon in a future study, in light of the lack of streptokinase-induced plasmin activity encountered in our present study. Collen et al.'s finding that streptokinase is unable to form a stable stoichiometric complex with (an unspecified) baboon plasminogen (Collen et al., 1993b) further reinforces the hypothesis that the SP domain of baboon plasminogen, in general, may be structurally different from its human counterpart.

Since tPA is able to elicit profound plasmin activity (and concomitant clot lysis) in Chacma baboon plasma (greater than can be induced in human plasma – also see section 7.4.2.6 and 7.4.2.7 below for discussion), it is probable that the Arg_{561}–Val_{562} bond is retained in the Chacma baboon, but conceivable that other structural differences in the SP domain may be present, which could lead to heightened enzymatic activity. Moreover, there may also be structural differences in either the kringle array (particularly in the KR5 domain, which is ultimately responsible for the last conformational change which exposes the Arg_{561}–Val_{562} cleavage site in the activation loop, after binding to fibrin (Law et al., 2012)) or the activation loop itself, which could allow tPA easier access to the cleavage site, and so augment tPA-induced plasminogen activation to levels above that seen in humans. This would also explain the peculiar observation that our high tPA-induced plasmin activity results (mean=178% of activity induced in human plasma, n=40 – also see section 7.4.2.6 below) were encountered in the absence of fibrin.

7.4.2.4 TAFI – a dissimilar indirect inhibitor of fibrinolysis in the Chacma baboon?

Since published mean concentrations for TAFI in various human populations range from 5.71 µg/mL (Fernandes et al., 2015) to 9.6 µg/mL (Mousa et al., 2004), and since TAFI is generally deemed to have a human reference interval of 4–15 µg/mL (70–275 nmol/L) (Bouma and Meijers, 2003, Bajzar et al., 1996, Mosnier et al., 1998), it is clear that virtually no TAFI was detected in our baboon plasma samples, even with the high sensitivity kit used in our study, which has a detection range of 5.00 to 320 ng/mL and a sensitivity of 3.00 ng/mL. Although the lower cut-off limit of the manufacturer-supplied

human reference interval for TAFI (1.7-20.0 µg/mL) is lower than the generally accepted human lower cut-off, the highest baboon value obtained (66.3 ng/mL) was still orders of magnitude below human ranges. This may indicate truly lower levels in the Chacma baboon, or more likely (similar to the ADAMTS13 Ag and plasminogen data), that the particular TAFI kit used may not be suitable for quantification of this protein in *Papio ursinus* plasma, as it was designed for use in humans.

Importantly, this again suggests possible structural differences between Chacma baboon TAFI and its human equivalent, which may or may not be functionally important. To better investigate this hypothesis, TAFI activity assays as described by Binette et al. (2007) in their study of TAFI activation during sepsis in Yellow baboons (*Papio cynocephalus*) and Olive baboons (*Papio anubis*) (Binette et al., 2007) may be useful. However, should Chacma baboon TAFI activities also be confirmed to be lower than those of humans, this may have significant implications for assumptions on the level of homology between the fibrinolytic systems of the Chacma baboon and man. Even its homology with other baboons may be diverse, since Yellow and Olive baboon activated TAFI (TAFIa) have already been shown to exert larger effects on clot lysis than human TAFIa does (Binette et al., 2007). Should Chacma baboons' TAFI levels and activities genuinely be very low, an absent or markedly reduced TAFI effect on clot lysis may partly explain Chacma baboons' short clot lysis times, as TAFI appears to be critical for clot lysis resistance in baboons. TAFIa maximally prolongs clot lysis by approximately 10-fold in clots formed from Yellow and Olive baboon plasma (Binette et al., 2007) but only results in a 3-fold prolongation in clots formed with human plasma (Bajzar et al., 1998). Please also see section 7.4.2.7 below for further elucidation of the potential role of TAFI in our clot lysis assay.

Since the immunogen for the capture and detection antibodies used in our ELISA kit consists of amino acids 115 – 423 of human TAFI (M Wong 2021, personal communication, 23 August), any part of the Chacma baboon TAFI protein corresponding to this portion in humans, could potentially be structurally different from human TAFI. This includes the following functionally essential elements: the Cardin–Weintraub motif (Trp_{210}-Ser_{221}; involved in heparin binding), the dynamic flap region (Phe_{297}-Trp_{350}; involved in maintaining TAFIa stability), all thrombin/plasmin cleavage sites (Arg_{302},

Lys_{327}, and Arg_{330}), a glutamine involved in factor XIIIa-mediated coupling to fibrin (Gln_{292}), numerous cysteines involved in structurally important disulphide bonds (Cys_{156}-Cys_{169}, Cys_{228}-Cys_{252}, and Cys_{243}-Cys_{257}), as well as residues involved in substrate hydrolysis (His_{159}, Glu_{162}, Arg_{217}, His_{288}, and Glu_{363}), substrate specificity (Asn_{234}-Arg_{235}, and Tyr_{341}), and (potentially) in substrate binding (Asn_{219}-Arg_{220}, Ser_{289}-Ser_{291}, Ser_{299}, Gly_{336}, Leu_{340}, and Asp_{348}-Asp_{349}) (Plug and Meijers, 2016). With the possibility of a potentially decreased TAFI effect on in vivo clot lysis and/or a possibly decreased propensity for activation by bovine thrombin compared with human TAFI (please also see section 7.4.2.7 below), the most likely areas of structural difference based on our data, would be the dynamic flap region, the thrombin/plasmin cleavage sites, areas with structurally important disulphide bonds, or those areas involved in substrate binding, specificity, or hydrolysis.

Significant interspecies differences have been demonstrated between bovine and human TAFI gene sequences (Anand et al., 2008) and between human, rat, and murine TAFI gene sequences (Sanglas et al., 2008). Bovine TAFI is 77% sequence identical and 86% sequence similar to human TAFI, with sequence comparisons suggesting that bovine TAFI is more stable than the human form (Anand et al., 2008). Rat and murine TAFI are reported to be 83% and 84% sequence identical, respectively, to human TAFI. Functional differences have also been shown between rat, murine and human TAFI (Hillmayer et al., 2006). Although the sequence of the Chacma baboon TAFI gene has not been formally studied and compared with human TAFI, it is possible that significant differences also exist across these two primate species, creating a potential future research opportunity.

7.4.2.5 Lower α2-antiplasmin concentrations – implications for therapeutic manipulation of the Chacma baboon fibrinolytic system

Although α2-antiplasmin concentrations/activities were not assessed in our pilot study (Joubert et al., 2021), nor in any of the other preclinical studies in the Chacma baboon aTTP model (Feys et al., 2010a, Feys et al., 2012, Callewaert et al., 2012, Tersteeg et al., 2017b), the substantially lower α2-antiplasmin concentrations found in our present study are consistent with results reported by Collen et al. (1993b) for an unspecified

baboon species, used to investigate the differing susceptibilities to activation by streptokinase and recombinant staphylokinase of the fibrinolytic systems of five representative mammalian species. In their experiments, α2-antiplasmin was present in baboon plasma at concentrations less than half that of human plasma, with α2-antiplasmin purification yielding concentrations of 8 µg/mL in baboon plasma, but 17 µg/mL in human plasma. Although baboon α2-antiplasmin was able to rapidly inhibit baboon plasmin, it did this at a rate >10 times slower than that of human α2-antiplasmin in a human system. Although α2-antiplasmin activities or inactivation rates were not measured in our present study, it is plausible that the lower α2-antiplasmin concentrations can also translate into lower rates of plasmin inactivation similar to the previously reported findings (Collen et al., 1993b).

This has important potential implications for the therapeutic manipulation of the Chacma baboon fibrinolytic system. Firstly, this may (at least partly) explain our observation that tPA is able to induce greater plasmin generation and shorter clot lysis times in Chacma baboon plasma than in human plasma (please see sections 7.4.2.6 and 7.4.2.7 below). Secondly, conclusions on the effects of the thrombolytic drugs in any future in vivo aTTP model experiments should be tempered with the knowledge that α2-antiplasmin-mediated plasmin inhibition may be much more profound in humans than in Chacma baboons, leading to a lesser in vivo plasmin effect on human VWF multimers for instance, than would be possible in the Chacma baboon. Thirdly, any differences between human and Chacma baboon plasmin activity (whether physiological or pharmacologically induced) may simply be a function of decreased α2-antiplasmin-mediated plasmin inactivation and not necessarily due to intrinsic plasmin/plasminogen differences between the species. However, this seems unlikely as a sole explanation for our observations, given the potential differences between human and Chacma baboon plasminogen already outlined in section 7.4.2.3 above. Whether the lower α2-antiplasmin levels in Chacma baboons are offset by higher α2-macroglobulin concentrations/activities is unknown and should be investigated in a future study.

7.4.2.6 Potential plasmin activity available for therapeutic utilisation

Regardless of whether Chacma baboon plasminogen may be structurally or even functionally different from human plasminogen as discussed in section 7.4.2.3 above, it is the overall plasmin activity that can potentially be therapeutically harnessed by the application of various drugs, that is ultimately of importance in the future use of thrombolytic drugs in this species in general, and in the aTTP model in particular. It is clear from our current data that streptokinase does not result in meaningful in vitro plasmin activity, even at a range of concentrations, confirming our pilot study findings that streptokinase is not the ideal thrombolytic drug to test the hypothesis that manipulation of the fibrinolytic system may have therapeutic utility in aTTP, in this model (Joubert et al., 2021).

Although there is no other published data on Chacma baboon streptokinase-induced plasmin activity, experiments in Hamadryas baboons (Lijnen et al., 1992, Collen et al., 1993a) and other unspecified baboon species (Hampton and Matthews, 1966, Collen et al., 1993b) have yielded similar results, with low or absent resultant plasmin activity. However, this observation is not universal, as Collen et al. (1993a) were able to show that streptokinase induces dose-dependent clot lysis of jugular vein thrombi in Hamadryas baboons. This suggests at least some meaningful streptokinase-induced plasminogen activation but also hints at possible interspecies differences within baboons. Although the presence of anti-streptokinase antibodies (which may have influenced the in vitro response to streptokinase) was not tested for due to cost constraints, anti-streptokinase antibodies as a sole explanation for the virtually non-existent plasmin activity, seems unlikely, since all forty baboons had almost identical poor in vitro responses to the drug. Moreover, none of the animals had been previously exposed to streptokinase, and an earlier *Papio hamadryas* study (Collen et al., 1993a) confirmed high antibody titres to only emerge after several weeks of repeated streptokinase treatment.

On the other hand, tPA led to clear activation of *Papio ursinus* plasminogen in our study, to activity levels higher than can be obtained in human plasma using the same amount of activator. Moreover, a tPA concentration of 1800 µg/mL (representing ~67% of the baseline concentration of 2700 µg/mL) rendered approximately the same amount of

plasmin activity in baboon plasma as the 2700 µg/mL concentration did in human plasma. Whether this is simply due to lower concentrations in Chacma baboon plasma of plasmin's principle inhibitor α2-antiplasmin is unclear (see also section 7.4.2.5).

In addition, escalating tPA concentrations led to clear dose-dependent activation of *Papio ursinus* plasminogen, reaching a plateau at either 5400 µg/mL (n=9) or 7200 µg/mL (n=31). This ability to generate convincing plasminogen activation in Chacma baboon plasma in vitro mirrored the in vivo experience of Franz et al. (2004) in the Chacma baboon. As also outlined in a later review (Franz, 2009), intravenous infusion of a recombinant human tPA (alteplase; Actilyse®) identical to that used in our present study resulted in significant fibrinolytic responses (as measured with rotational thromboelastography) to incremental tPA dosage, with 0.25 mg/kg being the lowest dose at which an identifiable response was noted. Increasing fibrinolytic activity could be appreciated at doses of 0.5 and 1 mg/kg, which was the maximum dose studied. Assuming a total blood volume of 70 mL/kg (Fortman et al., 2002) and a maximum possible haematocrit of 0.49 L/L (Melton and Melton, 1982), these doses would correspond roughly to concentrations of 7, 14, and 28 µg/mL plasma, respectively. Interestingly, the lowest concentration used in our in vitro experiments (28.125 µg/mL, which would correspond roughly to an in vivo dose of 1 mg/kg) was only able to induce low levels of plasminogen activation in vitro. However, when viewed in the context of the data from Franz et al. (2004), this may be sufficient to generate enough plasminogen activation in the Chacma baboon in vivo to be able to evaluate the effects of tPA in the aTTP model successfully.

It would be prudent to also correlate the maximum future in vivo doses in the aTTP model (and even other experiments) with the current in vitro activities data presented here. The maximum dose should probably not exceed 252 mg/kg, corresponding to the maximum in vitro concentration of 7200 µg/mL used in the present study. The tPA-induced VWF multimer degradation results indicate that the maximum dose should probably not exceed 63 mg/kg, which corresponds to the in vitro concentration (1800 µg/mL) at which tPA-induced plasmin activity resulted in maximal degradation of the high molecular weight VWF multimers (see sections 6.2.2.2 and 7.4.2.8). However, such mega doses, as was evaluated for streptokinase in our pilot study (Joubert et al., 2021), will probably not be

required. Since our data indicate that significant plasmin activity is present even at low concentrations, and early murine experiments with streptokinase showed that a dose representing 20% of the loading dose commonly used in humans to treat PE and stroke was able to successfully resolve aTTP (Tersteeg et al., 2014), a starting dose in the Chacma baboon aTTP model of as low as 0.3 mg/kg would be rational, as this corresponds to approximately 20% of the human tPA dose per kilogram body weight (100 mg in patients >67 kg body weight, i.e. a maximum dose of approximately 1.5 mg/kg). Moreover, since Franz et al. (2004) demonstrated plasmin activity in vivo at a tPA dose of 0.25 mg/kg, a wide range of doses (including the seemingly very low doses outlined above) should be considered in future experiments in this model to establish proof-of-concept, bearing the warning by Collen et al. (1993a) in mind that in vitro activity may not be representative of in vivo thrombolytic performance.

Our finding that human recombinant tPA is a more potent activator of Chacma baboon plasminogen than of human plasminogen is consistent with observations reported for the Olive baboon (Tarandovskiy et al., 2020). Using recombinant human tPA as plasminogen activator as part of a previously described plasmin generation assay (Tarandovskiy et al., 2019) with a similar plasmin substrate and method principle to our plasmin activity assay, Tarandovskiy et al. (2020) demonstrated a final mean plasmin peak in the ten Olive baboons studied that is approximately 20% higher than the corresponding human value. The difference in plasmin generation rates is even more dramatic, with Olive baboon plasmin generation rates 160% higher than human plasma. These data correlate broadly with our mean baseline Chacma baboon plasmin activity level of 178% (of human activity) and suggest that the plasminogen molecules of baboons as a broader group may be more susceptible to activation by human recombinant tPA than human plasminogen is.

Interestingly although Rhesus monkeys had plasmin generation rates more similar to human rates than Olive baboons had, their final mean plasmin peak was even higher than those of Olive baboons, suggesting that baboons may be a more suitable fibrinolysis model than Rhesus macaques. These authors caution that even within the most similar species (baboons, Rhesus macaques and humans), fibrinolytic system differences can be detected (Tarandovskiy et al., 2020), which could potentially also be applicable across different baboon species as well. Runge et al. (1996) investigated the in vivo use of

recombinant tPA as part of a study designed to evaluate the efficacy of a hybrid recombinant plasminogen activator (consisting of the anti-fibrin antibody 59D8 combined with low-molecular-weight single-chain uPA) in an unspecified baboon species (Runge et al., 1996). They showed that recombinant tPA affects both venous and arterial thrombi in their model, although tPA was inferior to the study drug. Remarkably, and also with implications for the Chacma baboon aTTP model, tPA significantly increased the bleeding time whereas the study drug did not, which could possibly be a feature of human tPA's more potent activation of baboon plasminogen, consistent with our observations. Moreover, in their model, tPA generated more systemic plasminogen activation than the study drug, and although this is to be expected since the study drug was designed to be absolutely fibrin-specific, systemic tPA-induced plasmin activity may be a beneficial feature of this drug in the context of TTP, where off-target effects (e.g. VWF or GPIbα degradation) may actually be desirable. This is also consistent with our observation that robust plasmin activity was demonstrable in our experiments in the absence of fibrin.

The greater plasmin activity generated by recombinant human tPA in baboon plasma compared with human plasma has several obvious implications for the use of recombinant tPA in the Chacma baboon aTTP model in terms of translatability. Firstly, any possible utility of the drug in the treatment of aTTP in humans may be overestimated since the same level of plasminogen activation (and the resultant possible effect on VWF multimer composition or GPIb interaction) achieved in the Chacma baboon aTTP model in vivo may not be attained in vivo in humans. However, our in vitro results (see section 7.4.2.8) indicate that unfurled Chacma baboon VWF may be less sensitive to degradation by plasmin than unfurled human VWF, despite Chacma baboon plasminogen's greater susceptibility to activation by tPA. Secondly, a different dosage may be required in vivo in humans than suggested by any future Chacma baboon model data. Should this drug be successful in treating aTTP in the Chacma baboon model, it is possible that a different dose will be required in humans to achieve the same effect, depending on whether the discrepant tPA-induced plasminogen activation potentials between the two species or the different sensitivities of baboon vs. human VWF to plasmin degradation (see section 7.4.2.8), is more important in vivo. Thirdly, the more potent plasmin activity has safety implications. Since these animals may now have a higher bleeding risk than expected

with a comparable dose in vivo in humans, any excessive bleeding in future experiments should be interpreted with caution and not summarily equated to a similarly increased risk in humans.

7.4.2.7 Actual clot lysis ability of tPA-activated Chacma baboon plasmin

The plasmin activity generated by tPA also resulted in actual clot lysis in vitro and not merely in plasmin activity measurable by chromogenic assay. This is an important in vitro validation of the effects of tPA before proceeding to any future in vivo study in this model. The greater plasmin activity generated by tPA in Chacma baboon plasma than human plasma also appears to translate into slightly quicker clot lysis times in vitro. This also confirms the implications for its use in the Chacma baboon aTTP model in terms of efficacy, dosage, and safety, already outlined in section 7.4.2.6 above. Interestingly, in the only other study to assess the clot lysis ability of the Chacma baboon (Schöchl et al., 2012), clots were found to be more resistant to fibrinolysis than human clots, although this study assessed fibrinolysis using thromboelastometry, and plasmin generation was not actively induced using a thrombolytic drug such as tPA. However, this does highlight the lytic potency of tPA in Chacma baboon plasma, given the apparent intrinsic resistance of Chacma baboon clots to fibrinolysis. In contrast, Cynomolgus monkeys (*Macaca fascicularis*) and humans are reported to have similar Maximum Lysis percentages on thromboelastometry (Spiezia et al., 2010), again highlighting the dangers of assuming uniform clot lysis abilities across all primate species.

In a study of thrombin-thrombomodulin's activation of TAFI in a Yellow baboon and Olive baboon *Escherichia coli*–induced sepsis model, Binette and co-workers (2007) performed clot lysis time assays using a method similar (although not identical) to ours. Interestingly, they found that when activated baboon TAFI was neutralised by adding potato tuber carboxypeptidase inhibitor (PTCI), clot lysis times were 89% lower in the PTCI-exposed plasma than in controls, suggesting a significant role for activated baboon TAFI in rendering clots resistant to tPA-induced fibrinolysis. The very low levels of TAFI in Chacma baboon plasma (if accurate) may therefore possibly also contribute to quicker clot lysis compared with human plasma, which, in combination with the Chacma baboon's

propensity for greater tPA-induced plasminogen activation, culminates in an overall increased susceptibility to tPA-induced fibrinolysis, than would be achieved in humans. Moreover, as also discussed in section 7.4.2.4, lower Chacma baboon TAFI concentrations may be particularly important in this context, as it appears that baboon TAFIa may have a larger effect on clot lysis than human TAFIa does. TAFIa was shown to maximally prolong Yellow baboon and Olive baboon clot lysis by a factor of almost 10 (Binette et al., 2007). In contrast, only a 3-fold prolongation was observed in clots formed with human plasma (Bajzar et al., 1998).

Another possibility to consider is that Chacma baboon TAFI is not readily activated by the bovine thrombin used in our modified clot lysis assay so that TAFI's potential inhibition of fibrinolysis is negated. Although TAFI can also be triggered by other proteases (Kawamura et al., 2002, Tan and Eaton, 1995, Eaton et al., 1991), including plasmin (Mao et al., 1999, Marx et al., 2002, Wang et al., 1994) which is present during the clot lysis assay reaction, these are not as potent as thrombin, especially when they are acting in isolation (Binette et al., 2007). Therefore, even if TAFI is indeed present in sufficient quantities, it may conceivably remain inactivated (or be only partially activated) in our assay, rendering it unable to significantly inhibit fibrinolysis – a potential theoretical limitation of our modified clot lysis assay. However, the situation may be markedly different in vivo, as more prolonged thrombin generation in vivo (discussed in section 7.3 above) could actually lead to greater activation of TAFI in the Chacma baboon than in humans, with a concomitant greater inhibition of fibrinolysis.

7.4.2.8 tPA-induced degradation of Chacma baboon VWF multimers – a possible treatment option worth exploring in the aTTP model?

Of all the data generated by the present study, the VWF multimer pattern results, which demonstrate the effect of tPA-induced plasmin generation on VWF in the unfurled (non-globular) state in the absence of ADAMTS13 activity, arguably have the most considerable implications for the future investigation of thrombolytic drugs in the Chacma baboon aTTP model.

Firstly, the apparent lack of effect on native (globular) VWF in both Chacma baboons and humans (Figure 15) is a desirable safety feature of tPA. Since any tPA-induced plasmin activity will therefore spare native VWF, the pool of globular VWF available for normal haemostasis should be unaffected by tPA therapy in the aTTP model. This mirrors earlier studies on human VWF–plasmin(ogen) interactions, where plasminogen was shown to bind to a binding site in the VWF A1 domain that is cryptic in globular VWF (Tersteeg et al., 2014) and that this occurs at the Lys_{1491}-Arg_{1492} peptide bond within the VWF A1-A2 linker region in a shear- and glycan-dependent manner (Brophy et al., 2017). Although plasmin is able to gradually cleave globular human VWF (Berkowitz et al., 1987, Wohner et al., 2012), it binds with limited affinity and the resultant cleavage is not rapid. Unfolding of VWF strongly enhances this process, so much so that plasmin (whether activated by urokinase or streptokinase) was able to destroy platelet–VWF complexes on endothelial cells and in suspension in a matter of seconds in earlier studies, indicating that the efficiency of human VWF cleavage by plasmin is a function of its conformation (Tersteeg et al., 2014). Our results confirm that the same principle holds true for Chacma baboon VWF–plasmin(ogen) interactions, i.e., globular Chacma baboon VWF is insensitive to degradation by plasmin, whereas unfolded VWF is readily cleaved.

Secondly, in the absence of ADAMTS13, tPA appears to have a distinct effect on the culprit high molecular weight multimers (involved in forming the pathological platelet-VWF-rich microthrombi) when they are in an unfurled conformation, in both human and Chacma baboon plasma (Figure 17). This effect appears more prominent in human plasma than in Chacma baboon plasma. Since tPA-induced plasmin activity is more remarkable in Chacma baboon plasma than in human plasma, the more exaggerated effect of tPA on human VWF is likely due to an increased susceptibility of human VWF to human plasmin degradation, compared with baboon VWF. Therefore, although this experiment provides in vitro evidence of tPA-induced degradation of Chacma baboon VWF, suggesting possible efficacy of tPA in the treatment of aTTP in the Chacma baboon model, this effect may be even more pronounced in humans, and should be considered when interpreting future Chacma baboon aTTP model data from experiments involving tPA. In light of tPA's greater plasminogen activation potential in Chacma baboons than in humans (see section 7.4.2.6), future investigators must consider that Chacma baboon

VWF may, in fact, be much less susceptible to plasmin degradation than apparent from this experiment, since, despite the more significant plasmin activity achievable in Chacma baboon plasma, the resultant VWF degradation was still less impressive than seen in human plasma. Thus, tPA doses that may successfully attenuate aTTP in humans may not necessarily be effective in the Chacma baboon aTTP model in vivo.

Thirdly, it is also important to note that the in vitro effect on the VWF multimers observed here occurred in the absence of any fibrin, suggesting that tPA's plasminogen activation is sufficiently non-fibrin-specific to potentially have therapeutic utility in aTTP. Streptokinase would possibly be a better choice of thrombolytic drug to treat aTTP in humans since its activation of plasminogen is non-fibrin-specific. However, streptokinase has now been confirmed to be unable to activate Chacma baboon plasminogen sufficiently to establish proof-of-concept in this model. Although tPA is relatively fibrin-specific (Collen and Lijnen, 2005), it would appear from our results that it does still lead to sufficient off-target plasminogen activation in Chacma baboon plasma to be a viable treatment option worth exploring in the aTTP model. Whether the tPA-induced, plasmin-mediated VWF degradation evident in vitro will be sufficient to ameliorate aTTP in the Chacma baboon model in vivo will have to be confirmed experimentally as part of a future in vivo study.

In terms of possibly using even fibrin-selective thrombolytic drugs such as tPA to treat aTTP in humans, Federici et al. (1992) have demonstrated that plasma VWF is indeed degraded during thrombolytic therapy in patients with acute myocardial infarction, but that the degree of degradation depends on the type of thrombolytic agent, being greater for streptokinase than for the fibrin-selective drugs tPA and urokinase (Federici et al., 1992). Ultimately, tPA may be used in the Chacma baboon aTTP model to establish proof-of-concept, but streptokinase may be a better therapeutic option in humans once the utility of thrombolytic drugs is confirmed in principle in the Chacma baboon model. However, since streptokinase has been shown to result in a two- to threefold rise in VWF antigen and activity within 2 hours of a single dose (with the greatest increase seen among the largest multimers) (Hamilton et al., 1985), tPA may be a more rational option in the context of aTTP. Plasmin's ability to cleave the human platelet receptor GPIb (Adelman et al., 1985) through plasmin's lysine binding regions (Adelman et al., 1986), may also

contribute to its possible therapeutic effect in aTTP, even in the Chacma baboon model, given that Chacma baboon and human GPIb are similar (Janse van Rensburg, 2016).

Interestingly, higher tPA concentrations had a less prominent effect on both human and Chacma baboon VWF multimers. There are several possible explanations for this, all of which are ultimately speculative and may be investigated in future studies. One possible reason is that the excess tPA activates all available plasminogen to such an extent that competitive inhibition between active plasmin molecules ensues, which manifests as decreased degradation of VWF, with concentrations of 7200 µg/mL and above. This is consistent with the plasmin activity assay data (see sections 6.2.2.2 and 7.4.2.6), where a plateau in plasmin activity was evident in most animals (n=31, 77.5%) at a concentration of 7200 ug/mL. It is also conceivable that massive initial activation of plasmin leads to a rapid breakdown of tPA, leaving too little tPA to carry the rest of the reaction. Increasing α2-antiplasmin inhibition of the generated plasmin is unlikely, but increasing α2-macroglobulin activation (and subsequent plasmin inhibition) cannot be excluded. Elevated PAP complex levels or other plasmin-induced breakdown products (including VWF breakdown products) may also be inhibiting plasmin-mediated VWF breakdown. Regardless of the underlying cause(s) for this observation, it leads to the fourth critical implication of these results for the future investigation of tPA in the Chacma baboon aTTP model: any future in vivo study should not exceed a dose of 63 mg/kg, which corresponds to the concentration of 1800 µg/mL found here to lead to maximal VWF multimer degradation in vitro.

Although it is possible that VWF immediately reverted to its globular state in the 3-hour incubation specimens before the addition of tPA, the curious observation that longer incubation times led to a less prominent effect on the VWF multimer patterns of both human and Chacma baboon plasma seems to indicate that tPA-induced, plasmin-mediated, VWF degradation may be reversible, implying only temporary disruption of the multimer patterns at baseline and 30 minutes incubation, with possible gradual re-multimerization occurring after 3 hours. α2-antiplasmin's remarkable ability to neutralise plasmin within 10 ms (Medcalf, 2015) could contribute to the short-lived effect. In addition, it is also possible that any excess plasmin not neutralised by α2-antiplasmin or α2-macroglobulin, eventually assumes an inactive open conformation, conceptually similar

to what has been reported for ADAMTS13 (Roose et al., 2020), adding to the temporary effect on the multimers. A more likely scenario, however, is that this may simply represent an in vitro artefact. Since, in contrast to previous experiments demonstrating the effects of plasmin on VWF (Tersteeg et al., 2014), these samples were not subjected to flow/shear forces/conditions during incubation (which, in vivo, will disperse all recently cleaved VWF), the resultant, accumulating, low molecular weight multimers could still gradually adhere to one another after plasmin activity had ceased, and can conceivably then migrate as large, loosely associated clumps during electrophoresis, despite not being formally assembled in their native high molecular weight multimer structures anymore. Furthermore, the urea concentration needed to keep Chacma baboon VWF in an unfolded state indefinitely is unknown, and can perhaps be investigated in a future study. Nevertheless, this is the fifth possibly important implication of these results: tPA administration may have to be repeated frequently in any future in vivo aTTP model experiments to maintain a persistent effect and may have to be administered at intervals as short as 3 hours or shorter.

Irrespective of the essential caveats outlined above, these results provide the first direct in vitro experimental evidence that human recombinant tPA can induce degradation of Chacma baboon high molecular weight VWF multimers, to such an extent that a repeat of our pilot study using tPA as plasminogen activator (instead of streptokinase) is probably justified.

7.5 ABO and Rh blood groups and their influence

Fascinatingly, in contrast to a previous study in South Africa of ABO blood groups in two Chacma baboon subspecies (*Papio ursinus orientalis* and *P.u. occidentalis*), where no baboon was found to be of blood group O in either of the two subspecies (Downing et al., 1975), the majority (n=30; 75%) of animals in our study were of blood group O. No baboons in our study typed as group B or AB either, while other studies reported the distinct presence of the B allele, similar to humans (Downing et al., 1973, Downing et al., 1975). This may be a consequence of the fact that only purpose-bred baboons from the UFS Animal Research Centre’s breeding colony were used for this study without the

addition of any wild-caught animals, which may have limited the genetic diversity of the group.

In contrast to observations in humans (Mannucci et al., 2004), baboons with blood group O had lower ADAMTS13 antigen levels than non-O baboons (p=0.0010). Still, since ADAMTS13 antigen levels were very low in all animals tested, this may merely be secondary to assay limitations (see section 7.2.1) and should be interpreted with caution. Nevertheless, as is recommended in humans, it is probably prudent to interpret any Chacma baboon ADAMTS13 data within the context of the ABO blood group, and future experimental designs in this species should take this observation into account.

A vital novel finding which has implications for any future experiment involving haemostasis in this species (not just in the aTTP model but also in sepsis models) is the significantly higher fibrinogen levels (p=0.0355) found in blood group O baboons. Since fibrinogen concentrations are reported not to be affected by ABO blood groups in various human populations (Kremers et al., 2015, Wang et al., 2017), this is an essential interspecies difference that should be taken into consideration when planning future Chacma baboon model experiments.

Although thrombin generation appears to be increased in humans of non-O ABO blood group (Bloemen et al., 2019), this was not observed in our study of the Chacma baboon. Ultimately, the bulk of the parameters of importance in TTP (and its potential treatment with thrombolytic drugs) were unaffected by ABO blood group, including parameters derived from assays with an experimental component or some form of external specimen manipulation.

The effect of Rh blood group could not be determined since all animals were Rh-positive. This is at odds with the results of a previous study of non-ABO blood group systems conducted by Ramis et al. (2013) in several non-human primate species, where none of the nine Chacma baboons included in their study expressed any Rh antigens (D, C, c, E or e) (Ramis et al., 2013). Although this may also be a consequence of the limited genetic diversity in the UFS Animal Research Centre’s breeding colony, it remains a curious and somewhat unexpected finding, which could be the subject of future research.

7.6 Influence of animals' sex

Individual animals' sex influenced Chacma baboon thrombin generation capability. The majority of animals were female (n=26; 65%, males: n=14; 35%) but males overall had less potent thrombin generation, with longer lag times (p=0.0475), lower peak thrombin concentrations (p=0.0203), and, notably, lower ETPs (p=0.0238). This is consistent with observations in humans, where thrombin generation was also reported to be increased in women (Marchi et al., 2015, Calzavarini et al., 2019, Bloemen et al., 2019). Whether this is due to sex-related differences in FVIII, antithrombin, or free protein S, as is reported for humans (Calzavarini et al., 2019, Bloemen et al., 2019), remains to be elucidated.

In addition, in contrast to humans where no sex-related difference is evident (Marchi et al., 2015, Calzavarini et al., 2019, Bloemen et al., 2019), fibrinogen concentrations were significantly lower in male baboons (p=0.0134) so that male animals almost appear "primed to bleed" (or female baboons seem "primed to clot"). This has implications for the selection of individual test animals for any future experiments in this species and the sex-based composition of any larger groups and subgroups, which, depending on the experimental design, should now arguably include both male and female animals.

Similar to humans (Budkowska et al., 2019), male baboons had significantly higher PAP complex concentrations (p=0.0188), which possibly indicate higher baseline background fibrinolytic activity than females, although there is no supporting evidence for this from the plasmin activity or modified clot lysis time data. Fundamentally, however, most of the key role players in TTP (and its potential treatment with thrombolytic drugs) were uninfluenced by the animals' sex.

7.7 Consolidated reference intervals and comparison with human biological variation

A summary of all the novel reference intervals calculated in the Results Chapters above is provided in Tables 19-21 below for those parameters where meaningful results could be generated, i.e. where assays were not suspected of being affected by significant interspecies differences (e.g. TAFI). One of the major strengths of these reference

intervals compared with other studies (and indeed of this entire thesis) is the large number of animals (n=40) included in the calculations. Other studies in the Chacma baboon, which focused specifically on establishing reference intervals for other haemostatic parameters such as platelet function and thromboelastometry, included only 24 (Ponschab et al., 2016) or 25 (Schöchl et al., 2012) animals, respectively. Moreover, unlike in our study, both these studies only included male baboons, which may bias the results. Nevertheless, in accordance with the CLSI guidelines for the establishment and verification of reference intervals (CLSI., 2008), ABO-specific and sex-specific reference intervals were not calculated using our data due to the limited numbers of animals per subgroup, even for those parameters where differences were apparent.

The interindividual variation, represented by the CV, is also compared in Tables 19-21 with a selection of published human interindividual (between-subject; CV_g) biological variation values, where available. All parameters of the VWF-ADAMTS13 axis and thrombin generation capability of the Chacma baboon and many of its fibrinolytic system components, displayed interindividual variation comparable to that of humans. This is encouraging in terms of the continued suitability of this species as an animal model of human haemostatic disease and the translatability of results. Thrombin generation, in particular, had interindividual variation remarkably similar to that of humans, although the differences outlined in other sections above need to be considered when interpreting results, such as the markedly different reference interval for ETP, for instance.

Fibrinogen and especially α2-antiplasmin (see sections 7.4.1 and 7.4.2.5 above) showed greater variability than in humans, and results from future experiments in this species should be interpreted with caution and with this higher interindividual variability in mind. The Chacma baboon's extremely high interindividual CV for α2-antiplasmin (270.5%) is somewhat unexpected since both interindividual (CV_g) and intraindividual (CV_i) human biological variation are reported to be well below 10%, at 7.1% (Wada et al., 2004) and 6.6% (Costongs et al., 1985b, Costongs et al., 1985a), respectively. Intraindividual variation was not assessed in the present study but may be the subject of future research.

Chacma baboon interindividual variation for tPA was lower than human interindividual variation. This could be explained by the sampling times of the specimens. Human tPA

levels display a prominent circadian rhythm, characterized by low early morning levels and high afternoon peaks, resulting in a peak-to-trough difference of almost 150% (Kluft et al., 1988). Since all baboon specimens were collected at roughly the same time of day (mid-morning), the effect of such a circadian rhythm (if also present in Chacma baboons) would have been negated, leading to less interindividual variation than reported in humans.

Table 19: Consolidated reference intervals and interindividual variation in the Chacma baboon (n=40), of the various ultimately testable quantitative parameters of the VWF-ADAMTS13 axis, compared with the local human reference intervals and published human interindividual biological variation respectively, where available.

Parameter	Chacma Baboon Reference interval (2.5%–97.5%)	Human reference interval for assay	%CV	Human $\%CV_g$, with reference
ADAMTS13 Act (%)	23-69	40-130	27	9.63 (Kilercik et al., 2014) 26.69 (van Marle et al., 2019)
VWF:Ag (%)	47-92	50-150	23	22.59 (Kilercik et al., 2014) 28.6 (de Maat et al., 2016) 31.71 (Shou et al., 2016) 36.09 (van Marle et al., 2019)
VWF:RCo (%)	76-275	50-150	33	18.5 (Kilercik et al., 2014) 31.2 (de Maat et al., 2016)
VWF:CB (%)	26-76	51-143	34	28.0 (de Maat et al., 2016)

%CV = Coefficient of Variation; $\%CV_g$ = Interindividual (between-subject) biological variation.

Table 20: Consolidated reference intervals and interindividual variation in the Chacma baboon (n=40), of fibrinogen and the various ultimately testable quantitative parameters of the fibrinolytic system, compared with the local human reference intervals and published human interindividual biological variation respectively, where available.

Parameter	Chacma Baboon Reference interval (2.5%–97.5%)	Human reference interval for assay	%CV	Human $\%CV_g$, with reference
Fibrinogen (g/L)	1.28-4.00	1.70-4.20	***33.67***	17.0 (de Maat et al., 2016) 20.2 (Sakkinen et al., 1999) 21.8 (Bloemen et al., 2019)
tPA (ng/mL)	1.6-3.0	2-8	***24.9***	47.8 (de Maat et al., 1996) 45.6 (Sakkinen et al., 1999)
PAI-1 (ng/mL)	2.6-30.1	7-43	63.1	70.9 (Sakkinen et al., 1999) 91* (Horne et al., 2002)
PAP complex (ng/mL)	109.0-215.8	0-514	28.8	26.0 (Wada et al., 2004) 29.3 (Sakkinen et al., 1999)
α2-antiplasmin (µg/mL)	1.2-21.8	25-50	***270.5***	7.1 (Wada et al., 2004)
tPA-induced plasmin activity (%)	124-261	±100	26	N/A
tPA-induced modified clot lysis time (minutes)	8.5-12.6	±13.4	11.6	10.2 (Talens et al., 2012)

%CV = Coefficient of Variation; %CVg = Interindividual (between-subject) biological variation; N/A = Not Available.

*Postmenopausal women.

%CVs that differ by >10 from the closest human $\%CV_g$ are shown in bold italics.

Table 21: Consolidated reference intervals and interindividual variation in the Chacma baboon (n=40), of the various ultimately testable quantitative parameters of thrombin generation, compared with the local human reference intervals and published human interindividual biological variation respectively, where available.

Parameter	Chacma Baboon Reference interval (2.5%–97.5%)	Human reference interval for assay	%CV	Human $\%CV_g$, with reference
Lag-time (minutes)	4.0-5.0	1.8–6.3	11.4	13.9 (Bloemen et al., 2019) 14.7 (Zekavat et al., 2014)
Peak-height (nM/L)	70.5-184.5	35.3–661.7	29.8	27.3 (Bloemen et al., 2019) 38.0 (Zekavat et al., 2014)
Time-to-peak (minutes)	11.0-19.0	4.1–12.1	16.6	14.3 (Bloemen et al., 2019) 56.1 (Zekavat et al., 2014)
Velocity index (nM/L/minute)	5.9-23.1	24.6 – 270.7	42.6	48.2 (Bloemen et al., 2019)
ETP (nM/L.minute)	4252.7-7796.8	1565.4–3975.0	18.8	16.4 (Bloemen et al., 2019) 34.3 (Zekavat et al., 2014)

%CV = Coefficient of Variation; %CVg = Interindividual (between-subject) biological variation.

Chapter 8 - **Dissertation Synopsis**

8.1 Overall conclusions

8.1.1 Introduction

This study generally succeeded in its aim to characterise and quantify those components of the Chacma baboon haemostatic system that are relevant to the pathophysiology of aTTP (and its possible treatment with thrombolytic drugs). It has provided novel insights that can be applied not only to the Chacma baboon model of aTTP and the possible treatment of TTP with thrombolytic drugs but also to any other experimental model in this species that involves or affects the haemostatic system. These components of the Chacma baboon haemostatic system were generally comparable to those of the human haemostatic system, with notable exceptions outlined below. Most of these exceptions do not fundamentally affect the utility of this species to function as a model of human haemostasis, where a few others (such as the more potent tPA-induced plasmin generation) actually represent opportunities to experimentally assess novel treatment approaches to an extent not possible with human in vitro studies.

8.1.2 The VWF-ADAMTS13 axis

The *Papio ursinus* VWF-ADAMTS13 axis likely has structural differences from its human counterpart, which can affect the assay values obtained to such an extent that ADAMTS13 antigen ELISA kits designed for use in humans may not be able to generate results, and that other results should be interpreted within the context of species-specific reference intervals. However, it is functionally similar enough to the human VWF-ADAMTS13 axis to function as a model of this component of human haemostasis. Moreover, none of the possible structural differences invalidates the aTTP model or its potential use for the investigation of thrombolytic drugs in aTTP. The induction of aTTP in vivo is not affected, nor is the ability of plasmin (when activated by tPA) to degrade non-globular VWF in the absence of ADAMTS13 activity in vitro.

8.1.3 Thrombin generation ability

The thrombin generation capability of *Papio ursinus* plasma outperforms that of human plasma, particularly in terms of the overall ETP of especially female Chacma baboons, but still appears to be closer to human values than those of many other commonly used laboratory animal species are. This greater thrombin generation potential may have an ameliorative effect in the aTTP model, but can also theoretically lead to more profound abnormalities in DIC/sepsis models than would be encountered in humans under similar conditions.

8.1.4 Fibrinogen and the fibrinolytic system

Fibrinogen, along with most role players of the fibrinolytic system, have ranges and characteristics comparable to their human equivalents. Plasminogen and TAFI, however, likely have structural/functional differences which can affect the assay values obtained but can also affect the treatment of aTTP with thrombolytic drugs in this model. While streptokinase cannot be used, recombinant human tPA may be an option although it might cause more prominent effects in Chacma baboons than in humans. Whether the more potent plasmin activity induced by recombinant human tPA in the Chacma baboon is simply due to lower α2-antiplasmin concentrations, is unclear but unlikely.

Critically, regardless of the reason for its greater plasminogen activation in the Chacma baboon, recombinant human tPA induces sufficient plasmin activity in *Papio ursinus* plasma to not only lead to brisk clot lysis but also to degradation (even in the absence of fibrin) of the unfolded high molecular weight VWF multimers implicated in the pathogenesis of TTP. This confirms tPA's potential utility in the treatment of aTTP in this model. Since significant plasmin activity was present even at low tPA concentrations, and considering that a streptokinase dose equivalent to 20% of the human PE treatment dose was effective in resolving aTTP in mice (Tersteeg et al., 2014), a low tPA dose of 0.3 mg/kg followed by escalating doses (up to 63 mg/kg if needed), should be attempted in the Chacma baboon aTTP model in vivo.

8.1.5 ABO and Rh blood groups and their influence

The local Chacma baboon population of the UFS Animal Research Centre, which is widely used for animal model research, does not have the same ABO or Rh blood group composition patterns and variability as reported for wild (Downing et al., 1973, Downing et al., 1975) or other captive (Ramis et al., 2013) Chacma baboon populations. This indicates limited genetic variation in the breeding colony, which may also impact other parameters. However, since wild-caught baboons are generally not used in modern animal model research, this observation is probably trivial and should not impact the continued use of the aTTP model.

Although group O baboons may be slightly more suitable for use in the aTTP model due to their higher fibrinogen concentrations and (possibly) lower ADAMTS13 antigen levels, all other parameters of importance in TTP (and its potential treatment with thrombolytic drugs) were unaffected by ABO blood group.

8.1.6 Influence of animals' sex

Unexpectedly, fibrinogen concentrations were significantly lower in male baboons. In light of their less potent thrombin generation (which may have an ameliorative effect in TTP) and possibly higher baseline background fibrinolytic activity, male baboons may potentially be better suited for use in the aTTP model to investigate the utility of thrombolytic drugs like tPA. However, the bulk of the parameters of importance in TTP (and its potential treatment with fibrinolytics) were unaffected by the animals' sex.

8.1.7 Consolidated reference intervals and biological variation

Where meaningful results could be obtained, most Chacma baboon reference intervals were generally comparable to human intervals. However, data from future experiments can now be interpreted more rationally and with greater confidence using these intervals. Ideally, species-specific reference intervals should be obtained and applied when analysing any animal model data, as these may differ from human reference intervals.

The relationship of the various Chacma baboon reference intervals to the human reference intervals for each of the quantitative parameters are summarized in Table 22 below. Irrespective of the relationships elucidated in Table 22, all intervals were generally comparable with human ranges.

Fibrinogen and especially α2-antiplasmin were subject to greater interindividual variability than reported in humans, whereas tPA was less variable.

Table 22: Summary of relationship between Chacma baboon reference intervals and human reference intervals/values.

Parameters with intervals/activities/times LOWER/SHORTER than humans		
ADAMTS13 Activity	VWF:Ag	VWF:CB
PAP complex	tPA	PAI-1
α2-antiplasmin	tPA-induced modified clot lysis time	Thrombin generation velocity index
Parameters with intervals/patterns/times SIMILAR/IDENTICAL to humans		
Fibrinogen	Thrombin generation lag time	Thrombin generation peak height
VWF multimer pattern		
Parameters with intervals/activities/times HIGHER/LONGER than humans		
VWF:RCo	tPA-induced plasmin activity	Thrombin generation time to peak
Thrombin generation endogenous thrombin potential		

The following assays were not included in this table, based on the suspicion that they were not able to generate meaningful results due to possible structural differences with the human protein: ADAMTS13 antigen, plasminogen, TAFI.

8.2 Summarized conclusion

The central thesis emerging from this research is that the components of the Chacma baboon's haemostatic system that are pertinent to the pathogenesis of aTTP and its treatment with thrombolytic drugs are similar enough to their human counterparts to enable continued use of this species as a model of human haemostasis, provided quantitative results are interpreted within the context of the novel reference intervals reported above and the limitations and interspecies differences outlined above are considered. The most important of these limitations and differences (none of which invalidate the aTTP model) would be the findings that streptokinase does not activate the Chacma baboon fibrinolytic system but that recombinant human tPA elicits even greater plasminogen activation in *Papio ursinus* than it does in humans, that Chacma baboon thrombin generation is more potent than human thrombin generation (particularly in female animals), that sex-related and ABO blood group-related differences are imperative when measuring fibrinogen in this species, that assays designed to measure human plasminogen and TAFI may not be usable in this species, and that fibrinogen and especially α2-antiplasmin are subject to considerable interindividual biological variation in these animals.

From this central thesis also flows the critical conclusion that, similar to humans, Chacma baboon plasmin, when activated by recombinant human tPA, is able to degrade unfolded *Papio ursinus* VWF to such an extent that tPA's use should be explored further in the Chacma baboon aTTP model, to provide proof-of-concept for the possible use of thrombolytic drugs in the treatment of aTTP in humans.

Chapter 9 - Future Studies, Recommendations and Limitations

9.1 Future studies

While the present study has answered many questions regarding the Chacma baboon aTTP model, it has also opened up multiple new potential research lines.

Firstly, it would be interesting and of practical value to repeat some of the assays using different methods or after the assays have been reconfigured or adjusted. It would be useful to repeat the VWF:CB assay utilising a variety of different collagen types to define the exact collagen type that will yield the most accurate and reliable assessment of Chacma baboon VWF:CB activity. The development of specific anti-Chacma baboon VWF and ADAMTS13 mAbs that can be used as capture or detection antibodies as part of species-specific ELISAs, would greatly improve the accuracy of these assays in this species and should be a long-term research priority in this model. It would be essential to confirm our plasminogen concentration results in a repeat future study using the appropriate proteinase inhibitors (such as aprotinin or benzamidine) before *Papio ursinus* plasminogen is definitively concluded to be structurally non-homologous to its human equivalent.

Perhaps the most pertinent opportunities lie in the sequencing of the genes of proteins found to have such discrepant results with human reference intervals to suggest fundamentally different structures. If alternative/reconfigured assays render similar results, sequencing of the following proteins' genes should be attempted to define and better understand any differences on a genetic level:

- ADAMTS13
- VWF (at least the A3 domain but possibly other domains as well)
- Plasminogen
- TAFI

Investigating the level of homology of these genes between the various commonly used baboon species and humans may also indicate whether any other baboon species may be superior to *Papio ursinus* as a model of human haemostasis.

In light of their greater thrombin generation potentials, a complete characterisation of the Chacma Baboon's natural anticoagulant system is indicated, which would include estimations of *Papio ursinus* antithrombin, protein C, protein S, protein Z, protein Z-dependent protease inhibitor, soluble thrombomodulin, and TFPI activities and concentrations. Establishing reference intervals for coagulation factor activities, especially for FVIII, will also clarify whether the elevated ETP of *Papio ursinus* is a consequence of a more prominent procoagulant drive or of a less potent natural anticoagulant system with concomitant lower thrombin inhibition.

Several components related to fibrinolysis and the fibrinolytic system also remain to be explored. Reference intervals for factor XIII, PAI-2, α2-macroglobulin, and baseline D-dimer concentrations will all add value to future studies. In addition, the measurement of TAFI, PAI-1 and α2-antiplasmin activities will help contextualise the present study's results in terms of the data available from other studies. Whether Chacma baboon plasmin is also able to cleave Chacma baboon ADAMTS13 as in humans can also be investigated.

The thought-provoking and somewhat unexpected findings that no animals expressed the ABO blood group B antigen or were Rh (D) antigen-negative could be the subject of future research. Not only does this have bearing on the aTTP model, but it speaks to the genetic diversity of the UFS Animal Research Centre's Chacma baboons, to such an extent that it may perhaps be time for the introduction of a limited number of wild-caught baboons into the breeding colony, to bolster genetic diversity.

Intraindividual variation (as opposed to interindividual variation) was not assessed for any of the parameters in the present study and may be a beneficial future research endeavour. In addition, it would be valuable to know whether Chacma baboons are also subjected to the same diurnal variations reported in humans for parameters such as the tPA concentration.

Perhaps the most vital potential future study arising from our data is a possible repeat of our pilot study, but using recombinant human tPA as thrombolytic drug. This should be done using a range of tPA dosages, starting at a low dose of 0.3 mg/kg, to provide proof-of-concept that thrombolytic drugs (perhaps even at very low doses) can be used to treat aTTP in humans. However, before embarking on an in vivo study, the remarkable in vitro observation that higher tPA concentrations led to a less prominent effect on both human and Chacma baboon VWF multimers should be investigated and clarified in a set of in vitro experiments designed to determine the minimum tPA concentration at which plasmin-mediated VWF multimer breakdown initiates.

9.2 Recommendations

Although the results of this study generally validated the Chacma baboon aTTP model, it also led to many valuable recommendations for the improvement, reconfiguration and more rational future use of the model, as well as for the future investigation of thrombolytic drugs in this species:

- The following assay kits are not recommended for use in this species:
    - Technozym® ADAMTS13 Antigen ELISA kit (Technoclone GmbH, Vienna, Austria)
    - Technozym® Glu-Plasminogen ELISA kit (Technoclone GmbH, Vienna, Austria)
    - Elabscience® Human TAFI ELISA kit (Elabscience®, Houston, Texas, USA)
- For future in vivo experiments, all quantitative VWF and ADAMTS13 antigen and activity testing should use individual animals' baseline (pre-injection of 3H9) values as the 100% reference point, rather than only a value derived from the calibration reference curve based on human plasma, especially since the intra-individual trend (and not the absolute value) will mostly be the compelling factor.

- Should an absolute value (not relative to baseline) for these parameters be required, pooled normal Chacma baboon plasma (now available after this study) should rather be used to derive the calibration reference curve instead of human plasma.
- If this is not possible or desirable, and a human plasma-derived calibration reference curve is chosen without comparison to baseline, the species-specific reference intervals calculated from the present study's data should be used to interpret results.
- The VWF:CB assay should be reconfigured using different types of collagen.
- The VWF multimer pattern assay can be used unaltered in its current configuration and interpreted *prima facie.*
- All other assays utilised in this study (apart from the ADAMTS13, plasminogen, and TAFI antigen ELISAs) can also be used as-is, but results should be interpreted within the context of their newly derived species-specific reference intervals.
- Where available, ELISAs using polyclonal anti-human antibodies should be used in the Chacma baboon model, over assays employing monoclonal anti-human antibodies, to mitigate the impact of possible subtle interspecies differences in protein structure.
- In the long-term, the development of in-house, species-specific ELISAs incorporating specific anti-Chacma baboon mAbs for use in this model should be considered, although it may have limited application outside of the aTTP model.
- Future studies in this species where fibrinogen concentrations are important should consist of both male and female baboons and animals from O and non-O ABO blood groups.
- The use of streptokinase is not recommended in this species for any application since it does not lead to meaningful plasminogen activation.

- Future experiments in the aTTP model to investigate the potential utility of thrombolytic drugs in the treatment of TTP should consider recombinant human tPA, possibly at a wide range of doses.

9.3 Limitations of the study

The main limitation of this study is that all the assays used to characterise the various Chacma baboon haemostatic components of interest were designed, developed or intended for use in humans, with the possible exception of the modified clot lysis time, which was modified to be suitable for use with Chacma baboon plasma. Although this is an inherent limitation of the aTTP model (and indeed of all animal models in general), it still limits the accuracy with which actual Chacma baboon haemostasis can be assessed. However, conversely, using human assays in the aTTP model can also be considered mandatory for two reasons. Firstly, since these are the assays that would ultimately be used in humans to assess the potential effects of any thrombolytic drug treatment in TTP, it would be prudent to use these very same assays already during the preclinical testing phase, so that assay variability is at least excluded when data are analysed and extrapolated for later human application. Secondly, since these are accepted, commonly used, generally widely available assays, the use of esoteric, species-specific assays may not only be detrimental to the translatability of any results but may also be impractical due to their possibly limited application outside of the Chacma baboon aTTP model. Moreover, the observations that some of the human-specific assays were not able to generate meaningful results also led to valuable conclusions in this study on possible structural differences between the ADAMTS13, plasminogen and TAFI molecules, for instance, of *Papio ursinus* and humans.

Even though this is the largest number of individual Chacma baboons ever included in a study involving parameters of haemostasis, the study is still limited by the low number of animals tested. Ideally, as also stipulated by the CLSI (CLSI., 2008), at least 120 participants are required to calculate reference intervals from non-parametric data. Nevertheless, and since such a large number of participants is simply not feasible, the reference intervals calculated from the current data now give researchers a much better

idea of the broader context of their quantitative results in this species. Similarly, the low number of males and animals of non-O ABO blood group included in the study is an (unexpected) limitation. Perhaps more pertinently, this observation (reinforced by the complete lack of Rh-negative, and ABO group B or AB baboons) also implies limited genetic diversity of the UFS breeding colony, which can in itself be seen as a significant limitation of this study. However, it can be argued that this limitation is of no practical importance since these are the actual animals used for experimentation. Conversely, should wild-caught baboons be introduced or used in the future, the results of the present study may not be as relevant anymore, creating a potential future limitation.

Finally, the presence of anti-streptokinase antibodies, which may have influenced the streptokinase-induced plasmin activity assays, was not excluded since none of the animals had been exposed previously to streptokinase.

These limitations fortunately do not void the central thesis of this research and can be addressed in future studies. Reconfiguration and refinement of the assays used will address many of the present study's shortcomings. Molecular genetic analysis will clarify some of the uncertainties surrounding the assay discrepancies encountered, and the limited genetic diversity of the UFS Animal Research Centre's baboon breeding colony can be counterbalanced by the inclusion of wild-caught baboons, or animals from other research facilities, in future studies.

Ultimately, although some may argue that animal models remain mere models of human disease, with levels of similarity that cannot ever be concretely measured or scientifically defined, and results that will never be completely transferrable to humans, the continued use of this particular species to investigate human haemostatic disease appears justified by the results of the present study.

Chapter 10 - **References**

ABCAM 2019. Human alpha-2 antiplasmin SimpleStep ELISA® Kit Package Insert. Cambridge, United Kingdom: Abcam.

ADELMAN, B., MICHELSON, A., GREENBERG, J. & HANDIN, R. 1986. Proteolysis of platelet glycoprotein Ib by plasmin is facilitated by plasmin lysine-binding regions. *Blood,* 68**,** 1280-1284.

ADELMAN, B., MICHELSON, A., LOSCALZO, J., GREENBERG, J. & HANDIN, R. 1985. Plasmin effect on platelet glycoprotein Ib-von Willebrand factor interactions. *Blood,* 65**,** 32-40.

AĬSINA, R. B. & MUKHAMETOVA, L. I. 2014. Structure and function of plasminogen/plasmin system. *Russian Journal of Bioorganic Chemistry,* 40**,** 590-605.

ALONSO-COELLO, P., BELLMUNT, S., MCGORRIAN, C., ANAND, S. S., GUZMAN, R., CRIQUI, M. H., AKL, E. A., VANDVIK, P. O., LANSBERG, M. G., GUYATT, G. H. & SPENCER, F. A. 2012. Antithrombotic Therapy in Peripheral Artery Disease. *Chest,* 141**,** e669S-e690S.

AMOROSI, E. L. & ULTMANN, J. E. 1966. Thrombotic thrombocytopenic purpura: report of 16 cases and review of the literature. *Medicine,* 34**,** 139-160.

ANAND, K., PALLARES, I., VALNICKOVA, Z., CHRISTENSEN, T., VENDRELL, J., WENDT, K. U., SCHREUDER, H. A., ENGHILD, J. J. & AVILÉS, F. X. 2008. The Crystal Structure of Thrombin-activable Fibrinolysis Inhibitor (TAFI) Provides the Structural Basis for Its Intrinsic Activity and the Short Half-life of TAFIa. *Journal of Biological Chemistry,* 283**,** 29416-29423.

ANDERSON, H. V. & WILLERSON, J. T. 1993. Thrombolysis in acute myocardial infarction. *The New England Journal of Medicine,* 329**,** 703-9.

ANDERSON, P. J., KOKAME, K. & SADLER, J. E. 2006. Zinc and calcium ions cooperatively modulate ADAMTS13 activity. *Journal of Biological Chemistry,* 281**,** 850-7.

ASADA, Y., SUMIYOSHI, A., HAYASHI, T., SUZUMIYA, J. & KAKETANI, K. 1985. Immunohistochemistry of vascular lesion in thrombotic thrombocytopenic purpura,

with special reference to factor VIII related antigen. *Thrombosis Research,* 38**,** 469-79.

ASTRUP, T. & PERMIN, P. M. 1947. Fibrinolysis in the animal organism. *Nature,* 159**,** 681.

AZUMA, H., SUGIMOTO, M., RUGGERI, Z. M. & WARE, J. 1993. A role for von Willebrand factor proline residues 702-704 in ristocetin-mediated binding to platelet glycoprotein Ib. *Thrombosis and Haemostasis,* 69**,** 192-6.

BAJZAR, L., MANUEL, R. & NESHEIM, M. E. 1995. Purification and characterization of TAFI, a thrombin-activable fibrinolysis inhibitor. *Journal of Biological Chemistry,* 270**,** 14477-84.

BAJZAR, L., NESHEIM, M., MORSER, J. & TRACY, P. B. 1998. Both Cellular and Soluble Forms of Thrombomodulin Inhibit Fibrinolysis by Potentiating the Activation of Thrombin-activable Fibrinolysis Inhibitor*. *Journal of Biological Chemistry,* 273**,** 2792-2798.

BAJZAR, L., NESHEIM, M. & TRACY, P. 1996. The profibrinolytic effect of activated protein C in clots formed from plasma is TAFI-dependent. *Blood,* 88**,** 2093-2100.

BEL, A., BORIK, W., DAVIDSON, S., HELIES, J.-M., STIMMER, L., FREMES, S., ZELENKOFSKE, S., RUSCONI, C., ALEXANDER, J., ALEXANDER, D., MENASCHÉ, P. & PEPPER, J. 2015. Inhibition of factor IXa by the pegnivacogin system during cardiopulmonary bypass: a potential substitute for heparin. A study in baboons. *European Journal of Cardio-Thoracic Surgery,* 49**,** 682-689.

BERKOWITZ, S. D., DENT, J., ROBERTS, J., FUJIMURA, Y., PLOW, E. F., TITANI, K., RUGGERI, Z. M. & ZIMMERMAN, T. S. 1987. Epitope mapping of the von Willebrand factor subunit distinguishes fragments present in normal and type IIA von Willebrand disease from those generated by plasmin. *The Journal of Clinical Investigation,* 79**,** 524-531.

BERNDT, M. C., WARD, C. M., BOOTH, W. J., CASTALDI, P. A., MAZUROV, A. V. & ANDREWS, R. K. 1992. Identification of aspartic acid 514 through glutamic acid 542 as a glycoprotein Ib-IX complex receptor recognition sequence in von Willebrand factor. Mechanism of modulation of von Willebrand factor by ristocetin and botrocetin. *Biochemistry,* 31**,** 11144-51.

BINETTE, T. M., TAYLOR, F. B., JR, PEER, G. & BAJZAR, L. 2007. Thrombin-thrombomodulin connects coagulation and fibrinolysis: more than an in vitro phenomenon. *Blood,* 110**,** 3168-3175.

BIVARD, A., LIN, L. & PARSONS, M. W. 2013. Review of Stroke Thrombolytics. *Journal of Stroke,* 15**,** 90-98.

BLOEMEN, S., HUSKENS, D., KONINGS, J., KREMERS, R. M., MISZTA, A., DE LAAT, B. & KELCHTERMANS, H. 2019. Interindividual Variability and Normal Ranges of Whole Blood and Plasma Thrombin Generation. *The Journal of Applied Laboratory Medicine,* 2**,** 150-164.

BONNEFOY, A., ROMIJN, R. A., VANDERVOORT, P. A. H., VAN ROMPAEY, I., VERMYLEN, J. & HOYLAERTS, M. F. 2006. von Willebrand factor A1 domain can adequately substitute for A3 domain in recruitment of flowing platelets to collagen. *Journal of Thrombosis and Haemostasis,* 4**,** 2151-2161.

BOUCHAMA, A., AL-MOHANNA, F., ASSAD, L., BATURCAM, E., ELDALI, A., OWAIDAH, T. & DEHBI, M. 2012. Tissue factor/factor VIIa pathway mediates coagulation activation in induced-heat stroke in the baboon. *Critical Care Medicine,* 40**,** 1229-1236.

BOUCHAMA, A., KUNZELMANN, C., DEHBI, M., KWAASI, A., ELDALI, A., ZOBAIRI, F., FREYSSINET, J.-M. & PROST, D. D. 2008. Recombinant Activated Protein C Attenuates Endothelial Injury and Inhibits Procoagulant Microparticles Release in Baboon Heatstroke. *Arteriosclerosis, Thrombosis, and Vascular Biology,* 28**,** 1318-1325.

BOUMA, B. N. & MEIJERS, J. C. M. 2003. Thrombin-activatable fibrinolysis inhibitor (TAFI, plasma procarboxypeptidase B, procarboxypeptidase R, procarboxypeptidase U). *Journal of Thrombosis and Haemostasis,* 1**,** 1566-1574.

BOWEN, D. J. & COLLINS, P. W. 2004. An amino acid polymorphism in von Willebrand factor correlates with increased susceptibility to proteolysis by ADAMTS13. *Blood,* 103**,** 941-7.

BRILL, A., FUCHS, T. A., SAVCHENKO, A. S., THOMAS, G. M., MARTINOD, K., DE MEYER, S. F., BHANDARI, A. A. & WAGNER, D. D. 2012. Neutrophil extracellular

traps promote deep vein thrombosis in mice. *Journal of Thrombosis and Haemostasis,* 10**,** 136-44.

BRINK, S. & STEYTLER, J. G. 1974. Effects of puff-adder venom on coagulation, fibrinolysis and platelet aggregation in the baboon. *South African Medical Journal,* 48**,** 1205-13.

BRINKMANN, V., REICHARD, U., GOOSMANN, C., FAULER, B., UHLEMANN, Y., WEISS, D. S., WEINRAUCH, Y. & ZYCHLINSKY, A. 2004. Neutrophil extracellular traps kill bacteria. *Science,* 303**,** 1532-5.

BROPHY, T. M., WARD, S. E., MCGIMSEY, T. R., SCHNEPPENHEIM, S., DRAKEFORD, C., O'SULLIVAN, J. M., CHION, A., BUDDE, U. & O'DONNELL, J. S. 2017. Plasmin Cleaves Von Willebrand Factor at K1491-R1492 in the A1-A2 Linker Region in a Shear- and Glycan-Dependent Manner In Vitro. *Arteriosclerosis, Thrombosis, and Vascular Biology,* 37**,** 845-855.

BRUNSKILL, S. J., TUSOLD, A., BENJAMIN, S., STANWORTH, S. J. & MURPHY, M. F. 2007. A systematic review of randomized controlled trials for plasma exchange in the treatment of thrombotic thrombocytopenic purpura. *Transfusion Medicine,* 17**,** 17-35.

BRYAN, J. 2014. The rise and fall of the clot buster. *The Pharmaceutical Journal,* 293**,** 1-8.

BUDDE, U. & SCHNEPPENHEIM, R. 2014. Interactions of von Willebrand factor and ADAMTS13 in von Willebrand disease and thrombotic thrombocytopenic purpura. *Hamostaseologie,* 34**,** 215-25.

BUDKOWSKA, M., LEBIECKA, A., MARCINOWSKA, Z., WOŹNIAK, J., JASTRZĘBSKA, M. & DOŁĘGOWSKA, B. 2019. The circadian rhythm of selected parameters of the hemostasis system in healthy people. *Thrombosis Research,* 182**,** 79-88.

BUZZA, M. S., DYSON, J. M., CHOI, H., GARDINER, E. E., ANDREWS, R. K., KAISERMAN, D., MITCHELL, C. A., BERNDT, M. C., DONG, J. F. & BIRD, P. I. 2008. Antihemostatic activity of human granzyme B mediated by cleavage of von Willebrand factor. *Journal of Biological Chemistry,* 283**,** 22498-504.

CALLEWAERT, F., ROODT, J., ULRICHTS, H., STOHR, T., JANSE VAN RENSBURG, W., LAMPRECHT, S., ROSSENU, S., PRIEM, S., WILLEMS, W. & HOLZ, J.-B.

2012. Evaluation of efficacy and safety of the anti-VWF Nanobody ALX-0681 in a preclinical baboon model of acquired thrombotic thrombocytopenic purpura. *Blood,* 120**,** 3603-3610.

CALZAVARINI, S., BRODARD, J., QUARROZ, C., MAIRE, L., NÜTZI, R., JANKOVIC, J., ROTONDO, L. C., GIABBANI, E., FIEDLER, G. M., NAGLER, M. & ANGELILLO-SCHERRER, A. 2019. Thrombin generation measurement using the ST Genesia Thrombin Generation System in a cohort of healthy adults: Normal values and variability. *Research and Practice in Thrombosis and Haemostasis,* 3**,** 758-768.

CESARMAN-MAUS, G. & HAJJAR, K. A. 2005. Molecular mechanisms of fibrinolysis. *British Journal of Haematology,* 129**,** 307-21.

CHAUHAN, A. K. 2014. Degradation of platelet-von Willebrand factor complexes by plasmin: an alternative/backup mechanism to ADAMTS13. *Circulation,* 129**,** 1273-5.

CHAVIN, S. I. 1984. Factor VIII: structure and function in blood clotting. *American Journal of Hematology,* 16**,** 297-306.

CHEN, J., LING, M., FU, X., LÓPEZ, J. A. & CHUNG, D. W. 2012. Simultaneous Exposure of Sites in von Willebrand Factor for Glycoprotein Ib Binding and ADAMTS13 Cleavage. *Arteriosclerosis, Thrombosis, and Vascular Biology* 32**,** 2625-2630.

CHUNG, D. W. 2019. Hairpin and allosteric regulation in ADAMTS13. *Blood,* 133**,** 1800-1801.

CLSI. 2008. Defining, Establishing and Verifying Reference Intervals in the Clinical Laboratory; Approved Guideline–Third Edition. *CLSI document EP28-A3c.* Wayne, PA: Clinical and Laboratory Standards Institute.

CLSI. 2010. Method Comparison and Bias Estimation Using Patient Samples; Approved Guideline- Second Edition (Interim Revision). *CLSI document EP09-A2-IR.* Wayne, PA: Clinical and Laboratory Standards Institute.

COLEMAN, P. L., BAROUSKI, P. A. & GELEHRTER, T. D. 1982. The dexamethasone-induced inhibitor of fibrinolytic activity in hepatoma cells. A cellular product which specifically inhibits plasminogen activation. *Journal of Biological Chemistry,* 257**,** 4260-4.

COLLEN, D., DE COCK, F. & STASSEN, J. M. 1993a. Comparative immunogenicity and thrombolytic properties toward arterial and venous thrombi of streptokinase and recombinant staphylokinase in baboons. *Circulation,* 87**,** 996-1006.

COLLEN, D. & DE MAEYER, L. 1975. Molecular biology of human plasminogen. I. Physiochemical properties and microheterogeneity. *Thrombosis et Diathesis Haemorrhagica,* 34**,** 396-402.

COLLEN, D. & LIJNEN, R. H. 2005. Thrombolytic agents. *Thrombosis and Haemostasis,* 93**,** 627-630.

COLLEN, D., VAN HOEF, B., SCHLOTT, B., HARTMANN, M., GUHRS, K. H. & LIJNEN, H. R. 1993b. Mechanisms of activation of mammalian plasma fibrinolytic systems with streptokinase and with recombinant staphylokinase. *European Journal of Biochemistry,* 216**,** 307-14.

COLLEN, D. & WIMAN, B. 1979. Turnover of antiplasmin, the fast-acting plasmin inhibitor of plasma. *Blood,* 53**,** 313-24.

CONRADIE, C. 2015. Coagulation testing at NHLS Universitas Academic Laboratory. Standard Operating Procedure GPL4115v2.

COSTONGS, G. M., JANSON, P. C., BAS, B. M., HERMANS, J., BROMBACHER, P. J. & VAN WERSCH, J. W. 1985a. Short-term and long-term intra-individual variations and critical differences of haematological laboratory parameters. *Journal of Clinical Chemistry and Clinical Biochemistry,* 23**,** 69-76.

COSTONGS, G. M. P. J., BAS, B. M., JANSON, P. C. W., HERMANS, J., BROMBACHER, P. J. & WERSCH, J. W. J. 1985b. Short-Term and Long-Term Intra-Individual Variations and Critical Differences of Coagulation Parameters. *Journal of Clinical Chemistry and Clinical Biochemistry,* 23**,** 405-410.

COX, L. A., JETT, C. & HIXSON, J. E. 1998. Molecular basis of an apolipoprotein[a] null allele: a splice site mutation is associated with deletion of a single exon. *Journal of Lipid Research,* 39**,** 1319-26.

CRAWLEY, J. T., LAM, J. K., RANCE, J. B., MOLLICA, L. R., O'DONNELL, J. S. & LANE, D. A. 2005. Proteolytic inactivation of ADAMTS13 by thrombin and plasmin. *Blood,* 105**,** 1085-93.

CRAWLEY, J. T. B., DE GROOT, R., XIANG, Y., LUKEN, B. M. & LANE, D. A. 2011. Unraveling the scissile bond: how ADAMTS13 recognizes and cleaves von Willebrand factor. *Blood,* 118**,** 3212-3221.

CRAWLEY, J. T. B. & SCULLY, M. A. 2013. Thrombotic thrombocytopenic purpura: basic pathophysiology and therapeutic strategies. *Hematology,* 2013**,** 292-299.

CRUZ, M. A., HANDIN, R. I. & WISE, R. J. 1993. The interaction of the von Willebrand factor-A1 domain with platelet glycoprotein Ib/IX. The role of glycosylation and disulfide bonding in a monomeric recombinant A1 domain protein. *Journal of Biological Chemistry,* 268**,** 21238-21245.

DANO, K., ANDREASEN, P. A., GRONDAHL-HANSEN, J., KRISTENSEN, P., NIELSEN, L. S. & SKRIVER, L. 1985. Plasminogen activators, tissue degradation, and cancer. *Advances in Cancer Research,* 44**,** 139-266.

DE CRISTOFARO, R., PEYVANDI, F., BARONCIANI, L., PALLA, R., LAVORETANO, S., LOMBARDI, R., DI STASIO, E., FEDERICI, A. B. & MANNUCCI, P. M. 2006. Molecular mapping of the chloride-binding site in von Willebrand factor (VWF): energetics and conformational effects on the VWF/ADAMTS-13 interaction. *Journal of Biological Chemistry,* 281**,** 30400-11.

DE MAAT, M. P., DE BART, A. C., HENNIS, B. C., MEIJER, P., HAVELAAR, A. C., MULDER, P. G. & KLUFT, C. 1996. Interindividual and intraindividual variability in plasma fibrinogen, TPA antigen, PAI activity, and CRP in healthy, young volunteers and patients with angina pectoris. *Arteriosclerosis, Thrombosis, and Vascular Biology,* 16**,** 1156-62.

DE MAAT, M. P. M., VAN SCHIE, M., KLUFT, C., LEEBEEK, F. W. G. & MEIJER, P. 2016. Biological Variation of Hemostasis Variables in Thrombosis and Bleeding: Consequences for Performance Specifications. *Clinical Chemistry,* 62**,** 1639-1646.

DECKMYN, H. & VANHOORELBEKE, K. 2006. When collagen meets VWF. *Blood,* 108**,** 3628-3628.

DEFORCHE, L., TERSTEEG, C., ROOSE, E., VANDENBULCKE, A., VANDEPUTTE, N., PAREYN, I., DE COCK, E., ROTTENSTEINER, H., DECKMYN, H., DE MEYER, S. & VANHOORELBEKE, K. 2016. Generation of Anti-Murine ADAMTS13

Antibodies and Their Application in a Mouse Model for Acquired Thrombotic Thrombocytopenic Purpura. *PLoS One,* 11**,** e0160388.

DOLAN, G., NEAL, K., COOPER, P., BROWN, P. & PRESTON, F. E. 1994. Protein C, antithrombin III and plasminogen: effect of age, sex and blood group. *British Journal of Haematology,* 86**,** 798-803.

DOWNING, H. J., BURGERS, L. E. & GETLIFFE, F. M. 1975. A-B-O blood groups of two subspecies of chacma baboon (*Papio ursinus*) in South Africa. *Journal of Medical Primatology,* 4**,** 103-7.

DOWNING, H. J., MILNER, L. V., ROGERS, V. J. & SKINNER, D. D. 1973. A-B-O groups of Chacma baboons (*Papio ursinus*, Kerr) in South Africa. *American Journal of Physical Anthropology,* 38**,** 783-787.

EATON, D. L., FLESS, G. M., KOHR, W. J., MCLEAN, J. W., XU, Q. T., MILLER, C. G., LAWN, R. M. & SCANU, A. M. 1987. Partial amino acid sequence of apolipoprotein(a) shows that it is homologous to plasminogen. *Proceedings of the National Academy of Sciences of the United States of America,* 84**,** 3224-8.

EATON, D. L., MALLOY, B. E., TSAI, S. P., HENZEL, W. & DRAYNA, D. 1991. Isolation, molecular cloning, and partial characterization of a novel carboxypeptidase B from human plasma. *Journal of Biological Chemistry,* 266**,** 21833-21838.

EIKENBOOM, J., FEDERICI, A. B., DIRVEN, R. J., CASTAMAN, G., RODEGHIERO, F., BUDDE, U., SCHNEPPENHEIM, R., BATLLE, J., CANCIANI, M. T., GOUDEMAND, J., PEAKE, I. & GOODEVE, A. 2013. VWF propeptide and ratios between VWF, VWF propeptide, and FVIII in the characterization of type 1 von Willebrand disease. *Blood,* 121**,** 2336-2339.

ELABSCIENCE 2019. Human TAFI (Thrombin Activatable Fibrinolysis Inhibitor) ELISA Kit. Package Insert. USA.

ELLIS, V., BEHRENDT, N. & DANO, K. 1991. Plasminogen activation by receptor-bound urokinase. A kinetic study with both cell-associated and isolated receptor. *Journal of Biological Chemistry,* 266**,** 12752-8.

FAVALORO, E. J. 2000. Detection of von Willebrand disorder and identification of qualitative von Willebrand factor defects. Direct comparison of commercial ELISA-

based von Willebrand factor activity options. *American Journal of Clinical Pathology,* 114**,** 608-18.

FAZLEABAS, A. T., EVERLY, S. L. & LOTTENBERG, R. 1991. Immunological and Molecular Characterization of Plasminogen Activator Inhibitors 1 and 2 in Baboon (*Papio Anubis*) Placental Tissues1. *Biology of Reproduction,* 45**,** 49-56.

FEDERICI, A. B., BERKOWITZ, S. D., ZIMMERMAN, T. S. & MANNUCCI, P. M. 1992. Proteolysis of von Willebrand factor after thrombolytic therapy in patients with acute myocardial infarction. *Blood,* 79**,** 38-44.

FEDERICI, A. B., ELDER, J. H., DE MARCO, L., RUGGERI, Z. M. & ZIMMERMAN, T. S. 1984. Carbohydrate moiety of von Willebrand factor is not necessary for maintaining multimeric structure and ristocetin cofactor activity but protects from proteolytic degradation. *Journal of Clinical Investigation,* 74**,** 2049-55.

FENG, S., LIANG, X., CRUZ, M. A., VU, H., ZHOU, Z., PEMMARAJU, N., DONG, J. F., KROLL, M. H. & AFSHAR-KHARGHAN, V. 2013. The Interaction between Factor H and Von Willebrand Factor. *PLoS One,* 8.

FERNANDES, A. B., LIMA, L. M., SOUSA, M. O., TOLEDO, V. D. P. C., KAZMI, R. S., LWALEED, B. A. & CARVALHO, M. D. G. 2015. Impaired Fibrinolysis in Angiographically Documented Coronary Artery Disease. *Advances in Hematology,* 2015**,** 214680.

FEYS, H. B., LIU, F., DONG, N., PAREYN, I., VAUTERIN, S., VANDEPUTTE, N., NOPPE, W., RUAN, C., DECKMYN, H. & VANHOORELBEKE, K. 2006. ADAMTS-13 plasma level determination uncovers antigen absence in acquired thrombotic thrombocytopenic purpura and ethnic differences. *Journal of Thrombosis and Haemostasis,* 4**,** 955-62.

FEYS, H. B., ROODT, J., VANDEPUTTE, N., PAREYN, I., LAMPRECHT, S., VAN RENSBURG, W. J., ANDERSON, P. J., BUDDE, U., LOUW, V. J., BADENHORST, P. N., DECKMYN, H. & VANHOORELBEKE, K. 2010a. Thrombotic thrombocytopenic purpura directly linked with ADAMTS13 inhibition in the baboon (*Papio ursinus*). *Blood,* 116**,** 2005-2010.

FEYS, H. B., ROODT, J., VANDEPUTTE, N., PAREYN, I., MOTTL, H., HOU, S., LAMPRECHT, S., VAN RENSBURG, W. J., DECKMYN, H. & VANHOORELBEKE,

K. 2012. Inhibition of von Willebrand factor-platelet glycoprotein Ib interaction prevents and reverses symptoms of acute acquired thrombotic thrombocytopenic purpura in baboons. *Blood,* 120**,** 3611-4.

FEYS, H. B., VANDEPUTTE, N., PALLA, R., PEYVANDI, F., PEERLINCK, K., DECKMYN, H., LIJNEN, H. R. & VANHOORELBEKE, K. 2010b. Inactivation of ADAMTS13 by plasmin as a potential cause of thrombotic thrombocytopenic purpura. *Journal of Thrombosis and Haemostasis,* 8**,** 2053-62.

FISCHER, J., HIGHAM, J. P., ALBERTS, S. C., BARRETT, L., BEEHNER, J. C., BERGMAN, T. J., CARTER, A. J., COLLINS, A., ELTON, S., FAGOT, J., FERREIRA DA SILVA, M. J., HAMMERSCHMIDT, K., HENZI, P., JOLLY, C. J., KNAUF, S., KOPP, G. H., ROGERS, J., ROOS, C., ROSS, C., SEYFARTH, R. M., SILK, J., SNYDER-MACKLER, N., STAEDELE, V., SWEDELL, L., WILSON, M. L. & ZINNER, D. 2019. Insights into the evolution of social systems and species from baboon studies. *eLife,* 8**,** e50989.

FORTMAN, J. D., HEWETT, T. A. & BENNETT, B. T. 2002. Important Biological Features. *The Laboratory Nonhuman Primate.* 1 ed. Boca Raton: CRC Press.

FRANCHINI, M., CAPRA, F., TARGHER, G., MONTAGNANA, M. & LIPPI, G. 2007. Relationship between ABO blood group and von Willebrand factor levels: from biology to clinical implications. *Thrombosis Journal,* 5**,** 14.

FRANCHINI, M., CRESTANI, S., FRATTINI, F., SISSA, C. & BONFANTI, C. 2014. ABO blood group and von Willebrand factor: biological implications. *Clinical Chemistry and Laboratory Medicine,* 52**,** 1273-6.

FRANZ, R. C. 2009. ROTEM analysis: A significant advance in the field of rotational thrombelastography. *South African Journal of Surgery,* 47**,** 2-6.

FRANZ, R. C., JOUBERT, J. E. H., BRIEDENHANN, S. & MEINTJES, C. J. 2004. The use of the rotational thrombelastograph (ROTEG) in the quantification of the 'lytic state' during thrombolytic therapy. A comparative analysis in the subhuman primate. *South African Journal of Surgery,* 42**,** 56.

FROST, S. R., MARCUS, L. F., BOOKSTEIN, F. L., REDDY, D. P. & DELSON, E. 2003. Cranial allometry, phylogeography, and systematics of large-bodied papionins (primates: Cercopithecinae) inferred from geometric morphometric analysis of

landmark data. *The Anatomical Record Part A: Discoveries in Molecular, Cellular, and Evolutionary Biology,* 275A**,** 1048-1072.

FUJIMURA, Y., USAMI, Y., TITANI, K., NIINOMI, K., NISHIO, K., TAKASE, T., YOSHIOKA, A. & FUKUI, H. 1991. Studies on anti-von Willebrand factor (vWF) monoclonal antibody NMC-4, which inhibits both ristocetin- and botrocetin-induced vWF binding to platelet glycoprotein Ib. *Blood,* 77**,** 113-120.

GAFFNEY, P. J. 2001. Fibrin Degradation Products. *Annals of the New York Academy of Sciences,* 936**,** 594-610.

GALBUSERA, M., NORIS, M. & REMUZZI, G. 2006. Thrombotic Thrombocytopenic Purpura—Then and Now. *Seminars In Thrombosis And Hemostasis,* 32**,** 81-89.

GALLINARO, L., CATTINI, M. G., SZTUKOWSKA, M., PADRINI, R., SARTORELLO, F., PONTARA, E., BERTOMORO, A., DAIDONE, V., PAGNAN, A. & CASONATO, A. 2008. A shorter von Willebrand factor survival in O blood group subjects explains how ABO determinants influence plasma von Willebrand factor. *Blood,* 111**,** 3540-3545.

GANDO, S., NANZAKI, S., SASAKI, S. & KEMMOTSU, O. 1998. Significant correlations between tissue factor and thrombin markers in trauma and septic patients with disseminated intravascular coagulation. *Thrombosis and Haemostasis,* 79**,** 1111-5.

GEORGE, J. N. 2006. Clinical practice. Thrombotic thrombocytopenic purpura. *The New England Journal of Medicine,* 354**,** 1927-35.

GEORGE, J. N. & NESTER, C. M. 2014. Syndromes of thrombotic microangiopathy. *The New England Journal of Medicine,* 371**,** 654-66.

GILLMAN, T., PILLAY, R. A. & NAIDOO, S. S. 1959. Serum mucoprotein, plasma fibrin and fibrinolytic activity during the menstrual cycle in baboons. *Journal of Endocrinology,* 19**,** 303.

GIRMA, J. P., TAKAHASHI, Y., YOSHIOKA, A., DIAZ, J. & MEYER, D. 1990. Ristocetin and botrocetin involve two distinct domains of von Willebrand factor for binding to platelet membrane glycoprotein Ib. *Thrombosis and Haemostasis,* 64**,** 326-32.

GRAHAM, C. H., FITZPATRICK, T. E. & MCCRAE, K. R. 1998. Hypoxia Stimulates Urokinase Receptor Expression Through a Heme Protein-Dependent Pathway. *Blood,* 91**,** 3300-3307.

GUISASOLA, J. A., COCKBURN, C. G. & HARDISTY, R. M. 1978. Plasmin digestion of factor VIII: characterization of the breakdown products with respect to antigenicity and von Willebrand activity. *Thrombosis and Haemostasis,* 40**,** 302-15.

GUNTHER, K., GARIZIO, D. & DHLAMINI, B. 2006. The pathogenesis of HIV-related thrombotic thrombocytopenic purpura - is it different? *ISBT Science Series,* 1**,** 246-250.

GURMAN, P., MIRANDA, O. R., NATHAN, A., WASHINGTON, C., ROSEN, Y. & ELMAN, N. M. 2015. Recombinant tissue plasminogen activators (rtPA): a review. *Clinical Pharmacology and Therapeutics,* 97**,** 274-85.

HAJJAR, K. A. 1995. Cellular receptors in the regulation of plasmin generation. *Thrombosis and Haemostasis,* 74**,** 294-301.

HAJJAR, K. A. 2003. The molecular basis of fibrinolysis. *In:* NATHAN, D. G., ORKIN, S. H., GINSBURG, D. & LOOK, A. T. (eds.) *Hematology of Infancy and Childhood.* Philadelphia, U.S.A. : W.B. Saunders Co.

HAMILTON, K. K., FRETTO, L. J., GRIERSON, D. S. & MCKEE, P. A. 1985. Effects of plasmin on von Willebrand factor multimers. Degradation in vitro and stimulation of release in vivo. *Journal of Clinical Investigation,* 76**,** 261-70.

HAMPTON, J. W. & MATTHEWS, C. 1966. Similarities between baboon and human blood clotting. *Journal of Applied Physiology,* 21**,** 1713-1716.

HEEB, M. J., MARZEC, U., GRUBER, A. & HANSON, S. R. 2012. Antithrombotic activity of protein S infused without activated protein C in a baboon thrombosis model. *Thrombosis and Haemostasis,* 107**,** 690-8.

HERBERT, J. M., HÉRAULT, J. P., BERNAT, A., SAVI, P., SCHAEFFER, P., DRIGUEZ, P. A., DUCHAUSSOY, P. & PETITOU, M. 2001. SR123781A, a Synthetic Heparin Mimetic. *Thrombosis and Haemostasis,* 85**,** 852-860.

HILLMAYER, K., MACOVEI, A., PAUWELS, D., COMPERNOLLE, G., DECLERCK, P. J. & GILS, A. 2006. Characterization of rat thrombin-activatable fibrinolysis inhibitor

(TAFI) – a comparative study assessing the biological equivalence of rat, murine and human TAFI. *Journal of Thrombosis and Haemostasis,* 4**,** 2470-2477.

HOLLESTELLE, M. J., LAI, K. W., VAN DEUREN, M., LENTING, P. J., DE GROOT, P. G., SPRONG, T. & BOVENSCHEN, N. 2011. Cleavage of von Willebrand factor by granzyme M destroys its factor VIII binding capacity. *PLoS One,* 6**,** e24216.

HORNE, M. K., 3RD, MCCLOSKEY, D. J., CULLINANE, A. M., MERRYMAN, P. K., RICK, M. E., HORTIN, G. L., WACLAWIW, M. A. & CANNON, R. O., 3RD 2002. Parameters of coagulant and fibrinolytic capacity and activity in postmenopausal women: within-subject variability. *Thrombosis Research,* 107**,** 229-33.

HOSLER, G. A., CUSUMANO, A. M. & HUTCHINS, G. M. 2003. Thrombotic thrombocytopenic purpura and hemolytic uremic syndrome are distinct pathologic entities. A review of 56 autopsy cases. *Archives of Pathology & Laboratory Medicine,* 127**,** 834-9.

HOYLAERTS, M., RIJKEN, D. C., LIJNEN, H. R. & COLLEN, D. 1982. Kinetics of the activation of plasminogen by human tissue plasminogen activator. Role of fibrin. *Journal of Biological Chemistry,* 257**,** 2912-9.

HUCK, V., SCHNEIDER, M. F., GORZELANNY, C. & SCHNEIDER, S. W. 2014. The various states of von Willebrand factor and their function in physiology and pathophysiology. *Thrombosis and Haemostasis,* 111**,** 598-609.

ISO 2012. International Standard ISO:15189. Medical laboratories — Requirements for quality and competence. Switzerland: ISO (The International Organization for Standardization).

ISRAELS, E. D. & ISRAELS, L. G. 2013. Fibrinogen, factor XIII and fibrinolysis. *In:* ISRAELS, L. G. & ISRAELS, E. D. (eds.) *Mechanisms in Hematology.* 4 ed. Ontario, Canada: Core Health Services Inc.

JACQUEMIN, M., STASSEN, J. M., SAINT-REMY, J.-M., VERHAMME, P., LAVEND'HOMME, R., VAN DER ELST, L., MEIRING, M., PIETERS, H., LAMPRECHT, S., ROODT, J. & BADENHORST, P. 2009. A human monoclonal antibody inhibiting partially factor VIII activity reduces thrombus growth in baboons. *Journal of Thrombosis and Haemostasis,* 7**,** 429-437.

JANSE VAN RENSBURG, W. J. 2016. Comparison of common platelet receptors between the chacma baboon (*Papio ursinus*) and human for use in pre-clinical human-targeted anti-platelet studies. *Platelets,* 27**,** 322-332.

JANSE VAN RENSBURG, W. J. 2019. Molecular suitability of the chacma baboon in human-targeted Von Willebrand factor directed studies. *Journal of Medical Primatology,* 48**,** 171-175.

JANSE VAN RENSBURG, W. J., BADENHORST, P. N. & ROODT, J. P. 2015. The Cape Chacma baboon is not suitable for evaluating human targeted anti-GPVI agents. *Platelets,* 26**,** 552-7.

JENKINS, P. V. & O'DONNELL, J. S. 2006. ABO blood group determines plasma von Willebrand factor levels: a biologic function after all? *Transfusion,* 46**,** 1836-44.

JOUBERT, J., MEIRING, S. M., CONRADIE, C., LAMPRECHT, S. & JANSE VAN RENSBURG, W. J. 2021. The effects of streptokinase in a Chacma baboon (*Papio ursinus*) model of acquired thrombotic thrombocytopenic purpura. *Clinical and Experimental Medicine,* 21**,** 663–674.

KAWAMURA, T., OKADA, N. & OKADA, H. 2002. Elastase from Activated Human Neutrophils Activates Procarboxypeptidase R. *Microbiology and Immunology,* 46**,** 225-230.

KAWANO, T., MORIMOTO, K. & UEMURA, Y. 1970. Partial purification and properties of urokinase inhibitor from human placenta. *Journal of Biochemistry,* 67**,** 333-42.

KEARON, C., AKL, E. A., COMEROTA, A. J., PRANDONI, P., BOUNAMEAUX, H., GOLDHABER, S. Z., NELSON, M. E., WELLS, P. S., GOULD, M. K., DENTALI, F., CROWTHER, M. & KAHN, S. R. 2012. Antithrombotic Therapy for VTE Disease. Antithrombotic Therapy and Prevention of Thrombosis, 9th ed: American College of Chest Physicians Evidence-Based Clinical Practice Guidelines. *Chest,* 141**,** e419S–e494S.

KELDERMAN, M. & MEIRING, M. 2011. Standard operating procedure document no. SHL 5 - Von Willebrand factor multimer analysis. Bloemfontein, Free State, South Africa: Specialised Haemostasis Laboratory, Department of Haematology and Cell Biology, University of the Free State.

KENNEDY, S. S., ZACHARSKI, L. R. & BECK, J. R. 1980. Thrombotic thrombocytopenic purpura: analysis of 48 unselected cases. *Seminars In Thrombosis And Hemostasis,* 6**,** 341-349.

KESHARI, R. S., SILASI, R., LUPU, C., TAYLOR, F. B., JR. & LUPU, F. 2017. In vivo-generated thrombin and plasmin do not activate the complement system in baboons. *Blood,* 130**,** 2678-2681.

KILERCIK, M., COSKUN, A., SERTESER, M., INAN, D. & UNSAL, I. 2014. Biological variations of ADAMTS13 and von Willebrand factor in human adults. *Biochemia Medica (Zagreb),* 24**,** 138-45.

KLUFT, C., JIE, A. F., RIJKEN, D. C. & VERHEIJEN, J. H. 1988. Daytime fluctuations in blood of tissue-type plasminogen activator (t-PA) and its fast-acting inhibitor (PAI-1). *Thrombosis and Haemostasis,* 59**,** 329-32.

KOKAME, K., MATSUMOTO, M., FUJIMURA, Y. & MIYATA, T. 2004. VWF73, a region from D1596 to R1668 of von Willebrand factor, provides a minimal substrate for ADAMTS-13. *Blood,* 103**,** 607-12.

KOKAME, K., NOBE, Y., KOKUBO, Y., OKAYAMA, A. & MIYATA, T. 2005. FRETS-VWF73, a first fluorogenic substrate for ADAMTS13 assay. *British Journal of Haematology,* 129**,** 93-100.

KOPP, G. H., ROOS, C., BUTYNSKI, T. M., WILDMAN, D. E., ALAGAILI, A. N., GROENEVELD, L. F. & ZINNER, D. 2014. Out of Africa, but how and when? The case of hamadryas baboons (*Papio hamadryas*). *Journal of Human Evolution,* 76**,** 154-164.

KREMER HOVINGA, J. A. & GEORGE, J. N. 2019. Hereditary Thrombotic Thrombocytopenic Purpura. *The New England Journal of Medicine,* 381**,** 1653-1662.

KREMER HOVINGA, J. A. & LÄMMLE, B. 2012. Role of ADAMTS13 in the pathogenesis, diagnosis, and treatment of thrombotic thrombocytopenic purpura. *Hematology. American Society of Hematology. Education Program,* 2012**,** 610-6.

KREMERS, R. M. W., MOHAMED, A. B. O., PELKMANS, L., HINDAWI, S., HEMKER, H. C., DE LAAT, H. B., HUSKENS, D. & AL DIERI, R. 2015. Thrombin Generating

Capacity and Phenotypic Association in ABO Blood Groups. *PLoS One,* 10**,** e0141491.

KRIZEK, D. R. & RICK, M. E. 2000. A rapid method to visualize von willebrand factor multimers by using agarose gel electrophoresis, immunolocalization and luminographic detection. *Thrombosis Research,* 97**,** 457-62.

KROON, M. E., KOOLWIJK, P., VAN DER VECHT, B. & VAN HINSBERGH, V. W. 2000. Urokinase receptor expression on human microvascular endothelial cells is increased by hypoxia: implications for capillary-like tube formation in a fibrin matrix. *Blood,* 96**,** 2775-83.

KRUITHOF, E. K., MESTRIES, J. C., GASCON, M. P. & YTHIER, A. 1997. The coagulation and fibrinolytic responses of baboons after in vivo thrombin generation--effect of interleukin 6. *Thrombosis and Haemostasis,* 77**,** 905-10.

KUNAMNENI, A., ABDELGHANI, T. T. & ELLAIAH, P. 2007. Streptokinase--the drug of choice for thrombolytic therapy. *Journal of Thrombosis and Thrombolysis,* 23**,** 9-23.

LAFFAN, M. 2006. Investigation of haemostasis. *In:* LEWIS, S. M., BAIN, B. J. & BATES, I. (eds.) *Dacie and Lewis Practical Haematology.* 10 ed. Philadelphia, PA: Churchill Livingstone Elsevier.

LANKHOF, H. L., VAN HOEIJ, M., SCHIPHORST, M. E., BRACKE, M., WU, Y.-P., IJSSELDIJK, M. J. W., VINK, T., DE GROOT, P. G. & SIXMA, J. J. 1996. A3 Domain Is Essential for Interaction of von Willebrand Factor with Collagen Type III. *Thrombosis and Haemostasis,* 75**,** 950-958.

LAW, R. H. P., ABU-SSAYDEH, D. & WHISSTOCK, J. C. 2013. New insights into the structure and function of the plasminogen/plasmin system. *Current Opinion in Structural Biology,* 23**,** 836-841.

LAW, RUBY H. P., CARADOC-DAVIES, T., COWIESON, N., HORVATH, ANITA J., QUEK, ADAM J., ENCARNACAO, JOANNA A., STEER, D., COWAN, A., ZHANG, Q., LU, BERNADINE G. C., PIKE, ROBERT N., SMITH, A. I., COUGHLIN, PAUL B. & WHISSTOCK, JAMES C. 2012. The X-ray Crystal Structure of Full-Length Human Plasminogen. *Cell Reports,* 1**,** 185-190.

LEADLEY, R. J., CHI, L., REBELLO, S. S. & GAGNON, A. 2000. Contribution of in vivo models of thrombosis to the discovery and development of novel antithrombotic agents. *Journal of Pharmacological and Toxicological Methods,* 43**,** 101-116.

LEVI, M., DE BOER, J. P., ROEM, D., TEN CATE, J. W. & HACK, C. E. 1992. Plasminogen activation in vivo upon intravenous infusion of DDAVP. Quantitative assessment of plasmin-alpha 2-antiplasmin complex with a novel monoclonal antibody based radioimmunoassay. *Thrombosis and Haemostasis,* 67**,** 111-6.

LEVY, G. G., NICHOLS, W. C., LIAN, E. C., FOROUD, T., MCCLINTICK, J. N., MCGEE, B. M., YANG, A. Y., SIEMIENIAK, D. R., STARK, K. R., GRUPPO, R., SARODE, R., SHURIN, S. B., CHANDRASEKARAN, V., STABLER, S. P., SABIO, H., BOUHASSIRA, E. E., UPSHAW, J. D., JR., GINSBURG, D. & TSAI, H. M. 2001. Mutations in a member of the ADAMTS gene family cause thrombotic thrombocytopenic purpura. *Nature,* 413**,** 488-94.

LIJNEN, H. R., DE COCK, F., MATSUO, O. & COLLEN, D. 1992. Comparative fibrinolytic and fibrinogenolytic properties of staphylokinase and streptokinase in plasma of different species in vitro. *Fibrinolysis,* 6**,** 33-37.

LIJNEN, H. R., STASSEN, J. M., VANLINTHOUT, I., FUKAO, H., OKADA, K., MATSUO, O. & COLLEN, D. 1991. Comparative fibrinolytic properties of staphylokinase and streptokinase in animal models of venous thrombosis. *Thrombosis and Haemostasis,* 66**,** 468-73.

LOSKUTOFF, D. J., VAN MOURIK, J. A., ERICKSON, L. A. & LAWRENCE, D. 1983. Detection of an unusually stable fibrinolytic inhibitor produced by bovine endothelial cells. *Proceedings of the National Academy of Sciences of the United States of America,* 80**,** 2956-2960.

MAGA, T. K., NISHIMURA, C. J., WEAVER, A. E., FREES, K. L. & SMITH, R. J. 2010. Mutations in alternative pathway complement proteins in American patients with atypical hemolytic uremic syndrome. *Human Mutation,* 31**,** E1445-60.

MAJERUS, E. M., ZHENG, X., TULEY, E. A. & SADLER, J. E. 2003. Cleavage of the ADAMTS13 propeptide is not required for protease activity. *Journal of Biological Chemistry,* 278**,** 46643-8.

MALAK, S., WOLF, M., MILLOT, G. A., MARIOTTE, E., VEYRADIER, A., MEYNARD, J. L., KORACH, J. M., MALOT, S., BUSSEL, A., AZOULAY, E., BOULANGER, E., GALICIER, L., DEVAUX, E., ESCHWÈGE, V., GALLIEN, S., ADRIE, C., SCHLEMMER, B., RONDEAU, E., COPPO, P. & FOR THE RÉSEAU D'ETUDE DES MICROANGIOPATHIES, T. 2008. Human Immunodeficiency Virus-Associated Thrombotic Microangiopathies: Clinical Characteristics and Outcome According to ADAMTS13 Activity. *Scandinavian Journal of Immunology,* 68**,** 337-344.

MANNUCCI, P. M. 1998. von Willebrand factor: a marker of endothelial damage? *Arteriosclerosis, Thrombosis, and Vascular Biology,* 18**,** 1359-62.

MANNUCCI, P. M., CAPOFERRI, C. & CANCIANI, M. T. 2004. Plasma levels of von Willebrand factor regulate ADAMTS-13, its major cleaving protease. *British Journal of Haematology,* 126**,** 213-8.

MAO, S.-S., COOPER, C. M., WOOD, T., SHAFER, J. A. & GARDELL, S. J. 1999. Characterization of Plasmin-mediated Activation of Plasma Procarboxypeptidase B: Modulation by Glycosaminoglycans. *Journal of Biological Chemistry,* 274**,** 35046-35052.

MARCHI, R., MARCOS, L. & PARADISI, I. 2015. Comparison by sex between thrombin generation and fibrin network characteristics in a healthy population. *Clinica Chimica Acta,* 441**,** 86-9.

MARX, P. F., DAWSON, P. E., BOUMA, B. N. & MEIJERS, J. C. M. 2002. Plasmin-Mediated Activation and Inactivation of Thrombin-Activatable Fibrinolysis Inhibitor. *Biochemistry,* 41**,** 6688-6696.

MATSUI, T., TITANI, K. & MIZUOCHI, T. 1992. Structures of the asparagine-linked oligosaccharide chains of human von Willebrand factor. Occurrence of blood group A, B, and H(O) structures. *Journal of Biological Chemistry,* 267**,** 8723-31.

MCCLINTOCK, D. K. & BELL, P. H. 1971. The mechanism of activation of human plasminogen by streptokinase. *Biochemical and Biophysical Research Communications,* 43**,** 694-702.

MCLEAN, J. W., TOMLINSON, J. E., KUANG, W. J., EATON, D. L., CHEN, E. Y., FLESS, G. M., SCANU, A. M. & LAWN, R. M. 1987. cDNA sequence of human apolipoprotein(a) is homologous to plasminogen. *Nature,* 330**,** 132-7.

MEDCALF, R. L. 2015. What drives "fibrinolysis"? *Hamostaseologie,* 35**,** 303-10.

MEIRING, M. 2011a. Standard operating procedure document no. SHL 1 - von Willebrand factor antigen (vWF:Ag). Bloemfontein, Free State, South Africa: Specialised Haemostasis Laboratory, Department of Haematology and Cell Biology, University of the Free State.

MEIRING, M. 2011b. Standard operating procedure document no. SHL 2 - Collagen binding assay of von Willebrand factor (vWF:CB). Bloemfontein, Free State, South Africa: Specialised Haemostasis Laboratory, Department of Haematology and Cell Biology, University of the Free State.

MEIRING, M. 2011c. Standard operating procedure document no. SHL 3 version 2 - Ristocetin co-factor assay. Bloemfontein, Free State, South Africa: Specialised Haemostasis Laboratory, Department of Haematology and Cell Biology, University of the Free State.

MEIRING, M., BADENHORST, P. N. & KELDERMAN, M. 2007. Performance and utility of a cost-effective collagen-binding assay for the laboratory diagnosis of Von Willebrand disease. *Clinical Chemistry and Laboratory Medicine,* 45**,** 1068-1072.

MEIRING, M., BADENHORST, P. N. & KELDERMAN, M. 2009. Laboratory Diagnosis of von Willebrand Disease. *European Oncology and Haematology,* 3**,** 33-36.

MEIRING, M., WEBB, M., GOEDHALS, D. & LOUW, V. 2012. HIV-associated Thrombotic Thrombocytopenic Purpura - What We Know So Far. *European Oncology and Haematology,* 8**,** 89-91.

MEIRING, S. M. 2017. Validation of the thrombin generation assay in haemostatic disorders. UFS Special Haemostasis Laboratory Validation Report.

MELTON, D. A. & MELTON, C. L. 1982. Blood parameters of the wild chacma baboon, *Papio ursinus*. *South African Journal of Zoology,* 17**,** 85-90.

MESTRIES, J. C., KRUITHOF, E. K., GASCON, M. P., HERODIN, F., AGAY, D. & YTHIER, A. 1994. In vivo modulation of coagulation and fibrinolysis by

recombinant glycosylated human interleukin-6 in baboons. *European Cytokine Network,* 5**,** 275-281.

MILES, L. A. & PARMER, R. J. 2013. Plasminogen Receptors: The First Quarter Century. *Seminars in Thrombosis and Hemostasis,* 39**,** 329-337.

MOAKE, J. L. 2002. Thrombotic microangiopathies. *The New England Journal of Medicine,* 347**,** 589-600.

MOAKE, J. L., RUDY, C. K., TROLL, J. H., WEINSTEIN, M. J., COLANNINO, N. M., AZOCAR, J., SEDER, R. H., HONG, S. L. & DEYKIN, D. 1982. Unusually large plasma factor VIII:von Willebrand factor multimers in chronic relapsing thrombotic thrombocytopenic purpura. *The New England Journal of Medicine,* 307**,** 1432-5.

MONTEAGUDO, J., PEREIRA, A., REVERTER, J. C., PIJOAN, J., TUSELL, J., PUIG, L., ORDINAS, A. & CASTILLO, R. 1991. Thrombin generation and fibrinolysis in the thrombotic thrombocytopenic purpura and the hemolytic-uremic syndrome. *Thrombosis and Haemostasis,* 66**,** 515-519.

MOSNIER, L. O., VON DEM BORNE, P. A. K., MEIJERS, J. C. M. & BOUMA, B. N. 1998. Plasma TAFI Levels Influence the Clot Lysis Time in Healthy Individuals in the Presence of an Intact Intrinsic Pathway of Coagulation. *Thrombosis and Haemostasis,* 80**,** 829-835.

MOTTO, D. G., CHAUHAN, A. K., ZHU, G., HOMEISTER, J., LAMB, C. B., DESCH, K. C., ZHANG, W., TSAI, H. M., WAGNER, D. D. & GINSBURG, D. 2005. Shigatoxin triggers thrombotic thrombocytopenic purpura in genetically susceptible ADAMTS13-deficient mice. *Journal of Clinical Investigation,* 115**,** 2752-61.

MOUSA, H. A., DOWNEY, C., ALFIREVIC, Z. & TOH, C. H. 2004. Thrombin activatable fibrinolysis inhibitor and its fibrinolytic effect in normal pregnancy. *Thrombosis and Haemostasis,* 92**,** 1025-31.

MUIA, J., GAO, W., HABERICHTER, S. L., DOLATSHAHI, L., ZHU, J., WESTFIELD, L. A., COVILL, S. C., FRIEDMAN, K. D. & SADLER, J. E. 2013. An optimized fluorogenic ADAMTS13 assay with increased sensitivity for the investigation of patients with thrombotic thrombocytopenic purpura. *Journal of Thrombosis and Haemostasis,* 11**,** 1511-1518.

MUIA, J., ZHU, J., GRECO, S. C., VANHOORELBEKE, K., GUPTA, G., WESTFIELD, L. A. & SADLER, J. E. 2019. Phylogenetic and functional analysis of ADAMTS13 identifies highly conserved domains essential for allosteric regulation. *Blood,* 133**,** 1899-1908.

MUIA, J., ZHU, J., GUPTA, G., HABERICHTER, S. L., FRIEDMAN, K. D., FEYS, H. B., DEFORCHE, L., VANHOORELBEKE, K., WESTFIELD, L. A., ROTH, R., TOLIA, N. H., HEUSER, J. E. & SADLER, J. E. 2014. Allosteric activation of ADAMTS13 by von Willebrand factor. *Proceedings of the National Academy of Sciences of the United States of America,* 111**,** 18584-18589.

MURRAY, G. P., POST, S. R. & POST, G. R. 2020. ABO blood group is a determinant of von Willebrand factor protein levels in human pulmonary endothelial cells. *Journal of Clinical Pathology,* 73**,** 347-349.

NATIONAL DEPARTMENT OF HEALTH, S. A. 2019. *Hospital Level (Adults) Standard Treatment Guidelines and Essential Medicines List,* Pretoria, Republic of South Africa, The National Department of Health.

NESHEIM, M. & BAJZAR, L. 2005. HISTORICAL SKETCH: The discovery of TAFI. *Journal of Thrombosis and Haemostasis,* 3**,** 2139-2146.

NEWMAN, T. K., JOLLY, C. J. & ROGERS, J. 2004. Mitochondrial phylogeny and systematics of baboons (*Papio*). *American Journal of Physical Anthropology,* 124**,** 17-27.

NOLASCO, L., NOLASCO, J., FENG, S., AFSHAR-KHARGHAN, V. & MOAKE, J. 2013. Human complement factor H is a reductase for large soluble von Willebrand factor multimers--brief report. *Arteriosclerosis, Thrombosis, and Vascular Biology,* 33**,** 2524-8.

O'DONNELL, J. & LAFFAN, M. A. 2001. The relationship between ABO histo-blood group, factor VIII and von Willebrand factor. *Transfusion Medicine,* 11**,** 343-51.

O'GARA, P. T., KUSHNER, F. G., ASCHEIM, D. D., CASEY, D. E., JR., CHUNG, M. K., DE LEMOS, J. A., ETTINGER, S. M., FANG, J. C., FESMIRE, F. M., FRANKLIN, B. A., GRANGER, C. B., KRUMHOLZ, H. M., LINDERBAUM, J. A., MORROW, D. A., NEWBY, L. K., ORNATO, J. P., OU, N., RADFORD, M. J., TAMIS-HOLLAND, J. E., TOMMASO, C. L., TRACY, C. M., WOO, Y. J., ZHAO, D. X., ANDERSON,

J. L., JACOBS, A. K., HALPERIN, J. L., ALBERT, N. M., BRINDIS, R. G., CREAGER, M. A., DEMETS, D., GUYTON, R. A., HOCHMAN, J. S., KOVACS, R. J., KUSHNER, F. G., OHMAN, E. M., STEVENSON, W. G. & YANCY, C. W. 2013. 2013 ACCF/AHA guideline for the management of ST-elevation myocardial infarction: a report of the American College of Cardiology Foundation/American Heart Association Task Force on Practice Guidelines. *Circulation,* 127**,** e362-425.

O'DONNELL, J., BOULTON, F. E., MANNING, R. A. & LAFFAN, M. A. 2002. Amount of H Antigen Expressed on Circulating von Willebrand Factor Is Modified by ABO Blood Group Genotype and Is a Major Determinant of Plasma von Willebrand Factor Antigen Levels. *Arteriosclerosis, Thrombosis, and Vascular Biology,* 22**,** 335-341.

OPIE, J. 2012. Haematological complications of HIV infection. *South African Medical Journal,* 102**,** 465-8.

OWEN, J., FRIEDMAN, K. D., GROSSMAN, B. A., WILKINS, C., BERKE, A. D. & POWERS, E. R. 1988. Thrombolytic Therapy With Tissue Plasminogen Activator or Streptokinase Induces Transient Thrombin Activity. *Blood,* 72**,** 616-620.

PARRY, M. A., ZHANG, X. C. & BODE, I. 2000. Molecular mechanisms of plasminogen activation: bacterial cofactors provide clues. *Trends in Biochemical Sciences,* 25**,** 53-59.

PETRI, A., KIM, H. J., XU, Y., DE GROOT, R., LI, C., VANDENBULCKE, A., VANHOORELBEKE, K., EMSLEY, J. & CRAWLEY, J. T. B. 2019. Crystal structure and substrate-induced activation of ADAMTS13. *Nature Communications,* 10**,** 3781.

PEYVANDI, F., PALLA, R., LOTTA, L. A., MACKIE, I., SCULLY, M. A. & MACHIN, S. J. 2010. ADAMTS-13 assays in thrombotic thrombocytopenic purpura. *Journal of Thrombosis and Haemostasis,* 8**,** 631-40.

PEYVANDI, F., SCULLY, M., KREMER HOVINGA, J. A., CATALAND, S., KNOBL, P., WU, H., ARTONI, A., WESTWOOD, J. P., MANSOURI TALEGHANI, M., JILMA, B., CALLEWAERT, F., ULRICHTS, H., DUBY, C. & TERSAGO, D. 2016. Caplacizumab for Acquired Thrombotic Thrombocytopenic Purpura. *The New England Journal of Medicine,* 374**,** 511-22.

PIETERS, M., PHILIPPOU, H., UNDAS, A., DE LANGE, Z., RIJKEN, D. C. & MUTCH, N. J. 2018. An international study on the feasibility of a standardized combined plasma clot turbidity and lysis assay: communication from the SSC of the ISTH. *Journal of Thrombosis and Haemostasis,* 16**,** 1007-1012.

PIZZO, S. V., SCHWARTZ, M. L., HILL, R. L. & MCKEE, P. A. 1973. The effect of plasmin on the subunit structure of human fibrin. *Journal of Biological Chemistry,* 248**,** 4574-83.

PLUG, T. & MEIJERS, J. C. M. 2016. Structure-function relationships in thrombin-activatable fibrinolysis inhibitor. *Journal of Thrombosis and Haemostasis,* 14**,** 633-644.

POITOUT-BELISSENT, F., CULANG, D., POULIN, D., SAMADFAN, R., COTTON, S. & BÉDARD, C. 2020. Monitoring Compound-Related Effects on Coagulability in Rats and Cynomolgus and Rhesus Monkeys by Thrombin Generation Kinetic Measurement. *International Journal of Toxicology,* 39**,** 207-217.

PONSCHAB, M., VAN GRIENSVEN, M., HEITMEIER, S., LAUX, V., SCHLIMP, C. J., CALATZIS, A., BAHRAMI, S., REDL, H. & SCHÖCHL, H. 2016. Platelet function in baboons and humans — A comparative study of whole blood using impedance platelet aggregometry (Multiplate®). *Thrombosis Research,* 147**,** 115-121.

PUY, C., NGO, A. T. P., PANG, J., KESHARI, R. S., HAGEN, M. W., HINDS, M. T., GAILANI, D., GRUBER, A., LUPU, F. & MCCARTY, O. J. T. 2019. Endothelial PAI-1 (Plasminogen Activator Inhibitor-1) Blocks the Intrinsic Pathway of Coagulation, Inducing the Clearance and Degradation of FXIa (Activated Factor XI). *Arteriosclerosis, Thrombosis, and Vascular Biology,* 39**,** 1390-1401.

RAIFE, T. J., CAO, W., ATKINSON, B. S., BEDELL, B., MONTGOMERY, R. R., LENTZ, S. R., JOHNSON, G. F. & ZHENG, X. L. 2009. Leukocyte proteases cleave von Willebrand factor at or near the ADAMTS13 cleavage site. *Blood,* 114**,** 1666-74.

RAMIS, G., MARTÍNEZ-ALARCON, L., QUEREDA, J. J., MROWIEC, A., FUNES, C., RÍOS, A., RAMÍREZ, P., MUÑOZ, A. & MAJADO, M. J. 2013. Non-ABO blood group systems phenotyping in non-human primates for blood banking laboratory and xenotransplantation. *Laboratory Animals,* 47**,** 100-105.

RAYES, J., ROUMENINA, L. T., DIMITROV, J. D., REPESSE, Y., ING, M., CHRISTOPHE, O., JOKIRANTA, T. S., HALBWACHS-MECARELLI, L., BOREL-DERLON, A., KAVERI, S. V., FREMEAUX-BACCHI, V. & LACROIX-DESMAZES, S. 2014. The interaction between factor H and VWF increases factor H cofactor activity and regulates VWF prothrombotic status. *Blood,* 123**,** 121-5.

REDL, H., SCHLAG, G., SCHIEßER, A. & DAVIES, J. 1995. Thrombomodulin Release in Baboon Sepsis: Its Dependence on the Dose of Escherichia coli and the Presence of Tumor Necrosis Factor. *The Journal of Infectious Diseases,* 171**,** 1522-1527.

REDL, H. R., MARTIN, U., KHADEM, A., PELINKA, L. E. & VAN GRIENSVEN, M. 2005. Anti-L-selectin antibody therapy does not worsen the postseptic course in a baboon model. *Critical Care,* 9**,** R735-44.

RICK, M. E., POPOVSKY, M. A. & KRIZEK, D. M. 1985. Degradation of factor VIII coagulant antigen by proteolytic enzymes. *British Journal of Haematology,* 61**,** 477-486.

ROGERS, J., RAVEENDRAN, M., HARRIS, R. A., MAILUND, T., LEPPÄLÄ, K., ATHANASIADIS, G., SCHIERUP, M. H., CHENG, J., MUNCH, K., WALKER, J. A., KONKEL, M. K., JORDAN, V., STEELY, C. J., BECKSTROM, T. O., BERGEY, C., BURRELL, A., SCHREMPF, D., NOLL, A., KOTHE, M., KOPP, G. H., LIU, Y., MURALI, S., BILLIS, K., MARTIN, F. J., MUFFATO, M., COX, L., ELSE, J., DISOTELL, T., MUZNY, D. M., PHILLIPS-CONROY, J., AKEN, B., EICHLER, E. E., MARQUES-BONET, T., KOSIOL, C., BATZER, M. A., HAHN, M. W., TUNG, J., ZINNER, D., ROOS, C., JOLLY, C. J., GIBBS, R. A. & WORLEY, K. C. 2019. The comparative genomics and complex population history of *Papio* baboons. *Science Advances,* 5**,** eaau6947.

ROOSE, E., SCHELPE, A.-S., TELLIER, E., SINKOVITS, G., JOLY, B. S., DEKIMPE, C., KAPLANSKI, G., LE BESNERAIS, M., MANCINI, I., FALTER, T., VON AUER, C., FEYS, H. B., RETI, M., ROSSMANN, H., VANDENBULCKE, A., PAREYN, I., VOORBERG, J., GREINACHER, A., BENHAMOU, Y., DECKMYN, H., FIJNHEER, R., PROHÁSZKA, Z., PEYVANDI, F., LÄMMLE, B., COPPO, P., DE MEYER, S. F., VEYRADIER, A. & VANHOORELBEKE, K. 2020. Open ADAMTS13, induced

by antibodies, is a biomarker for subclinical immune-mediated thrombotic thrombocytopenic purpura. *Blood,* 136**,** 353-361.

ROSSITER, D. 2012. Antithrombotic agents. *South African Medicines Formulary.* 10th ed. Rondebosch, South Africa: Health and Medical Publishing Group.

RUNGE, M. S., HARKER, L. A., BODE, C., RUEF, J., KELLY, A. B., MARZEC, U. M., ALLEN, E., CABAN, R., SHAW, S.-Y., HABER, E. & HANSON, S. R. 1996. Enhanced Thrombolytic and Antithrombotic Potency of a Fibrin-Targeted Plasminogen Activator in Baboons. *Circulation,* 94**,** 1412-1422.

SAGRIPANTI, A., CARPI, A., BAICCHI, U., MORGANTI, M., ROSAIA, B., NICOLINI, A. & MITTERMAYER, C. 1996. Plasmatic parameters of coagulation activation in thrombotic microangiopathy. *Biomedicine & Pharmacotherapy,* 50**,** 357-62.

SAKKINEN, P. A., MACY, E. M., CALLAS, P. W., CORNELL, E. S., HAYES, T. E., KULLER, L. H. & TRACY, R. P. 1999. Analytical and biologic variability in measures of hemostasis, fibrinolysis, and inflammation: assessment and implications for epidemiology. *American Journal of Epidemiology,* 149**,** 261-7.

SANGLAS, L., VALNICKOVA, Z., AROLAS, J. L., PALLARÉS, I., GUEVARA, T., SOLÀ, M., KRISTENSEN, T., ENGHILD, J. J., AVILES, F. X. & GOMIS-RÜTH, F. X. 2008. Structure of Activated Thrombin-Activatable Fibrinolysis Inhibitor, a Molecular Link between Coagulation and Fibrinolysis. *Molecular Cell,* 31**,** 598-606.

SAVAGE, B., SALDIVAR, E. & RUGGERI, Z. M. 1996. Initiation of platelet adhesion by arrest onto fibrinogen or translocation on von Willebrand factor. *Cell,* 84**,** 289-97.

SCHÖCHL, H., SOLOMON, C., LAUX, V., HEITMEIER, S., BAHRAMI, S. & REDL, H. 2012. Similarities in thromboelastometric (ROTEM(R)) findings between humans and baboons. *Thrombosis Research,* 130**,** e107-12.

SCHÖCHL, H., VAN GRIENSVEN, M., HEITMEIER, S., LAUX, V., KIPMAN, U., ROODT, J., BAHRAMI, S. & REDL, H. 2017. Dual inhibition of thrombin and activated factor X attenuates disseminated intravascular coagulation and protects organ function in a baboon model of severe Gram-negative sepsis. *Critical Care,* 21**,** 51.

SCULLY, M., CATALAND, S. R., PEYVANDI, F., COPPO, P., KNÖBL, P., KREMER HOVINGA, J. A., METJIAN, A., DE LA RUBIA, J., PAVENSKI, K., CALLEWAERT, F., BISWAS, D., DE WINTER, H. & ZELDIN, R. K. 2019. Caplacizumab Treatment

for Acquired Thrombotic Thrombocytopenic Purpura. *The New England Journal of Medicine,* 380**,** 335-346.

SCULLY, M., HUNT, B. J., BENJAMIN, S., LIESNER, R., ROSE, P., PEYVANDI, F., CHEUNG, B. & MACHIN, S. J. 2012. Guidelines on the diagnosis and management of thrombotic thrombocytopenic purpura and other thrombotic microangiopathies. *British Journal of Haematology,* 158**,** 323–335.

SHOU, W., CHEN, Q., WU, W. & CUI, W. 2016. Biological Variations of Lupus Anticoagulant, Antithrombin, Protein C, Protein S, and von Willebrand Factor Assays. *Seminars In Thrombosis And Hemostasis,* 42**,** 87-92.

SILASI, R., KESHARI, R. S., LUPU, C., VAN RENSBURG, W. J., CHAABAN, H., REGMI, G., SHAMANAEV, A., SHATZEL, J. J., PUY, C., LORENTZ, C. U., TUCKER, E. I., GAILANI, D., GRUBER, A., MCCARTY, O. J. T. & LUPU, F. 2019. Inhibition of contact-mediated activation of factor XI protects baboons against *S aureus*-induced organ damage and death. *Blood Advances,* 3**,** 658-669.

SILLER-MATULA, J. M., PLASENZOTTI, R., SPIEL, A., QUEHENBERGER, P. & JILMA, B. 2008. Interspecies differences in coagulation profile. *Thrombosis and Haemostasis,* 100**,** 397-404.

SINGER, K., BORNSTEIN, F. P. & WILE, S. A. 1947. Thrombotic thrombocytopenic purpura; hemorrhagic diathesis with generalized platelet thromboses. *Blood,* 2**,** 542-54.

SINNAEVE, P. R., ARMSTRONG, P. W., GERSHLICK, A. H., GOLDSTEIN, P., WILCOX, R., LAMBERT, Y., DANAYS, T., SOULAT, L., HALVORSEN, S., ORTIZ, F. R., VANDENBERGHE, K., REGELIN, A., BLUHMKI, E., BOGAERTS, K. & VAN DE WERF, F. 2014. ST–Segment-Elevation Myocardial Infarction Patients Randomized to a Pharmaco-Invasive Strategy or Primary Percutaneous Coronary Intervention: Strategic Reperfusion Early After Myocardial Infarction (STREAM) 1-Year Mortality Follow-Up. *Circulation,* 130**,** 1139-1145.

SOBEL, G. W., MOHLER, S. R. & JONES, N. W. 1952. Urokinase: an activator of plasma profibrinolysin extracted from urine. *American Journal of Physiology***,** 768–769.

SODETZ, J. M., PAULSON, J. C. & MCKEE, P. A. 1979. Carbohydrate composition and identification of blood group A, B, and H oligosaccharide structures on human Factor VIII/von Willebrand factor. *Journal of Biological Chemistry,* 254**,** 10754-60.

SOUTH, K., FREITAS, M. O. & LANE, D. A. 2017. A model for the conformational activation of the structurally quiescent metalloprotease ADAMTS13 by von Willebrand factor. *Journal of Biological Chemistry,* 292**,** 5760-5769.

SPIEZIA, L., BERTINI, D., BOLDRIN, M., RADU, C., BULATO, C., GAVASSO, S., COZZI, E. & SIMIONI, P. 2010. Reference values for thromboelastometry (ROTEM®) in cynomolgus monkeys (*Macaca fascicularis*). *Thrombosis Research,* 126**,** e294-7.

STEYN, D. G., HAMILTON-BRUCE, R. J., ZUURMOND, T. J. & PHARO, R. 1975. Standard serum chemical and haematological values in the chacma baboon (*Papio ursinus*). *Journal of the South African Veterinary Association,* 46**,** 191-196.

SUTTON, D. M., NAIR, R. C. & ROCK, G. 1989. Complications of plasma exchange. *Transfusion,* 29**,** 124-127.

TAKAHASHI, H., TATEWAKI, W., WADA, K. & SHIBATA, A. 1989. Thrombin generation in patients with thrombotic thrombocytopenic purpura. *American Journal of Hematology,* 32**,** 255-7.

TALENS, S., MALFLIET, J. J., RUDEŽ, G., SPRONK, H. M., JANSSEN, N. A., MEIJER, P., KLUFT, C., DE MAAT, M. P. & RIJKEN, D. C. 2012. Biological variation in tPA-induced plasma clot lysis time. *Thrombosis and Haemostasis,* 108**,** 640-6.

TAN, A. K. & EATON, D. L. 1995. Activation and characterization of procarboxypeptidase B from human plasma. *Biochemistry,* 34**,** 5811-6.

TARANDOVSKIY, I. D., RAJABI, A. A., KARNAUKHOVA, E. & BUEHLER, P. W. 2019. Contradictory to its effects on thrombin, C1-inhibitor reduces plasmin generation in the presence of thrombomodulin. *Journal of Thrombosis and Thrombolysis,* 48**,** 81-87.

TARANDOVSKIY, I. D., SHIN, H. K. H., BAEK, J. H., KARNAUKHOVA, E. & BUEHLER, P. W. 2020. Interspecies comparison of simultaneous thrombin and plasmin generation. *Scientific Reports,* 10**,** 3885-3885.

TECHNOCLONE 2011. Technozym® ADAMTS13 Activity ELISA kit package insert. Vienna, Austria.

TECHNOCLONE 2012a. Technozym® ADAMTS13 Antigen ELISA kit package insert. Vienna, Austria.

TECHNOCLONE 2012b. Technozym® PAI-1 Antigen ELISA kit package insert. Vienna, Austria.

TECHNOCLONE 2012c. Technozym® PAP Complex ELISA kit package insert. Vienna, Austria.

TECHNOCLONE 2012d. Technozym® t-PA Antigen EDTA ELISA kit package insert. Vienna, Austria.

TECHNOCLONE 2014. Technozym® Glu-Plasminogen ELISA kit package insert. Vienna, Austria.

TERSTEEG, C., DE MAAT, S., DE MEYER, S. F., SMEETS, M. W., BARENDRECHT, A. D., ROEST, M., PASTERKAMP, G., FIJNHEER, R., VANHOORELBEKE, K., DE GROOT, P. G. & MAAS, C. 2014. Plasmin cleavage of von Willebrand factor as an emergency bypass for ADAMTS13 deficiency in thrombotic microangiopathy. *Circulation,* 129**,** 1320-31.

TERSTEEG, C., FIJNHEER, R., PASTERKAMP, G., DE GROOT, P. G., VANHOORELBEKE, K., DE MAAT, S. & MAAS, C. 2016. Keeping von Willebrand Factor under Control: Alternatives for ADAMTS13. *Seminars In Thrombosis And Hemostasis,* 42**,** 9-17.

TERSTEEG, C., JOLY, B. S., GILS, A., LIJNEN, R., DECKMYN, H., DECLERCK, P. J., PLAIMAUER, B., COPPO, P., VEYRADIER, A., MAAS, C., MEYER, S. F. D. & VANHOORELBEKE, K. 2017a. Amplified endogenous plasmin activity resolves acute thrombotic thrombocytopenic purpura in mice. *Journal of Thrombosis and Haemostasis,* 15**,** 2432-2442.

TERSTEEG, C., ROODT, J., VAN RENSBURG, W. J., DEKIMPE, C., VANDEPUTTE, N., PAREYN, I., VANDERBULCKE, A., PLAIMAUER, B., LAMPRECHT, S., DECKMYN, H., LOPEZ, J. A., DE MEYER, S. F. & VANHOORELBEKE, K. 2017b. N-acetylcysteine in preclinical mouse and baboon models of thrombotic thrombocytopenic purpura. *Blood,* 129**,** 1030-1038.

TILLETT, W. S. & GARNER, R. L. 1933. The fibrinolytic activity of hemolytic streptococci. *Journal of Experimental Medicine,* 58**,** 485-502.

TOH, C. H. & DENNIS, M. 2003. Disseminated intravascular coagulation: old disease, new hope. *BMJ,* 327**,** 974-7.

TURNER, N., NOLASCO, L., NOLASCO, J., SARTAIN, S. & MOAKE, J. 2014. Thrombotic microangiopathies and the linkage between von Willebrand factor and the alternative complement pathway. *Seminars In Thrombosis And Hemostasis,* 40**,** 544-50.

ULRICHTS, H., SILENCE, K., SCHOOLMEESTER, A., DE JAEGERE, P., ROSSENU, S., ROODT, J., PRIEM, S., LAUWEREYS, M., CASTEELS, P., VAN BOCKSTAELE, F., VERSCHUEREN, K., STANSSENS, P., BAUMEISTER, J. & HOLZ, J.-B. 2011. Antithrombotic drug candidate ALX-0081 shows superior preclinical efficacy and safety compared with currently marketed antiplatelet drugs. *Blood,* 118**,** 757-765.

VAN MARLE, A. C., JOUBERT, J. & MEIRING, S. M. 2019. Comparison of ADAMTS13 and Von Willebrand factor levels and activities, and plasminogen levels, in plasma products currently available for the treatment of thrombotic thrombocytopenic purpura in South Africa. *Transfusion and Apheresis Science,* 58**,** 72-78.

VAN ZYL, W. B., PRETORIUS, G. H. J., LAMPRECHT, S., ROODT, J. P. & KOTZÉ, H. F. 2000. PLATSAK, a Potent Antithrombotic and Fibrinolytic protein, Inhibits Arterial and Venous Thrombosis in a Baboon Model. *Thrombosis Research,* 98**,** 435-443.

VANHOORELBEKE, K. & DE MEYER, S. F. 2013. Animal models for thrombotic thrombocytopenic purpura. *Journal of Thrombosis and Haemostasis,* 11**,** 2-10.

VENTER, C. S., NEL, C. J., VORSTER, H. H., JERLING, J. C., OOSTHUIZEN, W., VELDMAN, F. J., KELLERMAN, J. A., SMUTS, C. M., VERMAAK, W. J. H., VAN DER NEST, D. G. & RIDDER, J. H. 1997. Soluble-fibre concentrate lowers plasminogen activator inhibitor-1 in baboons (*Papio ursinus*). *British Journal of Nutrition,* 78**,** 625-637.

VENTER, E. H., HILDEBRAND, L. & VERMEULEN, M. E. G. 2018. NHLS Standard Operating Procedure UNII0014. ABO Blood grouping. National Health Laboratory Service.

VESELY, S. K., GEORGE, J. N., LAMMLE, B., STUDT, J. D., ALBERIO, L., EL-HARAKE, M. A. & RASKOB, G. E. 2003. ADAMTS13 activity in thrombotic thrombocytopenic purpura-hemolytic uremic syndrome: relation to presenting features and clinical outcomes in a prospective cohort of 142 patients. *Blood,* 102**,** 60-8.

VEYRADIER, A. 2016. Von Willebrand Factor — A New Target for TTP Treatment? *The New England Journal of Medicine,* 374**,** 583-585.

WADA, H., MORI, Y., SHIMURA, M., HIYOYAMA, K., IOKA, M., NAKASAKI, T., NISHIKAWA, M., NAKANO, M., KUMEDA, K., KANEKO, T., NAKAMURA, S. & SHIKU, H. 1998. Poor outcome in disseminated intravascular coagulation or thrombotic thrombocytopenic purpura patients with severe vascular endothelial cell injuries. *American Journal of Hematology,* 58**,** 189-194.

WADA, Y., KURIHARA, M., TOYOFUKU, M., KAWAMURA, M., IIDA, H., KAYAMORI, Y., KINOSHITA, S. & HAMASAKI, N. 2004. Analytical goals for coagulation tests based on biological variation. *Clinical Chemistry and Laboratory Medicine,* 42**,** 79-83.

WANG, W., HENDRIKS, D. F. & SCHARPÉ, S. S. 1994. Carboxypeptidase U, a plasma carboxypeptidase with high affinity for plasminogen. *Journal of Biological Chemistry,* 269**,** 15937-15944.

WANG, X., LIN, X., LOY, J. A., TANG, J. & ZHANG, X. C. 1998. Crystal Structure of the Catalytic Domain of Human Plasmin Complexed with Streptokinase. *Science,* 281**,** 1662-1665.

WANG, Y., LIU, Q., ZHU, J., YUAN, Z. & MA, X. 2002. Procoagulant effects of thrombolytic therapy in acute myocardial infarction. *Chinese Medical Sciences Journal,* 17**,** 36-9.

WANG, Z., DOU, M., DU, X., MA, L., SUN, P., CAO, H., YE, S., JIANG, P., LIU, F., LIN, F., ZHANG, R. & LI, C. 2017. Influences of ABO blood group, age and gender on plasma coagulation factor VIII, fibrinogen, von Willebrand factor and ADAMTS13 levels in a Chinese population. *PeerJ,* 5**,** e3156-e3156.

WILDMAN, D. E., BERGMAN, T. J., AL-AGHBARI, A., STERNER, K. N., NEWMAN, T. K., PHILLIPS-CONROY, J. E., JOLLY, C. J. & DISOTELL, T. R. 2004.

Mitochondrial evidence for the origin of hamadryas baboons. *Molecular Phylogenetics and Evolution,* 32**,** 287-296.

WOHL, R. C., SINIO, L., SUMMARIA, L. & ROBBINS, K. C. 1983. Comparative activation kinetics of mammalian plasminogens. *Biochimica et Biophysica Acta,* 745**,** 20-31.

WOHNER, N., KOVÁCS, A., MACHOVICH, R. & KOLEV, K. 2012. Modulation of the von Willebrand factor-dependent platelet adhesion through alternative proteolytic pathways. *Thrombosis Research,* 129**,** e41-6.

YARRANTON, H., LAWRIE, A. S., PURDY, G., MACKIE, I. J. & MACHIN, S. J. 2004. Comparison of von Willebrand factor antigen, von Willebrand factor-cleaving protease and protein S in blood components used for treatment of thrombotic thrombocytopenic purpura. *Transfusion Medicine,* 14**,** 39-44.

ZAAR, M., HERZIG, M. C., FEDYK, C. G., MONTGOMERY, R. K., PRAT, N., PARIDA, B. K., HINOJOSA-LABORDE, C., MUNIZ, G. W., SHADE, R. E., BAUER, C., DELACRUZ, W., BYNUM, J. A., CONVERTINO, V. A., CAP, A. P. & PIDCOKE, H. F. 2020. Similar hemostatic responses to hypovolemia induced by hemorrhage and lower body negative pressure reveal a hyperfibrinolytic subset of non-human primates. *PLoS One,* 15**,** e0234844.

ZEKAVAT, O. R., HAGHPANAH, S., DEHGHANI, J., AFRASIABI, A., PEYVANDI, F. & KARIMI, M. 2014. Comparison of thrombin generation assay with conventional coagulation tests in evaluation of bleeding risk in patients with rare bleeding disorders. *Clinical and Applied Thrombosis/Hemostasis,* 20**,** 637-44.

ZHENG, X., CHUNG, D., TAKAYAMA, T. K., MAJERUS, E. M., SADLER, J. E. & FUJIKAWA, K. 2001. Structure of von Willebrand Factor-cleaving Protease (ADAMTS13), a Metalloprotease Involved in Thrombotic Thrombocytopenic Purpura. *Journal of Biological Chemistry,* 276**,** 41059-41063.

ZHU, J., MUIA, J., GUPTA, G., WESTFIELD, L. A., VANHOORELBEKE, K., TOLIA, N. H. & SADLER, J. E. 2019. Exploring the “minimal” structure of a functional ADAMTS13 by mutagenesis and small-angle X-ray scattering. *Blood,* 133**,** 1909-1918.

ZIPFEL, P. F., EDEY, M., HEINEN, S., JÓZSI, M., RICHTER, H., MISSELWITZ, J., HOPPE, B., ROUTLEDGE, D., STRAIN, L., HUGHES, A. E., GOODSHIP, J. A.,

LICHT, C., GOODSHIP, T. H. J. & SKERKA, C. 2007. Deletion of Complement Factor H–Related Genes CFHR1 and CFHR3 Is Associated with Atypical Hemolytic Uremic Syndrome. *PLoS Genetics,* 3**,** e41.

Chapter 11 - **Appendices**

11.1 Appendix A – Published pilot study paper

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ORIGINAL ARTICLE

The effects of streptokinase in a Chacma baboon (*Papio ursinus*) model of acquired thrombotic thrombocytopenic purpura

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Abstract

TTP is a life-threatening disorder with limited pharmaceutical treatment options. Recently, the potential of streptokinase in the treatment of acquired TTP was demonstrated in humans in vitro, and in vivo in a mouse model. We aimed to determine the in vitro and in vivo effects of streptokinase in an established *Papio ursinus* model of acquired TTP. In vitro: VWF activities & multimer patterns and thromboelastograms were assessed with increasing concentrations of streptokinase. In vivo: After induction of TTP, escalating streptokinase doses (ranging from 50,000 to 900,000 IU) were administered, and the effects of streptokinase assessed on peripheral blood counts, fibrinolysis, VWF activities & multimer patterns and thromboelastograms. In an extension of the study, high-dose streptokinase (1,500,000–3,000,000 IU) was administered to another baboon. After spiking, fibrinolysis with loss of large VWF multimers was observed at [2200 IU/mL]—roughly equivalent to 1,500,000 IU. However, administration of escalating intravenous streptokinase doses had no in vivo effect on the TTP phenotype, and in vivo increases in plasmin activity were mild when compared with baseline, even at high doses. Minimal effect on VWF multimer patterns was observed but only at doses ≥ 1500,000 IU. Streptokinase is not effective in resolving TTP in a *Papio ursinus* model of TTP, possibly due to limited activation of the baboon fibrinolytic system. Modifications to this model, the use of alternative higher animal models, or alternative thrombolytics, should be considered to establish proof-of-concept.

Keywords Thrombotic thrombocytopenic purpura · Fibrinolysis · Streptokinase · Baboon model

Introduction

Thrombotic thrombocytopenic purpura (TTP) is a severe thrombotic disease caused by a deficiency of the enzyme ADAMTS13 (a disintegrin and metalloproteinase with a thrombospondin type I motif, member 13) [1]; the plasma protease that cleaves large Von Willebrand factor (VWF) multimers into smaller, less adhesive forms [2]. As a consequence of this deficiency, ultra-large VWF (ULVWF) multimers accumulate, resulting in abnormal platelet aggregation, micro-thrombus formation and microvascular occlusion [3], with subsequent thrombocytopenia and an associated fragmentation haemolysis [2]. It is characterised by the classical diagnostic pentad of thrombocytopenia, microangiopathic haemolytic anaemia, neurological signs, renal impairment and fever [4], although it can present without the full pentad [2].

Congenital TTP, or Upshaw–Schulman syndrome, is a rare disorder caused by mutations of the ADAMTS13 gene on chromosome 9q34, which results in an inherited deficiency of the enzyme [1]. Acute acquired idiopathic TTP is the most common form of TTP and is characterised by autoantibodies (usually IgG), directed against ADAMTS13 [2]. It is considered an autoimmune disease [5], although the underlying process leading to its manifestation remains obscure.

Patients with congenital TTP are, however, only intermittently symptomatic, and patients with acquired ADAMTS13 deficiency can achieve remission of TTP without recovery

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of ADAMTS13 levels. These observations suggest the presence of additional factors affecting the pathophysiology of TTP [6], some of which may be amenable to therapeutic manipulation.

The role of plasmin as an ADAMTS13 backup mechanism for proteolytic cleavage of VWF multimers in the development of TTP was consequently investigated by Tersteeg et al. [7] and reported in a 2014 paper that also received editorial comment [6]. They showed that plasminogen binds to the VWF A1 domain in a lysine-dependent manner, to a binding site that remains cryptic when VWF is in a globular state. Moreover, plasminogen activation, mediated through a urokinase-type plasminogen activator receptor (uPAR)-dependent mechanism, was shown to result in rapid cleavage of platelet-VWF complexes on activated endothelial cells in the absence of ADAMTS13, also in a lysine-dependent manner [7]. Another recent study confirmed that globular VWF is resistant to plasmin cleavage under static conditions, but is readily cleaved by plasmin under shear stress [8], and that this occurs at the Lys_{1491}–Arg_{1492} peptide bond within the VWF A1–A2 linker region in a shear- and glycan-dependent manner, and not at the Tyr_{1605}–Met_{1606} ADAMTS13 proteolytic site in the A2 domain [8].

During acute TTP episodes, plasmin-α_2-antiplasmin (PAP) complexes, a marker of plasminogen activation, were found to be elevated in proportion to the degree of thrombocytopenia and, thus, the extent of the disease [7]. In addition, hypoxia has been shown to augment endothelium-mediated plasminogen activation through upregulation of uPAR [9, 10]. Tersteeg et al. [7] hypothesised that the ischaemia following microvascular thrombosis prompts plasminogen activation, with subsequent plasmin-mediated degradation of platelet-VWF complexes and concluded that plasmin, independent of other enzymes, aids in the local clearance of microvascular obstruction, as seen in TTP. In a murine model of acquired TTP, Tersteeg et al. [11] also subsequently showed that by amplifying endogenous plasmin activity through inhibition of PAI-1 in *α2-antiplasmin*$^{-/-}$ mice, acute TTP could be resolved.

In light of recent findings that plasmin is activated during microangiopathy and could substitute for ADAMTS13 to degrade platelet–VWF complexes, the induction of plasminogen activation by non-fibrin-selective thrombolytic agents was investigated in vitro and in a murine model in vivo [7]. Tersteeg et al. [12] explored the hypothesis that thrombolytic agents may be effective in TTP, by administering streptokinase and human plasminogen to *Adamts13*$^{-/-}$ mice, in which TTP had been induced via injection of high-dose recombinant human VWF. A single dose roughly equivalent to 20% of the loading dose used to treat pulmonary embolism and stroke in humans was found to attenuate the symptoms of TTP and to correct the thrombocytopenia [7]. Moreover, histochemistry indicated that the appearance of microvascular thrombi in the liver tissue of challenged mice was prevented by thrombolytic treatment [7], suggesting a possible role for drugs such as u-PA, streptokinase, or even tissue-type plasminogen activator (tPA), as adjunctive or alternative therapies for acquired TTP [6].

There is a large body of clinical experience with streptokinase in the treatment of myocardial infarction [13], but not in the treatment of TTP. Before human trials can be conducted, however, data from non-human primates would be preferred, due to the greater homology between non-human primates and man [14].

A Chacma baboon (*Papio ursinus*) model of acquired TTP has previously been developed and established locally [15]. Although this model has been used successfully to investigate various drugs with therapeutic potential in TTP [16–18], it has not been used yet to investigate the potential effects of thrombolytic drugs.

The aim of this pilot study was to establish proof-of-concept of the potential clinical utility of streptokinase in the treatment of acquired TTP, by determining the in vitro effects of streptokinase on the coagulation profile and fibrinolytic system of *Papio ursinus*, as well as the in vivo effects of low, intermediate and high-dose streptokinase treatment in a *Papio ursinus* model of acquired TTP.

Methodology

Ethics approval was granted by the Interfaculty Animal Ethics Committee of the University of the Free State for both the in vitro (UFS-AED2016/0083) and in vivo (UFS-AED2016/0056) studies. Protocols are evaluated against the South African National Standard for the care and management of laboratory animals (SANS10386:2008). Three animals, all healthy, purpose-bred, adult *Papio ursinus* males, were utilised for this study—one for phlebotomy for the in vitro experiments, and two for the in vivo experiments. None had been used for any previous study. Housing (with environmental enrichment in the form of a perch, interconnected cages and a foraging tray, as well as visual, auditory and olfactory contact with at least one social partner), routine care, phlebotomy, and streptokinase/3H9 injections were conducted at the Animal Research Facility of the University of the Free State (Bloemfontein, South Africa). All animals were anaesthetized by an intramuscular injection of 10 mg/kg ketamine hydrochloride, for all blood withdrawals and streptokinase/3H9 injections, which were performed by venepuncture of the femoral/great saphenous vein, with aseptic technique. All 3H9 injections were given at a dose of 600 µg/kg every 48 h. All streptokinase doses were administered as bolus injections at 12 hourly intervals, after reconstitution in 10 mL 0.9% saline. No animals died or were

euthanized after conclusion of the study, or had any signs of bleeding, apart from purpura related to thrombocytopenia.

In vitro study

For the in vitro study, we collected venous blood from the 13 kg baboon into 5 mL BD Vacutainer® tubes containing 3.2% sodium citrate (BD Biosciences, Franklin Lakes, New Jersey, USA). We determined the prothrombin time (PT), activated partial thromboplastin time (aPTT), thrombin time (TT), fibrinogen level, fibrinogen/fibrin degradation products, Platelet Function Analyser-100 (PFA-100) closure times, VWF activities & multimer patterns, plasmin activity and thromboelastograms (TEGs) at two incubation timepoints (30 min, and 3 h), after spiking the citrated baboon whole blood with increasing concentrations of streptokinase (Streptase®; Mirren pharmaceuticals, Houghton, South Africa). The final incubation concentrations (Low; 440 IU/mL, Intermediate; 700 IU/mL, High; 2200 IU/mL) correlated with the intravenous doses used in the in vivo studies outlined below.

In vivo studies

Low and intermediate dose study

We used the baboon model of acquired TTP as described by Feys et al. [15] to evaluate the in vivo effect of streptokinase on TTP. Briefly, after induction of TTP with the anti-ADAMTS13 mAb 3H9 (obtained from IRF Life Sciences Laboratory for Thrombosis Research, Kulak Kortrijk Campus of KU Leuven, Kortrijk, Belgium), escalating streptokinase doses (ranging from 50,000 to 900,000 IU) were administered intravenously to the 13 kg baboon (Fig. 1), and the effects of these low and intermediate doses were assessed on peripheral blood counts, schistocytes (red cell fragments), routine coagulation tests, PFA-100 closure times, ADAMTS13 antigen & activity, fibrinolysis (fibrinogen, fibrin(ogen) degradation products (FDPs), plasminogen, PAP complexes, and plasmin activity), VWF activities & multimer patterns and TEGs.

High-dose study

In a subsequent, refined high-dose extension of this study (amendment of UFS-AED2016/0056) a second male baboon of 14.2 kg was treated with high-dose streptokinase (1,500,000 IU × 3; 3,000,000 IU × 1) after induction of TTP with the anti-ADAMTS13 mAb 3H9, as outlined in Fig. 2.

Specimens were collected to confirm TTP (Full blood count and schistocyte percentage), assess fibrinolysis (Fibrinogen, FDPs, plasminogen, PAP complexes, and plasmin activity), and evaluate VWF antigen concentrations and multimer patterns.

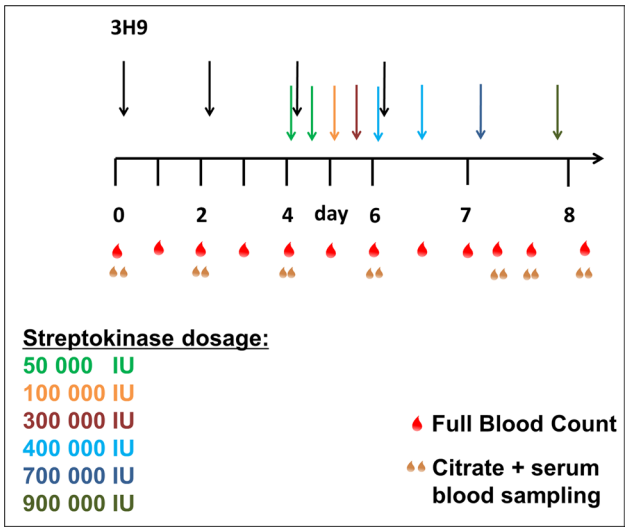


Fig. 1 Overall low and intermediate dose study outline, showing venous blood sampling, and injection of 3H9 and low and intermediate dose streptokinase, in a 13 kg baboon

Rationale for streptokinase doses and spiking concentrations

The starting dose (50,000 IU) in the low and intermediate dose in vivo study is approximately 20% of the dose used in humans *per kg* to treat acute myocardial infarction (MI) (1,500,000 IU) [19], where a 70 kg person would receive approximately 21,429 IU/kg. This was supported by recent in vivo murine data, where a dose representing approximately 20% of the loading dose generally administered to treat pulmonary embolism and stroke in humans was successfully used to reverse the TTP phenotype in a mouse model of acute TTP [7], while still keeping the risk for bleeding as low as possible. This was escalated to a dose of 300,000 IU the next day, which represents 20% of the *total* dose given in humans to treat acute MI. The 440 IU/mL concentration used in the in vitro spiking experiment is based on this dose, where 300,000 IU streptokinase would be distributed in a total blood volume of 700 mL in a 10 kg baboon for a concentration of ± 429 IU/mL, based on an assumed minimum blood volume of 70 mL/kg measured body weight [20].

The dose was further escalated to intermediate doses of 400,000 IU and 700,000 IU which spans the dose of 0.50 mg/kg (equivalent to 500,000 IU) found by Collen et al. to result in the shortest time to reflow in a *Papio hamadryas* arterial thrombosis model [20]. The concentration of 700 IU/mL used in the in vitro spiking experiment is based on a dose of 500,000 IU distributed in a total blood volume of

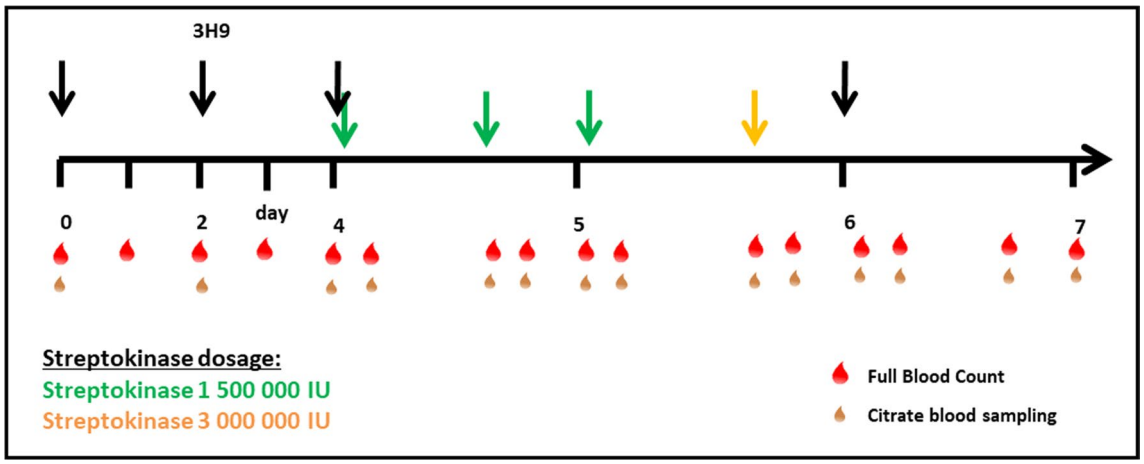


Fig. 2 Overall refined high-dose study outline, showing venous blood sampling, and injection of 3H9 and high-dose streptokinase, on a second male baboon of 14.2 kg

700 mL in a 10 kg baboon, for a concentration of ± 714 IU/mL. The in vivo dose was further escalated to 900,000 IU, representing 60% of the full total dose (1,500,000 IU) used in humans to treat acute myocardial infarction [19].

In the later, high-dose extension experiment, the full dose of 1,500,000 IU used in humans to treat acute MI was administered to a second baboon, followed by an ultra-high dose of double the usual total dose. The in vitro spiking experiment concentration of 2200 IU/mL is based on a dose of 1,500,000 IU distributed in a total blood volume of 700 mL, for a concentration of ± 2143 IU/mL.

Assays

Plasma used for VWF and coagulation testing, ELISAs and plasmin activity assays was obtained from whole blood centrifuged at 1500 × *g* for 15 min at room temperature. All coagulation assays were performed in an ISO15189 accredited laboratory, on a Sysmex CS-2100i® fully automated coagulation analyser (Sysmex, Kobe, Japan, supported locally by Siemens). All plasma samples not tested immediately were aliquoted and stored frozen at − 80 °C. No specimens were subjected to more than one freeze–thaw cycle.

Platelet function was assessed using the PFA-100® platelet function analyser (Siemens Healthcare GmbH, Erlangen, Germany), using Dade® PFA-100 Collagen/EPI as well as Dade® PFA-100 Collagen/ADP test cartridges.

Thromboelastography was performed on the TEG® 5000 Thromboelastograph® Haemostasis Analyser system (Haemonetics Corporation®, Boston, Massachusetts, USA) using kaolin and calcium chloride as activators. All traces were run for a minimum of 60 min.

Streptokinase-induced plasmin activity was assessed according to a chromogenic method obtained from the IRF Life Sciences Laboratory for Thrombosis Research, at the Kulak Kortrijk Campus of KU Leuven, Kortrijk, Belgium (C Tersteeg 2017, personal communication, 30 August). Briefly, a chromogenic plasmin substrate (TOS-Gly-Pro-Lys-4-NA) is added to 2 µL buffered test plasma. Streptokinase (10 µL of a 400 IU/mL solution) is added, and plasmin activity is measured kinetically for 2 h with an ELISA reader at 405 nm, set at > 37 °C. Readings were compared with a standard curve obtained from commercially available pooled buffered normal human plasma and reported as a percentage.

VWF antigen, VWF collagen binding activity (VWF:CB) and VWF ristocetin cofactor activity (VWF:RCo) were assessed according to methods previously described [21]. VWF multimer patterns were determined using the rapid visualization method described by Krizek and Rick [22]. Briefly, following submerged horizontal agarose gel electrophoresis, VWF is transferred onto a polyvinylidine fluoride membrane for immune-localisation and subsequent luminographic visualisation of the multimer pattern. The multimer density was then determined using a gel-documentation system.

Peripheral blood cell counts were performed on a Siemens ADVIA® 2120i automated haematology analyser (Siemens Healthcare GmbH, Erlangen, Germany) in an ISO15189 accredited laboratory, on specimens collected in BD Vacutainer® tubes containing ethylenediaminetetraacetic acid (EDTA) (BD Biosciences, Franklin Lakes, New Jersey, USA). Schistocytes were counted manually on peripheral blood smear morphology as previously described [23] and expressed as a percentage. Similar to humans [23], fragmentation haemolysis is deemed to be present in this model if the schistocyte percentage is above 1% [15].

Plasminogen levels were determined using a Technozym® Glu-Plasminogen ELISA kit according to the manufacturer's instructions (Technoclone GmbH, Vienna, Austria). Glu-plasminogen is the predominant native plasminogen

that is converted to active plasmin through two consecutive cleavage actions by certain plasma proteases [24]. Plasmin-mediated modification of Glu-plasminogen exposes lysine binding sites to form Lys-plasminogen that, with its superior fibrin binding and activation capacity, constitutes a positive feedback mechanism for enhanced conversion of plasminogen to plasmin [24]. By testing Glu-plasminogen instead of Lys-plasminogen or plasmin, the total potential pool available for plasmin formation is therefore considered.

PAP complex levels were measured using a Technozym® PAP complex ELISA kit according to the manufacturer's instructions (Technoclone GmbH, Vienna, Austria).

ADAMTS13 activity and antigen levels were determined according to the manufacturer's instructions (Technoclone GmbH, Vienna, Austria), using the Technozym® ADAMTS-13 Activity ELISA and the Technozym® ADAMTS-13 Antigen ELISA, respectively.

All ELISAs were measured using an ELISA reader with a 450 nm filter (Synergy HT reader, Biotek, USA), and concentrations read from a calibration reference curve. All ELISA data should be interpreted semi-quantitatively since all baboon plasma levels were determined against curves derived from human plasma.

FDPs were measured semi-quantitatively in plasma by latex agglutination, using a Stago FDP Plasma kit, performed according to the manufacturer's instructions (Stago, Asnières-sur-Seine, France).

Lactate dehydrogenase (LDH) and haptoglobin levels were determined in an ISO15189 accredited laboratory on a Roche Cobas 6000 C501 automated analyser, using a UV photometric and an immunoturbidimetric assay, respectively.

Results

In vitro study

The results of the in vitro study are summarised in Table 1 and Fig. 3. None of the streptokinase spiking concentrations or incubation time points had any effect on the PT, PTT, TT, fibrinogen level, PFA-100, VWF antigen, or VWF collagen binding activity (VWF:CB), compared with baseline. Interestingly, at 8%, the baseline VWF:CB activity was much lower than would be expected for normal human plasma (51%–143%) [25]. VWF ristocetin cofactor activity (VWF:RCo) values fell within or above the reference range for human plasma (50%–150%), at baseline (156%) and across all concentrations and time points (100%–164%, mean; 133%). Although still within the human reference range, VWF:RCo was reduced at the 3-h incubation time point compared with baseline, at both the low and high concentrations (109% and 100% activity, respectively, compared with 156% at baseline).

As indicated in Fig. 3a, there was moderate loss of large VWF multimers at high streptokinase concentrations. TEGs showed evidence of primary fibrinolysis at the intermediate and high streptokinase concentrations, but not at low concentrations (Fig. 3b) and a concentration-dependent escalation in plasmin activity could be appreciated (Fig. 3c).

Table 1 In vitro spiking experiment

Incubation time	Baseline (0 min; No added SK)	30 min	180 min	30 min	180 min	30 min	180 min
SK concentration [IU/mL]	N/A	440		700		2200	
PFA-100 Col/Epi (sec)	90	87	86	96	89	87	86
PFA-100 Col/ADP (sec)	59	54	65	63	70	61	56
VWF antigen (%)	70	65	63	60	65	58	59
VWF CBA (%)	8	6	7	7	6	5	5
VWF RICO (%)	156	150	109	136	139	164	100
Fibrinogen (g/L)	1.52	1.49	1.47	1.49	1.51	1.48	1.62
FDPs (µg/mL)	Negative	Negative	Negative	Negative	Negative	Negative	Negative
TT (sec)	19.4	19.3	19.1	19.3	19.3	19.7	18.8
PT (sec)	11.4	11.5	12.1	11.7	12.2	11.6	12.2
PTT (sec)	33.7	33.6	35.1	34.3	35.4	34.3	34.9

The effects of escalating streptokinase concentrations on various haemostatic parameters in *Papio ursinus*, at different incubation time-points

SK streptokinase, *PFA* platelet function analyser, *Col/Epi* collagen/epinephrine, *Col/ADP* collagen/adenosine diphosphate, *VWF* Von Willebrand factor, *CBA* collagen binding activity, *RICO* ristocetin cofactor activity, *FDPs* fibrin(ogen) degradation products, *TT* thrombin time, *PT*; prothrombin time, *PTT* partial thromboplastin time, *N/A* not applicable, *sec* seconds

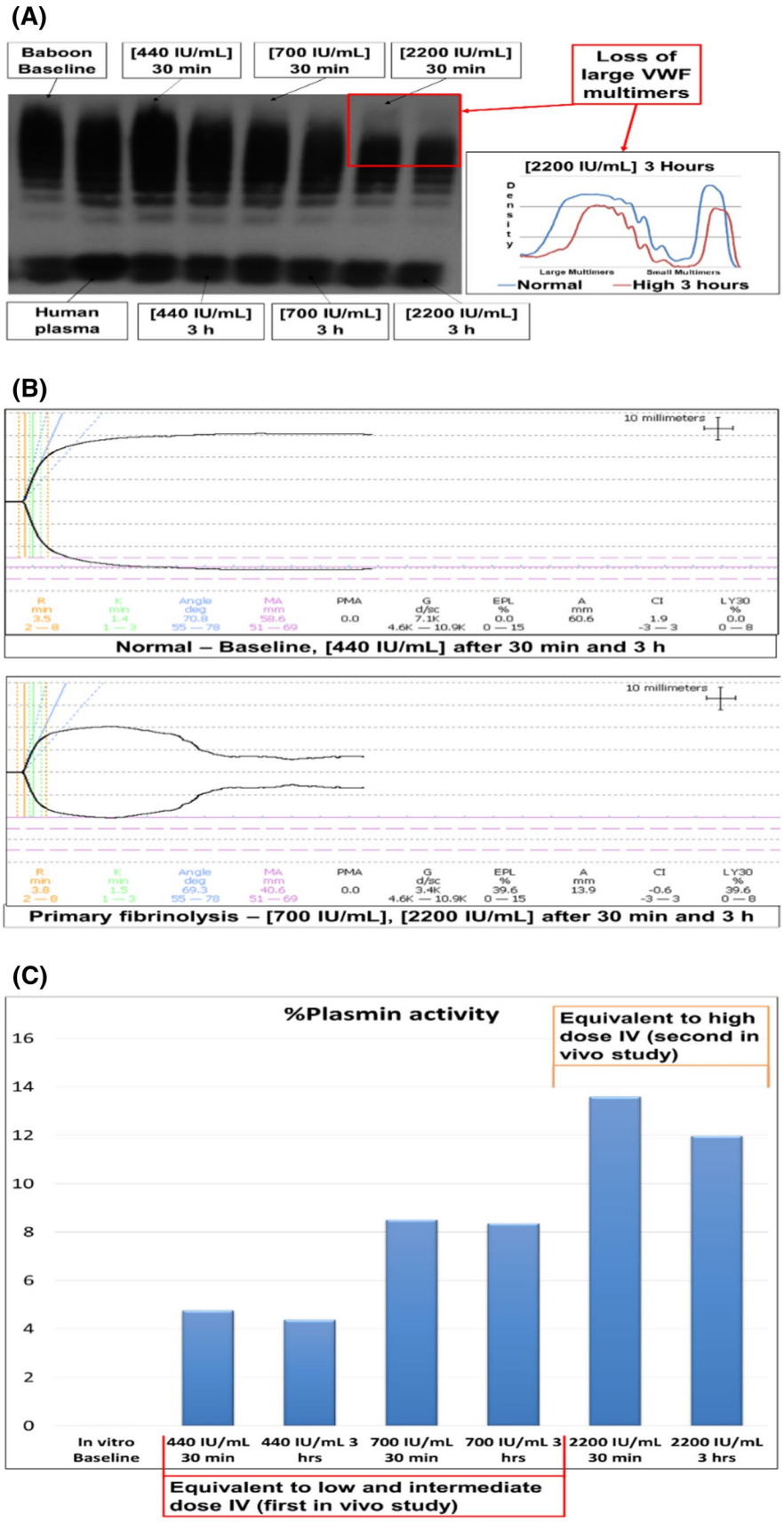


Fig. 3 In vitro spiking experiment. Von Willebrand (VWF) multimer patterns **(A)**, thromboelastograms (**B**; generally representative of the various traces obtained at the stated concentrations and time points), and plasmin activity (**C**; compared with normal human plasmin activity) obtained after incubating citrated *Papio ursinus* whole blood (spiked with increasing concentrations of streptokinase) for 30 min and 3 h

In vivo studies

Low and intermediate dose study

The results of the low and intermediate dose study are summarised in Table 2. Consistent with previous reports in this model [15, 17], thrombocytopenia and schistocytes were present on day 3. Although there was no increase in bleeding, in vivo administration of escalating intravenous streptokinase doses also had no effect on the TTP phenotype (mild mucocutaneous bleeding), platelet counts, haemoglobin concentration, schistocyte percentage, D-dimer levels, standard coagulation times, fibrinogen levels, or VWF antigen levels, activities and multimer patterns. TEGs and PFAs essentially only showed features of thrombocytopenia. Since no clinical effect or effect on platelet count was apparent, streptokinase treatment was gradually increased to a maximum of 900,000 IU and testing frequency intensified on

Table 2 The in vivo effects of low and intermediate dose streptokinase in the *Papio ursinus* model of acute acquired TTP

Test	D0 (Baseline)	D1	D2	D3	D4 am Pre SK	D5 am Pre SK	D6 am Pre SK	D6 pm Pre SK	D7 am Pre SK	D7 am 30 minutes post SK	D7 am 90 minutes post SK	D8
SK dose am	**N/A**	**N/A**	**N/A**	**N/A**	**50,000**	**100,000**	**400,000**			**700,000**		**N/A**
SK dose pm	**N/A**	**N/A**	**N/A**	**N/A**	**50,000**	**300,000**		**400,000**			**900,000**	**N/A**
Haemoglobin (g/dL)	14.2	15.1	13.6	14	12.4	9.9	9.8	9	8.3	7.6	7	6
Platelets (x 10^9/L)	257	196	86	8	8	9	15	14	13	16	17	15
Fragments (%)	0	0	0	0.9	1.9	4.7	5.6	3.8	8.4	10.2	12.8	11
LDH (U/L)	272		348		734		1295					1369
Haptoglobin (g/L)	0.46		0.13		<0.10		<0.10					<0.10
PFA-100 Col/Epi (sec)	157		108		>238		>228					>171
PFA-100 Col/ADP (sec)	74		94		>283		>227					>264
TEG	Normal		Mildly ↓MA, mildly ↑K time.		Markedly ↓ MA, moderately ↓ angle		Markedly ↓ MA, mildly ↑K time.					Markedly ↓ MA, mildly ↑K time.
ADAMTS13 Ag (%)	8		7		5		5			3	2	2
ADAMTS13 activity (%)	33		1		1		19			13	0	0
Plasminogen (µg/mL)	180		48		1623		1716			1119	757	268
Plasmin activity (%)	0		0		0		0			0	0	0
PAP complexes (ng/mL)	112		88		168		286			1431	1017	297
VWF antigen (%)	30		29		25		32			38	34	43
VWF multimer patterns (note no loss of large VWF multimers) Large Intermediate Small												
VWF CBA (%)	7		14		10		13			8	7	13
VWF RICO (%)	73		82		59		70			84	56	80
Fibrinogen (g/L)	1.12		1.34		1.34		1.7					1.95
FDPs (µg/mL)	Neg		Neg		Neg		Neg			≥ 5 < 10	≥ 10 < 20	≥ 10 < 20
D-Dimers (mg/L)	<0.10		<0.10		<0.10		<0.10					<0.10
TT (sec)	20.3		19		18		18					17
PT (sec)	10.9		11.3		11.2		12.9					12.1
PTT (sec)	35.7		33.2		38		37.7					33.7

Since no clinical effect or effect on platelet count was observed, streptokinase treatment was gradually increased to a maximum of 900,000 IU and testing frequency intensified on day 7, in order to assess any possible transient effects at the intermediate dosages

SK streptokinase, *LDH* lactate dehydrogenase, *PFA* platelet function analyser, *TEG* thromboelastogram, *PAP* plasmin-antiplasmin, *VWF* Von Willebrand factor, *CBA* collagen binding activity, *RICO* ristocetin cofactor activity, *FDPs* fibrin(ogen) degradation products, *TT* thrombin time, *PT* prothrombin time, *PTT* partial thromboplastin time, *MA* maximum amplitude, *N/A* not applicable, *am* morning, *pm* evening

day 7, in order to assess any possible transient effects at the intermediate dosages. Plasminogen levels first decreased approximately fourfold from baseline at day 2, then followed an upward trend, increasing approximately tenfold by day 6, as TTP was progressively established. PAP-complex levels showed a very similar pattern up to day 6. This suggests that PAP-complexes may be formed by endogenous plasminogen activation as a function of rising plasminogen levels (possibly secondary to TTP), rather than significant streptokinase-induced plasminogen activation with associated plasminogen consumption and PAP-complex formation, at these doses. However, on day 7, a significant decrease in plasminogen levels (suggesting possible consumption) with a concomitant increase in PAP-complex levels and FDPs can be appreciated compared with day 6, suggesting possible activation of fibrinolysis at doses above 700,000 IU. Although plasminogen levels continued to decrease on day 8 suggesting possible consumption, this effect appears transient, as PAP-complex levels reverted back to day 6 levels, on day 8. Ultimately, actual plasmin activity was undetectable (0%) throughout the experiment, confirming a lack of streptokinase-induced plasminogen activation in vivo, at these dosages.

High-dose study

With the high-dose extension study, TTP was again predictably induced as shown in Table 3, with a platelet count nadir of 18×10^9/L, reached on day 3. Schistocytes also appeared on day 3, reaching a peak of 30.9% on day 6 (Table 3). Although there was no increase in bleeding observed, high-dose streptokinase had no effect on the TTP phenotype (mild mucocutaneous bleeding), platelet counts, haemoglobin concentration, or schistocyte percentage (Table 3). High-dose streptokinase appeared to have only a minimal effect on VWF, resulting in only mild decreases from baseline, in the number of high molecular weight VWF multimers present, as well as VWF antigen levels (Table 3). Fibrinogen levels dropped from a baseline concentration of 5.3 g/L to an average of 3.8 g/L during the streptokinase portion of the study, suggesting possible fibrinogenolysis. This was, however, not confirmed by FDP levels, which only rose to a maximum concentration of 40 µg/mL during streptokinase treatment (average of 18 µg/mL during treatment), from a baseline level of 5 µg/mL. The high-dose study included more focused pre- and post-dose sampling, in order to assess any transient effects of streptokinase. As shown in Table 3, streptokinase treatment generally resulted in lower plasminogen levels after dosing, possibly suggesting consumption during activation of fibrinolysis. This effect was transient, however, with a generally upward trend in plasminogen levels since the onset of TTP. In contrast to findings in the low and intermediate dose study, PAP-complex levels remained virtually unchanged from baseline (or decreased slightly) after streptokinase administration, indicating minimal free plasmin activity. Concordant with observations in the in vitro spiking experiment (Fig. 3), high-dose streptokinase was able to induce plasmin activity only up to a maximum level of 12%. As shown in Table 3, repeated dosing, as well as ultra-high-dose streptokinase (3,000,000 IU), were not able to induce meaningful plasmin activity.

Discussion

Our results indicate that streptokinase is not effective in resolving TTP in a *Papio ursinus* model of acquired TTP, possibly due to limited activation of the baboon fibrinolytic system by streptokinase. Although our in vitro data would indicate (at least some) plasminogen activation by streptokinase, with a concomitant effect on VWF multimer composition, this was not convincingly demonstrated in the in vivo experiments, echoing earlier findings that in vitro observations may not be representative of in vivo fibrinolytic potential [26], and reinforcing the importance of in vivo studies to evaluate any novel therapy.

However, this lack of effective streptokinase-induced plasminogen activation in vivo indicates significant differences between the human and *Papio ursinus* haemostatic systems, rather than proof-of-concept that thrombolytic drugs are ineffective in the treatment of acute TTP. As streptokinase is a potent activator of human plasminogen [27], the relatively low plasmin activity seen in our experiments may indicate intrinsic fibrinolytic system differences between *Papio ursinus* and man. Although there is a dearth of streptokinase (and indeed thrombolytic) experience in *Papio ursinus* in vivo, other baboon models (most notably *Papio hamadryas*) have been used to investigate the effects of many of the thrombolytic drugs (including streptokinase), often with conflicting results. As far back as 1966, high-dose urokinase was found to be able to activate fibrinolysis in an unnamed baboon species to an extent comparable with humans, whereas streptokinase was not [28]. In a later study in a *Papio hamadryas* arterial and venous thrombosis model [26], however, streptokinase was found to lead to progressive dose-dependent clot lysis in baboons with jugular vein thrombosis, indicating meaningful in vivo fibrinolytic system activation [26]. It is conceivable that there may not only be fibrinolytic system differences between *Papio ursinus* and man, but also differences across the various *Papio spp*.—a potential research opportunity.

There was also variation between the fibrinolytic system data obtained from our two in vivo studies. Baseline PAP-complex levels differed from 112 ng/mL (and 88 ng/mL on day 2) in the low and intermediate dose experiment to 390 ng/mL in the high-dose experiment, although

the normal human reference range for this assay is fairly wide (0–514 ng/mL) [29]. Baseline plasminogen levels also varied dramatically between the low and intermediate dose test animal (180 µg/mL) and the high-dose test animal (2562 µg/mL). The normal human reference range for this assay is 60–250 µg/mL [30]. The high-dose test animal, therefore, had a level far in excess of what is deemed normal in humans. Since there are no known reference ranges for these parameters in *Papio ursinus*, researchers' ability to correctly interpret data from this present study and other studies (including future, even unrelated, studies) is limited. In addition, the high-dose test animal had a baseline platelet count almost double that of the intermediate dose test animal (546 vs 257×10^9/L) indicating possibly significant, large inter-individual variation in this species, which makes direct comparison between the two animals difficult.

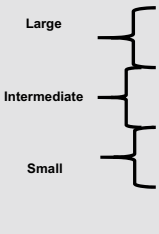
Although the in vitro experiment did confirm loss of high molecular weight VWF multimers at high concentrations streptokinase, this was not confirmed convincingly in the in vivo studies, where even high doses resulted in only minimally reduced VWF antigen levels and high molecular weight multimers (Table 3). However, although not a universal finding in this model [15], the induction of TTP with 3H9 generally results in the rapid appearance of ultra-large VWF multimers [16], which was not seen in either of the baboons tested in our study, suggesting at least a minimal streptokinase effect on the multimer composition. This is, however, still not consistent with previous data reported on the effects of streptokinase-activated plasmin on human VWF [7] and suggests possible resistance of *Papio ursinus* VWF to plasmin degradation, or (more likely) resistance of *Papio ursinus* plasminogen to activation by streptokinase, in vivo. Human globular VWF is resistant to plasmin cleavage under static conditions, but is readily cleaved by plasmin under shear [8] at the Lys_{1491}–Arg_{1492} peptide bond within the VWF A1–A2 linker region, and not at the Tyr_{1605}-Met_{1606} ADAMTS13 proteolytic site in the A2 domain [8]. The Lys_{1491}–Arg_{1492} bond is present and intact in the *Papio ursinus* VWF protein [31] and should therefore similarly be cleaved by plasmin at this site, suggesting intrinsic fibrinolytic system differences between the species (i.e. resistance of *Papio ursinus* to activation by streptokinase) rather than significant differences in the VWF molecule, as a possible explanation for our findings. There is, however, a marked discrepancy between the normal range for human VWF: CB activity (51%–143%) [25] and that observed at baseline in the in vitro (8%) and low and intermediate dose study (7%; Table 2), which does signal possible differences in VWF physiology as well. In humans, the administration of streptokinase has been found to lead to a two- to threefold rise in VWF antigen and platelet-agglutinating activity within 2 h, with the greatest increase seen among the largest multimers [32]. This is an important consideration for the potential use of streptokinase to treat TTP in humans, as an excess of large multimers is central to the pathogenesis of TTP.

In the low and intermediate dose in vivo study, the baseline ADAMTS13 antigen and activity levels were 8% and 33%, respectively; well below the human reference ranges of 70%–160% and 40%–130% for these assays [33]. As this is possibly a reflection of the species incompatibility of the specific kits used, these assays were omitted in the subsequent, refined, high-dose study, especially since thrombocytopenia and fragmentation were convincingly and predictably induced in both animals, and since acquired ADAMTS13 deficiency is a reliable feature of this model [15]. However, the low baseline ADAMTS13 levels found here may also suggest important interspecies differences regarding the reference range for these assays in *Papio ursinus* that may be particularly relevant to TTP, since the ADAMTS13 activity results obtained at baseline in the present study are not consistent with data reported in another *Papio ursinus* study, where near-human values were obtained at baseline, using the same assay kit [17]. Regardless, it is unlikely that clinically meaningful ADAMTS13 activity was still present in the circulation after day 3 in either animal, in the face of the overwhelming thrombocytopenia and fragmentation haemolysis.

Environmental, genetic and physiologic differences between mice, baboons, and humans can hamper direct comparison and extrapolation of data obtained in different species [14]. Recently, direct comparisons between *Papio ursinus* and human platelet receptors found *Papio ursinus* to be a suitable animal model for the evaluation of human-targeted anti-platelet agents directed against the P2Y12, GPIIb/IIIa and GPIbα receptors [34], but not for the evaluation of human-targeted anti-GPVI agents [35]. Aggregation responses to different platelet agonists are generally lower in *Papio ursinus* than in humans [36], and although thromboelastometric assays indicate broad similarity between humans and baboons, blood clots formed by baboons are more resistant to fibrinolysis than human clots [37], suggesting fundamental differences that require further investigation.

The present study has highlighted fundamental gaps in our knowledge of *Papio ursinus* haemostasis and reiterated the need for a deeper characterisation of this model. Moreover, it has signalled the presence of possible fundamental differences between the human and *Papio ursinus* haemostatic systems, which also have implications for the broader applicability, translational value and limitations of this model.

Table 3 The in vivo effects of high-dose streptokinase in the *Papio ursinus* model of acute acquired TTP

Test	D0	D1	D2	D3	D4 am Pre SK	D4 am 30 minutes Post SK	D4 pm Pre SK	D4 pm 30 minutes Post SK	D5 am Pre SK	D5 am 30 minutes post SK	D5 pm Pre SK	D5 pm 30 minutes post SK	D6 am (Completion)	D6 pm (Completion)
Streptokinase dose (IU)	N/A	N/A	N/A	N/A	1,500,000		1,500,000		1,500,000		3,000,000		N/A	N/A
Haemoglobin (g/dL)	12.8	12.5	12.6	12.7	11.8	11.4	9.6	9.5	8.3	8.6	8	7.7	5.6	5.4
Platelets (x 10^9/L)	546	512	272	18	23	23	21	21	26	33	28	38	45	49
Schistocytes (%)	0	0	0.4	1.9	5.9	6.3	11.4	10.1	10.2	10	19.3	18.5	23.8	30.9
Plasminogen (ng/mL)	2526		2575		1964	2599	2085	1071	3068	1673	3616	2747	1770	2159
PAP complexes (ng/mL)	390		396		325	398	339	221	453	291	516	416	302	347
Plasmin activity (%)	1		0		3	11	3	12	0	0	1	0	8	8
VWF multimer patterns (note only minimal loss of large VWF multimers) 														
VWF Ag (%)	76		72		75	77	59	62	58	60	59	63	65	64
Fibrinogen (g/L)	5.3		4.7		4.5	4.3	4	4.2	3.7	3.8	3.8	3.8	3.3	3.6
FDPs (μg/mL)	5		5		5	10	40	20	20	10	10	10	20	20

SK streptokinase, *PAP* plasmin-antiplasmin, *VWF* Von Willebrand factor, *FDPs* fibrin(ogen) degradation products, *N/A* not applicable, *am* morning, *pm* evening

Conclusion

Streptokinase is not effective in resolving TTP in a *Papio ursinus* model of acquired TTP, possibly due to limited activation of the baboon fibrinolytic system by streptokinase. This suggests fundamental differences between the *Papio ursinus* and human fibrinolytic systems, which require further investigation. The use of alternative higher animal models of TTP, or alternative thrombolytic drugs in this model, should possibly be considered to establish proof-of-concept, before streptokinase can be tested in human in vivo trials, or discarded as a therapeutic option. Modifications to the present model (such as the concomitant infusion of human plasminogen) can also be considered. A large case–control study involving multiple baboons to investigate the clinical utility of streptokinase in the treatment of acute TTP does not appear feasible or ethical in this model in its current configuration.

Limitations

Many haemostatic aspects of the *Papio ursinus* model (including aspects directly relating to TTP) remain to be elucidated. There are no or very limited published data on normal reference ranges in these animals for the levels and activities of the key role-players in TTP and fibrinolysis, and how these differ from man.

In the spirit of the three Rs of humane animal research (Replacement, Reduction and Refinement), and as this was essentially a pilot study performed in a well-validated model to determine the feasibility of a large case–control study, no control animal was included for either of the two in vivo studies, as this would result in unwarranted animal wastage.

The presence of anti-streptokinase antibodies, which may have influenced the response to streptokinase, was not tested for due to cost constraints. Anti-streptokinase antibodies were deemed unlikely to be present in significant

quantities, however, since none of the animals had been previously exposed to streptokinase, and a previous *Papio hamadryas* study confirmed partial resistance to clot lysis to only occur within a week after streptokinase administration, and high antibody titres to only emerge after several weeks of repeated streptokinase treatment [26].

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Authors' contribution JJ, SMM, and WJVR designed research, performed experiments, collected, analysed, and interpreted data, and co-wrote the manuscript; CC performed experiments, collected and analysed data, and reviewed the manuscript for technical content; SL designed research and performed experiments.

Declarations

Conflict of interest The authors declare no competing financial interests.

References

1. Kremer Hovinga JA, Lämmle B. Role of ADAMTS13 in the pathogenesis, diagnosis, and treatment of thrombotic thrombocytopenic purpura. Hematology Am Soc Hematol Educ Program. 2012;2012:610–6.
2. Scully M, Hunt BJ, Benjamin S, Liesner R, Rose P, Peyvandi F, et al. Guidelines on the diagnosis and management of thrombotic thrombocytopenic purpura and other thrombotic microangiopathies. Br J Haematol. 2012;158:323–35.
3. Moake JL. Thrombotic microangiopathies. N Engl J Med. 2002;347(8):589–600.
4. Amorosi EL, Ultmann JE. Thrombotic thrombocytopenic purpura: report of 16 cases and review of the literature. Medicine. 1966;34(2):139–60.
5. Malak S, Wolf M, Millot GA, Mariotte E, Veyradier A, Meynard JL, et al. Human immunodeficiency virus-associated thrombotic microangiopathies: clinical characteristics and outcome according to ADAMTS13 activity. Scand J Immunol. 2008;68(3):337–44.
6. Chauhan AK. Degradation of platelet-von Willebrand factor complexes by plasmin: an alternative/backup mechanism to ADAMTS13. Circulation. 2014;129(12):1273–5.
7. Tersteeg C, de Maat S, De Meyer SF, Smeets MW, Barendrecht AD, Roest M, et al. Plasmin cleavage of von Willebrand factor as an emergency bypass for ADAMTS13 deficiency in thrombotic microangiopathy. Circulation. 2014;129(12):1320–31.
8. Brophy TM, Ward SE, McGimsey TR, Schneppenheim S, Drakeford C, O'Sullivan JM, et al. Plasmin cleaves Von Willebrand Factor at K1491–R1492 in the A1–A2 Linker region in a shear- and glycan-dependent manner in vitro. Arterioscler Thromb Vasc Biol. 2017;37(5):845–55.
9. Kroon ME, Koolwijk P, van der Vecht B, van Hinsbergh VW. Urokinase receptor expression on human microvascular endothelial cells is increased by hypoxia: implications for capillary-like tube formation in a fibrin matrix. Blood. 2000;96(8):2775–83.
10. Graham CH, Fitzpatrick TE, McCrae KR. Hypoxia stimulates urokinase receptor expression through a heme protein-dependent pathway. Blood. 1998;91(9):3300–7.
11. Tersteeg C, Joly BS, Gils A, Lijnen R, Deckmyn H, Declerck PJ, et al. Amplified endogenous plasmin activity resolves acute thrombotic thrombocytopenic purpura in mice. J Thromb Haemost. 2017;15(12):2432–42.
12. Tersteeg C, Fijnheer R, Pasterkamp G, de Groot PG, Vanhoorelbeke K, de Maat S, et al. Keeping von Willebrand factor under control: alternatives for ADAMTS13. Semin Thromb Hemost. 2016;42(1):9–17.
13. Bryan J. The rise and fall of the clot buster. Pharm J. 2014;293(7819):1–8.
14. Vanhoorelbeke K, De Meyer SF. Animal models for thrombotic thrombocytopenic purpura. J Thromb Haemost. 2013;11(Suppl. 1):2–10.
15. Feys HB, Roodt J, Vandeputte N, Pareyn I, Lamprecht S, Van Rensburg WJ, et al. Thrombotic thrombocytopenic purpura directly linked with ADAMTS13 inhibition in the baboon (*Papio ursinus*). Blood. 2010;116(12):2005–10.
16. Feys HB, Roodt J, Vandeputte N, Pareyn I, Mottl H, Hou S, et al. Inhibition of von Willebrand factor-platelet glycoprotein Ib interaction prevents and reverses symptoms of acute acquired thrombotic thrombocytopenic purpura in baboons. Blood. 2012;120(17):3611–4.
17. Callewaert F, Roodt J, Ulrichts H, Stohr T, Janse van Rensburg W, Lamprecht S, et al. Evaluation of efficacy and safety of the anti-VWF Nanobody ALX-0681 in a preclinical baboon model of acquired thrombotic thrombocytopenic purpura. Blood. 2012;120(17):3603–10.
18. Tersteeg C, Roodt J, Van Rensburg WJ, Dekimpe C, Vandeputte N, Pareyn I, et al. N-acetylcysteine in preclinical mouse and baboon models of thrombotic thrombocytopenic purpura. Blood. 2017;129(8):1030–8.
19. O'Gara PT, Kushner FG, Ascheim DD, Casey DE Jr, Chung MK, de Lemos JA, et al. 2013 ACCF/AHA guideline for the management of ST-elevation myocardial infarction: a report of the American College of Cardiology Foundation/American Heart Association Task Force on Practice Guidelines. Circulation. 2013;127(4):e362-425.
20. Fortman JD, Hewett TA, Bennett BT. Important biological features The Laboratory Nonhuman Primate. 1st ed. Boca Raton: CRC Press; 2002. p. 1–34.
21. Meiring M, Badenhorst PN, Kelderman M. Laboratory diagnosis of von Willebrand Disease. Eur Oncol Haematol. 2009;3(1):33–6.
22. Krizek DR, Rick ME. A rapid method to visualize von willebrand factor multimers by using agarose gel electrophoresis, immunolocalization and luminographic detection. Thromb Res. 2000;97(6):457–62.
23. Zini G, d'Onofrio G, Briggs C, Erber W, Jou JM, Lee SH, et al. ICSH recommendations for identification, diagnostic value, and quantitation of schistocytes. Int J Lab Hematol. 2012;34(2):107–16.
24. Israels LG, Israels ED. Fibrinogen, factor XIII and fibrinolysis. In: Mechanisms in Hematology. 3rd Edition. Ontario, Canada: Core Health Services Inc; 2002.
25. Meiring M, Badenhorst PN, Kelderman M. Performance and utility of a cost-effective collagen-binding assay for the laboratory diagnosis of Von Willebrand disease. Clin Chem Lab Med. 2007;45(8):1068–72.
26. Collen D, De Cock F, Stassen JM. Comparative immunogenicity and thrombolytic properties toward arterial and venous thrombi

of streptokinase and recombinant staphylokinase in baboons. Circulation. 1993;87(3):996–1006.
27. Lijnen HR, De Cock F, Matsuo O, Collen D. Comparative fibrinolytic and fibrinogenolytic properties of staphylokinase and streptokinase in plasma of different species in vitro. Fibrinolysis. 1992;6(1):33–7.
28. Hampton JW, Matthews C. Similarities between baboon and human blood clotting. J Appl Physiol. 1966;21:1713–6.
29. Technoclone. Technozym® PAP Complex ELISA Kit Package Insert. Technoclone GmbH, Vienna, Austria. 2012.
30. Technoclone. Technozym® Glu-Plasminogen ELISA Package Insert. Technoclone GmbH, Vienna, Austria. 2014.
31. Janse van Rensburg WJ. Molecular suitability of the chacma baboon in human-targeted Von Willebrand factor directed studies. J Med Primatol. 2019;48(3):171–5.
32. Hamilton KK, Fretto LJ, Grierson DS, McKee PA. Effects of plasmin on von Willebrand factor multimers. Degradation in vitro and stimulation of release in vivo. J Clin Invest. 1985;76(1):261–70.
33. Peyvandi F, Palla R, Lotta LA, Mackie I, Scully MA, Machin SJ. ADAMTS-13 assays in thrombotic thrombocytopenic purpura. J Thromb Haemost. 2010;8(4):631–40.
34. Janse van Rensburg WJ. Comparison of common platelet receptors between the chacma baboon (Papio ursinus) and human for use in pre-clinical human-targeted anti-platelet studies. Platelets. 2016;27(4):322–32.
35. Janse van Rensburg WJ, Badenhorst PN, Roodt JP. The Cape Chacma baboon is not suitable for evaluating human targeted anti-GPVI agents. Platelets. 2015;26(6):552–7.
36. Ponschab M, van Griensven M, Heitmeier S, Laux V, Schlimp CJ, Calatzis A, et al. Platelet function in baboons and humans—a comparative study of whole blood using impedance platelet aggregometry (Multiplate®). Thromb Res. 2016;147:115–21.
37. Schochl H, Solomon C, Laux V, Heitmeier S, Bahrami S, Redl H. Similarities in thromboelastometric (ROTEM(R)) findings between humans and baboons. Thromb Res. 2012;130(3):e107–12.

11.1 Appendix B – Ethics approval letter

UNIVERSITY OF THE FREE STATE
UNIVERSITEIT VAN DIE VRYSTAAT
YUNIVESITHI YA FREISTATA
UFS UV

Animal Research Ethics

18-Apr-2019

Dear Dr Jaco Joubert

Student Project Number: UFS-AED2019/0054

Project Title: CHARACTERISATION OF THE FIBRINOLYTIC SYSTEM AND THE VON WILLEBRAND FACTOR-ADAMTS13 AXIS IN THE CHACMA BABOON.

Department: Haematology and Cell Biology Department (Bloemfontein Campus)

You are hereby kindly informed that, at the meeting held on 18-Apr-2019, the Interfaculty Animal Ethics Committee approved the above project.

Kindly take note of the following:

1.
A progress report with regard to the above study has to be submitted Annually and on completion of the project. Reports are submitted by logging in to RIMS and completing the report as described in SOP AEC007: Submission of Protocols, Modifications, Amendments, Reports and Reporting of Adverse Events which is available on the UFS intranet.

2.
Researchers that plan to make use of the Animal Experimentation Unit must ensure to request and receive a quotation from the Head, Mr. Seb Lamprecht.

3.
Fifty (50%) of the quoted amount is payable when you receive the letter of approval.

Yours Sincerely

Mr. Gerhard Johannes van Zyl
Chair: Animal Research Ethics Committee