The Role of Thrombin Activatable Fibrinolysis Inhibitor in Abdominal Aortic Aneurysms

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Intellectual Property and Publication Statements

The candidate confirms that the work submitted is her own, except where work which has formed part of jointly authored publications has been included. The contribution of the candidate and the other authors to this work has been explicitly indicated below. The candidate confirms that appropriate credit has been given within the thesis where reference has been made to the work of others.

Publications which have been used as part of this thesis are:

- ***Bridge KI**, Philippou H, Ariëns RAS. Clot properties and cardiovascular disease (Review). Thrombosis and Haemostasis 2014 Nov 3;112(5):901-8.* An updated diagram from this paper, as well as material covered in the review, appears in Chapter 1 (Introduction) on pages 17-24. I co-authored this review with Helen Philippou and Robert Ariëns. I wrote the review, created the diagrams and submitted the manuscript for publication. Robert Ariëns was involved in the planning of the review and the revision of the manuscript. Helen Philippou was involved in the revision of the manuscript.

- ***Bridge KI**, Macrae FL, Bailey MA, Johnson AB, Philippou H, Scott DJA, Ariëns RAS. The Arg407Lys polymorphism of Alpha-2-Anti-plasmin is negatively associated with Abdominal Aortic Aneurysm. Journal of Thrombosis Research, 2014 Sep;134(3):723-8.* Figure 5 (Page 25) is based upon a figure from the manuscript. Methods from this paper appear in the methods section on human aneurysm disease (Chapter 2, Experimental Design, Materials and Methods, pages 48, 66-67). Results from this paper

appear in the results and discussion of Chapter 3, Results – The role of TAFI in human AAA, pages 115-123 and 139-141. For this section of work, I extracted the DNA from blood samples along with Fraser Macrae. After instruction by Fraser Macrae, I did all of the genotyping for this study. I performed the data analysis, prepared the manuscript and submitted the manuscript for publication. Anne Johnson recruited the patients. Marc Bailey, Helen Philippou and Julian Scott reviewed the manuscript. Robert Ariëns helped with planning the work for the paper, the data analysis and interpretation, and reviewed the manuscript.

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Finally, I would like to offer my sincerest thanks to the British Heart Foundation for funding my project through a Clinical Research Fellowship Award.

Abstract

Thrombin-activatable fibrinolysis inhibitor (TAFI) prevents the breakdown of fibrin clots through its action as an indirect inhibitor of plasmin. Patients with abdominal aortic aneurysms (AAA), in common with a number of cardiovascular diseases, display an altered fibrin clot structure, with denser clots that have smaller pores and an increased resistance to lysis. Murine studies have implicated a potential role for TAFI in AAA disease. This study aimed to investigate the role of TAFI in human AAA, and to complement this with an investigation into the effect of TAFI inhibition on AAA development and progression *in-vivo.*

By measuring the plasma levels of TAFI zymogen, activated and inactivated TAFI, and the TAFI activation peptide in plasma samples, and using clot lysis assays to determine *in-vitro* TAFI activity, this study indicated increased turnover of TAFI in patients with AAA. Further to this, it was determined that the delay in lysis previously demonstrated in patients with AAA could be corrected through the inhibition of TAFI.

Inhibition of TAFI *in-vivo* resulted in a decrease in mortality in the Angiotensin II model of AAA. The site of action of the inhibitor determined the effect on AAA formation, however, with inhibition of plasmin-mediated TAFI activation resulting in a decrease in AAA formation rates, whilst inhibition of all active TAFI resulted in an increase in AAA formation rates. Delayed TAFI inhibition following the formation of AAA had no effect on AAA progression.

This study demonstrated evidence of increased TAFI turnover in patients with AAA, whilst *in-vivo* studies indicate a role for TAFI early in the development of AAA disease.

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Abbreviations

-/-	Homozygote knock-out
-/+	Heterozygote knock-out
°C	Degrees centigrade
AAA	Abdominal Aortic Aneurysm
ACE	Angiotensin converting enzyme
Ag	Antigen
Ala	Alanine
AngII	Angiotensin II
ApoE	Apolipoprotein-E
ARB	Angiotensin Receptor Blocker
Arg	Arginine
BHF	British Heart Foundation
BMI	Body mass index
BP	Blood pressure
Bpm	Beats per minute
BSA	Bovine Serum Albumin
CaCl$_2$	Calcium Chloride
CAD	Coronary Artery Disease
CVA	Cerebrovascular accident

CVD	Cardiovascular Disease
cm	Centimetres
CPN	Carboxypeptidase-N
CRP	C-reactive protein
DBP	Diastolic blood pressure
DM	Diabetes mellitus
DNA	Deoxyribonucleic acid
dH$_2$O	Distilled water
ECG	Electrocardiogram
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
ETOH	Ethanol
FITC	Flurorecein isotriocyanate
g	Grams
g	G-force/Relative centrifugal force
G	Gauge
Gln	Glutamine
GWAS	Genome wide association studies
H+E	Haematoxylin and eosin
H$_2$O$_2$	Hydrogen peroxide

H$_2$SO$_4$	Sulphuric acid
HMWK	High molecular weight kininogen
HR	Heart rate
HRP	Horseradish peroxidase
IgG	Immunoglobulin G
Ile	Isoleucine
ILT	Intra-luminal Thrombus
IV	Intra-venous
IVC	Inferior Vena Cava
kDA	Kilo-Dalton
kg	kilogram
KO	Knockout
LDL	Low-density Lipoprotein
LDLR-1	Low density lipoprotein receptor related protein 1
LEADS	Leeds Aneurysm Development Study
Leu	Leucine
LI60	Lysis Index at 60 minutes
LICAMM	Leeds Institute of Cardiovascular and Metabolic Medicine
LT	Lysis Time
Lys	Lysine

M	Molar
MA	Maximum absorbance
MAP	Mean arterial pressure
MCF	Maximum clot firmness
mg	milligram
MHz	Megahertz
MI	Myocardial Infarction
Min	Minute
ml	Millilitres
mm	Millimetres
mmHg	Millimetres of mercury
MMP	Matrix Metalloproteinase
MSB	Martius Scarlet Blue
MVG	Miller van Geison
NaCl	Sodium Chloride
ng	Nanogram
NHS	National Health Service
nm	Nanometres
nM	Nanomolar
NPP	Normal Pooled Plasma

OD	Optical density
OPD	O-Phenylenediamine dihydrochloride
OPN	Osteopontin
OR	Odds Ratio
PAI-1	Plasminogen Activator Inhibitor-1
PAI-2	Plasminogen Activator Inhibitor-2
PAPs	Plasmin-antiplasmin complexes
PBS	Phosphate buffered saline
PCI	Potato (tuber) Carboxypeptidase Inhibitor
PCR	Polymerase Chain Reaction
PFA	Paraformaldehyde
PhD	Doctor of Philosophy
PPE	Porcine pancreatic elastase
PSR	Picosirus red
RA	Rheumatoid Arthritis
RNA	Ribonucleic acid
ROTEM	Rotational thromboelastrometry
SBP	Systolic blood pressure
SORT-1	Sortilin-1
TAT	Thrombin-Anti-thrombin complex

TAFI	Thrombin Activatable Fibrinolysis Inhibitor
TAFIa	Activated Thrombin activatable fibrinolysis inhibitor
TAFIai	Inactivated Thrombin activatable fibrinolysis inhibitor
TAFI-AP	Thrombin activatable fibrinolysis inhibitor activation peptide
TBS	Tris-buffered saline
TF	Tissue Factor
Thr	Threonine
TIA	Transient ischaemic attack
TMB	Tetramethylbenzidine
tPA	Tissue plasminogen activator
T-TM	Thrombin-thrombomodulin
Tyr	Tyrosine
UK	United Kingdom
uPA	Urine plasminogen activator (Urokinase)
USS	Ultra sound scan
Val	Valine
VPR	Validated pressure recording
vWF	Von WIllebrand Factor
WT	Wild type
X^2	Chi-squared

α2AP	Alpha-2-Antiplasmin
µg	Micrograms
µl	Microlitres
µm	Micrometres
3D	Three dimensional

Chapter 1   Introduction

Cardiovascular disease is a major global health problem, accounting for almost a quarter of all deaths, or 160,000 deaths per annum, in the UK alone (BHF, 2014). Despite technological and scientific advances in the prevention, diagnosis and treatment of this disease, its prevalence continues to increase both in the western world, but also in developing countries. One type of cardiovascular disease which remains poorly understood is Abdominal Aortic Aneurysm (AAA). The multi-faceted pathogenesis of this condition has meant that research into potential medical interventions for AAA have hitherto failed to make any significant advances, with surgery remaining the mainstay of treatment. Even with advances in surgical technique, equipment and materials, such that endovascular intervention has now replaced the need for open surgical repair in almost all cases, surgical intervention of any kind continues to carry significant risks of morbidity and mortality. Changes in coagulation and fibrinolysis are common to many cardiovascular diseases, although the aetiology of this remains poorly understood; in many cases, whether such changes are cause or effect is yet to be fully determined. In line with all cardiovascular disease, the fibrin clot structure is altered in patients with AAA, and they show increased resistance to lysis (Scott et al., 2011). This thesis aims to further explore 1) Why fibrinolysis is altered in patients with AAA, 2) The specific role of the fibrinolysis inhibitor Thrombin Activatable Fibrinolysis Inhibitor (TAFI) in these changes, and 3) The potential for new therapeutic interventions affecting fibrinolysis in treating patients with AAA.

1.1 **Abdominal Aortic Aneurysms**

An Abdominal Aortic Aneurysm (AAA) is a permanent, localised dilatation of the descending abdominal aorta, exceeding the normal diameter by more than 50%, or being greater than 3 cm (Johnston et al., 1991). It most commonly occurs in men over the age of 65 years, and affects up to 1 in 25 men in this age group (Collin et al., 1988, Ashton et al., 2002). In the UK, men over the age of 65 years are the focus of an abdominal aortic aneurysm screening programme, which was rolled out nationally in 2014, and invites all men to have a single ultrasound scan of the abdomen during the year of their 65th birthday. Interestingly, the detection rate of AAA in this screening programme has thus far been lower than anticipated, with a prevalence of just 1.4% of all men screened (NAAASP, 2014). This may be a reflection of broad lifestyle changes in the UK population, that is, decreasing smoking rates, and the increasing use of secondary preventative medications for cardiovascular disease, such as aspirin, antihypertensive medications, and statins. In fact, a recent observational study of AAA patients identified an apparently protective effect of both Angiotensin Converting Enzyme (ACE) inhibitors and Angiotensin Receptor Blockers (ARBs), with a reduction in mortality in patients taking either medication, and a decrease in the need for surgical intervention in those taking ACE inhibitors (Kristensen et al., 2015). Despite lower than expected detection rates, the screening programme remains cost effective (Glover et al., 2014), and so looks set to continue. This is because the implications of ruptured AAA remain serious and significant. The natural history of an untreated AAA is rupture, the risk of which increases significantly with increasing size (Brewster

et al., 2003). There are no medical treatments which can be used to prevent AAA formation, or to halt or slow progression once an AAA is established. Currently, the only management available is surgical intervention, which is offered electively once the AAA reaches the intervention threshold, dependent upon anatomy and pre-existing co-morbidities. The current intervention threshold is set at 5.5 cm, where the risk of rupture each year exceeds the risk of mortality from elective repair (Filardo et al., 2012). AAA rupture occurs when the stress on the aortic wall exceeds the wall strength. Ruptured AAA is associated with significant morbidity and mortality, with two thirds of patients dying before they reach hospital, and a mortality rate of 40-50% in those admitted for emergency treatment (Dillavou et al., 2006, NAAASP, 2014). Despite the introduction of the national screening programme, ruptured AAA is still responsible for over 6000 deaths per annum in the UK alone (NAAASP, 2014).

There are a number of established risk factors for AAA, including smoking, hypertension and male gender. Unlike in ischaemic heart disease and stroke, diabetes mellitus has been shown to be protective against AAA (Lederle et al., 1997), although the mechanism by which this occurs is not fully understood. There is also a strong suggestion of a genetic component, with a proportion of patients having so called 'familial AAA', recognised in those patients with one or more first degree relatives also affected by the condition. The incidence of familial AAA has been shown to range between 8% and 18% (Kent et al., 2010, Larsson et al., 2009, Linne et al., 2012), with the risk of siblings of patients with AAA developing an aneurysm being anywhere between two and five times that of the general population (Blanchard et al., 2000, Salo et al., 1999). The exact

mode of inheritance has not been established, and although a number of candidate genes have been investigated, no individual gene has accounted for the apparent familial presentation (Sandford et al., 2007). Genome wide association studies identified the genes for low-density lipoprotein receptor-related protein 1 (LDLR-1) and sortilin-1 (SORT-1) as being of potential interest, but these alone do not explain the incidence of AAA in the general population (Bown, 2014, Bown et al., 2011, Jones et al., 2013). Clearly, AAA disease is complex and multifactorial, and is likely the result of the interplay between a number of factors, both genetic and environmental.

1.1.1 Pathophysiology of AAA

How and why AAA formation is initiated in not understood. Histologically, there is degradation of the tunica media, with elimination of elastin and collagen, along with smooth muscle cell loss (Thompson et al., 1997), infiltration of lymphocytes and macrophages (Rizas et al., 2009) and neovascularisation (Thompson et al., 1996). Matrix metalloproteinases (MMPs) and other proteases are derived from macrophages and aortic smooth muscle cells, and are secreted into the extracellular matrix. This proteolytic activity promotes the deterioration of the elastin and collagen which normally gives the extracellular matrix of the aortic wall its structure and strength. Increased expression of collagenases MMP-1 and MMP-13 and elastases MMP-2, -9, and -12 (Annabi et al., 2002, Curci et al., 1998, Knox et al., 1997, Mao et al., 1999, Reeps et al., 2009) have been demonstrated in human AAA, and their role in AAA development have been further explored using animal models (Pyo et al., 2000). Another mechanism of interest relates to inflammation and immune

responses. An extensive transmural infiltration by macrophages and lymphocytes is present on aneurysm histology, and these cells may release a cascade of cytokines that subsequently activate a number of proteases. The trigger for the influx of these leucocytes is not known, although it is hypothesised that elastin degradation products within the aortic wall may act as a chemoattractant (Ailawadi et al., 2003). Deposition of immunoglobulins into the aortic wall has been demonstrated, and is thought to support the theory that autoimmune reactions could contribute to AAA formation (Brophy et al., 1991, Jagadesham et al., 2008). Another area of focus in understanding AAA pathogenesis relates to the biomechanical stress on the aortic wall. The reduction in elastin and increased deposition of collagen in the wall of the aneurysmal aorta results in a stiffer, less distensible vessel (He and Roach, 1994). The stress on the aortic wall is not only related to diameter, but also to asymmetry (Vorp et al., 1998), likely due to an increase in the turbulence of arterial flow. The presence of an intra-luminal thrombus also has a significant effect on the wall stress, altering the stress distribution, and has been shown to have a 'cushioning' effect on the underlying aortic wall (Wang et al., 2002). The intra-luminal thrombus is discussed in more detail below. A schematic representation of the processes involved in AAA development can be seen in Figure 1.
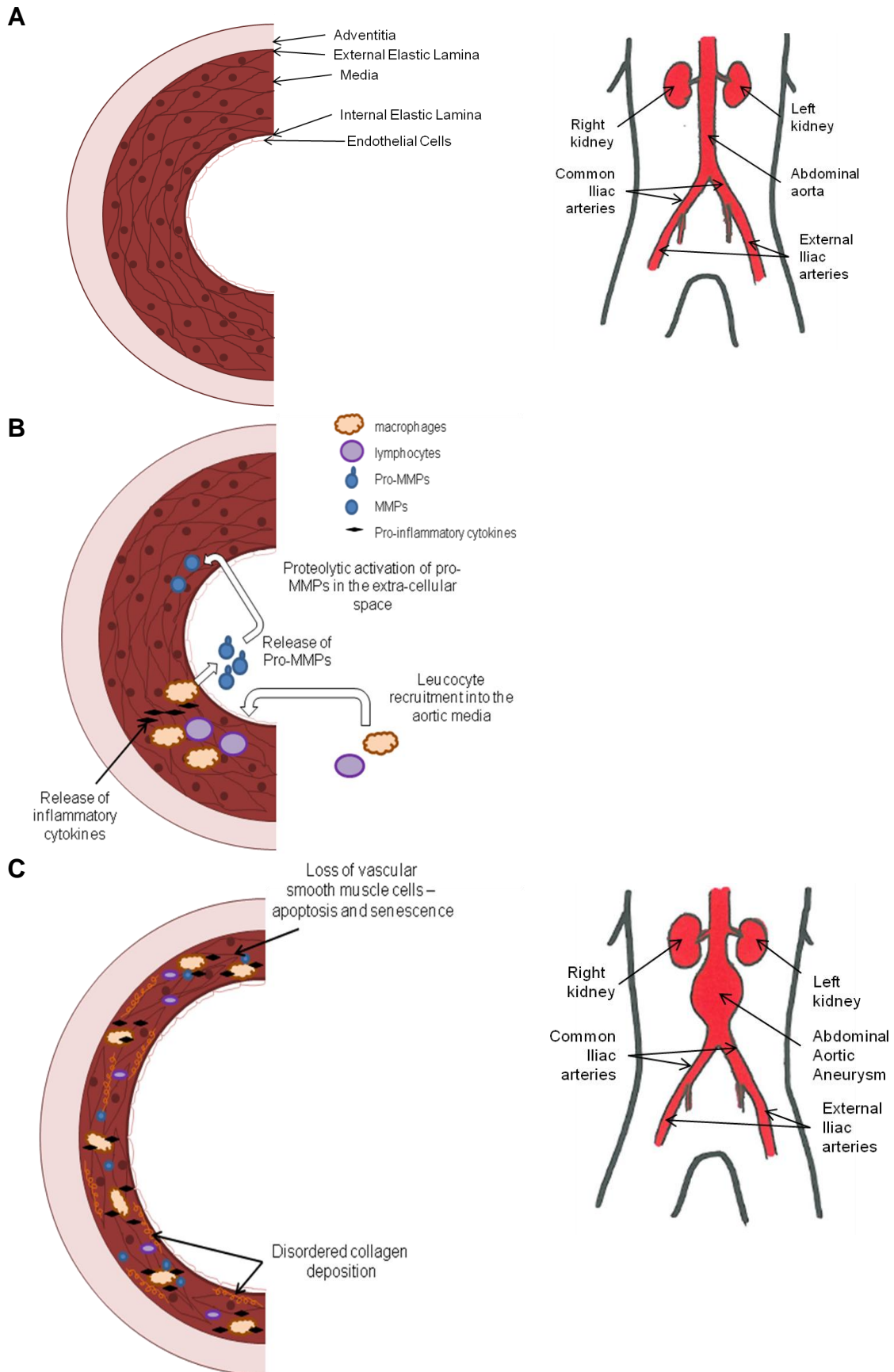
Figure 1. The Pathogenesis of Abdominal Aortic Aneurysm

A. The structure of the normal aortic wall, with the three main layers (intima, media and adventia). A well-organised structure of collagen, elastin and vascular smooth muscle cells are present.

B. Following insult or injury to the endothelial layer, either as result of, or in combination with, established risk factors for AAA, leads to the recruitment of macrophages and lymphocytes into the aortic media, and the production of pro-inflammatory cytokines. Activated macrophages, as well as producing inflammatory cytokines also produce pro-MMPs, which are activated to MMPs in the extracellular space. These MMPs are responsible for elastin and collagen degradation. Over a number of years, elastin degradation, elevated wall tension as a result of hypertension, and wall strain due to turbulent flow result in progressive aortic dilatation. Smooth muscle cells and fibroblasts in the aortic media promote some repair, resulting in the laying down of a disorganised collagen network, but overall smooth muscle cells become senescent and numbers deplete due to apoptosis.

C. The structure of the aortic wall in advanced AAA disease. The medial layer is thinner, with apoptosis and senescence of vascular smooth muscle cells, elastin breakdown and disordered collagen deposition. Inflammatory cells remain present within the wall, indicating a potential for an autoimmune element to AAA disease.

1.1.2 Intra-Luminal Thrombus

Larger aneurysms are often accompanied by the presence of intra-luminal thrombus (ILT) (Vorp et al., 2001), with thrombus being found in 75% of AAA (Wang et al., 2002). Historically, it was thought that the presence of ILT may serve as a protective mechanism against rupture, as it has been shown that the presence of an ILT alters the wall stress distribution and decreases peak wall stress (Inzoli et al., 1993, Mower et al., 1997, Wang et al., 2002). Aneurysm wall that is covered in thrombus has been shown to have decreased tensile strength (Vorp and Vande Geest, 2005), and histologically is thinner, with a reduction in elastin fibres and vascular smooth muscle cells (Kazi et al., 2003). The ILT is not a static structure; it is biologically active with constant remodelling of the luminal surface. *In-vitro* studies show active secretion of proteolytic factors, which although more active at the luminal surface, are still thought to contribute to the breakdown of the underlying vessel wall (Folkesson et al., 2011, Houard et al., 2007, Fontaine et al., 2002, Coutard et al., 2010). High levels of both plasmin anti-plasmin complexes (PAPs) and D-dimer, a plasmin mediated fibrin degradation product, are found within the ILT, the concentrations of which increase with proximity to the luminal layer of the thrombus, indicating ongoing remodelling of the luminal surface (Houard et al., 2007). A positive association has been demonstrated between ILT thickness, levels of smooth muscle cell apoptosis, levels of MMPs, and decreasing numbers of elastin fibres within the aneurysm wall (Koole et al., 2012). Increased concentrations of the plasminogen activators (tPA and uPA) have been demonstrated in AAA wall compared with normal aortic wall, and plasmin itself is a key activator of MMPs,

suggesting that production of plasmin by the ILT could be directly responsible for the activation of MMPs within the aneurysm wall, leading to increased breakdown of collagen and elastin, and acceleration of aneurysm expansion (Carrell et al., 2006). In addition, the ILT is thought to have further indirect effects on wall weakening, through local hypoxia related to thrombus thickness (Vorp et al., 2001). Observational studies have reported significant associations between the size of the ILT and both the rate of expansion and the risk of AAA rupture (Wolf et al., 1994, Stenbaek et al., 2000). In addition, a faster growth rate of the ILT itself is indicative of a higher risk of AAA rupture (Stenbaek et al., 2000). These findings, however, are merely the result of clinical observational studies; the mechanisms behind the association between ILT and AAA rupture have not been fully explored. What is clear, however, is that as the ILT is usually found at the point of rupture of an AAA (Simao da Silva et al., 2000), any biomechanical protection on the underlying wall offered by the ILT is far outweighed by the negative impact related to the increased proteolysis and hypoxia it is responsible for.

The main constituent of the ILT is fibrin, which is produced as a result of the coagulation cascade, in association with platelets, erythrocytes, leucocytes and neutrophils. Both macroscopically and microscopically, the ILT has three distinct layers. The luminal layer, which appears red, and is the main location for protease activity; the medial layer, which is white/yellow in colour; and the abluminal layer, which is dark yellow/brown. A continuous network of interconnected canniculi transverse from the luminal to the abluminal surface, and thus allow for the progression of proteases and other substances through

the ILT (Adolph et al., 1997). The ILT itself is also fluid permeable, with fluid and other small molecules passing through the thrombus network.

1.2 **The Coagulation Cascade**

Thrombus formation is the end point of a series of enzymatic reactions and protein interactions, commonly referred to as the Coagulation Cascade. Prior to formation of fibrin within a clot, primary haemostasis, resulting from the action of platelets, occurs, with the formation of a platelet plug. Secondary haemostasis refers to the formation of a fibrin scaffold, which is required to stabilise the platelet plug. This is achieved through the production of thrombin. *In-vivo*, there are two pathways which ultimately result in the formation of thrombin, and thus the cleavage of fibrinogen to fibrin. The 'Tissue Factor pathway' or the 'Extrinsic pathway', is the main pathway for the initiation of blood coagulation. Activation of the tissue factor pathway generates a 'thrombin burst', which ultimately results in platelet aggregation, fibrin formation and stabilisation, and the inhibition of fibrinolysis. The second pathway is the 'Contact Activation' or 'Intrinsic pathway', which begins with the formation of a primary complex on collagen by high molecular weight kininogen (HMWK), prekallikrein and Factor XII (FXII).

1.2.1 Primary Haemostasis and the Initiation of Coagulation

Following injury to the vessel wall (either mechanical, chemical or electrical), the endothelial cells surrounding the damaged area are activated. Upon activation, P-selectin is released to the endothelial cell surface from the Weibel-Palade storage bodies (McEver et al., 1989). Vessel wall injury also activates platelets, with further P-selectin released to the surface of the platelet from within the

alpha-granules. The receptor for P-selectin, P-selectin glycoprotein ligand-1 (PSGL-1), is held within the membranes of neutrophils and monocytes. Binding of P-selectin to its receptor thus aids the localisation of platelets and leucocytes, and thus thrombus formation, to the site of injury (Polgar et al., 2005). Alongside the release of P-selectin, von Willebrand Factor (vWF) is released from the body of endothelial cells and the α-granules of platelets. vWF has two roles in the initiation of coagulation; firstly, under conditions of high shear stress (as is found in arterial flow), vWF binds to platelet membrane glycoprotein Ibα and to connective tissue (collagen) exposed in the subendothelial matrix, thus supporting adhesion of platelets to the site of injury. Secondly, vWF binds to platelet glycoprotein IIb/IIIa, which enhances platelet to platelet aggregation. Tissue Factor is a transmembrane glycoprotein expressed on stromal fibroblasts and vascular smooth muscle cells. Tissue factor is exposed to the blood when the endothelium is disrupted and the blood comes into contact with these subendothelial cells. When Tissue Factor comes into contact with circulating Factor VII (FVII), it binds FVII, which undergoes auto-activation, and an active TF-FVIIa complex is formed. This complex then activates Factor X (FX) to Factor Xa (FXa). FXa then converts prothrombin to thrombin. This small amount of thrombin then propagates further thrombin generation in the amplification phase (Monroe and Hoffman, 2006).

1.2.2 The Amplification Phase

The trace amount of thrombin produced during the initiation of coagulation causes further activation of platelets, as well as Factors V and XI, to FVa and FXIa. vWF circulates in the plasma in complex with Factor VIII (FVIII). Thrombin

produced during the initiation phase of coagulation releases the FVIII from the vWF, and activates it to FVIIIa. Factor VIIIa binds with FIXa on the platelet surface, and this complex, in the presence of calcium, activates additional FX. FVIIIa-FIXa along with calcium are referred to as the 'tenase' complex.

1.2.3 The Propagation Phase

The formation of the 'tenase' complex leads to large-scale generation of FXa, which binds to FVa to form the prothrombinase complex. In the presence of calcium, this prothrombinase complex results in the 'Thrombin Burst' which is responsible for the conversion of fibrinogen to fibrin. The thrombin produced in the propagation phase continues to activate platelets, FV, FVIII and FXI, and thus results in further thrombin generation. Furthermore, thrombin activates FXI, which through activation of FIX further contributes to thrombin generation. Thrombin also activates FXIII and Thrombin-Activatable Fibrinolysis Inhibitor (TAFI) (further discussed below), which, through different mechanisms, both result in stabilisation of the fibrin clot.

1.2.4 Contact Pathway

The contact pathway of coagulation was previously thought to be of little physiological relevance, as deficiency of the initiator of this part of the coagulation cascade, Factor XII (FXII) causes no bleeding phenotype (Ratnoff and Colopy, 1955). However, mice deficient in FXII do show defective thrombus formation, and show resistance to thromboembolism compared with wild type controls (Renne et al., 2005), implying that this pathway does hold pathophysiological significance. The contact pathway is initiated when FXII comes into contact with collagen, or other negatively charged substances,

underlying the endothelium in the damaged blood vessel wall. FXII is autoactivated to FXIIa, which subsequently activates Factor XI (FXI) to FXIa. FXIa activates FIX, which, along with FVIII from platelets, activates FX. This part of the coagulation cascade also forms an important link with inflammatory pathways. As FXII accumulates at the blood vessel surface and becomes activated, it also activates XIIa dependent prekallikrein. Kallikrein cleaves HMWK to release bradykinin, a potent inflammatory stimulus.

Following activation by the contact or tissue factor pathways, the coagulation cascade is maintained in a prothrombotic state by continued activation of FV, FVIII and FIX, until it is down regulated by exogenous anticoagulants or by the fibrinolysis pathways. A summary of the tissue factor dependent coagulation cascade is shown in Figure 2.
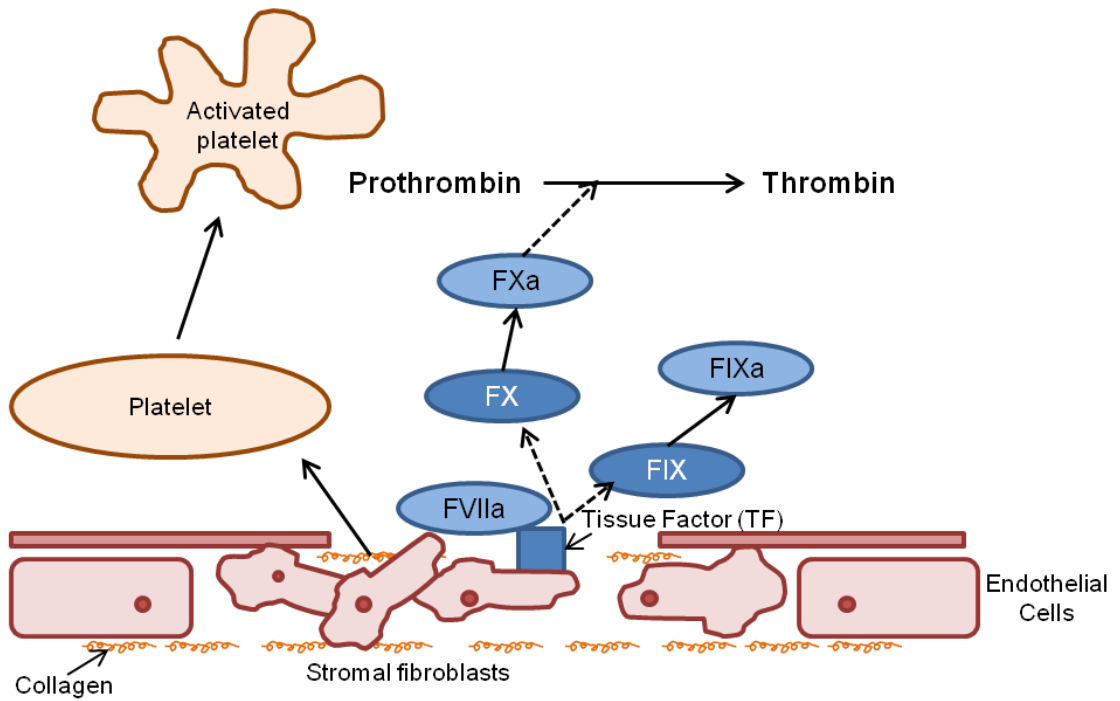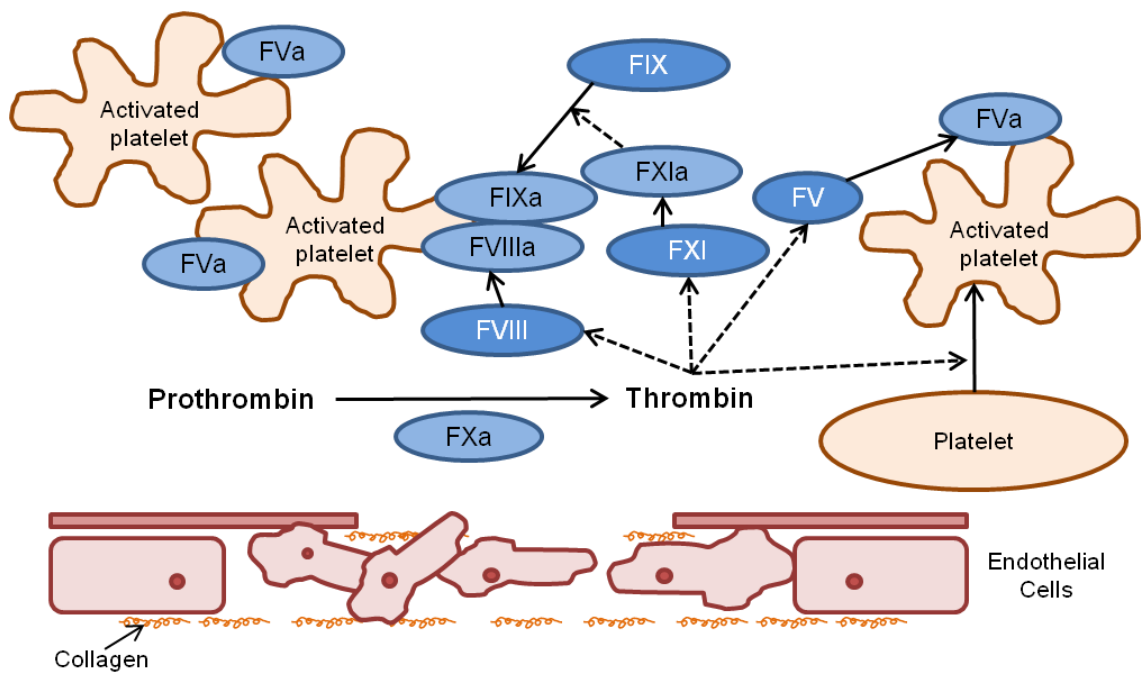
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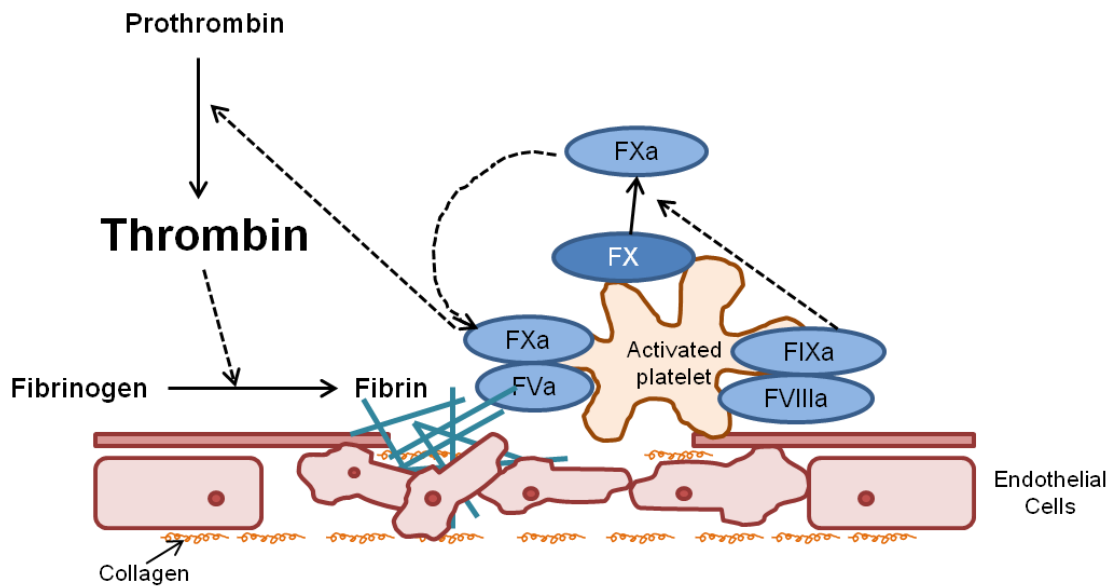
Figure 2. The Coagulation Cascade – Initiation, Amplification and Propagation of Coagulation

A. Initiation

Exposed collagen and subendothelial proteins trigger the adhesion and activation of platelets. Tissue Factor, expressed on the surface of stromal fibroblasts and vascular smooth muscle cells, comes into contact with circulating Factor VII (FVII), FVII is activated, and together they form an active TF-FVIIa complex. This then activates Factor IX (FIX) to Factor IXa (FIXa) and Factor X (FX) to Factor Xa (FXa). FV is slowly activated by FXa, and, by the amplification and propagation phases, the Fva and FXa bind together to form the prothrombinase complex.

B. Amplification

The trace amount of thrombin produced during the initiation of coagulation causes the further activation of platelets, FV and FXI, which then activates FIX to FIXa. vWF circulates in the plasma in complex with Factor VIII (FVIII). Thrombin produced during the initiation phase of coagulation releases the FVIII from the vWF, and activates it to FVIIIa. Factor VIIIa binds with FIXa on the platelet surface, and this complex, in the presence of calcium, activates additional FX. FVIIIa-FIXa along with calcium are referred to as the 'tenase' complex.

C. Propagation

The formation of the 'tenase' complex leads to large scale generation of FXa, which, with FVa forms a prothrombinase complex. The prothrombinase complex, in the presence of calcium, results in the 'Thrombin Burst' which is responsible for the conversion of fibrinogen to fibrin. The thrombin produced in the propagation phase continues to activate platelets, FV, FVIII and FXI, and thus results in further thrombin generation.

Adapted from (Wisler and Becker, 2012).

1.3 **Fibrinogen**

Fibrinogen is the third most abundant of all human plasma proteins, secondary only to albumin and the immunoglobulins, and has circulating plasma levels of 2-4 mg/ml under normal conditions. Fibrinogen is an acute phase protein, and, in response to pro-inflammatory agents such as interleukin-6 and other cytokines, expression is upregulated, gene transcription is increased and circulating plasma levels become elevated (Dalmon et al., 1993, Lane et al., 1991). High plasma levels of fibrinogen are associated with cardiovascular disease (Danesh et al., 2005, Scarabin et al., 2003, Tatli et al., 2009), stroke (Danesh et al., 2005), peripheral vascular disease (Bartlett et al., 2009) and pulmonary embolism (Klovaite et al., 2013). Previous work from our group has also found elevated levels of fibrinogen in patients with AAA (Parry et al., 2009). Despite multiple studies demonstrating a link between high fibrinogen levels and cardiovascular disease, whether this association represents cause or effect remains a subject of debate. In fact, a recent multi-ethnic meta-analysis failed to show strong evidence of a causal association between circulating fibrinogen levels and cardiovascular disease (Sabater-Lleal et al., 2013).

1.3.1 Fibrinogen Structure and Synthesis

Fibrinogen is a large glycoprotein of 340 kDa, and is comprised of two identical subsets, each consisting of three polypeptides (Aα-, Bβ- and γ- chains), supported by a total of twenty-nine disulphide bonds. The six chains all converge in the central part of the fibrinogen molecule, the E-region, where the cleavage sites for thrombin are located (Henschen et al., 1983, Weisel and

Medved, 2001). Away from the central E-region, the Aα- Bβ- and γ- chains intertwine and form alpha-helical coil structures, which expand outwards and connect the E regions to two D-regions. The D-regions contain the binding pockets which are important in polymerisation of the fibrinogen molecule (Yang et al., 2000). Unlike the Bβ- and γ- chains, which end in the D-region, the Aα-chain continues, and protrudes beyond the D-region, forming a long flexible αC region of over 350 amino acid residues (Tsurupa et al., 2009). The structure of fibrinogen is shown in Figure 3. Fibrinogen is primarily synthesised in the liver, where the chains are assembled rapidly in the endoplasmic reticulum, producing a complete molecule in as little as five minutes (Redman and Xia, 2001).
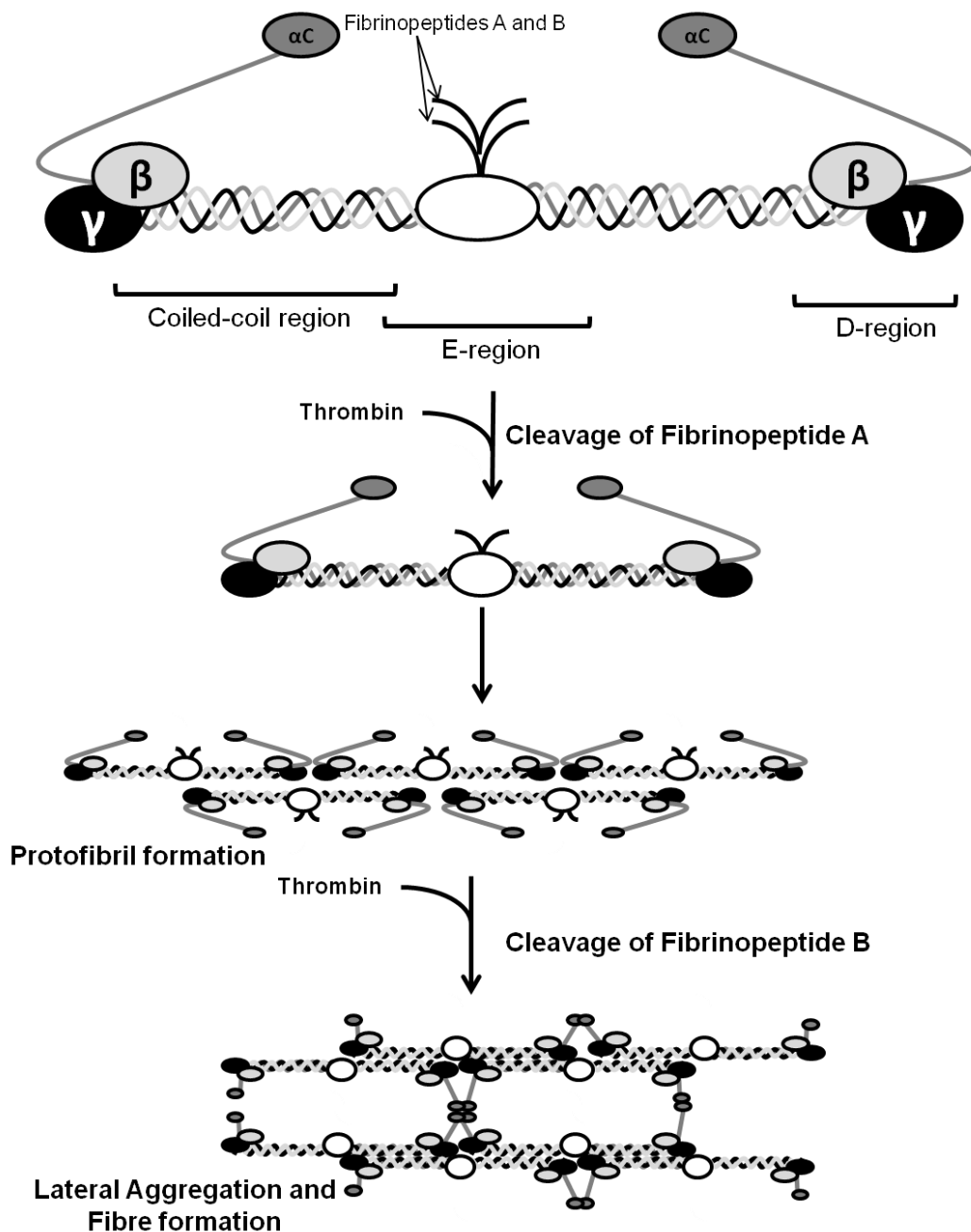
Figure 3. The Structure of Fibrinogen and Convesion of Fibrinogen into Fibrin

Fibrinogen consists of two identical subsets, each containing an alpha, beta and gamma chain. The molecules all converge in a central E-region, which contains the cleavage sites for thrombin (Fibrinopeptides A and B). The chains extend out of this central E-region through a coil-coiled region to the D-regions, where the beta and gamma chains reach their C-terminus. The alpha chain extends beyond this D-region, as the alpha-C chain. Thrombin cleaves Fibrinopeptide A, which exposes a binding site on the E-region which is specific for a binging pocket in the D-region, allowing fibrin(ogen) molecules to come together in a staggered protofibril, with each E-region bound to the D-region of two fibrinogen molecules. Cleavage of Fibrinopeptide B results in the exposure of a second binding site, and is associated with the release of the αC regions, the lateral aggregation of protofibrils molecules and fibrin fibre formation. *Adapted from Bridge et al. Thromb Haemost 2014 (Bridge et al., 2014b).*

1.4 **Fibrin Clot Formation**

Soluble fibrinogen is converted to insoluble fibrin by the thrombin cleavage of two fibrinopeptides A, and two fibrinopeptides B, from the Aα- and Bβ-chains respectively (Cilia La Corte et al., 2011, Yang et al., 2000). In brief, this exposes polymerisation sites, results in a change in solubility, and causes the fibrinogen molecules to polymerise and form fibrin fibres. The first fibrinopeptide to be cleaved by thrombin is fibrinopeptide A, which exposes a binding site on the E-region which is specific for a pocket in the D-region γ-chain. Due to the configuration of the fibrinogen molecule, with one central E-region and two peripheral D-regions, one fibrin molecule can bind to two other fibrin molecules, which in turn each can bind to another fibrin molecule and so on, producing a fibrin polymer, or protofibril, comprised of staggered and overlapping fibrin molecules. This is illustrated in Figure 3 (Ariens, 2013, Cilia La Corte et al., 2011, Blomback et al., 1978, Weisel, 1986). The cleavage of fibrinopeptide B by thrombin occurs at a much slower rate compared to the cleavage of fibrinopeptide A. Cleavage of fibrinopeptide B exposes a second binding site in the E-region (which is specific for a different binding pocket in the fibrinogen D-region β-chain), and also results in the release of the αC-region, such that this region of the fibrin α-chain becomes available for intermolecular interactions. These actions facilitate the lateral aggregation of the protofibrils into fibrin fibres (Weisel, 1986).

1.5 The role of Factor XIII

Whilst the majority of the blood coagulation proteins are serine proteases, factor XIII (FXIII) is a transglutaminase, which, when activated, plays a vital role in stabilising the fibrin clot. This is achieved through its ability to covalently cross-link fibrin, both to other fibrin molecules, and to proteins involved in the prevention of fibrinolysis, such as alpha-2-antiplasmin (α2AP). FXIII consists of two A subunits, which contain the active site of the enzyme, and two B subunits, which protect the hydrophobic A subunits. The activation of FXIII is a two step process. Thrombin first cleaves a 37 amino acid activation peptide from the FXIII-A subunit, before calcium induces dissociation of the B subunits, exposing the activated FXIII-A and producing the active enzyme (FXIIIa) (Ariens et al., 2002) (Figure 4). Once covalently cross-linked by FXIIIa, fibrin clots have increased density, reduced pore size and thinner fibres, and increased resistance to lysis (Hethershaw et al., 2013). FXIIIa cross-links the α- and γ-, but not the β-chains, of fibrin. γ-Chain cross-linking occurs first, soon after protofibril formation, and affects fibre density (Duval et al., 2014). Cross-linking of the γ-chains occurs within as little as 5-10 minutes, with isopeptide bonds forming between Gln398 or 399 on the γ-chain of one fibrin molecule and Lys406 on the γ-chain of another (Purves et al., 1987, Spraggon et al., 1997). α-Chain cross-linking increases fibre tautness, increases clot stiffness and reduces clot deformation (Duval et al., 2014), and, by reducing the accessibility of the coiled-coil regions to plasmin, plays an important role in the regulation of fibrinolysis (Gaffney and Whitaker, 1979, McDonagh et al., 1971, Mutch et al., 2010b). There are many residues involved in α-chain cross-linking, with Gln221, Gln237, Gln328 and Gln366 in one α-chain binding to Lys208, Lys219, Lys224,

Lys418, Lys427, Lys429, Lys446, Lys448, Lys508, Lys539, Lys556, Lys580, Lys583, Lys601 and Lys606 of another (Lorand, 2001, Sobel and Gawinowicz, 1996). Inter-chain cross-linking between the γ- and α-chain also occurs (Lorand, 2001). Other proteins that can be cross-linked into the fibrin clot by FXIIIa include α2AP (Fraser et al., 2011), plasminogen activator inhibitor-2 (PAI-2) (Ritchie et al., 2000, Ritchie et al., 2001, Ritchie et al., 1999), and TAFI (Valnickova and Enghild, 1998), which serve to help stabilise and maintain the fibrin clot structure. α2AP is cross-linked to Lys303 of the fibrin α-chain primarily via one major site, Gln2, but also three minor sites, Gln21, Gln419 and Gln447 (Lee et al., 2001). PAI-2 is cross-linked via Gln83 and Gln86 (Ritchie et al., 1999). TAFI has three known amine acceptor sites (Gln2, Gln5 and Gln83) (Valnickova and Enghild, 1998), but the receptor sites on fibrinogen have not yet been reported.
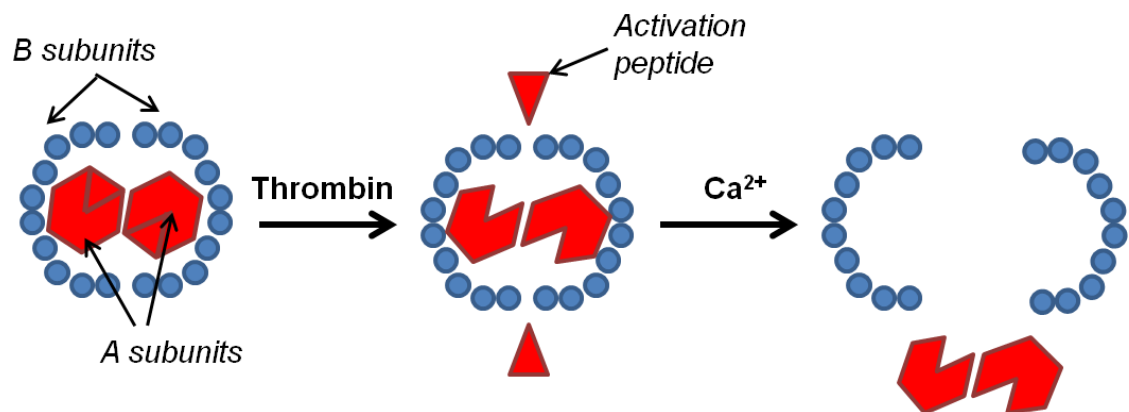
Figure 4. The Activation of Factor XIII

FXIII consists of two A subunits (shown in red), which contain the active site of the enzyme, and two B subunits (shown in blue), which protect the hydrophobic A subunits. Activation of FXIII occurs in a two stage process; thrombin cleaves a 37 amino acid activation peptide from the FXIII-A subunit, before calcium induces dissociation of the B subunits, exposing the activated FXIII-A and producing the active enzyme (FXIIIa).
Modified from (Ariens et al., 2002)

1.6 **Fibrinolysis**

Plasmin, generated from plasminogen by the action of tissue- or urokinase-type plasminogen activators, is the main enzyme responsible for lysis of the fibrin fibres in human plasma. The presence of fibrin enhances the conversion of plasminogen to plasmin by providing a catalytic surface to which both tissue plasminogen activator (tPA) and plasminogen bind. This co-localisation of plasminogen with tPA, to the C-terminal lysine residues of partially degraded fibrin, increases the catalytic activity of this reaction by over 1000-fold, with fibrin acting as a co-factor in its own breakdown. Fibrinolysis is largely inhibited by the inhibition of plasmin, which occurs directly, by α2AP, and indirectly, through the inhibition of plasmin generation, by plasminogen activator inhibitor-1 (PAI-1) and TAFI. The fibrinolysis pathway is shown in Figure 5.

Changes in the fibrin clot structure are associated with changes in rates of fibrinolysis; denser clots with smaller pores and more closely packed fibres lyse more slowly than clots with larger pores and more loosely packed fibres. The delay in fibrinolysis rates in densely packed clots with thinner fibres is likely due to a combination of mechanisms, including reduced permeation of the fibrinolytic enzymes into the centre of the clot, as well as reduced binding of tPA and plasminogen to densely packed, thin fibres (Longstaff et al., 2011, Mutch et al., 2010a).
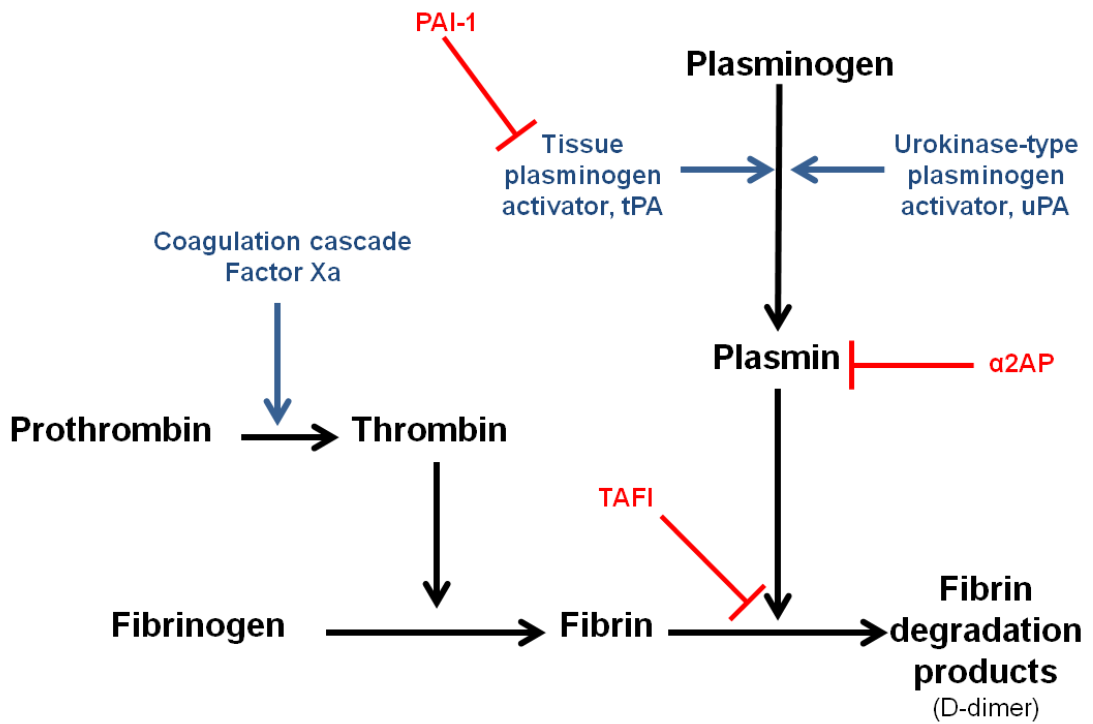
Figure 5. Fibrinolysis

Plasminogen is converted to plasmin through the action of tissue plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA). Plasmin is the main enzyme responsible for the lysis of the fibrin clot. Fibrinolysis is controlled in-vivo through the inhibition of plasmin, which occurs directly, through alpha-2-antiplasmin **(α2AP)**, or indirectly, though plasminogen activator inhibitor-1 **(PAI-1)** and thrombin activatable fibrinolysis inhibitor **(TAFI)**. Adapted from (Bridge et al., 2014a).

1.6.1 Fibrinolysis and AAA

Whilst the presence of ILT is more often associated with large aneurysms, there is evidence for a systemic change in clotting in patients with AAA. A potential connection between fibrinolysis and early AAA development was highlighted by studying fibrin degradation products. Higher levels of D-dimer (a plasmin-induced fibrin degradation product) were found in patients with small AAA compared to controls, suggesting that increased plasmin generation and fibrinolysis may be present even from the early stages of the disease (Parry et al., 2009). In addition, plasma levels of plasmin-antiplasmin complexes (PAPs) have been positively correlated with AAA expansion rates in patients with small aneurysms (Lindholt et al., 2001). The plasma levels of fibrinolysis proteins appear unaffected by treatment, with patients undergoing endovascular repair continuing to display high levels of thrombin-antithrombin (TAT), prothrombin fragments 1 and 2 (F1+2), and D-dimer up to 5 months after intervention (Bailey et al., 2013b). Patients undergoing open repair, however, did not continue to display high levels of these markers, suggesting that the ongoing presence of the ILT within the aneurysm sac, even when the aneurysm is stented successfully, continues to fuel the AAA related hypercoagulability observed in patients prior to intervention (Bailey et al., 2013b).

1.7 Thrombin-Activatable Fibrinolysis Inhibitor

Thrombin-activatable fibrinolysis inhibitor (TAFI), is a zinc dependent metalloprocarboxypeptidase with a molecular weight of 56 kDa (Bajzar et al., 1995), and is intimately involved in the regulation of fibrinolysis and the

maintenance of thrombus stability. Identified in parallel by a number of groups, it is also known as plasma pro-carboxypeptidase R (Campbell and Okada, 1989), pro-carboxypeptidase U (Hendriks et al., 1990), and pro-carboxypeptidase B (Eaton et al., 1991). The TAFI pre-propeptide is a molecule of 423 amino acids, and is synthesized by the liver and by megakaryocytes. The two pools of TAFI show differing glycosylation patterns, confirming that the platelet TAFI is not synthesized by the liver, but is instead produced within the megakaryocytes (Mosnier et al., 2003). After removal of the secretory peptide, the proenzyme contains 401 amino acids, and circulates in the blood as an inactive zymogen. The majority of the TAFI in blood circulates within the plasma, with a small amount (<0.1%) being carried in platelets (Mosnier et al., 2003). TAFI is released from the alpha granules of platelets upon platelet activation, and is thought to complement the TAFI already in circulation, particularly in arterial thrombosis (Schadinger et al., 2010).

The normal concentration of TAFI antigen in human plasma is between 4-15 µg/ml, or 73-275 nM (Colucci and Semeraro, 2012). Antigen levels can also be expressed as a percentage compared with pooled plasma of healthy subjects (which is assigned a concentration of 100% or 1 unit/ml). Levels of TAFI are normally distributed (van Tilburg et al., 2000), the variation in levels between individuals is wide, ranging from 41-259%, although individual variations are narrow (Chetaille et al., 2000). TAFI acts under a threshold dependent mechanism; that is, once TAFIa levels reach the minimum threshold level (estimated to be approximately 8 units/litre, that is, approximately 1% of the total plasma antigen concentration), fibrinolysis essentially ceases until they fall below the threshold level again (Leurs et al., 2004). With such a low

concentration of TAFIa required to actually prevent fibrinolysis, it is possible that the thrombin burst generated during the propagation phase of coagulation could be sufficient to activate enough TAFI to exceed the threshold level. High levels of TAFI (resulting in TAFIa concentrations which are persistently above the minimum threshold value) may lead to a hypofibrinolytic state, and an increased risk of thrombotic disease.

1.7.1   Activation of TAFI

TAFI is cleaved at Arg92 by either thrombin, thrombin-thrombomodulin (T-TM) complex, or plasmin, to yield the active molecule, TAFIa, and an activation peptide (Willemse and Hendriks, 2006). More recently, in-silico analysis has also revealed a second thrombin cleavage site at Arg12. The significance of this cleavage site has not yet been fully elucidated, although it has been speculated that cleavage at Arg12 may accelerate thrombin-mediated TAFI activation (Plug et al., 2014). TAFIa cleaves lysine residues from the C-terminal ends of partially degraded fibrin, eliminating the plasminogen binding sites involved in the fibrin-enhancement of plasminogen to plasmin conversion by tPA, thereby inhibiting plasmin generation and fibrinolysis (Bouma and Mosnier, 2006). This is illustrated in Figure 6. As a result of *in-vitro* studies, the major physiological activator of TAFI was, until recently, taken as being the T-TM complex, due to its 1250-fold higher catalytic activity compared to thrombin alone (Bajzar et al., 1996). However, *in-vivo*, thrombomodulin is localised to the endothelial cell surface, and as such, activation of TAFI by T-TM could only occur on the surface of intact endothelium. As a clot forms and extends into the lumen of the vessel, away from the endothelial surface, other activators of TAFI are likely to

come into play. Indeed, recent work with monoclonal antibodies specific to TAFI have demonstrated that plasmin itself is a significant TAFI activator both *in-vitro* and *in-vivo* (Vercauteren et al., 2011, Mishra et al., 2011).

Human TAFIa has a relatively short half-life in plasma of around 8-10 minutes at 37°C, although until activation, TAFI is relatively stable. The stability of TAFIa certainly seems to be largely temperature dependent, as the stability of TAFIa increases to approximately two hours at room temperature, and at 0°C it is almost completely stable (Boffa et al., 1998). Murine TAFIa has a markedly reduced half-life of 2-3 minutes (Marx et al., 2000b).
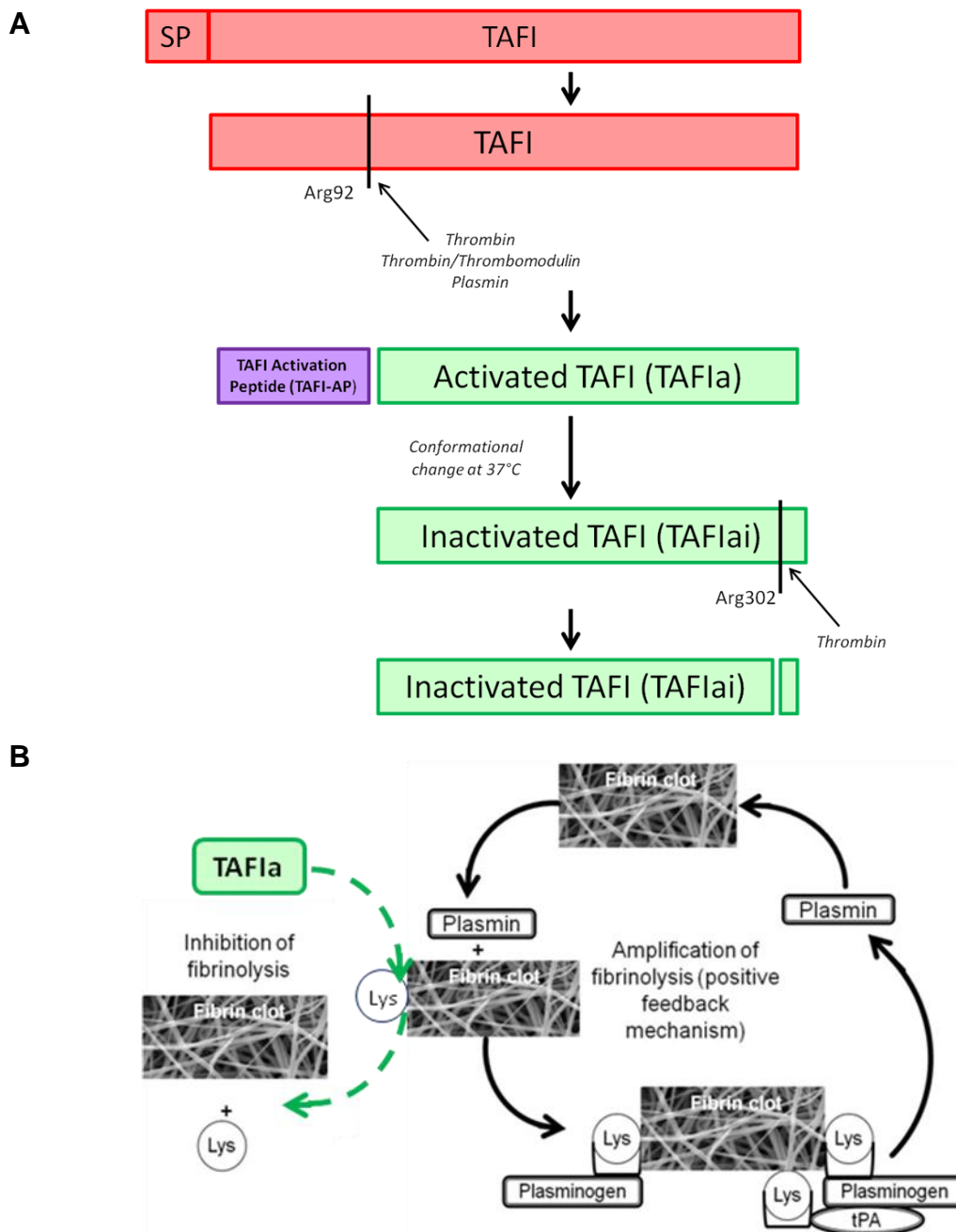
Figure 6. The Life-cycle of a TAFI Molecule and The Role of TAFI in Fibrinolysis

A - Following release of the secretory peptide (SP), TAFI circulates in the plasma as a 406aa zymogen. The intact TAFI zymogen is activated by thrombin, thrombin/thrombomodulin and plasmin. The zymogen is cleaved at Arginine 92 to release the active molecule (TAFIa) and the 92 amino acid activation peptide (TAFI-AP). The active molecule is then inactivated through a conformational change in the dynamic flap region of the TAFIa molecule, before being further cleaved by thrombin. **B** –TAFIa cleaves the C-terminal lysine residues from partially degraded fibrin. In doing so, it removes the binding sites for the co-localisation of plasminogen and tPA on the fibrin clot. This co-localisation is responsible for increasing the catalytic activity of the tPA/plasminogen reaction 1000-fold. Thus, the action of TAFI results in the decreased conversion of plasminogen to plasmin, and thus inhibits the plasmin-mediated breakdown of the fibrin clot. Adapted from (Bouma and Mosnier, 2006). Electron microscopy images courtesy of Dr Cedric Duval, University of Leeds.

1.7.2 TAFIa inactivation

TAFIa is inactivated by a conformational change, and is then cleaved at position Arg302 into an irreversibly inactivated molecule (Bajzar, 2000). Confirmation that the inactivation of TAFIa is the result of a conformational change rather than proteolytic cleavage came from studies of a TAFI mutant resistant to the secondary cleavage by thrombin (Arg302Gln mutant), that, following activation, displayed the same half-life as the wild type (Marx et al., 2000a). When the crystal structure of TAFI was first determined in 2008, it revealed a highly dynamic region consisting of amino acids 296-350 (Marx et al., 2008). This led to the hypothesis that the flexibility of this region induced a conformational change that subsequently eliminated the enzymatic activity of TAFIa. More weight was added to this theory through the study of TAFI molecules with mutations in this region. The naturally occurring polymorphism of TAFI, Thr325Ile, results in an increase in stability, with the half-life of the 325Ile form being double that of the more commonly occurring Thr325 form at room temperature (see below) (Brouwers et al., 2001). In addition, by creating point mutations in the region of amino acids 300 to 330, TAFI variants with significantly increased half-lives compared with the wild type protein have been produced (Ceresa et al., 2006b, Marx et al., 2004). Until recently, it was thought that the stability of the TAFI zymogen was related to the relationship between the activation peptide and the dynamic flap region of the TAFIa molecule. Prior to the cleavage of the activation peptide, these two regions lie in close proximity, and thus the activation peptide could stabilise the flexible region, and in doing so protect it from the conformational change that results in TAFIa's inactivation. It was suggested that this stabilisation was the result of

hydrophobic interactions between Val35 and Leu39 in the activation peptide and Tyr341 in the dynamic region (Marx et al., 2008). However, this has recently been disputed, following studies of another TAFI mutant, which focused on the activation peptide itself rather than the dynamic flap region. The activation peptide of TAFI is made up of 92 amino acids. The first 73 form a globular domain which shields the catalytic domain of TAFI from large substrates. The remaining 19 amino acids link this globular domain to the catalytic region. A mutant of TAFI which lacks the globular domain, but not amino acids 74-92 resulted in a molecule that was able to react with small substrates in a similar way to activated TAFI, but which maintained the stability of the TAFI zymogen. It was only when the remaining 19 amino acids of the activation peptide were removed that the mutant became 'destabilized' in a similar way to active TAFI. It has therefore been suggested that it is actually not the interaction between Val35, Leu39 and Tyr341 that stabilises the TAFI zymogen, but instead the 'connecting region' of the activation peptide, amino acids Arg74-Arg92 (Plug and Meijers, 2015, Zhou and Declerck, 2015). A pictorial representation of the activation and inactivation stages of TAFI is shown in Figure 6.

1.7.3 TAFI and inflammation

Aside from its effects on fibrinolysis, TAFIa also exhibits anti-inflammatory properties. It inactivates the anaphylatoxins C3a and C5a, thrombin cleaved osteopontin (OPN) and bradykinin (Campbell et al., 2002, Myles et al., 2003, Shinohara et al., 1994). The action of TAFIa on these substrates is similar to its action on fibrin, that is, it cleaves C-terminal lysine residues from C3a and C5a,

and C-terminal arginine residues from thrombin cleaved OPN. The cleaved products have a much lower inflammatory activity than the uncleaved substrates. This suggests that TAFIa is involved in a homeostatic mechanism, whereby TAFIa, once activated by thrombin, is able to regulate thrombin's inflammatory activity. C5a is a potent chemoattractant for neutrophils (Marder et al., 1985). Excessive C5a can have a negative effect, contributing to immunoparalysis and the dysregulation of coagulation and fibrinolysis. C3a and C4a are also generated when complement is activated, although their potency as chemoattractants are much lower than that of C5a. The role of TAFIa in relation to inflammation has previously been thought to be only relatively minor. Any activity it did have towards inflammatory substrates was largely masked by those of the only other known plasma carboxypeptidase, carboxypeptidase N (CPN). However, it is now known that TAFIa cleaves C5a, OPN and BK with an 8.7-26 fold higher affinity than CPN, and this suggests that TAFIa does play a significant role in attenuating inflammation (Campbell et al., 2002, Myles et al., 2003, Shinohara et al., 1994). This is supported by studies in a number of *In-vivo* models. TAFI-/- mice develop a much more extensive C5a induced alveolitis than wild type mice (Nishimura et al., 2007). In models of rheumatoid arthritis, a primarily autoimmune disease, TAFI-/- mice develop severe disease which is abolished by the addition of an anti-C5a antibody (Song et al., 2011). Even in models of osteo-arthritis, a predominantly 'wear and tear' condition, TAFI was shown to be protective against joint destruction (Lepus et al., 2014). In human studies, patients with severe rheumatoid arthritis (RA), an auto-immune inflammatory condition, were less likely to carry the TAFI 325Ile allele, and those with the 325Ile allele had a lower risk of developing the most severe

RA (Song et al., 2011). The majority of research into the links between TAFI and inflammation have focused on C5a as a substrate, as it is cleaved by TAFIa with a higher affinity compared with C3a. However, a recent study of a murine model of intra-abdominal sepsis in the TAFI-/- mouse revealed an important role for C3a rather than C5a, having shown an unexpected survival benefit in TAFI-/- mice compared with wild type controls, that could not be attributed to either increased C5a levels or enhanced fibrinolysis. The authors hypothesised that in TAFI-/- mice, the C3a is able to override the negative effects of excess C5a, exerting a greater protective effect, and causing an overall survival benefit. Differences in the effect of TAFIa in inflammatory disease *in-vivo* may be related to the site of the insult, and be related to the differing patterns of the receptors for C3a and C5a. C3a receptors are only found on peritoneal macrophages, with its primary role residing in the activation of peritoneal macrophages in order to limit dissemination of intra-abdominal infection. In contrast, C5a receptors are also expressed on neutrophils and other cell types, and thus lead to a widespread increased inflammatory response, which in the absence of TAFIa is allowed to propagate unregulated, and thus has a negative effect (Shao et al., 2015).

1.7.4 Polymorphisms of TAFI

The gene for TAFI is located on chromosome 13 locus q14.11 (Boffa et al., 1999). Two naturally occurring single nucleotide polymorphisms that result in amino acid substitutions have been identified. The first lies at position 147 where alanine is substituted for threonine (allele frequency 0.32-0.41) (de Bruijne et al., 2007, Morange et al., 2005, Verdu et al., 2008), although

functionally there appears to be no difference between the two genotypes (Zhao et al., 1998). The second lies at position 325, in the area of the gene that has been shown to be related to thermal stability (Boffa et al., 2000). Here, threonine is substituted for isoleucine (1040C/T, rs1926447), with an allele frequency of 0.28-0.30 (de Bruijne et al., 2007, Morange et al., 2005, Verdu et al., 2008). Unlike the Ala147Thr polymorphism, this Thr325Ile polymorphism results in a functional change of TAFIa, significantly prolonging its half-life due to increased thermal-stability (from 8 to 15 minutes in plasma at 37°C) and increasing anti-fibrinolytic potential by up to 60% (Schneider et al., 2002).

1.7.5 TAFI and Cardiovascular Disease

Other physiological inhibitors of plasmin, specifically PAI-1, are associated with an increased incidence of coronary artery disease (Scarabin et al., 1998), with polymorphisms that result in increased plasma PAI-1 levels being strongly associated with an increased risk of MI (Gong et al., 2012). The association between PAI-1 levels and MI may not be entirely genetically determined however, as PAI-1 plasma levels are also determined by insulin resistance markers, such as obesity (Juhan-Vague et al., 2000). In contrast, plasma TAFI levels are only minimally influenced by traditional CVD risk factors (Boffa and Koschinsky, 2007). A number of studies have investigated associations between TAFI levels, the Thr325Ile polymorphism and cardiovascular disease, most commonly focussing on coronary artery disease and stroke (de Bruijne et al., 2009, Fernandez-Cadenas et al., 2007, Kamal et al., 2011, Kozian et al., 2010, Morange et al., 2005, Segev et al., 2004, Tassies et al., 2009, Zorio et al., 2003). There is significant variability in the reported findings of these studies, as

is discussed in the following paragraphs. The reasons for some of this variability may be related to the difficulty in accurately measuring TAFI levels in plasma. Enzyme-linked immunosorbent assays (ELISAs) for TAFI use antibodies specific to TAFI or TAFIa. However, due to its intrinsic instability, measuring TAFIa in such a way is complicated. Also, many of the antibodies used for such ELISAs are unable to distinguish between TAFIa and TAFIai (Heylen et al., 2011). The majority of ELISAs are not able to distinguish between the different TAFI Thr325Ile isoforms (Schneider et al., 2002). Assays to target TAFI activity are also variable and not without problems, specifically due to the interaction of other plasma carboxypeptidases in these assays. One vital component in TAFIa measurement relates to the processes around sample collection. Samples should be collected onto ice to minimise sample degradation, and centrifuged at 4°C. The choice of anticoagulant is also important, as EDTA may interfere with TAFIa activity (Stromqvist et al., 2001).

1.7.6 Coronary Artery Disease and Acute Coronary Syndromes

Despite multiple studies, there is still no clear consensus as to the role of TAFI in coronary artery disease (CAD).

Elevated total TAFI antigen levels have been correlated with both an increased risk (Kamal et al., 2011, Santamaria et al., 2004), and a decreased risk of CAD (Brouwers et al., 2003), whilst a number of studies have failed to show any relationship (Morange et al., 2005, Paola Cellai et al., 2006). For activated TAFI (TAFIa), there is also no clear association; low levels have been found to correlate with an increased risk of myocardial infarction (MI) (Meltzer et al., 2009) whilst other studies have associated increased TAFIa levels with acute

MI in a young population (Zorio et al., 2003) as well as overall population levels of cardiovascular disease (CVD) (de Bruijne et al., 2009) and cardiovascular death (Tregouet et al., 2009).

The Thr325Ile polymorphism of TAFI has been investigated as a potential marker of 'high risk' patients with CAD, but with conflicting results. Early studies suggested there was no difference in the genotype of patients with myocardial infarction (MI) (Morange et al., 2002). Since then, a number of studies reported 'high risk' genotypes which seemed to vary with age (Zorio et al., 2003),(de Bruijne et al., 2009), nationality (Morange et al., 2005),(Kamal et al., 2011), and disease sub-type (Tassies et al., 2009). A recent large meta-analysis of genetic variations in TAFI in all cardiovascular disease, CVD, (specifically 19 studies of Ala147Thr consisting of 4977 CVD patients and 8082 controls, and 15 studies of Thr325Ile consisting of 4890 cases and 8311 controls) found that there was a 25% increase in the risk of CVD in those who were homozygote for the 325Ile allele compared with Ile/Thr and Thr/Thr, but no significant associations between the Ala147Thr polymorphism and CVD incidence were found (Shi et al., 2014).

1.7.7 Stroke

The association between ischaemic stroke and TAFI appears less controversial and more clearly delineated. The risk of ischaemic stroke is associated with increasing levels of both total TAFI and TAFIa (Jood et al., 2012, Ladenvall et al., 2007). In acute stroke, patients have significantly higher levels of TAFI at admission compared to controls (Rooth et al., 2007), with neurological deficit worsening as levels increase (Montaner et al., 2003). After the onset of acute

stroke, TAFI levels decreased over the first 72 hours, and then increased back to the baseline level by day 7, a pattern that became increasingly pronounced with stroke severity (Brouns et al., 2010). Furthermore, patients have been shown to have baseline TAFI levels that remained persistently higher than controls even at three months post-stroke, suggesting that the TAFI elevation does not merely represent an acute phase reaction (Leebeek et al., 2005, Santamaria et al., 2003). It has been shown separately that thrombolysis in the treatment of stroke resulted in a decrease in TAFI levels, whilst high levels of TAFI prior to thrombolysis have been correlated with a decrease in effectiveness of re-canalisation (Brouns et al., 2009). Possession of the isoleucine allele at position 325 was associated with both the incidence of stroke and lower age at onset of first stroke (Kozian et al., 2010). In addition, when thrombolysis of the middle cerebral artery was attempted, patients with the 325Ile allele were more resistant to re-canalisation (Fernandez-Cadenas et al., 2007).

1.7.8 TAFI Inhibitors

Although there are no known physiological inhibitors of TAFI, there are a number of naturally occurring inhibitors. These include potato tuber carboxypeptidase inhibitor (PCI), leech carboxypeptidase inhibitor (LCI) (Reverter et al., 1998) and tick carboxypeptidase inhibitor (TCI) (Arolas et al., 2005). These inhibitors have a biphasic effect on TAFI activity; at low concentrations they cause prolongation of clot lysis, whereas at high concentrations they cause an enhancement of lysis (Schneider and Nesheim, 2003). This biphasic effect is thought to be due to a TAFIa stabilizing effect at

low concentrations (Walker et al., 2003). The potential correlation between TAFI and thrombotic CVD has lead to interest in TAFI as a potential therapeutic target for the treatment of atherothrombotic disease, with a number of potential molecular targets affecting TAFI activity identified (Gils et al., 2005). A series of imidazolepropionic acids were designed that exhibited high potency and selectivity against TAFIa, without affecting other circulating carboxypeptidases. These proved effective in rabbit models at an intravenous dose of 100 μg/kg/min, resulting in increased thrombus dissolution (Bunnage et al., 2007). Further developments, giving rise UK-396082, lead to the characterisation of this inhibitor in rat models and humans (Owen et al., 2010). UK-396082 entered phase I clinical trials, but its development was discontinued in 2011 for unknown reasons. UK-396082 is a direct inhibitor of the active catalytic site of TAFI. Its chemical structure is shown in Figure 7. Another compound from Astrazenaca, AZD9684 (2-(6-aminopyridin-3-ylmethyl)-3-mercaptobutyric acid), progressed into phase II clinical trials, but its development was discontinued in 2007. More recently, nanobodies affecting various aspects of the TAFI pathway, from activation of TAFI by thrombin-thrombomodulin to the action of TAFIa, have been developed with the ongoing aim to develop a pro-fibrinolytic drug (Buelens et al., 2010). Substrate specific antibodies to TAFIa have been developed (TCK26D6 and TCK11A9), which in *in-vitro* functional assays are able to inhibit TAFIa when fibrin is used as a substrate, but not when osteopontin is used as a substrate (Semeraro et al., 2013). These antibodies have been developed to target specific areas in the TAFI activity cycle. MA-TCK26D6 modulates TAFIa by impairing the activation of TAFI mediated by thrombin and plasmin. It specifically prevents the interaction of TAFIa and fibrin, but has no effect on

other small proinflammatory substrates, such as osteopontin (Semeraro et al., 2013).
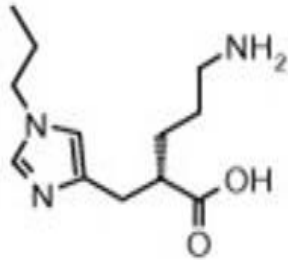
Figure 7. The chemical structure of UK-396082

UK-396082 is an imidazolepropionic acid, which is a known inhibitor of active TAFI (Bunnage et al., 2007).

1.7.9 TAFI and AAA

The only study specifically focussing on TAFI and AAA in humans was a small cohort based study of patients with AAA which showed that the activity of TAFI was significantly lower in patients with AAA compared with control subjects (Dubis et al., 2014). The main association between TAFI and the development of AAA has been inferred through work in the TAFI knockout mouse (TAFI-/-). In a study by Schultz et al (Schultz et al., 2010), animals underwent porcine pancreatic elastase (PPE) infusion into the abdominal aorta. TAFI-/- mice developed AAA sooner, and produced larger aneurysms which were more likely to rupture than their wild type controls. By day 3 post PPE infusion at a standard concentration of 15 units/ml, 66% had ruptured AAA, compared with no ruptures in the wild type AAA mice. Such changes were not apparent in TAFI-/+ heterozygote mice.

Furthermore, this group provided evidence for the role of plasmin in AAA development and rupture, as when TAFI-/- mice were given tranexamic acid (a potent inhibitor of plasmin formation from plasminogen), AAA formation was reduced and rupture was completely abolished.

Whilst murine models have been used extensively for the *in-vivo* study of TAFI, it is not clear how applicable findings related to murine TAFI may be to human disease. The amino acid sequence of mouse TAFI shares >85% identity with human TAFI, and includes conservation of the residues involved in glycosylation, zinc binding, substrate binding and substrate specificity (Hillmayer et al., 2006, Schatteman et al., 1999). In order to be able to better

model the effect of TAFI inhibitors on human TAFI *in-vivo*, transgenic mice which are devoid in murine TAFI but express human TAFI have been generated (the hTAFI mouse). These mice show delayed lysis time in whole blood rotational thromboelastometry (ROTEM) studies compared with the TAFI-/-mouse, but an increased lysis time compared with the wild type mouse with mTAFI. In addition, male hTAFI mice express hTAFI at 3.5 fold higher levels than female hTAFI mice (Mishra et al., 2014). Whilst the reasons for such gender disparities in this model are unknown, this is akin to the gender disparity reflected in other cardiovascular murine models, including the Angiotensin II model of AAA (see below).

1.8 Murine Models of AAA

Three main murine models of AAA have been described in the literature. Daugherty and Cassis have provided a useful review of these models (Daugherty and Cassis, 2004). A brief summary of the main features of each model is given below.

1.8.1 Elastase Infusion

This model involves infusion of elastase into the infra-renal segment of the aorta, and is based on the role of elastin degradation in AAAs. Originally used in rats (Anidjar et al., 1990), it has since been adapted for use in mice (Schultz et al., 2010, Pyo et al., 2000). In this model, the murine aorta is exposed, and a cannula inserted at the iliac bifurcation. A ligature is applied proximally (distal to the renal arteries) and distally (at the bifurcation itself). PPE type 1 is infused

intra-arterially (at a dose of 4-5 units/ml), at a constant pressure of 100 mmHg for 15 minutes. The ligatures are then removed and the arteriotomy closed with a single suture. The infusion itself causes an immediate and sustained dilatation of the aorta due to the mechanical effects of the procedure. By day 14, mice infused with elastase show destruction of the elastic lamellae, and the presence of inflammatory infiltrate (mainly macrophages) within the adventitia (Daugherty and Cassis, 2004).

1.8.2 Calcium Chloride

Initially developed in rabbits, application of calcium chloride to the arterial wall of hyperlipidaemic animals promoted the formation of AAA (Freestone et al., 1997). Adapted for use in mice, this method involves either placement of a gauze swab soaked in a calcium chloride solution, or direct placement of a concentrated solution (0.25-1 M), onto the infra-renal aorta (Chiou et al., 2001, Longo et al., 2002) for up to 20 minutes. This model also leads to structural disruption of the medial layer and inflammatory responses, but without any of the mechanical effects that are seen in the elastase infusion model (Daugherty and Cassis, 2004).

1.8.3 Angiotensin II Infusion

Subcutaneous infusion of Angiotensin II (Ang II) at doses of 500-1000 ng/kg/min, via subcutaneous osmotic mini pumps, into either LDL receptor -/- or ApoE-/- mice leads to the production of suprarenal AAAs (Daugherty et al., 2000, Zhang et al., 2012). This animal model is the only AAA model that also displays the sex dimorphism displayed in human AAA; that is, male ApoE-/- mice are up to 4 times more likely to develop AAA on infusion with Ang II than

females (Zhang et al., 2012). The pathophysiological mechanisms involved in aneurysm formation in this model have been studied by examining the aorta of ApoE-/- mice between days 1-56 post Ang II infusion. Within days of initiating Ang II infusion, there is macrophage accumulation in the media of the aortic region prone to AAA formation. Between 3-10 days, there is medial rupture, with dissection and dilatation of the aorta leading to haematoma/thrombus formation and subsequent inflammatory responses. This model of AAA formation is consistent with activation of an inflammatory response and the stimulation of a proteolytic cascade (Saraff et al., 2003), which then contributes to elastin degradation within the aortic wall.

Since its discovery over 25 years ago, Thrombin-Activatable Fibrinolysis Inhibitor has become a focus in the investigation of fibrinolysis in cardiovascular disease. Whilst a lot is already known about TAFI, there is still much to be established, both at a molecular level, and in relation to disease states. To my knowledge, there are only two previous studies that have investigated TAFI in relation to human AAA. The first measured TAFI zymogen levels using a commercially available ELISA kit in a small number of patients with ruptured (n=10) and non-ruptured (n=17) AAA. The validity of these results is uncertain, however, as the results quoted for TAFI levels in both groups were extremely high (in some cases >50 µg/ml), and fell outside the reference range of the ELISA (as quoted by the authors) of 12-20 µg/ml. No control group was included, and it is therefore unclear as to whether these values are a reflection of extremely high TAFI levels in patients with large AAA, or an error related to the assay (Hobbs et al., 2007). The second study to date compared TAFI activity in plasma from patients with AAA (n=32) and control subjects (n=45), and, using a commercially available chromogenic assay, found a reduction in TAFI activity in AAA patients compared to controls (Dubis et al., 2014). The experiments described in the following chapters aim to further identify the role of TAFI in AAA. This will be achieved through the use of both samples from humans with AAA, as well as *in-vivo* models.

1.8.4 Hypothesis

TAFI plays a role in the development and progression of AAA, and contributes to the alteration in the lysis of fibrin clots that has been demonstrated in patients with AAA. TAFI stabilises clot formation, and through the control of plasmin generation, regulates local proteolytic activity at the site of the aneurysm, as well as contributing to the systemically increased cardiovascular risk in this group of patients.

1.8.5 Aims

1. To investigate TAFI levels, TAFI activity and TAFI polymorphisms in patients with AAA in relation to AAA size, growth and clinical outcome.

2. To identify other proteins, aside from TAFI, which are involved in fibrinolysis and may contribute to the altered fibrinolysis seen in AAA.

3. To study the role of TAFI and TAFI inhibition in an *in-vivo* murine AAA model, and investigate fibrinolysis in plasma and aortic sections from this model.

Chapter 2   Experimental Design, Materials and Methods

The Assessment of the Role of TAFI in Human AAA

Given the evidence for a delay in the fibrinolysis in plasma samples of patients with AAA, and a link between TAFI and AAA formation in the porcine pancreatic elastase infusion model of AAA, a series of experiments were designed to establish if TAFI could be important in the regulation of lysis in patients with AAA.

2.1 Collection of plasma samples from patients with AAA

A large case-control cohort study of AAA patients and their controls, the Leeds Aneurysm Development Study (LEADS), was used to provide samples of DNA, plasma, whole blood and tissue for the study. The inclusion and exclusion criteria, as well as the study protocol, consent form and patient information sheet can be found in Appendices 1-3. Ethical approval for this study was granted by the Leeds East Research Ethics Committee (approval number 03/142). Patients were recruited and their samples collected by a senior research nurse, Mrs Anne Johnson.

2.2 Blood collection and the preparation of platelet poor plasma

Free flowing venous blood was collected from the antecubital vein of the non-dominant arm of AAA patients and their controls. Ten millilitres of blood was collected onto cold 0.1 M sodium citrate (9 parts of blood to 1 part of citrate), and then centrifuged at 4°C at 2,400 g for 20 minutes. The plasma supernatant was divided into 500 μl aliquots, flash frozen in liquid nitrogen and stored at -40°C until analysis.

DNA was extracted from whole blood. For this, 6 mls of blood was collected into a BD Vacutainer® K$_2$EDTA, and stored at -20°C until DNA extraction.

2.3 Normal pool plasma

For the optimisation of a number of assays, and also for the provision of a standard in the TAFI and TAFI-AP ELISAs, normal pool plasma (NPP) was used. This was produced by the collection of free-flowing venous blood from the antecubital fossa of the non-dominant arm of 25 healthy individuals. Collection of the NPP was approved by the Leeds NHS Trust Research Ethics Committee (HSLTM/12/045 and HSLT/09/020). Blood was again collected onto 0.1 M sodium citrate (9 parts blood to 1 part citrate), centrifuged for 20 minutes at 2,400 g to separate plasma, which was pooled together to create a mixture of all 25 individuals' plasmas. The plasma mix was then again divided into aliquots (0.5-1 ml) before being flash frozen using liquid nitrogen, and was stored at -80°C until required.

2.4 Measurement of TAFI antigen levels in plasma

Enzyme-linked immunosorbent assays (ELISA) were used to measure the plasma levels of intact TAFI (zymogen), TAFI activation peptide (TAFI-AP) and activated and inactivated TAFI in combination (TAFIa/TAFIai). The levels of TAFIa/TAFIai were measured using a commercially available ELISA kit (Asserachrom® TAFIa/TAFIai, ref 00616, Diagnostica Stago UK Ltd). Levels of intact TAFI and TAFI-AP were measured using ELISAs developed by the laboratory of Prof Ann Gils (KU Leuven). For these ELISAs, antibodies developed in KU Leuven were used. Plates were coated with MA-T12D11 (a

monoclonal antibody specific to the activation peptide of TAFI). For the intact TAFI ELISA, another monoclonal antibody, MA-T30E5A2-HRP was used as the secondary antibody. For the TAFI-AP ELISA, monoclonal antibody MA-T18A8-HRP was used as the secondary antibody. MA-T18A8 is also known to bind to the activation peptide. The site of binding of MA-T30E5A2 is not known, but during the development of these ELISAs, it was shown in combination with MA-T12D11 to preferentially detect the intact molecule, and not react with either the activation peptide or the activated TAFI (Ceresa et al., 2006a). The configuration of antibodies for each of these ELISAs are illustrated in Figure 8. Detailed protocols for each of the ELISAs used are described in the following sections.
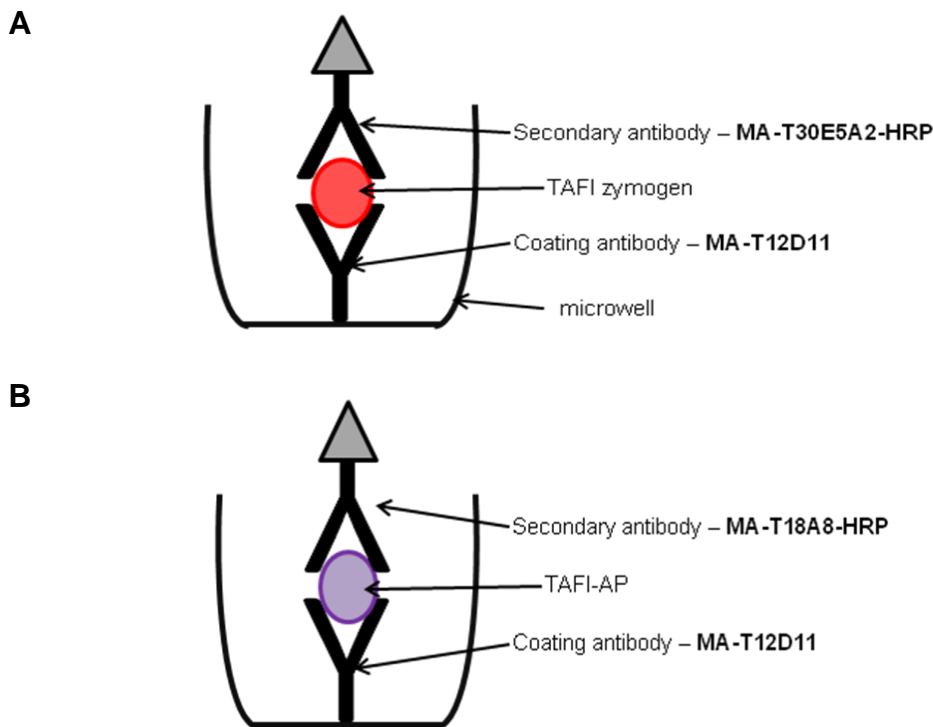
Figure 8. Antibody configuration in the TAFI and TAFI-AP ELISAs

Each well of a high-protein-binding plate is coated with a coating antibody which is specific to an epitope on the protein of interest. After an incubation period, each well is filled with a blocking buffer containing high protein levels, which bind to the remaining plate surface rendering it inaccessible to further protein binding. A plasma sample is added to each well, and the protein of interest binds to the coating antibody. Excess plasma is washed away, and a further antibody, specific to a different epitope of the protein of interest is added (Secondary Antibody, conjugated with HRP), and this binds to the protein already caught by the coating antibody. This conjugated antibody reacts with a peroxidase substrate to give a colour, which is proportional to the amount of protein in the plasma sample.

A – Antibody configuration for the intact TAFI ELISA. Plates are coated with MA-T12D11, which then binds to the activation peptide of TAFI present in the plasma sample. The secondary antibody MA-T30E5A2-HRP preferentially binds to the intact TAFI zymogen, so only intact TAFI zymogens are detected.

B – Antibody configuration for the TAFI activation peptide ELISA. Plates were coated with MA-T12D11, which binds to the activation peptide of TAFI. The secondary antibody in this case, MA-T18A8-HRP, also binds to the activation peptide, so only the TAFI activation peptide is detected.

2.4.1 TAFIa/TAFIai

Levels of TAFIa/TAFIai were measured using a commercially available ELISA (Asserachrom® TAFIa/TAFIai, ref 00616, Diagnostica Stago UK Ltd) according to the manufacturer's instructions. In brief, plasma samples and the high control were diluted 1:100 in dilution buffer. The low control was diluted 1:200 in dilution buffer. Samples (200 µl) were added to each well of the pre-coated microplate. Samples were incubated for 1 hour at room temperature, before the plate was emptied and the wells were washed five times using the wash buffer provided. Anti-TAFIa/TAFIai-peroxidase conjugate (200 µl) was added to each well, and was left to incubate for 1 hour at room temperature. The wells were emptied and washed five times, before 200 µl of tetremethylbenzidine, TMB, was added. This was left to incubate for exactly five minutes before the reaction was stopped with the addition of 50 µl of 1 M sulphuric acid, H_2SO_4. The absorbance was measured at 450 nm OD using a Molecular Devices SPECTRAmax Plus 384 plate reader (Molecular Devices LLC. USA).

2.4.2 TAFI and TAFI-AP

For both the intact TAFI ELISA and the TAFI-AP ELISA, the initial protocol is the same. Nunc-Immuno™ Maxisorp™ ELISA plates (Thermo Fisher Scientific Inc.) were coated with 200 µl of MA-T12D11 at a concentration of 4 µg/ml diluted in 0.01 M phosphate buffered saline, pH 7.4 (PBS, achieved by dissolving 5 tablets in 1 litre of distilled water, P4417 Sigma-Aldrich Co. LLC.). Plates were incubated for 72 hours at 4°C. After 72 hours, the wells were emptied and blocked with 300 µl per well of an albumin buffer (PBS containing

1% Bovine Serum Albumin, A8531 Sigma-Aldrich Co. LLC., BSA). The plates were incubated for two hours at room temperature. The wells were emptied, and washed four times with 300 µl of PBS buffer containing 0.002% TWEEN®-20 (P9416 Sigma-Aldrich Co. LLC.) using a BioTek MultiFlo automatic plate washer (BioTek Instruments Inc.). The wells were completely emptied by blotting onto absorbent paper, before being prepared for storage. Prior to storage, the wells were filled with 200 µl of mannitol-saccharose solution (100 g mannitol and 20 g saccharose per litre dH$_2$O), incubated for 2-4 minutes and then emptied completely by blotting on absorbent paper. The plates were covered in foil to protect them from moisture and light, and stored at -20°C until needed.

2.4.3 Intact TAFI ELISA

Immediately before use, the plates were allowed to reach room temperature, then washed four times with 300 µl of PBS containing 0.002% TWEEN®-20.

Plasma samples were collected and stored as described above. Plasma was diluted 1 part plasma in 160 parts PTAE buffer (0.01 M PBS containing 0.002% TWEEN®-20, 0.1% BSA and 5 mM Ethylenediaminetetraacetic acid, EDTA, E5134 Sigma-Aldrich Co. LLC.). The addition of EDTA was made in order to ensure inactivation of any TAFI in the sample via interaction with the zinc molecule present in the active site. Serial dilutions of this 1/160 sample in PTAE buffer were performed to give four concentrations for each plasma sample (1/160, 1/320, 1/640 and 1/1280). On each plate, two standard samples were included in order to test for inter-plate variability of the assay. These were a normal pool plasma sample, produced by pooling the plasma from 25 healthy

subjects, and a sample from a single healthy control (ABJ60). These standards were diluted to the same concentrations as the plasma samples to be tested (1/160-1/1280). Each plate also contained a standard curve, produced using purified TAFI (Enzyme Research Laboratories, Swansea, UK) diluted in PTAE buffer. The first point of the standard curve was diluted to 100 ng/ml, and then serial dilutions were performed to give a total of six dilutions (100, 50, 25, 12.5, 6.25 and 3.125 ng/ml respectively). A final well containing just PTAE buffer was used as a blank. Either plasma sample or standard solution (180 µl) was added to each well, and the plate was incubated overnight at 4°C. On all plates, equal numbers of samples from patients with AAA and control subjects were included. A sample plate lay out is shown in Figure 7.

Following overnight incubation, the wells were emptied, and washed four times using a 0.01 M PBS 0.002% TWEEN®-20 buffer. The wells were emptied completely by blotting against an absorbent paper. The antibody MA-T30E5A2-HRP was used as the capture antibody for intact TAFI. It was diluted 1/8000 in PTA buffer (0.01 M PBS containing 0.1% BSA and 0.002% TWEEN®-20), and 170 µl of this solution was added to each well. The plates were incubated for 2 hours at room temperature. Following incubation with conjugate, the wells were emptied, and washed four times using 0.01 M PBS 0.002% TWEEN®-20 buffer. After washing, the wells were again completely emptied by blotting on absorbent paper.

To produce a colorimetric reaction, 160 µl of a substrate solution containing *o*-Phenylenediamine (OPD) was added to each well. To prepare the substrate solution, a 20 mg OPD tablet (P7288 Sigma-Aldrich Co. LLC.) was dissolved in

30 mls of 0.05 M phosphate citrate buffer, pH 5.0 (containing 7.30 g Citric Acid, 251275 Sigma-Aldrich Co. LLC. and 11.87 g sodium phosphate dibasic, S7907 Sigma-Aldrich Co. LLC. in 1 litre dH_2O). For every 30 mls of substrate solution, 12 µl of 30% hydrogen peroxide, H_2O_2 (H1009 Sigma-Aldrich Co. LLC.), was added. The plates were then left to incubate for 45 minutes at room temperature. The plates were covered in foil during the incubation period to protect them from light. After 45 minutes, 50 µl of 4 M H_2SO_4 (320501, Sigma-Aldrich CO. LLC.) was added to each well to stop the reaction. The absorbance was measured using a Molecular Devices SPECTRAmax Plus 384 plate reader (Molecular Devices LLC. USA) at 492 nm OD. The TAFI content in the plasma samples was calculated using a standard curve generated by plotting the absorbance against the concentration of TAFI in the known standard TAFI samples. A sample standard curve is displayed in Figure 9.

A

	1	2	3	4	5	6	7	8	9	10	11	12	
A	TAFI 100 ng/ml	a	b	c	d	e	f	g	h	i	j	NPP	1/160
B	TAFI 50 ng/ml												1/320
C	TAFI 25 ng/ml												1/640
D	TAFI 12.5 ng/ml												1 /1280
E	TAFI 6.25 ng/ml	k	l	m	n	o	p	q	r	s	t	ABJ 60	1/160
F	TAFI 3.125 ng/ml												1/320
G	TAFI 0ng/ ml												1/640
H	B												1 /1280

B
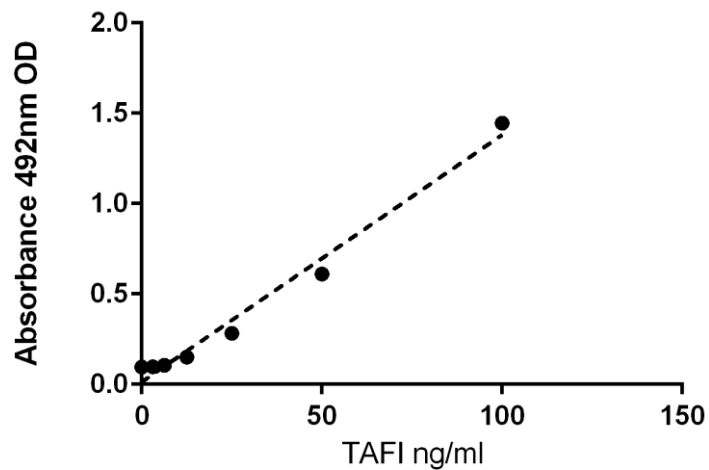
Figure 9. Intact TAFI ELISA

A – Sample layout of ELISA plates for the intact TAFI ELISA. A standard curve was produced using purified TAFI at known concentrations (column 1). Two standards were run on each plate (NPP and ABJ60), each at four concentrations between 1/160 and 1/1280. Twenty plasma samples, ten from patients and ten from controls, (a-t) were run on each plate, again at four concentrations between 1/160 and 1/1280.

B – Sample standard curve for the Intact TAFI ELISA, with known concentrations of TAFI plotted against absorbance at 492 nm OD.

2.4.4 Production of the TAFI-AP standard

A sample of TAFI-AP standard was provided by Prof Ann Gils, from the Laboratory for Therapeutic and Diagnostic Antibodies, KU Leuven. A stock of TAFI-AP standard was then produced in our laboratory using purified TAFI (Enzyme Research Laboratories, Swansea, UK) following her protocol, and this stock was compared to her original standard for TAFI-AP levels. Purified TAFI (final concentration 3.75 µg/ml) was incubated along with Calcium (CaCl$_2$, C1016 Sigma-Aldrich Co. LLC., final concentration 5 mM), Thrombin, (Human Thrombin from Plasma, 605190, Merck Chemicals Ltd., UK, final concentration 16 nM) and Thrombomodulin (Rabbit Lung Thrombomodulin, RP-43135, Thermo Scientific, USA, final concentration 15 nM), in a water bath at 37°C for exactly 13 minutes. The dilution buffer contained 25 mM HEPES (H3375 Sigma-Aldrich Co. LLC.), 137 mM NaCl (BP3581 Fisher Scientific, US) 3.5 mM KCl (10417460 Fisher Scientific, US), 3 mM CaCl$_2$ and 0.1% BSA, pH 7.4. After 13 minutes, D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (PPACK, sc-201291, Santa Cruz Biotechnology Inc.) was added to a final concentration of 30 µM in order to stop any further TAFI activation. The solution was then aliquoted and stored at -80°C until needed. This standard (from here on referred to as KB13), was compared to the standard prepared in the same way and used as a standard for the TAFI AP ELISA at KU Leuven (S13). Two standard curves were prepared on different plates for each standard using the ELISA protocol as laid out below, and they were compared to ensure the similarity of our standard (KB13) to the original standard (S13). These are shown in Figure 10.
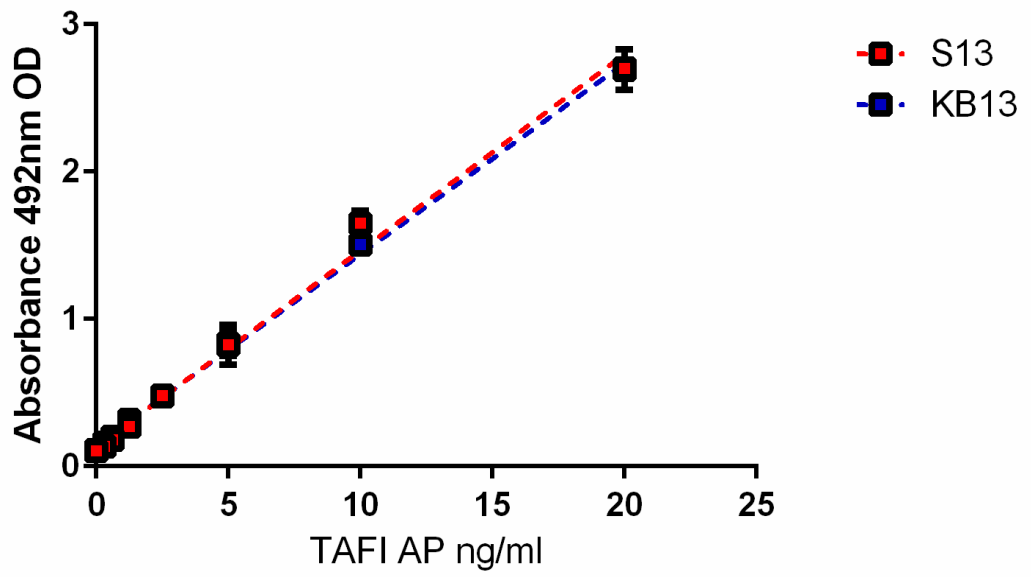
Figure 10. TAFI-AP standard production

KB13 was produced using the method for generation of the activation peptide standard provided by KU Leuven. This was compared to the Leuven standard (S13) in the TAFI-AP ELISA at a range of concentrations between 0-20 ng/ml.

2.4.5 TAFI-AP ELISA

Plasma samples were collected and stored as described above. Plasma was diluted 1 part plasma in 40 parts PTAE buffer (0.01 M PBS containing 0.002% TWEEN®-20, 0.1% BSA and 5 mM EDTA). Serial dilutions of this 1/40 sample in PTAE buffer were performed to give four concentrations for each plasma sample (1/40, 1/80, 1/160 and 1/320). On each plate, two standard samples were included in order to test for inter-plate variability of the assay. These were a NPP sample, produced by pooling the plasma from 25 healthy subjects, and a sample from a single healthy control (ABJ60). These standards were diluted to the same concentrations as the plasma samples to be tested (1/40-1/320). Each plate also contained a standard curve, produced using KB13, as described above, diluted in PTAE buffer. The first point of the standard curve was diluted to 20 ng/ml, and then serial dilutions were performed to give a total of six dilutions (20, 10, 5, 2.5, 1.25 and 0.65 ng/ml respectively). A final well containing just PTAE buffer was used as a blank. 180 µl of either plasma sample or standard solution was added to each well, and the plate was incubated overnight at 4°C. A sample of how the plate was laid out is shown in Figure 11.

Figure 11. TAFI-AP ELISA

Sample layout of the TAFI-AP ELISA plate. A standard curved was produced by diluting the activation peptide standard KB13 to a range of concentrations between 0-20 ng/ml (column 1). Two standards were run on each plate (NPP and ABJ60), each at four concentrations between 1/40 and 1/320. Twenty plasma samples (a-t) were run on each plate, again at four concentrations between 1/40 and 1/320.

Following overnight incubation, the wells were emptied, and were washed four times using a 0.01 M PBS 0.002% TWEEN®-20 buffer. Wells were emptied completely by blotting against an absorbent paper. The antibody MA-T18A8-HRP was used as the capture antibody for TAFI-AP. It was diluted 1/8000 in PTA buffer (0.01 M PBS containing 0.1% BSA and 0.002% TWEEN®-20), and 170 µl of this solution was added to each well. The plates were incubated for 2 hours at room temperature. Following incubation with conjugate, the wells were emptied, and were washed four times using 0.01 M PBS 0.002% TWEEN®-20 buffer. After washing, the wells were completely emptied by blotting on absorbent paper.

To produce a colorimetric reaction, 160 µl of a substrate solution containing *o*-Phenylenediamine (OPD) was added to each well. This was prepared as for the intact TAFI ELISA, described above. The plates were then left to incubate for 45 minutes at room temperature. The plates were covered in foil during the incubation period to protect them from light. After 45 minutes, 50 µl of 4 M H_2SO_4 was added to each well to stop the reaction. The absorbance was measured using a Molecular Devices SPECTRAmax Plus 384 plate reader (Molecular Devices LLC. USA) at 492 nm OD. The TAFI-AP content in the plasma samples was calculated using a standard curve generated by plotting the absorbance against the concentration of TAFI-AP in the standard TAFI-AP samples.

2.5   TAFI activity levels – Functional TAFI Assay

TAFI activity was measured using a modified version of the functional TAFI assay developed by Guimaraes et al (Guimaraes et al., 2005).

For the initial experiments, plasma samples prepared as above were diluted sequentially in TAFI-deficient plasma (Enzyme Research Laboratories, Swansea, UK). Diluted plasma samples (100 µl) were added to the wells of a Greiner bio-one microtitre plate containing 25 µl of the reaction mix, composed of human thrombin (0.5 units/ml), rabbit lung thrombomodulin (0.6 units/ml), recombinant tPA (Technoclone GmbH, Austria, 0.10 µg/ml), $CaCl_2$ (20 mM), and where stated, Potato-Carboxypeptidase Inhibitor (C0279 Sigma-Aldrich Co. LLC.), PCI (30 µg/ml), in 50 mM HEPES buffer, pH 7.4 with 0.1% BSA. Concentrations refer to the final concentrations in the assay solution. The plate was then immediately placed in a pre-warmed (37°C) incubation chamber of a Molecular Devices SPECTRAmax Plus384 plate reader (Molecular Devices LLC. USA). The optical density at 405 nm was measured every minute for 150 minutes. For each plasma sample, a control with PCI was included.

Lysis time (LT) is defined as the time point corresponding to a 50% decrease in optical density. The TAFI-related retardation of fibrinolysis was determined for each sample by subtracting the LT in the presence of PCI (LT_{+PCI}) from the LT in the absence of PCI (LT_{-PCI}). This is illustrated in Figure 12.

A standard curve using the known dilutions of normal pool plasma was used to determine a sensitive range for the determination of TAFI-related lysis retardation in future experiments. The dilution which gave the most sensitive and reliable results in these initial experiments was 1 parts plasma to 9 parts

diluent. For the screening of multiple plasma samples, Tris-buffered saline (TBS) was used as the diluent, containing 50 mM Tris-Base (BP1521, Thermo Scientific Ltd.), 100 mM NaCl, pH 7.4. Compared to TAFI-deficient plasma, using TBS as the diluent seemed to give more consistent results, especially once a lower concentration of thrombin was used (0.5 units/ml vs. 3.3 units/ml as per the Guimaraes et al. method). These curves are shown in Figure 12.

TAFI-specific anti-fibrinolytic activity was calculated by determining the ratio between TAFI-related retardation to lysis time (min) and TAFI antigen levels (µg/ml) as determined using the ELISA.

A

B
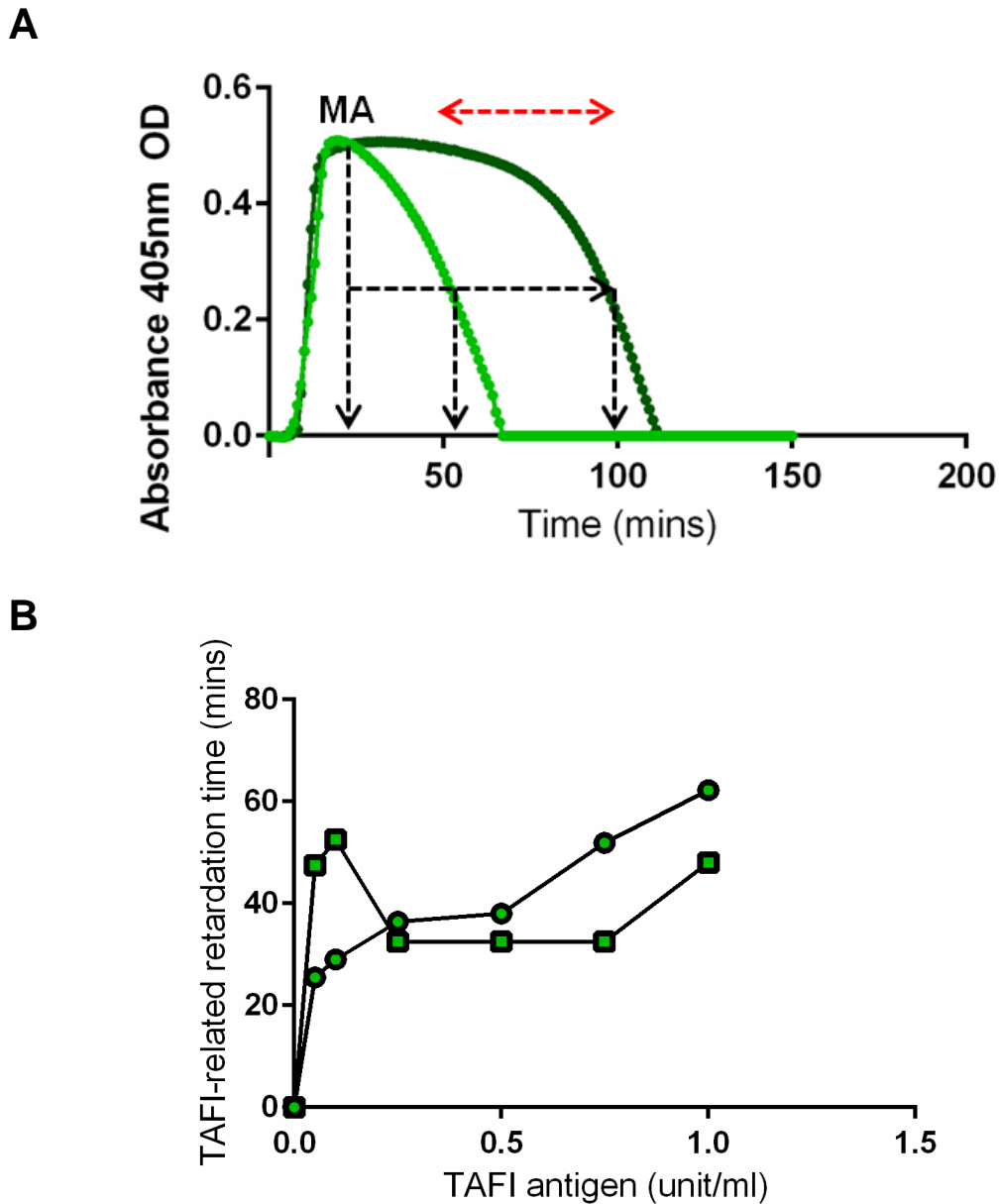
Figure 12. A functional assay of TAFI activity in plasma

A – Turbidity/lysis assays were performed for all samples in the presence (light green) and absence (dark green) of potato carboxypeptidase inhibitor (PCI). The time from the point of maximum absorbance (MA) until the time when MA reduced by half (or the time to half lysis) was measured. The difference in the time to half lysis in absence of PCI compared with the presence of PCI (red dotted arrow) represents the TAFI-related retardation to lysis time.

B – TAFI-related retardation to lysis time in samples of known concentrations of TAFI (0-1 unit/ml) generated using normal pooled plasma. The green squares represent the assay conducted as per Guimaraes et al., using TAFI-deficient plasma as a diluent and thrombin at a final concentration of 3.3 unit/ml. The green circles represent the assay performed using a modified protocol with TBS used as the dilutent and Thrombin at a final concentration of 0.5 unit/ml.

2.6 Turbidity-Lysis in patients with AAA

Previous work from our group has shown that there is a significant delay in the lysis of clots produced using the plasma of patients with AAA when compared with control subjects (Scott et al., 2011). In order to establish if this difference could be attributable to TAFI, serial turbidity lysis experiments were performed using plasma from patients, in which TAFI was inhibited using PCI, and controls, in which additional TAFI was added in order to enhance the effect of TAFI on lysis time.

Plasma samples were collected and stored as described above (Section 2.2). A sample of patients with large AAA (aneurysm diameter > 5 cm), (n=32) and control subjects (n=32) were selected at random from the LEADS database. In each well of a 96-well Greiner bio-one microtitre plate, 25 µl of plasma was added to 75 µl of TBS. Fibrin clot formation was achieved by the addition of 50 µl of an activation mix containing thrombin (0.2 units/ml), $CaCl_2$ (7.5 mM) and tPA (41 ng/ml). Concentrations shown are the final concentrations per well. All samples were run in duplicate. The absorbance was measured at 340 nm OD every 12 seconds for 250 minutes using a ThermoScientific MultiSkan™ Go 3.2 spectrophotometer (Thermo Fisher Scientific Inc. USA).

The experiment was repeated with the addition of 30 µg/ml (final concentration per well) PCI to the AAA samples, and the addition of purified TAFI (Enzyme Research Laboratories, Swansea, UK) to the control samples. TAFI was added to a final concentration of 15 µg/ml in the plasma (the upper limit of normal in human plasma), so that all samples had an excess of TAFI of 15 µg/ml over

their original TAFI concentration. Again, 50 µl of an activation mix containing thrombin (0.2 units/ml), $CaCl_2$ (7.5 mM) and tPA (41 ng/ml) was added, and the absorbance was read at 340 nm OD every 12 seconds for 250 minutes.

All samples were then repeated with the addition of Thrombomodulin (final concentration per well 0.6 units/ml) to the activation mix.

2.7 TAFI Thr325Ile, Alpha-2-Antiplasmin Arg6Trp and Arg407Lys, and tPA Polymorphisms

These four common polymorphisms of proteins involved in fibrinolysis were investigated in a large sample of patients and control subjects (n=602 and n=490 respectively). DNA was extracted from samples of whole blood using a commercially available kit (QIAamp Blood Maxi Kit, QIAGEN). DNA was extracted from blood with assistance from a laboratory technician, Mr Fraser Macrae. A full protocol can be found in Appendix 4.

The DNA concentration in the resulting solution was determined using a Nanodrop (ND-1000 Spectrophotometer, LabTech International), using the 260 nm/280 nm absorbance ratio and 2 µl of solution. Each 6 mls of blood yielded 2 mls of 70-200 ng/µl genomic DNA. All samples were diluted to 10 ng/µl using DNA and RNA free Tris EDTA buffer solution (93283, Sigma-Aldrich Co. LLC.), and stored at 4°C until genotyping.

TaqMan single nucleotide polymorphism (SNP) Genotyping Assays (Life Technologies) were used for each of the polymorphisms (TAFI Thr325Ile rs1926447, α2AP Arg6Trp rs2070863, Arg407Lys rs1057335 and tPA 7351C→T rs2020918). The probe was added to TaqMan Genotyping

MasterMix (Life Technologies, Thermo Fisher Scientific Ltd.) in a 20:1 ratio (henceforth referred to as MasterProbe). MasterProbe (2.7 µl) and 2.2 µl of the diluted DNA (10 ng/µl) were added to each well of a 384 well plate. Two no template controls (containing only MasterProbe) were included on each plate. A Roche LightCycler480 (Roche Diagnostics, Burgess Hill, UK) was used for programming the real time polymerase chain reaction, PCR, with each programme consisting of 50 amplifications to 95°C. End-point genotyping was used to determine genotype frequencies. Correct genotype was called in each plate with an accuracy of 96-100%; samples which were not called successfully on the first attempt were subsequently repeated. In addition to the no template controls, random samples were repeated on each plate to ensure reproducibility of the results. Only samples for which all four genotypes could be determined were included in the final analysis.

2.8 Histological Examination of the Intra-Luminal Thrombus of patients with AAA

Samples of ILT were collected from patients with AAA undergoing open AAA repair, according to the protocol of the LEADS study (see Appendix 1). Sections of full-thickness ILT were collected into normal saline (NaCl 0.9%) and kept on ice for transportation. On arrival in the laboratory, samples were transferred into a 4% paraformaldehyde solution, and incubated at 4°C for at least 24 hours. After a minimum of 24 hours, these samples were dehydrated using a 17 hour programme (detailed in Appendix 7) on a Leica TP1020 tissue processing carousel (Leica Microsystems) and embedded in paraffin (Sigma-Aldrich Co. LLC.). Blocks were sectioned using a Leica RM2125 microtome (Leica

Microsystems) into 5 µm sections, and collected onto slides (4951PLUS4 Superfrost™Plus, Thermo Scientific Ltd), left to dry at 37°C overnight, and stored before staining. Slides were stained with Martius Scarlet Blue in order to study the fibrin composition within the ILT. Details of this method are described in Section 2.22.5.

Inhibition of TAFI in a Murine Model of Abdominal Aortic Aneurysm

A murine model of AAA was used to further investigate the molecular mechanisms underpinning the role of TAFI in AAA. An overview of all the mice used in the study is displayed in Figure 13.

All murine studies were carried out within UK Home Office Regulations under Project Licence 40/3523 (Dr S Wheatcroft) and Personal Licence 70/25000 (Miss K Bridge).
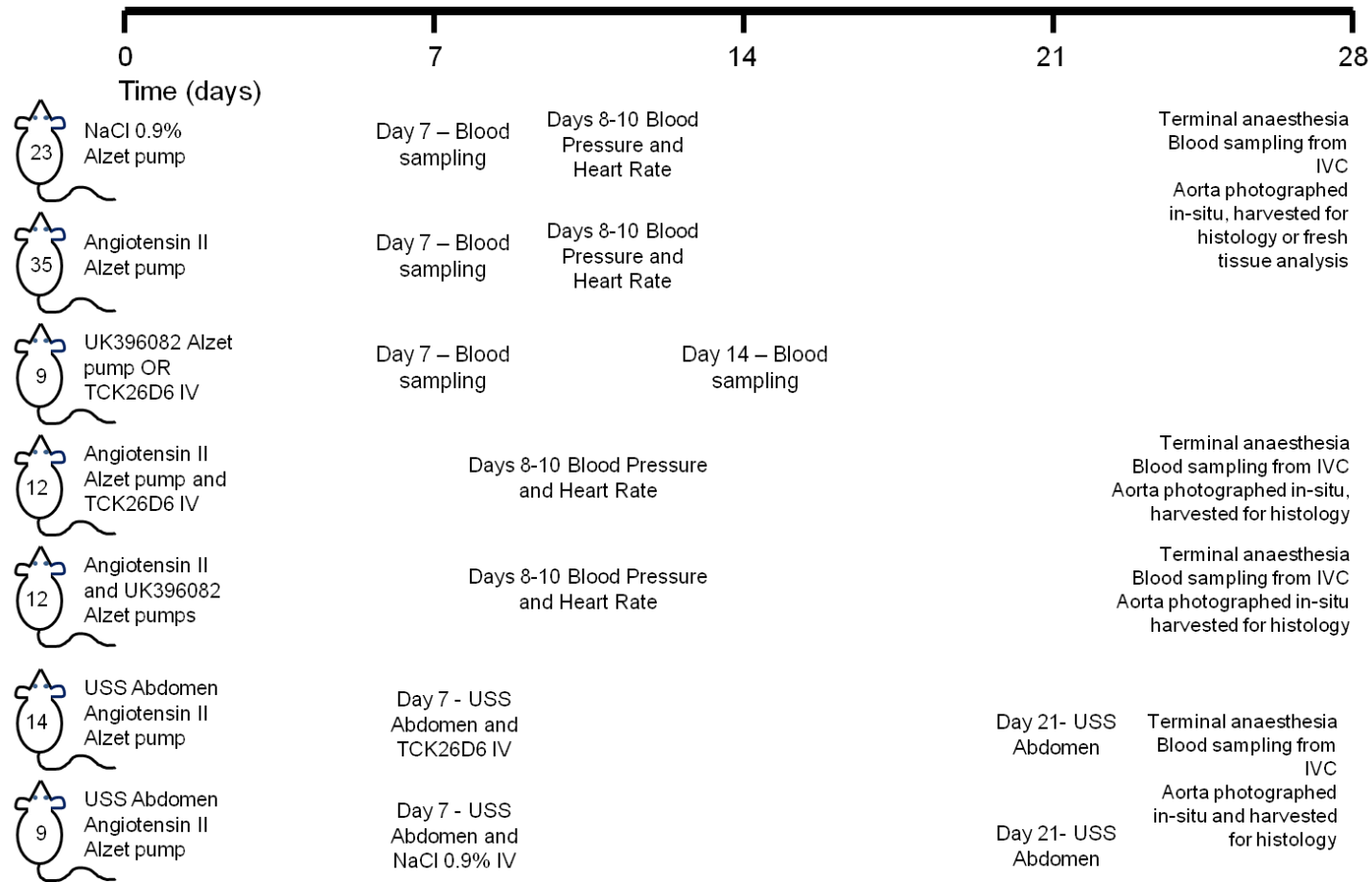
Figure 13. Overall Murine Plan

In total, seven separate experimental conditions were included; these groups are shown on the right hand side, with the numbers used for each condition displayed within the outline of the mouse. The different treatments included normal saline infusion (NaCl 0.9%), Angiotensin II infusion, TAFI inhibitors UK-396082 (delivered via subcutaneous mini-osmotic pump) and MA-TCK26D6 (delivered via intravenous, IV, injection). In later experiments, AAA development was monitored using ultra sound scanning (USS). IVC = inferior vena cava, IV = intravenous injection.

2.9 AAA formation in the Angiotensin II model of AAA

At 12-16 weeks of age, male Apolipoprotein-E knockout mice (ApoE-/-) backcrossed onto a C57BL/6 background, and maintained on standard sterilized laboratory diet and water, were administered subcutaneous infusions of Angiotensin II (A9525 Sigma-Aldrich Co. LLC.) for 28 days via mini-osmotic pumps (Alzet® model 1004, obtained from Charles River Laboratories, Germany), based on the method designed by Daugherty et al (Daugherty et al., 2000).

Initially, in order to optimise the experimental protocol and gather information about AAA formation rates using the Angiotensin II model in the absence of any TAFI inhibitor, two groups of mice were used. Mice were treated with either Angiotensin II (Ang II), at a dose of 750 ng/kg/min for 28 days, or a control pump containing normal saline (NaCl 0.9%).

2.9.1 Alzet® Mini Pump Insertion

In brief, mice were anaesthetised in an anaesthetic chamber using volatile isoflurane at an initial dose of 5 litres/min with 2 litres/min of oxygen. Once mice were anaesthetised, they were weighed, and then transferred onto a warmed operating table. Anaesthesia was maintained using isoflurane at 1.5-2 litres/min with 2 litres/min of oxygen. At induction, mice were administered with Vetergesic® (Reckitt Benckiser) as an intra-peritoneal injection (dose range 0.1-1 mg/kg) in order to minimise post operative pain. A small amount of Lubrithal™ Ophthalmic Eye Gel was applied to the eyes of the mice to prevent drying of the cornea during anaesthetic. Mice were then laid prone, and their position secured using Micropore™ surgical tape. A small area of the dorsal surface of

the neck was shaved using an electric razor, and the site of the incision cleaned using 70% ethanol. A small midline incision was made, and a subcutaneous channel created along the left dorsal flank, lateral to the spine, using closed needle holders. Alzet® mini-pumps were prepared containing sufficient drug concentrations for the weight of each animal (See Appendix 5). Pumps were inserted cap-first into the subcutaneous pouch and the incision closed using a continuous 6-0 vicryl® suture (Ethicon US LLC). Upon completion, mice were ear notched to allow for individual follow up (see Appendix 6), and given an intra-peritoneal bolus of warmed NaCl 0.9% (25 mls/kg) before recovery. Once awake, mice were placed in a warm, clean cage, and returned to their normal cage environment once fully recovered. The procedure for the insertion of a mini-pump is illustrated in Figure 14.
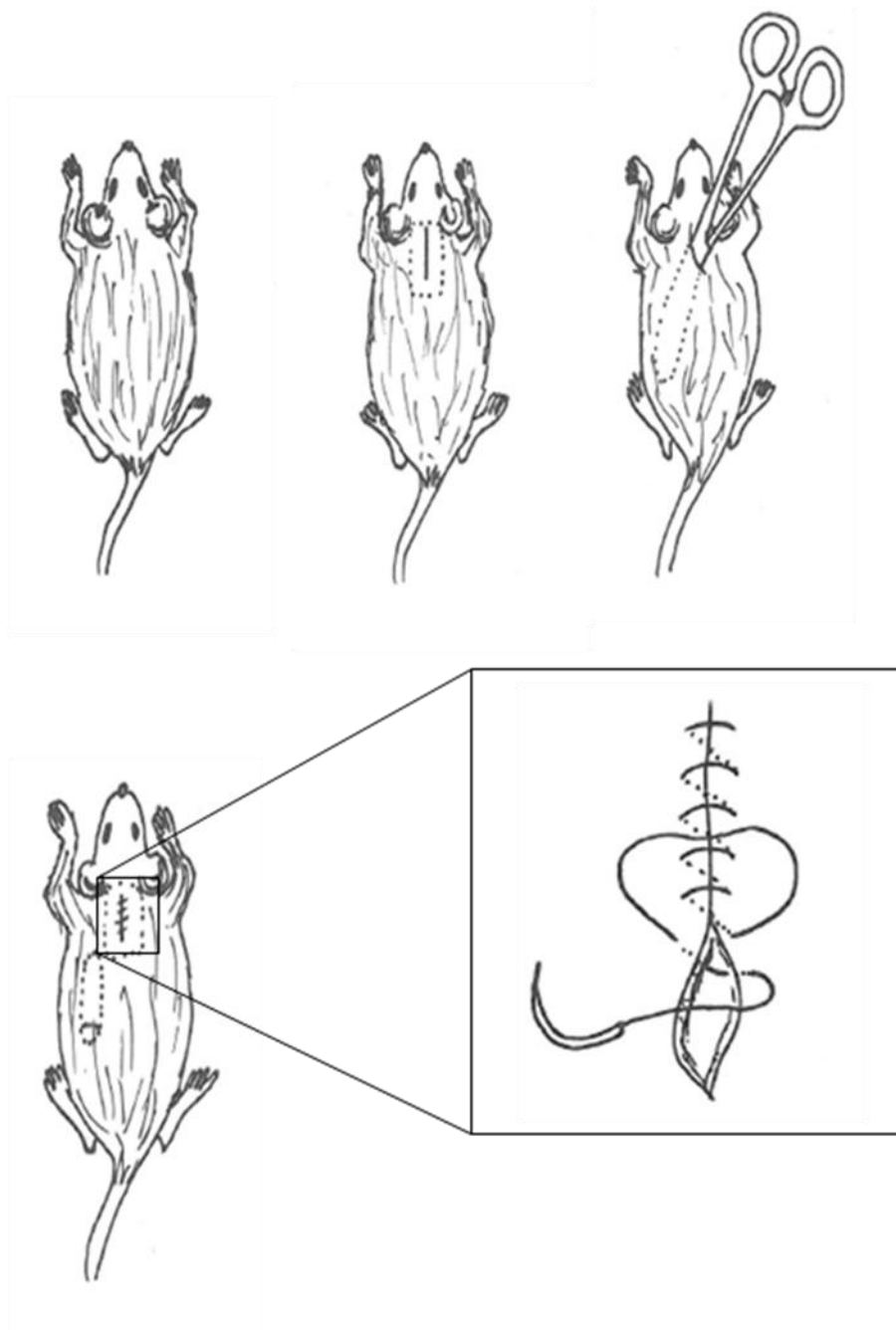
Figure 14. Insertion of an Alzet mini-osmotic pump under general anaesthesia

Mice were laid prone, and their position secured using micropore surgical tape. Anaesthesia was maintained using inhaled isoflourane 2 litres/min. Fur was removed from a small area between the shoulder blades, and a longitudinal incision made through the skin. A subcutaneous pouch was created using blunt dissection, by inserting a pair of closed needle holders through the incision and along the left flank. An Alzet mini-osmotic pump was inserted cap-first into the pouch, and the wound closed using a continuous suture of 6-0 vicryl.

Following pump insertion, mice were closely monitored. Their wounds were carefully observed to ensure there was no evidence of any postoperative infection. The established mortality rate related to this model is between 30-40%; this most commonly occurs between days 3-8 post-operatively and is due to aortic rupture. During this 'at risk' period, mice were observed at least twice daily, and any animals showing signs of pain or distress were terminated using Home Office Schedule 1 approved methods.

2.9.2 Non-invasive Blood Pressure and Heart Rate measurement by tail cuff plethysmography

Following mini-pump insertion, mice underwent non-invasive monitoring of their heart rate (HR) and blood pressure (BP) using the CODA device (Kent Scientific Corporation). In brief, animals were restrained using an animal nose cone holder and kept in a chamber warmed to 37°C. The occluder cuff (O-cuff) and the volume pressure recording (VPR) cuff were placed on the tail of the mouse, approximately 0.5 cm from the base of the tail, with the O-cuff lying proximal to the VPR cuff. The CODA device was set to record a total of 28 readings. The first ten readings of any session were set as acclimatisation readings and were discarded. Following this, there were three sets of six cycles, with 5 seconds between sets and 5 seconds rest between cycles. All mice underwent a 'training session' where blood pressure measurements were not recorded in order to allow them to become familiar with the environment and the test. On two subsequent occasions, the test was repeated, and measurements recorded. This was done to ensure that there was no false hypertension or tachycardia related to the stress of the examination. BP and HR readings over a session were analysed for quality. All readings within a single session that met the

quality criteria (flow volume was measured, the animal was not moving, the graph trace was deemed to be 'good') were then averaged to give a result of BP and HR for each mouse. A sample trace is shown in Figure 15.
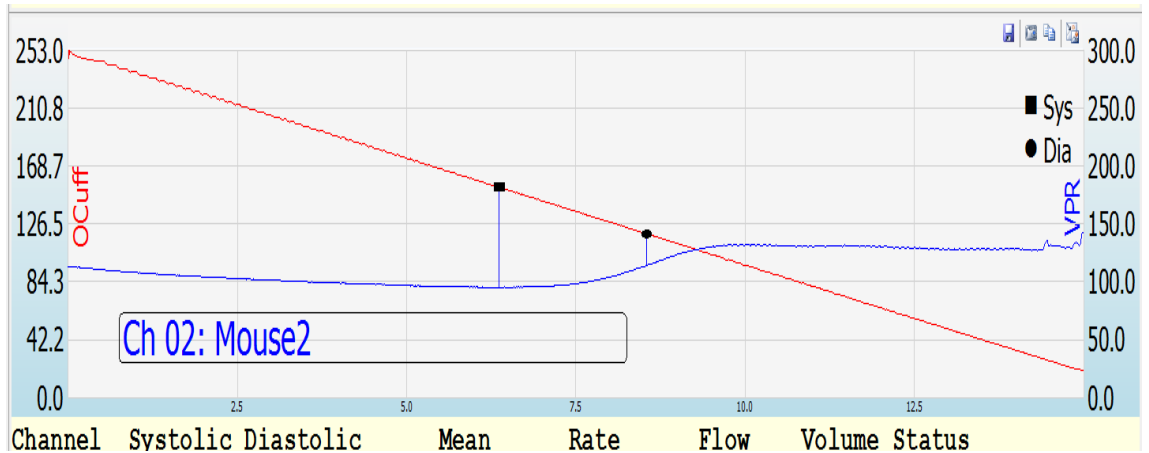
Figure 15. Sample reading from tail cuff plethysmography, used for the non-invasive measurement of heart rate and blood pressure in mice

In order to measure blood pressure and heart rate, mice were restrained using an animal nose cone device. They were kept at 37°C throughout the course of the experiment. Two tail cuffs were applied to the tail of the mouse, with the O-cuff proximal to the VPR cuff. For each session, there were 10 acclimatisation cycles, then 3 sets of 6 cycles when measurements were taken. The occlude trace (shown in red) falls steadily, whilst the VPR trace rises slowly. Where the VPR trace rises and before the two traces intersect, and measurement of blood pressure is taken. Heart rate, flow rates and volume are also measured, and in combination allow for an assessment of the quality of the trace.

2.9.3 Termination of the Experiment and Blood Collection

At 4 weeks post-pump implantation, mice were anaesthetised using inhaled isoflurane at 5 litres/min, then maintained at 2 litres/minute until the point of death. Mice were placed supine, the abdomen cleaned with 70% ethanol, and a midline incision made along the linea alba from pelvis to sternum. The abdominal contents were moved to the right to expose the inferior vena cava (IVC). IVC puncture was performed using a 26 G needle on a 1 ml syringe (BD Plastipak ^{TM}), and the whole blood volume was collected (up to 1 ml) onto 0.1 M sodium citrate at a ratio of 9 parts blood to 1 part citrate. Blood was then stored on ice until centrifugation and storage. Following exsanguination, the thorax was opened through a midline incision. The left ventricle was cannulated, and the circulation was perfused with 8-10 mls of phosphate buffered saline (PBS). If the aorta was to be taken for paraffin block fixation, this PBS infusion was then followed by 5 mls of 4% Paraformaldehyde, PFA (P6148, Sigma-Aldrich Co. LLC.). Macroscopic imaging of the aorta was undertaken using the OPMI-PICO videomicrometer (Carl Zeiss AG).

2.9.4 Processing of Blood Samples

Samples of whole blood collected from IVC were kept on ice until processing. They were then centrifuged at 14,500 rpm for 20 minutes using a table-top micro-centrifuge (Eppendorf MiniSpin plus, Scientific Laboratory Supplies Ltd.UK). The supernatant was collected into a clean Eppendorf tube, flash frozen in liquid nitrogen, and stored at -40°C until analysis.

2.9.5 Harvesting of the Aorta and preparation for *ex-vivo* study

The aorta was harvested en-bloc from the mid-thoracic level to below the left renal artery. For protein studies, a sample of liver (for control) and the aorta were flash frozen in liquid nitrogen. For histological examination, the aorta was placed in 4% PFA for a minimum of 24 hours before being processed. After a minimum of 24 hours, these samples were dehydrated using a 17 hour programme (detailed in Appendix 7) on a Leica TP1020 tissue processing carousel (Leica Microsystems) and embedded in paraffin (Sigma-Aldrich Co. LLC.). Blocks were sectioned using a Leica RM2125 microtome (Leica Microsystems) into 5 μm sections, and collected onto slides (4951PLUS4 Superfrost™Plus, Thermo Scientific Ltd), left to dry at 37°C overnight, and stored before staining.

2.10 Administration of TAFI inhibitors

Following optimisation of the Ang II model of AAA, mice were treated with inhibitors of TAFI. Two different inhibitors were used separately. Their site of action is shown in Figure 16. The first, UK-396082, a small molecule inhibitor of activated TAFI developed by Pfizer (Bunnage et al., 2007), was resynthesised by Dr Richard Foster and Dr Charlotte Revill of the School of Chemistry at the University of Leeds. The dosing of this inhibitor was based upon previous studies in rats (Owen et al., 2010). After discussion with the University Veterinarian, a dosing regimen for subcutaneous infusion taking into account bioavailability and half-life was selected. The drug was dissolved in a solution of normal saline, and was administered at a dose of 30 mg/kg/min via a subcutaneous Alzet® 1004 mini-osmotic pump. The drug was initially tested for

toxicity, before being administered in conjunction with Ang II. When given in combination, two mini-osmotic pumps were inserted under the same general anaesthetic. As described above, a pump containing Ang II was inserted on the left flank, and a second pump containing UK-396082 was implanted on the right flank.

The second inhibitor used in this model was MA-TCK26D6, a monoclonal antibody that targets the plasmin-mediated activation of TAFI. This inhibitor was provided as a generous gift from Prof Ann Gils (KU Leuven). In previous studies from the laboratory of Ann Gils, a dose based on a 26-fold antibody:TAFI ratio (26 mg/kg) was used. All of these experiments, however, were done under terminal anaesthesia, and so injection volume did not need to be limited. Indeed, the dose used in the laboratory of Prof Gils (26 mg/kg) required an IV injection volume which exceeds the amount permitted by the UK Home Office Licence for recovery experiments (which suggests that the maximum volume for administration should be no more than 200 µl in a single IV dose). Further work in their laboratory had also suggested that the half-life of antibody inhibitors was 14 days in murine models. Mice were therefore given a single IV dose of MA-TCK26D6 to last the duration of the experiment (28 days), with the volume injected set as the maximum amount permissible by the UK Home Office (200 µl IV of 2.179 mg/ml MA-TCK26D6, 435.8 µg per mouse, which for an average 25 g mouse is equivalent to 17 mg/kg). Injections were administered under direct vision into the femoral vein.

2.10.1 Femoral Vein Injection of MA-TCK26D6

Mice were anaesthetised in an anaesthetic chamber using volatile isoflurane at an initial dose of 5 litres/min with 2 litres/min of oxygen. Once fully anaesthetised, mice were weighed, and then transferred onto a warmed operating table. Anaesthesia was maintained using isoflurane at 1.5-2 litres/min with 2 litres/min of oxygen. At induction, mice were administered with Vetergesic® (Reckitt Benckiser) as an intra-peritoneal injection (dose range 0.1-1 mg/kg) in order to minimise post-operative pain. Mice were laid supine and their limbs held in position using Micropore™ surgical tape. Fur was removed from a small area over their left groin using Veet® hair removal cream. A small incision was made slightly inferior to the inguinal ligament, and the femoral vein exposed using blunt dissection. Using a 31 G needle (328280 BD ULTRA-FINE™ Needle Insulin Syringe, Beckton Dickinson and Company), 200 µl of MA-TCK26D6 or NaCl 0.9% (control) was injected under direct vision into the femoral vein. Pressure was applied to the site of venepuncture immediately after removal of the needle using a small piece of sterile gauze for two minutes. Once haemostasis was ensured, the gauze was carefully removed by dampening with NaCl 0.9% to allow a smooth removal without dislodging any clot that may have formed. The wound was then washed using 0.9% NaCl. The fat was closed over the vein using a single stitch of 8-0 Vicryl®, before the skin was closed using continuous suturing of 8-0 Vicryl®. The procedure for femoral vein injection is illustrated in Figure 17.

Prior to administration in combination with Ang II, MA-TCK26D6 was given in isolation to 8 mice. Mice were sacrificed at 7, 10 and 28 days, and blood taken from the IVC for ROTEM analysis. There was no evidence of any toxicity or

adverse effects of MA-TCK26D6. Mice behaved normally, and showed no spontaneous bleeding.

Mice were then given Ang II and MA-TCK26D6 in combination. Under a single general anaesthetic, a subcutaneous mini-osmotic pump containing Ang II (750 ng/kg/min) and an IV injection of MA-TCK26D6 (200 µl) were administered as described above.
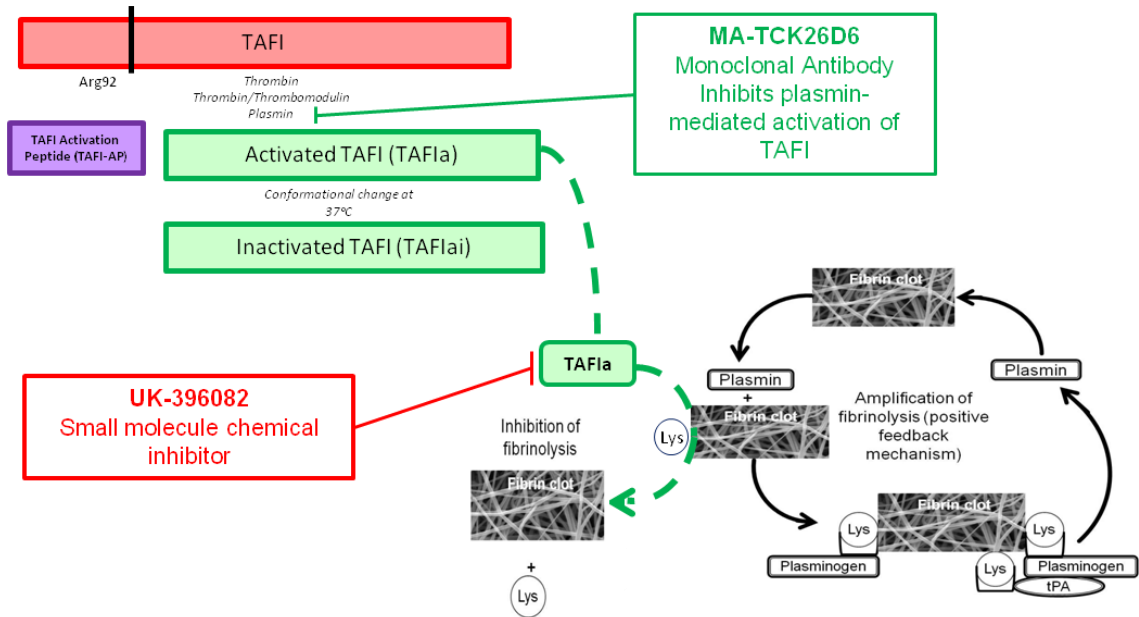
Figure 16. Site of action of TAFI inhibitors MA-TCK26D6 and UK-396082

Two inhibitors of TAFI were used. MA-TCK26D6 is a monoclonal antibody which inhibits plasmin-mediated activation of TAFI to TAFIa, and spares TAFIa's anti-inflammatory properties. UK-396082 is a small molecule inhibitor which directly binds and inhibits the catalytic site of TAFIa, leading to inhibition of all TAFI activity in the circulation.
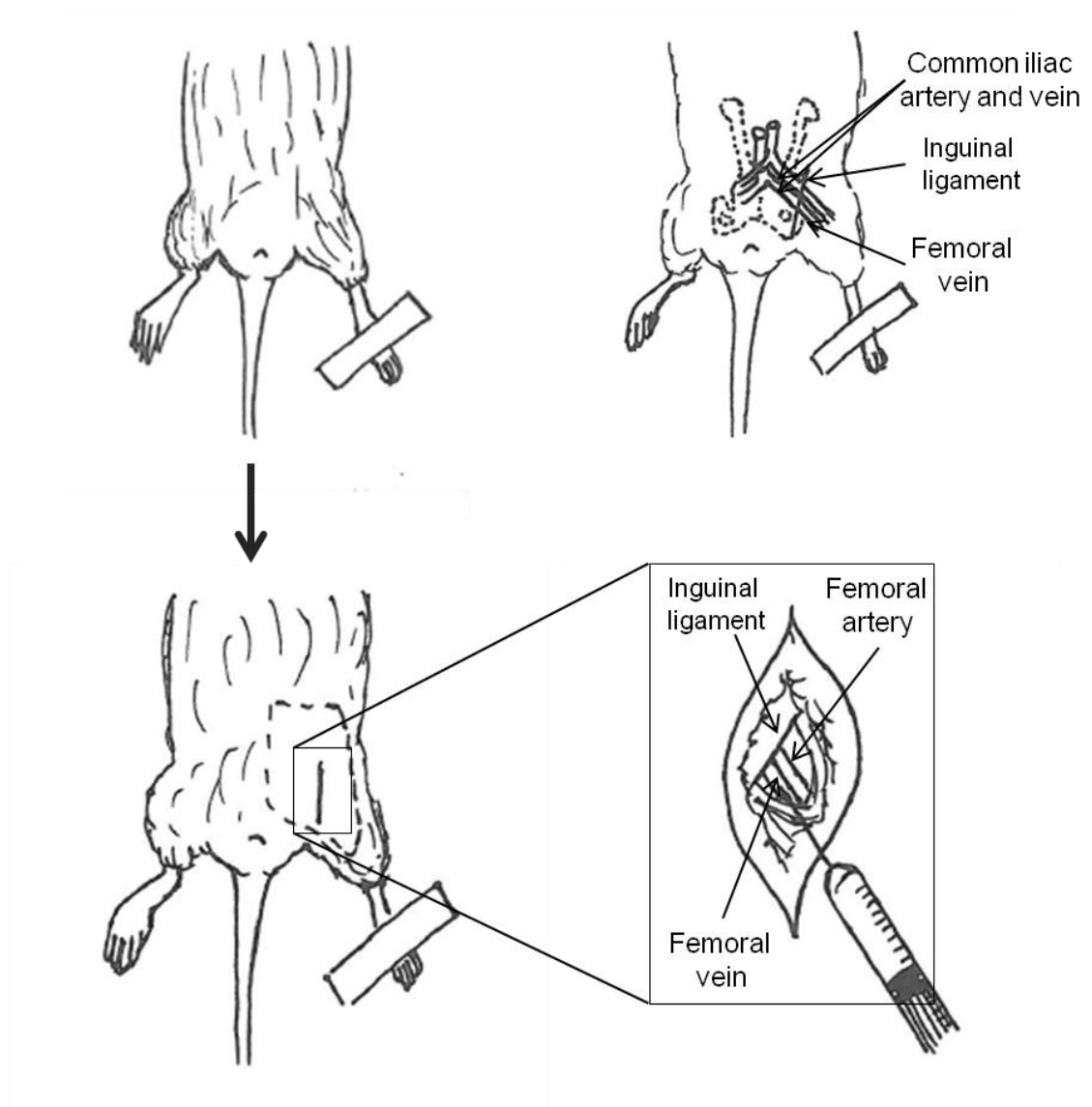
Figure 17. Femoral vein injection under general anaesthesia

Anaesthesia was maintained using inhaled isofluorane at 2 litres/min. The mouse was laid supine, and its position secured using micropore surgical tape. The left leg was held in extension. Fur was removed using veet hair removal cream from a small area of skin overlying the left groin, and a longitudinal incision was made. Careful, blunt dissection deep to this incision revealed the femoral artery and vein as they emerged from below the inguinal ligament. The vein was injected using a 31G needle. Following injection, pressure was applied using sterile gauze. Once haemostasis was ensured, the wound was closed with a continuous 8-0 vicryl suture.

2.11 Late Treatment with MA-TCK26D6

After analysis of the effect of MA-TCK26D6 on mortality rates and AAA formation in the Ang II model of AAA, the effect of late administration of the inhibitor, once the AAA disease process had been established, was investigated. To do this, mice underwent a baseline ultrasound scan (USS) using the Vevo®2100 pre-clinical scanner (FUJIFILM VisualSonics Inc.), before an Ang II pump was implanted as described in Section 2.9. The mice then underwent a second scan after 1 week, after which they were given a single IV dose of MA-TCK26D6 as described in Section 2.10.1. They were then maintained in normal laboratory conditions, and were scanned again 14 days later to observe any effect of the MA-TCK26D6 on AAA progression. The experiment was terminated after 28 days, blood taken, and the aortas imaged and collected for histology as described above. A control group were treated with the same protocol, but given an injection of 0.9% NaCl into their left femoral vein at 7 days, rather than an MA-TCK26D6 injection.

2.12 Vevo®2100 Ultrasound Imaging

For the Vevo®2100 USS imaging, mice were anaesthetised in an anaesthetic chamber using volatile isoflurane at an initial dose of 5 litres/min with 2 litres/min of oxygen. Once the mouse was anaesthetised, they were transferred onto a pre-warmed scanning table platform, and anaesthesia maintained using volatile isoflurane at 2 litres/min with 2 litres/min of oxygen. The animals were positioned supine, and their arms and legs were secured onto the electrode pads using Micropore™ surgical tape, to allow for electrocardiogram (ECG) and

respiratory monitoring throughout. Good contact was ensured using an electrode cream (SignaCreme ElectrodeCream, Parker Labs). A rectal probe (RET-3, Kent Scientific Corporation) was used to continuously monitor the animals temperature. The fur was removed from the entire abdomen using an electronic razor followed by Veet® hair removal cream, in order to ensure good contact between the skin and the USS probe. The abdomen was covered with USS scanning gel (MediSupplies Ltd.). A MS550D MicroScan™ transducer at 22-55 MHz was used. Images were focused at a depth of 4 mm. A series of images of the abdominal aorta were taken, both in cross section and longitudinally. Colour Doppler and pulse wave Doppler were used to ensure the positioning of the abdominal aorta. A 3D USS image of the suprarenal aorta was obtained for the 10.5 mm superior to the right renal artery, in the position where any AAA would be expected to be located. An electrocardiogram-gated (ECG-gated) image was obtained of the longitudinal aorta in order to assess distensibility. Following the scan, the mice were recovered in a warm clean cage, before being returned to their usual maintenance environment.

Scan images were processed remotely using VevoLab 1.7.0 and VevoVasc software. The aortic diameter was measured in cross section at a position 5 mm proximal to the right renal artery. Longitudinally, the aortic diameter was measured in its maximal diameter using an ECG-gated image at maximal systole. Distensibility was calculated using an ECG-gated image and VevoVasc software. The aortic volume was calculated using a 3D reconstruction of the 10.5 mm of the abdominal aorta proximal to the right renal artery, which is the site of any AAA formation.

2.13 Turbidity and Turbidity-Lysis

The turbidity and turbidity-lysis protocols which have previously been used in humans (Scott et al., 2011) were adapted for use in murine plasma. Plasma samples were collected under terminal anaesthesia and processed as described above. Prior to use, plasma samples were defrosted and allowed to reach room temperature.

Murine plasma samples (100 µl, diluted 1:4 in 50 mM HEPES buffer with 0.1% BSA, pH 7.4) were added to each well of a 96-well Greiner bio-one microtitre plate. An activation mix (25 µl) containing murine thrombin (MCT-5020-50, Cambridge Biosciences, UK) 0.2 units/ml, 10 mM $CaCl_2$ +/- tPA 50 ng/ml in a 50 mM HEPES buffer, were added to each well. Concentrations shown are the final concentrations per well. Plasma samples were run in duplicate without any tPA, and in duplicate with tPA. The absorbance was measured every 12 seconds for 90 minutes at 405 nm using a Molecular Devices SPECTRAmax Plus 384 plate reader (Molecular Devices LLC. USA).

2.14 Confocal Microscopy

Plasma samples were collected and stored under terminal anaesthesia as described above. For each sample, a clot containing murine fluorescein isothiocyanate-labelled (FITC-labelled) fibrinogen was prepared. In order to do this, a mixture containing 30 µl of plasma, $CaCl_2$ (final concentration 20 mM) and murine FITC-fibrinogen (Oxford Biomedical Research, USA, final concentration 50 µg/ml) was made in TBS up to a total volume of 54 µl. A reaction mix (6 µl) containing murine thrombin (MCT-5020-50, Cambridge Biosciences, UK), final concentration in plasma 0.5 units/ml, was added to the

samples. Thirty microlitres of this mixture was immediately transferred into one side of a capillary confocal slide (80601 Thistle Scientific Ltd. UK), and the sample monitored to ensure it crossed onto the other side of the slide by capillary action. Once prepared, the slides were kept in a dark humidity chamber at room temperature for 2-4 hours in order to ensure that the clot was completely formed. Slides were then imaged using a Zeiss LSM-700 inverted confocal microscope at 63x magnification. Images were obtained from at least 4 different parts of the clot in order to ensure an accurately representative sample. Images were then analysed using Image J.

2.15 Rotational Thromboelastometry

Whole blood samples were used for Rotational Thromboelastometry (ROTEM) analysis. Samples of whole blood were collected from the IVC onto sodium citrate, and kept on ice until analysis. The ROTEM®-delta system (ROTEM, Tem International GmBH), with ROTEM® mini-cups were used. In brief, 105 µl of blood, 7 µl of star-tem® reagent and 7 µl ex-tem® reagent were added to each mini cup. For studies of lysis, exogenous tPA (Technoclone GmbH, Austria) was added to each sample in a range of concentrations from 2 nM to 20 nM, and lysis monitored over a 2 hour period. There are a number of parameters that can be measured using ROTEM. In this study, measures were taken of the Maximum Clot Firmness (MCF), which is measured in millimetres, and reports the greatest vertical amplitude of the ROTEM trace. It reflects the absolute strength of the fibrin and platelet clot, and can be decreased with reduced platelet number/function or decreased fibrinogen level. The Lysis Index

at 60 minutes (LI60) was also measured, and describes the stability of the clot remaining after 60 minutes in relation to the MCF.

2.16 **Murine D-dimer ELISA**

Plasma levels of D-dimer, a fibrin degradation product, were measured in the plasma of mice that developed AAA, and compared with mice receiving a control infusion of NaCl 0.9%. Levels of D-dimer were measured using a commercially available kit (Cat No. 024579, US Biological Life Sciences) according to the manufacturer's instructions. In brief, a standard curve was produced using known concentrations of D-dimer from 0-5000 ng/ml. Fifty microlitres of standard or plasma sample was added to each well, followed by 50 µl of detection reagent A. The plate was incubated for 1 hour at 37°C. The wells were emptied and washed 3 times. 100 µl of detection reagent B was then added to each well, and the plate was incubated for 30 minutes at 37°C. It was emptied and washed five times. TMB substrate (90 µl) was added to each well, and incubated for 15 minutes at 37°C, after which 50 µl of stop solution was added to each well, and the absorbance was read at 450 nm using a ThermoScientific MultiSkan™ Go 3.2 spectrophotometer. Rather than a standard sandwich ELISA, this kit uses the approach of a competitive inhibition reaction, with the D-dimer in the plasma samples (or standards) competitively binding to the pre-coated ELISA plate at the same time as biotin labelled D-dimer in the detection reagent A. The higher the D-dimer in the plasma sample, the lower the amount of biotin labelled D-dimer that can bind to the plate. Avadin-bound HRP (detection reagent B) is only able to bind to the biotin labelled D-dimer, and so the absorbance following the addition of TMB is

negatively associated with the amount of D-dimer in the plasma sample. A standard curve was produced using the known standard samples (as provided by the manufacturers), and the levels of plasma D-dimer calculated using this curve.

2.17 **Murine TAFI ELISA**

Levels of total TAFI antigen in the murine plasma samples were measured using an in-house ELISA which was kindly shared with us by Prof Ann Gils (KU Leuven). Nunc-Immuno™ Maxisorp™ ELISA plates (Thermo Fisher Scientific Inc.) were coated with 200 µl of RT-36A2F5 at a concentration of 4 µg/ml diluted in 0.01 M phosphate buffered saline (PBS). Plates were incubated for 72 hours at 4°C. After 72 hours, the wells were emptied and blocked with 300 µl per well of an albumin buffer (0.01 M PBS containing 1% BSA). The plates were incubated for two hours at room temperature. The wells were emptied, and washed four times with 300 µl of 0.01 M PBS buffer containing 0.002% TWEEN®-20 using a BioTek MultiFlo automatic plate washer (BioTek Instruments Inc.). The wells were completely emptied by blotting onto absorbent paper, before being prepared for storage. Prior to storage, the wells were filled with 200 µl of mannitol-saccharose solution (100 g mannitol and 20 g saccharose per litre dH$_2$O), incubated for 2-4 minutes, and then emptied completely by blotting on absorbent paper. The plates were then covered in foil to protect them from moisture and light, and stored at -20°C until needed. Prior to application of the plasma samples, the plates were allowed to warm to room temperature, and were washed four times using 0.01 M PBS with 0.002% TWEEN®-20. Plasma samples were collected and stored as described above.

Plasma was diluted 1 part plasma in 500 parts PTAE buffer (0.01 M PBS containing 0.002% TWEEN®-20, 0.1% BSA and 5 mM EDTA). Serial dilutions of this 1/500 sample in PTAE buffer were then performed to give four concentrations for each plasma sample (1/500, 1/1000, 1/2000 and 1/4000). On each plate, two standard samples were included in order to test for inter-plate variability of the assay. These were samples taken from two normal mice (one male and one female) that had not undergone any experimental procedures. These standards were diluted to the same concentrations as the plasma samples to be tested (1/500-1/4000). Each plate also contained a standard curve, produced using purified murine TAFI which was a kind gift from Prof Ann Gils (KU Leuven). The first point of the standard curve was diluted to 30 ng/ml in PTAE buffer, and then serial dilutions were performed to give a total of six dilutions (30, 15, 7.5, 3.25, 1.625 and 0.8125 ng/ml respectively). A final well containing just PTAE buffer was used as a blank. Either plasma sample or standard solution (180 µl) was added to each well, and the plate was incubated overnight at 4°C.

Following overnight incubation, the wells were emptied, and were again washed four times using a 0.01 M PBS 0.002% TWEEN®-20 buffer, after which, the wells were emptied completely by blotting against absorbent paper. The antibody RT-82F12-HRP was used as the capture antibody for this ELISA. It was diluted 1/4000 in PTA buffer (0.01 M PBS containing 0.1% BSA and 0.002% TWEEN®-20), and 170 µl of this solution was added to each well. The plates were incubated for 2 hours at room temperature. Following incubation, the wells were emptied, and were washed four times using 0.01 M PBS 0.002%

TWEEN®-20 buffer. After washing, the wells were again completely emptied by blotting on absorbent paper.

To produce a colorimetric reaction, 160 µl of a substrate solution containing o-Phenylenediamine (OPD) were added to each well. To prepare the substrate solution, a 20 mg OPD tablet (P7288 Sigma-Aldrich Co. LLC.) was dissolved in 30 mls of 0.05 M phosphate citrate buffer pH 5.0 (containing 7.30 g Citric Acid, 251275 Sigma-Aldrich Co. LLC. and 11.87 g sodium phosphate dibasic, S7907 Sigma-Aldrich Co. LLC. in 1 litre dH_2O). For every 30 mls of substrate solution, 12 µl of 30% H_2O_2 (H1009 Sigma-Aldrich Co. LLC.) was added. The plates were incubated for 60 minutes at room temperature, during which time they were covered in foil to protect them from light. After 60 minutes, 50 µl of 4 M H_2SO_4 was added to each well to stop the reaction. The absorbance was measured using a ThermoScientific MultiSkan™ Go 3.2 spectrophotometer (Thermo Fisher Scientific Inc. USA) at 492 nm OD. The TAFI content in the plasma samples was then calculated using a standard curve generated by plotting the absorbance against the concentration of TAFI in the standard TAFI samples.

2.18 Murine CRP ELISA

The levels of C-reactive protein (CRP) in the plasma of mice were measured at 28 days following initiation of Ang II and TAFI inhibitor administration using a commercially available ELISA kit (ab157712 CRP PTX1 Mouse ELISA kit, Abcam® plc). The ELISA was conducted according to the manufacturer's instructions. In brief, a standard curve was produced by diluting a calibrator of known concentration to a range of concentrations between 0-25 ng/ml. Plasma

samples were diluted 1/10 in the sample diluent. All samples were run in duplicate. Sample or standard (100 μl) was added to each well of a pre-coated microwell plate, and incubated at room temperature for 10 minutes. The wells were emptied and washed four times. One hundred microlitres of enzyme-antibody conjugate was added to each well. The plate was incubated for 10 minutes in the dark at room temperature. The wells were washed and 100 μl of TMB substrate was added to each well. The plate was incubated for precisely 5 minutes before 100 μl of stop solution was added. The absorbance was measured using a ThermoScientific MultiSkan™ Go 3.2 spectrophotometer at 450 nm OD. A standard curve was produced using the standards of known concentration as provided by the manuacturers, and the levels of CRP in the plasma samples were calculated from this curve.

2.19 Murine PAP ELISA

The plasma levels of the plasmin-anti-plasmin (PAP) complex were measured after 28 days of treatment with Ang II, with or without a TAFI inhibitor, using a commercially available ELISA kit (Bioassay Technology Laboratory Cat No E0286 Mo, Shanghai Crystal Day Biotech Co. Ltd, assay range 5-600 ng/ml). The assay was performed according to the manufacturer's instructions. In brief, a standard curve was produced using the supplied standard at a range between 0-640 ng/ml. For the standards, 50 μl of standard and 50 μl of streptavidin-HRP was added to each well. For the plasma samples, 40 μl sample, 10 μl PAP antibodies and 50 μl of streptavidin-HRP was added to each well. The plate was then incubated at 37°C for 1 hour. The wells were emptied, washed and blotted onto absorbent paper. Fifty microlitres of chromagen solution A and 50 μl of

chromagen solution B was added to each well. The plate was incubated in the dark at 37°C for 10 minutes, and 50 µl of stop solution was added to each well. The absorbance was read at 450 nm OD using a ThermoScientific MultiSkan™ Go 3.2 spectrophotometer. A standard curve was produced using the standards of known concentrations as provided by the manufacturers, and the levels of PAP in the plasma samples were calculated from this curve.

2.20 Analysis of Macroscopic Images of AAA using Image J

Images were analysed using Image J software. Aortic images taken at 10x magnification were inverted using Image J to allow for clearer identification of the margins of the aorta (see Figure 18 for sample images). Three measurements were taken from the widest part of the abdominal aorta, and three measurements were taken from the normal thoracic aorta. The average of these three measurements was then used to calculate the aortic ratio (abdominal aortic size (mm)/thoracic aorta size (mm), ratio of greater than or equal to 1.5 taken as being aneurysmal).
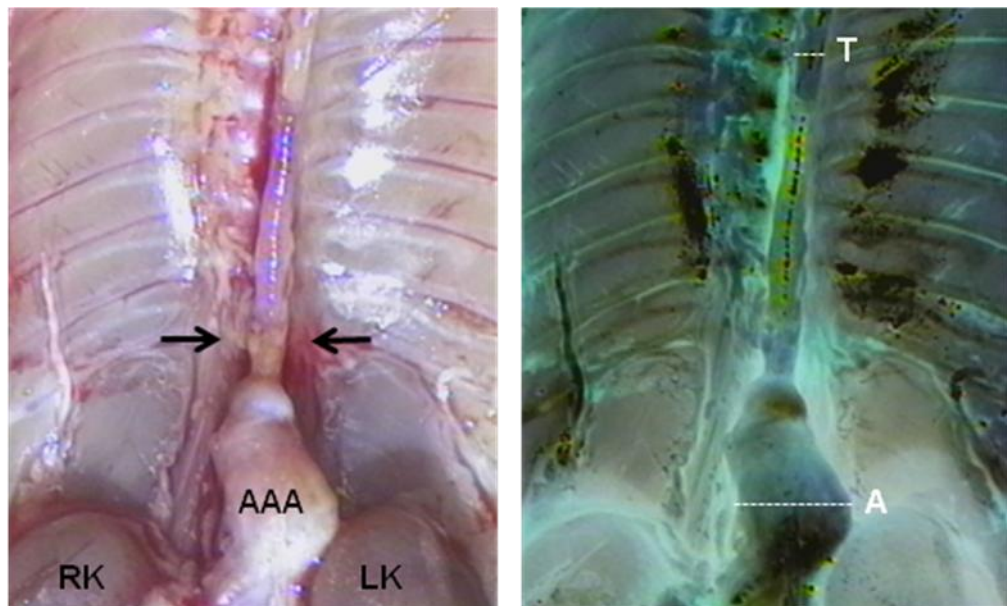
Figure 18. Measurement of aortic size and calculation of the aortic ratio using ImageJ

In-situ photographs were taken of the aortas of mice at 4x, 6x and 10x magnification using a OPMI-PICO videomicrometer (Carl Zeiss AG). Using ImageJ, these images were inverted in order to be able to more accurately determine the edges of the aorta. Using an image taken at 10x magnification, three diameter measurements were taken in a section of normal thoracic aorta (T) and three measurements were taken across the widest section of the suprarenal abdominal aorta (A). These measurements were averaged, and the aortic ratio calculated by dividing the size of abdominal aorta (mm) by the thoracic aorta (mm). RK=right kidney, LK=left kidney, AAA=abdominal aortic aneurysm, Black arrow – denotes the position of the diaphragm.

2.21 Quantification of TAFI in Aortic Tissue

Fresh aortas were harvested from mice treated with Ang II that developed aneurysms (n=8), and saline control treated mice (n=8) as described above. The tissue was immediately flash frozen on liquid nitrogen and kept at -40°C until analysis. The protocol was first optimised using liver samples (which were expected to have a relatively higher TAFI content) before being repeated using aortas.

2.21.1 Homogenisation of Tissue

Tissue samples were allowed to warm to room temperature. A small amount of tissue (approximately 50 µg) was placed into the bottom of a 2 ml round bottomed Eppendorf tube. Lysis buffer (120 mM NaCl, 50 mM Tris, 20 mM Sodium Fluoride, NaF, 1 mM Benzamidine, 1 mM EDTA, 6 mM ethylene glycol tetraacetic acid, EGTA, 15 mM PPi, 55.6 mM glycerophosphate, 1% Nonidet P-40 (Sigma-Aldirch Co. LLC.), 0.1 mM phenylmethanesulfonylfluoride, PMSF, 10 µg/L leupeptin, 10 µg/l aprotinin, 1 ml/l phosphatase inhibitor cocktail 2 (P5726 Sigma-Aldrich Co. LLC.) and 1 ml/l phosphatase inhibitor cocktail 3 (P0044 Sigma-Aldrich Co. LLC.) was added to each Eppendorf; 100 µl for aorta samples and 500 µl for liver samples. The samples were incubated in the lysis buffer on ice for 30 minutes. A lysis bead (Retsch stainless steel cone balls 6mm, 22.455.0003C) was added to each Eppendorf. Samples were transferred into the QIAGEN TissueLyser II (83500 QIAGEN, Netherlands) and lysed at 30 Hz (1800 oscillations/min) for 2 minutes. The samples were then centrifuged using a table top microcentrifuge (Eppendorf MiniSpin plus, Scientific

Laboratory Supplies Ltd.UK) at 14,500 rpm for 10 minutes, and the supernatant was collected into a clean Eppendorf tube.

2.21.2 Total Protein content in the tissue homogenate

Total protein concentration in the supernatant was measured using a Pierce™ BCA Protein Assay kit (Thermo Fisher Scientific Inc.) following the manufacturer's protocol. In brief, a series of standards of known protein concentration (range 0-2000 µg/ml) were produced using serial dilutions of a 2 mg/ml BSA stock solution. Samples of aortic homogenate were diluted 1 part homogenate to 8 parts dH_2O and samples of liver homogenate were diluted 1/50 in dH_2O. Sample or standard (25 µl) were added in duplicate to each well of a 96-well plate (Greiner bio-one microtitre plate, Greiner Bio One International GmbH). Protein assay solution (200 µl) was added to each well (Reagent A:Reagent B mixed in a 50:1 ratio) using a multi-channel pipette. The plate was covered using a microplate adhesive cover (Thermo Fisher Scientific Ltd.) to prevent any evaporation, and the plate was incubated at 37°C for 30 minutes. The absorbency was read using a Dynex Revelation 4.21 microplate reader (Dynex Technologies Ltd. UK) at 540 nm OD. A standard curve was plotted using the absorbencies of the BSA standards, and the total concentration of protein in the homogenates was extrapolated from this graph.

2.21.3 Measurement of TAFI in the tissue homogenate

The murine TAFI ELISA was performed using the aortic tissue homogenates. The protocol was based upon the ELISA for plasma TAFI (see section 2.17). The standard curve was prepared as described previously. In order to assess the best dilutions for the homogenate samples, the ELISA was performed using

the homogenate of murine liver samples and a single control aortic sample. The total protein level was measured in these homogenates using the protocol described above. A liver sample with high total protein (25.3 mg/ml) and a sample with low total protein (10.3 mg/ml) were selected for optimisation of the ELISA. These samples, along with a sample from a single control aorta were serially-diluted starting with 1/50 to give a range of 8 dilutions (1/50, 1/100, 1/200, 1/400, 1/800, 1/1600, 1/3200 and 1/6400). The absorbencies yielded by these samples are shown in Figure 19. Based on this, four dilutions of 1/25, 1/50, 1/100 and 1/200 were used when measuring TAFI in the aorta homogenates. For each sample, the total TAFI level in the tissue samples was measured, and the TAFI concentration as a percentage of the total protein in the tissue was calculated.
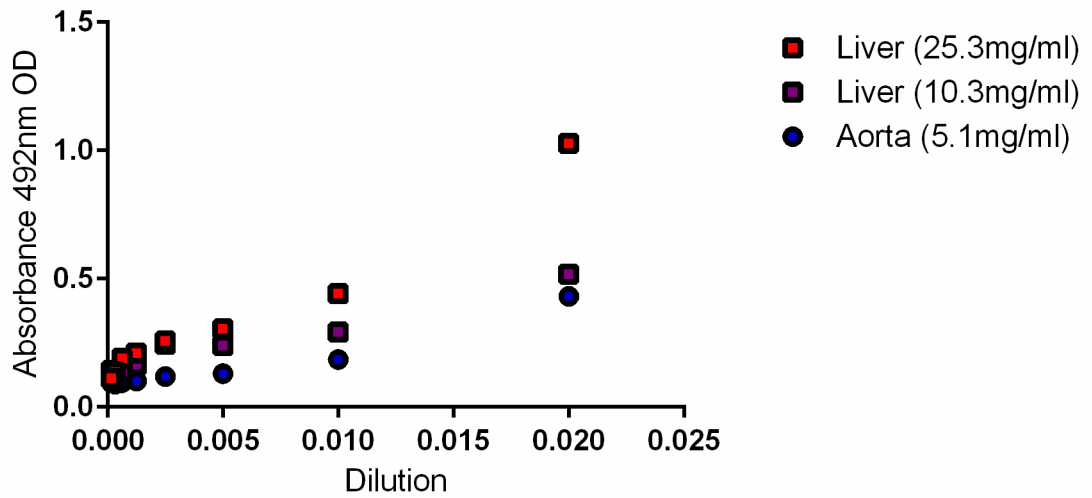
Figure 19. Dilution profiles of TAFI in liver and aortic samples

Levels of TAFI in the homogenate of tissue from control mice was measured using ELISA. Homogenates from livers with high total protein content (red square) and low total protein content (purple square), as well as from a sample aorta (blue circle) were serially diluted to give a range between 1/50 to 1/6400. Based on the absorbencies of these dilutions, a dilution profile for the tissue ELISA was selected, with homogenate samples being diluted to 1/25, 1/50, 1/100 and 1/200.

2.22 Histological examination of murine AAA

Sections from murine aorta were stained for general morphology (haematoxylin and eosin, H+E), collagen (Picosirus red, PSR), elastin, collagen and smooth muscle cells (Millers Van Gieson, MVG) and fibrin (Martius Scarlet Blue, MSB).

2.22.1 Preparation of slides for staining

Prior to staining, samples were first deparaffinised and rehydrated. They were deparaffinised by soaking in xylene (534056 Sigma-Aldrich Co. LLC.) for five minutes, then a second, clean xylene bath for a further 5 minutes. Samples were transferred into 100% ethanol for 5 minutes, before being transferred into clean 100% ethanol for a further 5 minutes. Samples were rehydrated by moving them through a series of ethanol solutions; 70% ethanol for 3 minutes, 50% for 3 minutes, 25% for 3 minutes, before being washed in water for 2 minutes.

2.22.2 Haematoxylin and Eosin

Samples were deparaffinised and rehydrated as described above. Haematoxylin stain for nuclei was applied first (7 g/L Harris Haematoxylin Stain80, Sigma-Aldrich Co. LLC.), with slides incubated in the stain at room temperature for 10 minutes. Slides were then washed under running water for 10 minutes. The colour was optimised by reducing the background colour or increasing the 'blue' as appropriate. The background was reduced by incubating the slides in a 1% HCl and 70% ETOH solution for 30 seconds before washing under running water for a further 3 minutes. Colour was increased by incubating in a 0.2% ammonium sulphate solution for 60 seconds, before washing under

running water for a further 3 minutes. Once the stain appeared acceptable, the slides were dipped into 3 solutions of 70% ethanol prior to staining with eosin. A stock of eosin stain was produced by adding 1.25 g of Eosin Y (E4009 Sigma-Aldrich Co. LLC.) to 100 mL of 90% ethanol and 25 mL of molecular grade dH_2O, before the solution was diluted 1:4 by adding a further 300 mL of 100% ETOH, 75 mL of molecular grade dH_2O and 2.5 mL of 40% glacial acetic acid. The slides were incubated in this stain for 30 seconds, then rinsed three times in molecular grade dH_2O.

2.22.3 Picosirus Red

Slides were stained for haematoxylin as described above. They were then counterstained with picosirus red in order to visualise the collagen. One gram of Direct Red 80 (365548 Sigma-Aldrich Co. LLC.) was added to 1 litre of a saturated aqueous solution of picric acid (197378 Sigma-Aldrich Co. LLC.). Slides were incubated in this solution for 60 minutes before being washed in running water for 5 minutes, and then left to dry for 20 minutes at 70°C.

2.22.4 Millers Van Gieson

Following deparaffinisation and rehydration as described above, samples were oxidised by soaking them in a 0.5% solution of potassium permanganate (223468 Sigma-Aldrich Co.LLC.) for 10 minutes. Slides were washed in running water for two minutes, before being soaked in a 2% solution of oxalic acid (247537 Sigma-Aldrich Co.LLC.) for 1 minute in order to reduce any subsequent background during staining. Next, slides were washed in running water for 2 minutes, before being dipped in 3 serial solutions of 96% ETOH. The samples were incubated in the Miller's stain for 1 hour. Slides were washed in 4

serial 100% ETOH solutions for 2 minutes at a time, in order to 'de-stain' the Miller's stain from the slide, before being washed under running water for 5 minutes. Slides were transferred into the Van Geison stain and incubated for 30 minutes at room temperature. Slides were washed in molecular grade dH$_2$O until the water was clear, and left to dry at 70°C for 20 minutes.

2.22.5 Martius Scarlet Blue

Samples were stained for fibrin using Martius Scarlet Blue (MSB) (Lendrum et al., 1962) using a kit from Atom Scientific (RRSK2, Atom Scientific Ltd, UK) according to the manufacturer's instructions. In brief, samples were stained in Weigerts Iron Haematoxylin for 10 minutes. They were then washed in running water, before being de-stained by incubating for 3 minutes in a 1% HCl and 70% ETOH solution. They were washed again in running water, and stained with a 0.5% Martius Yellow solution for 3 minutes. After another wash in running water, they were transferred into 1% Crystal Ponceau in 1% acetic acid for 5 minutes, washed again and transferred into 1% Phophotungstic acid for 5 minutes. After a further wash in running water, they were placed into a Methyl Blue solution (1% methyl blue in 1% acetic acid) for 2-5 minutes. Slides were finally washed in running water, and left to dry at room temperature.

2.22.6 Dehydration and Mounting of Slides

After staining, samples were dehydrated by serial soaking first in 96% ethanol for 2 x 2 minutes, then xylene for 2 x 2 minutes. Slides were allowed to air dry for 15 minutes before microscope cover slips (C9056, Sigma-Aldrich Co. LLC.) were applied using DPX mounting medium (06522, Sigma-Aldrich Co. LLC.).

Slides were imaged using an Olympus BX40 Dual View Microscope and Image Pro-Plus 8.0 at 4-20x magnifications.

2.23 Data Analysis and Sample Size selection

Raw data obtained in the laboratory was initially collected into Microsoft Excel. GraphPad Prism v6 was used to represent the data graphically. Statistical tests were performed using IBM SPSS Statistics v20 (SPSS Inc. Chicago, Illinois, USA) and GraphPad Prism v6. Statistically significant results were taken as $p < 0.05$.

The samples sizes for the various plasma assays were determined by using pilot data. For the intact TAFI ELISA, with a standard deviation in each group of 4 μg/ml with 90% power at $p < 0.01$, 23 per group were required to detect a significant difference in the mean values. As the variation was higher in the pilot data for the TAFIa/TAFIai (standard deviation up to 19 ng/ml) and TAFI-AP ELISAs (standard deviation up to 250 ng/ml), when the same conditions were applied, 105 and 163 respectively were required per group. For the TAFI activity assay, due to wide inter-group variation, with standard deviation of up to 16 minutes, under the same conditions, 129 were needed per group. Therefore, the aim was to perform each assay on >200 per group. For the assays relating to turbidity-lysis in patient samples, sample size was based upon the difference previously detected in lysis in this group of patients (Scott et al., 2011). The aim was to test if the delay in lysis could be corrected by altering TAFI levels, and to do this with 90% power at $p < 0.01$, only 9 samples were needed per group. As a result, 32 patients were used per group, to allow for the detection of an effect that was subtle than a complete correction in the change in lysis.

For the data on polymorphisms in proteins involved in fibrinolysis, genotype distribution was compared with the Hardy-Weinberg Equilibrium. The sample size used for these gene studies provided >80% power to detect a genotype relative risk of 1.3 for an average allele frequency of 0.2, with a disease prevalence of 8% and $\alpha = 0.05$ (Menashe et al., 2008). The difference in the distribution of genotypes between groups was analysed using Pearsons Chi-Squared testing.

For the murine work investigating TAFI inhibition *in-vivo*, the sample size was based upon pilot data relating to the Angiotensin II model in our institution, with Angiotensin II given at a dose of 750 ng/kg/min. Using our results, in order to detect a change in AAA ratio of 1.3 (reverting from the mean for AAA in our pilot data 2.3 ± 0.9 to the normal 1.0 ± 0.2), with 90% power at p<0.05, 6 animals would be needed to appreciate an effect. Given the known mortality of this model, 12 mice were used per group.

For the data on TAFI in plasma in human AAA, normality was tested using Shapiro-Wilk. A normal distribution could not be achieved by transforming data using Log_{10} or natural Log, and so data was analysed as non-parametric data. Data on turbidity and turbidity lysis in human plasma was also tested for normality using Shapiro-Wilk, and was transformed using Log_{10}, and subsequently analysed as parametric data. Non-parametric data was analysed using Mann-Whitney U testing, and parametric data was analysed using Student T-testing. Non-parametric data is presented as median (Inter-quartile range), and parametric data as mean ± standard deviation. For patient demographics, continuous data is presented as median (Inter-quartile range) and categorical data as number of subjects (% of total), with differences

between the groups analysed initially using student T-test for continuous data and Pearsons Chi-Squared for categorical data, prior to binary logistic regression analysis. For murine studies, groups were compared using student T-test, and data presented as mean ± standard deviation.

Chapter 3   Results – The Role of TAFI in Human AAA

The role of TAFI in human AAA was investigated using samples of plasma and DNA from patients with AAA and control subjects who were recruited as part of the Leeds Aneurysm Development Study (ethical approval granted by Leeds East Research Ethics Committee 03/142). Plasma samples were collected and processed as described in Section 2.2, page 48. Plasma levels of intact TAFI zymogen, TAFI activation peptide (TAFI-AP) and activated and inactivated TAFI (TAFIa/TAFIai) were measured using ELISA. Plasma TAFI activity was determined using a modified clot lysis assay. Patients with AAA and control subjects were genotyped for four common polymorphisms of proteins involved in fibrinolysis (TAFI Thr325Ile, α2AP Arg6Trp and Arg407Lys, and tPA 7351C→T). Finally, the role of TAFI in the delayed lysis seen in patients with AAA was explored using turbidity-lysis assays with exogenous TAFI and an inhibitor of TAFI, PCI.

3.1 Plasma levels of activated and inactivated TAFI, and the TAFI activation peptide, are higher in patients with AAA than control subjects

Plasma levels of intact TAFI zymogen, TAFIa/TAFIai measured in combination, and TAFI-AP were all measured using ELISA techniques as described on Page 49. The assays were performed on a total of 259 patients with AAA and 227 control subjects. The basic demographics of these two groups are shown in Table 1. The two groups were compared for compounding risk factors, such as cardiovascular disease and smoking. As might be expected, there were some differences in the incidence of MI (27.0% in AAA patients vs. 7% in controls, $p<0.001$), hypertension (62.5% vs. 39.7%, $p<0.001$) and smoking status (current smokers 21.2% vs. 11.9%, $p=0.005$), but after binary logistic regression, only aortic diameter remained different between the two groups (AAA 4.9(3.8-6.0) cm vs. control 1.8(1.6-2.1) cm, $p<0.001$).

Table 1. Demographics of AAA patients and control subjects in whom levels of plasma TAFI, TAFIa/TAFIai and TAFI-AP were measured

		Patients (AAA) n=259	Controls (no AAA) n=227	p-value
Aorta size (cm)		4.9 (3.8-6.0)	1.8 (1.6-2.1)	<0.0001
BMI (weight kg/height m^2)		27.6 (24.4-30.7)	27.8 (24.7-30.4)	0.98
Age (years)		74.0 (64.0-79.0)	69.0 (63.0-75.0)	<0.0001
Male n(%)		211 (81.5%)	166 (73.1%)	0.03
Drug history n(%)	Aspirin	182 (70.3%)	80 (35.2%)	<0.0001
	Statin	210 (81.1%)	105 (46.3%)	<0.0001
Past Medical History n(%)	DM	38 (14.7%)	27 (11.9%)	0.42
	MI	70 (27.0%)	16 (7.0%)	<0.0001
	Angina	61 (23.6%)	18 (7.9%)	<0.0001
	CVA/TIA	35 (13.5%)	27 (11.9%)	0.59
	↑BP	162 (62.5%)	86 (37.9%)	<0.0001
Smoking Status n(%)	Current	55 (21.2%)	26 (11.5%)	0.007
	Ever	229 (88.4%)	150 (66.1%)	<0.0001

DM - Diabetes Mellitus, MI - Myocardial Infarction, CVA/TIA - Cerebrovascular Accident or Transient Ischaemic Attack, ↑BP – Hypertension, AAA – abdominal aortic aneurysm, BMI – body mass index. Results are shown as median (interquartile range) and number (percentage of patients). Differences between groups were analysed using Pearsons Chi-Squared (categorical data) and Student T-test (continuous data).

The levels of intact TAFI zymogen were measured using an in-house ELISA from the laboratory of Prof Ann Gils in KU Leuven. MA-T12D11 was used as the coating antibody, with MA-T30E5A2-HRP used as the detection antibody. Results are shown as median (inter-quartile range). All samples were diluted to four different concentrations, and all plates were run at least twice to ensure an adequate number of duplicates for each sample. This is described in detail on Page 53. The quality control data for this ELISA is shown in Figure 20 (panels A-B). There was no difference in the levels of intact TAFI zymogen between patients with AAA and control subjects (13.1 (11.0-15.7) µg/ml vs. 12.7 (10.6-15.2) µg/ml respectively, p=0.37, panel A, Figure 21).
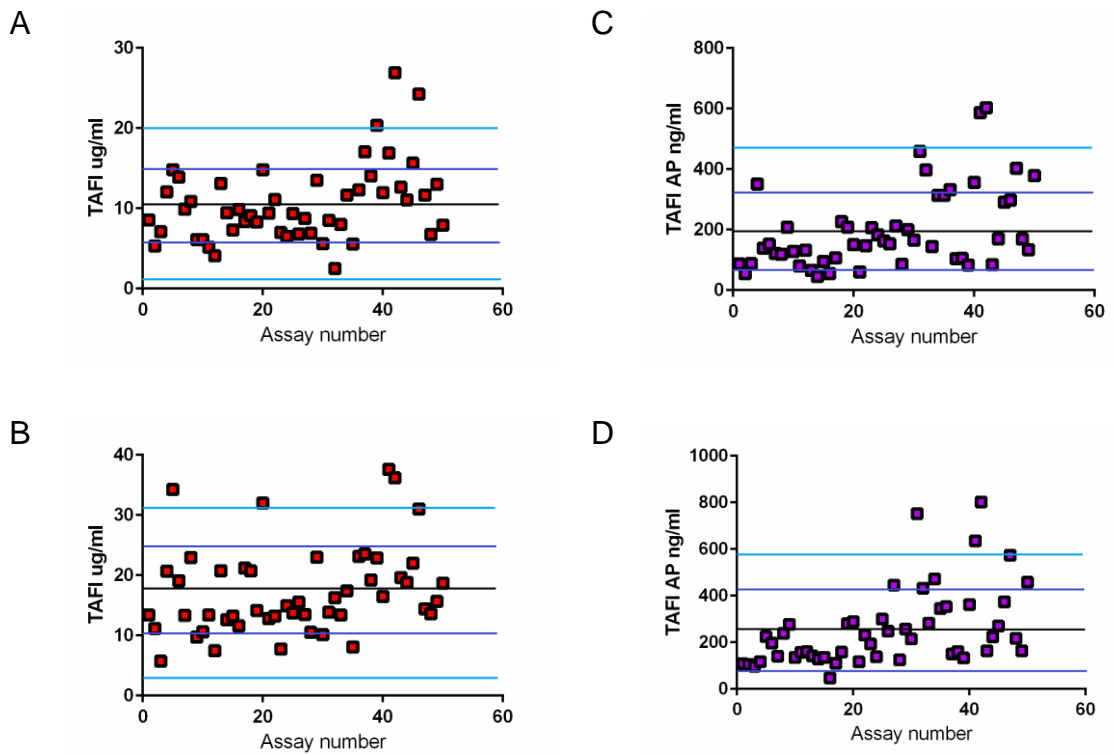
Figure 20. Intra-assay variability for the Intact TAFI and TAFI-AP ELISAs

Each point represents the mean value for each sample per plate, generated using 4 different dilutions of the standards on a single plate. The black line represents the mean value for that standard across all plates. The dark blue lines represent 1-standard deviation from the mean, with the light blue line(s) showing 2-standard deviations from the mean. All plates containing a control sample that fell > 2 standard deviations away from the mean were repeated.

A – Variation in the level of intact TAFI in ABJ60, a single patient standard over time

B – Variation in level of intact TAFI in NPP, a standard based on 25 healthy individuals, over time

C – Variation in the level of the TAFI-AP in ABJ60 over time

D – Variation in the level of the TAFI-AP in NPP over time

	AAA	Control	Large AAA	Small AAA
Intact TAFI µg/ml	13.1 (11.0-15.7)	12.7 (10.6-15.2)	13.1 (11.2-15.5)	13.2 (10.9-16.1)
TAFIa/TAFIai ng/ml	20.5 (14.5-33.4)	14.2 (10.9-19.2)	22.4 (14.8-39.0)	19.1 (13.9-31.9)
TAFI AP ng/ml	318.7 (195.4-483.3)	248.6 (193.4-325.0)	321.4 (207.5-509.4)	320.6 (193.5-478.8)
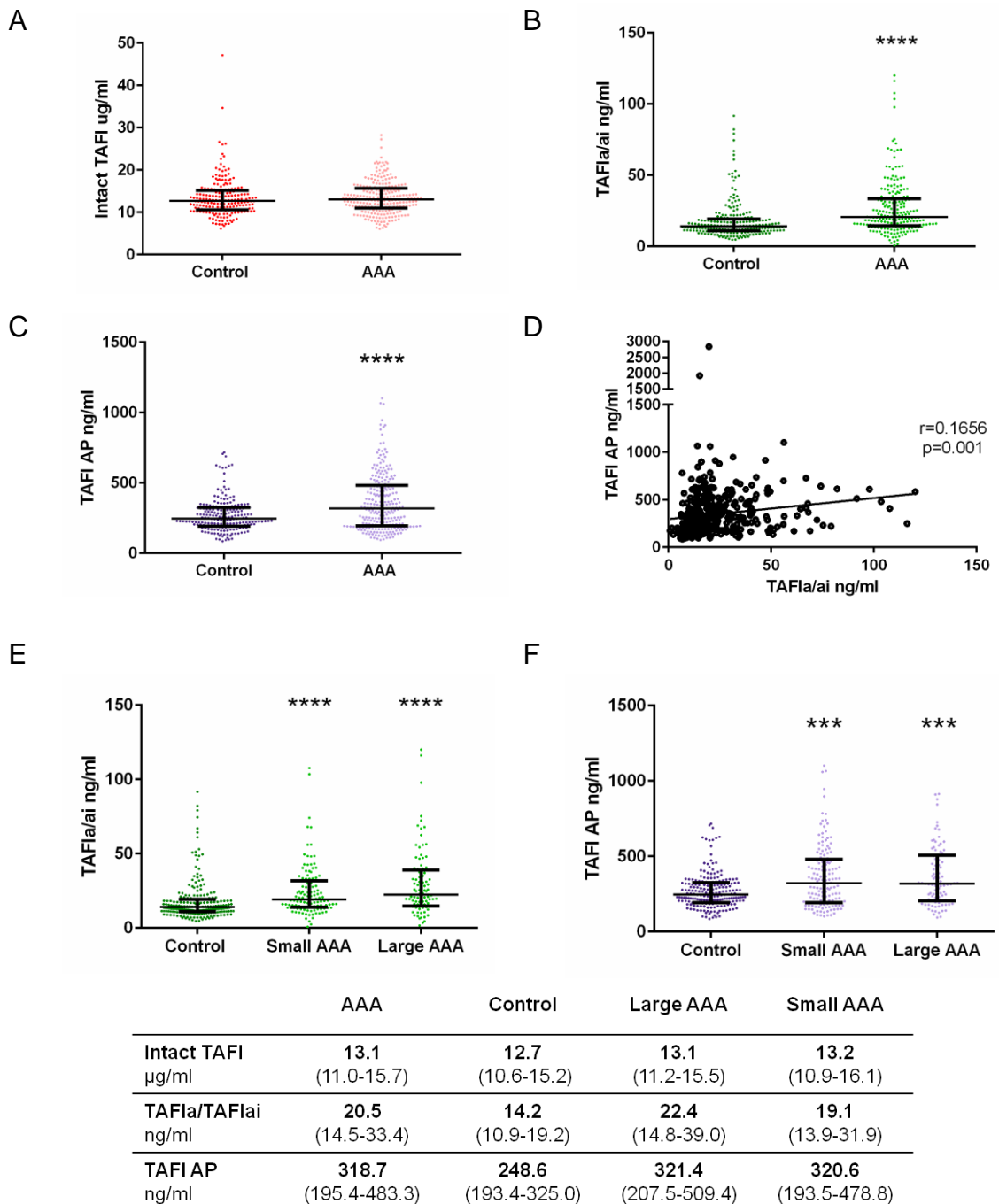
Figure 21. Plasma levels of TAFIa/TAFIai and TAFI-AP are increased in patients with AAA, and are associated with aneurysm size

Levels of TAFI, TAFIa/TAFIai and TAFI-AP were measured in the plasma of patients with AAA (n=259) and control subjects (n=227) using ELISAs. There was no difference in the plasma levels of total TAFI zymogen in patients with AAA compared with controls (p=0.37, Panel A). The levels of TAFIa/TAFIai and TAFI-AP were significantly increased in patients with AAA compared with control subjects; TAFIa/TAFIai, p<0.0001, Panel B, and TAFI-AP, p<0.0001, Panel C. Levels of TAFIa/TAFIai and TAFI-AP were positively correlated (r=0.1656, p=0.001), Panel D. The increase in plasma levels was associated with AAA size; patients with large AAA (>5cm, n=154) had the highest levels, even compared to patients with small AAA (3-5cm, n=93), Panels E and F. Data are presented as median (IQR).

The levels of TAFI-AP were also measured using an in-house ELISA from KU Leuven. MA-T12D11 was used as the coating antibody and MA-T18A8-HRP was used as the detection antibody. Again, all samples were diluted to four different concentrations, and all plates were run at least twice to ensure an adequate number of duplicates for each sample as described on Page 59. The quality control data for this ELISA is shown in Figure 20 (panels C and D). Results are shown as median (inter-quartile range). The levels of TAFI-AP were significantly higher in plasma of patients with AAA compared with plasma from control subjects (318.7 (195.4-483.3) ng/ml vs. 248.6 (193.4-325.0) ng/ml, $P<0.0001$, panel C, Figure 21).

The levels of TAFIa/TAFIai were measured in combination using a commercially available ELISA (Asserachrom® TAFIa/TAFIai, ref 00616, Diagnostica Stago UK Ltd). Quality control testing was done for each plate using a high and low control as per the manufacturer's instructions. Results are shown as median (inter-quartile range). Again, the levels of TAFIa/TAFIai were significantly higher in patients with AAA compared with control subjects (20.5 (14.5-33.4) ng/ml vs. 14.2 (10.9-19.2) ng/ml, $P<0.0001$, panel B, Figure 21).

Plasma levels of TAFIa/TAFIai and TAFI-AP were positively correlated ($r=0.1656$, $p=0.001$), indicating that the results of these ELISAs are both indicators of the amount of TAFI that has been activated in the plasma samples (panel D, Figure 21).

 AAA subjects were divided into two groups based on the size of their AAA; small AAA (3-5 cm, n=93) or large AAA (>5 cm, n=154). Both the levels of TAFI-AP and TAFIa/TAFIai increased with the size of the AAA. TAFI-AP levels were

320.6 (193.5-478.8) ng/ml in patients with small AAA, increasing to 321.4 (207.5-509.4) ng/ml in patients with large AAA (p=0.0002). TAFIa/TAFIai levels were 19.1 (13.9-31.9) ng/ml in the plasma of patients with small AAA, increasing to 22.4 (14.8-39.0) ng/ml in patients with large AAA (p<0.0001). There was no difference in the levels of the intact TAFI zymogen between patients with small and large AAA (13.2 (10.9-16.1) µg/ml vs. 13.1 (11.2-15.5) µg/ml, panels E and F, Figure 21).

3.2 TAFI Activity is decreased in the plasma of patients with AAA compared with control subjects

In order to measure just active TAFI in the plasma samples, a modified clot lysis assay was used. Clot lysis assays were performed with and without the presence of PCI, a potent inhibitor of TAFI. The difference in the time to half lysis when PCI is present is a proxy of the activity of TAFI in the sample, and is termed the TAFI-related retardation to lysis time. This is illustrated in Figure 12. Results are presented as median (inter-quartile range). The TAFI-related retardation to lysis time was significantly lower in patients with AAA compared with control subjects (44.25 (34.25-54.25) mins vs. 52.38 (39.06-63.31) mins, p<0.0001, Figure 22, panel A). In order to adjust for differences in the intact zymogen levels of TAFI in the samples prior to its activation with thrombin and thrombomodulin, the TAFI-specific anti-fibrinolytic activity was calculated for each sample, which was taken as a ratio of the TAFI-related retardation to lysis divided by the intact zymogen levels (as established by the intact TAFI ELISA). The TAFI-specific anti-fibrinolytic activity in plasma from patients with AAA was

significantly lower than the activity in samples from control subjects (3.15 (2.42-4.15) vs. 3.94 (2.76-5.24), p<0.0001, Figure 22, panel B).

	TAFI related retardation to lysis time (mins)	Intact TAFI level (µg/ml)	TAFI-specific anti-fibrinolytic activity
AAA	44.25 (34.25-54.25)	13.07 (11.0-15.67)	3.15 (2.42-4.15)
Control	52.38 (39.06-63.31)	12.69 (10.60-15.18)	3.94 (2.76-5.24)
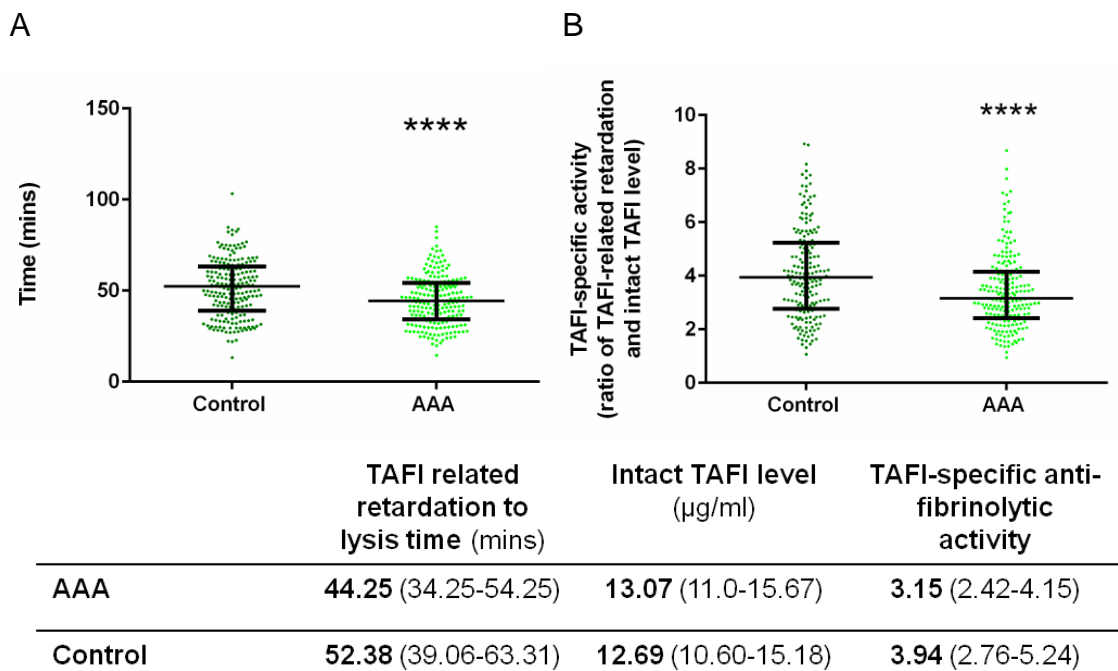
Figure 22. TAFI-related retardation to lysis and TAFI-specific anti-fibrinolytic activity is decreased in patients with AAA compared with control subjects

Using a modified clot lysis assay, the TAFI-related retardation to lysis was measured in the plasma of patients with AAA (n=214) and control subjects (n=210). Results are presented as median (IQR). The TAFI-related retardation to lysis time was decreased in patients with AAA compared with control subjects (p<0.0001, Panel A). By comparing these results with the levels of intact TAFI (as measured by ELISA), the TAFI-specific anti-fibrinolytic activity was calculated. The TAFI-specific anti-fibrinolytic activity was decreased in patients with AAA compared with control subjects (p<0.0001, Panel B).

3.3 The correlation between polymorphisms of proteins involved in fibrinolysis and AAA

DNA was extracted from the whole blood of patients with AAA (n=602) and control subjects (n=490), and Taqman genotyping assays were used to analyse four polymorphisms in proteins related to fibrinolysis; TAFI Thr325Ile, α2AP Arg6Trp, α2AP Arg407Lys and tPA 7351C→T. These methods are described in full in Chapter 2.7. Larger numbers of subjects were included for this part of the thesis in order to ensure that genotyping results had significant power. The basic demographics of these patients are shown in Table 2. As was the case when a smaller population was used for the studies of plasma TAFI levels, there were some differences in relation to evidence of cardiovascular disease between patients with AAA and control subjects, but again, after binary logistic regression analysis, only aortic size remained significantly different between the two groups. Results are shown as a percentage of subjects in each group being in possession of the rare allele, followed by the odds ratio (95% confidence intervals).

All genotype distributions were in keeping with the Hardy-Weinberg equilibrium. In addition, the allele frequencies for all four of the polymorphisms are in the range expected based on previously published results of these polymorphisms in disease states and in healthy populations (Brouwers et al., 2001, Christiansen et al., 2007, Uitte de Willige et al., 2011). The allele frequencies and comparison of the results with the Hardy-Weinberg equilibrium are shown in Table 3. A sample of the output generated using PCR and end-point genotyping is shown in Figure 23.

Table 2. The Demographics of patients with AAA and control subjects in whom genotyping was undertaken to establish the presence of polymorphisms of TAFI, α2AP and tPA

		Patients (AAA) n=602	Controls (no AAA) n=490	p-value
Aorta size (cm)		4.69 (4.57-4.81)	1.94 (1.90-1.98)	<0.0001
Family History n(%)		50 (8.3%)	42 (8.6%)	0.91
BMI (weight kg/height m^2)		27.09 (26.70-27.48)	27.64 (27.26-28.03)	0.06
Waist-hip ratio		0.95 (0.94-0.96)	0.93 (0.92-0.94)	<0.0001
Age (years)		74.7 (73.7-75.0)	70.0 (69.3-70.7)	<0.0001
Male n(%)		497 (82.8%)	377 (76.9%)	0.02
Drug history, n(%)	Aspirin	396 (65.9%)	214 (43.7%)	<0.0001
	Statin	438 (72.9%)	239 (48.8%)	<0.0001
Past Medical History, n(%)	DM	85 (14.1%)	62 (12.7%)	0.923
	MI	159 (26.5%)	48 (9.8%)	<0.0001
	Angina	142 (23.6%)	76 (15.5%)	0.02
	CVA/TIA	106 (17.6%)	63 (12.9%)	0.04
	↑BP	343 (57.1%)	198 (40.4%)	<0.0001
Smoking Status, n(%)	Current	136 (22.7%)	78 (15.9%)	0.006
	Ever	543 (90.1%)	339 (69.2%)	<0.0001

DM - Diabetes Mellitus, MI - Myocardial Infarction, CVA/TIA - Cerebrovascular Accident or Transient Ischaemic Attack, ↑BP – Hypertension, AAA – abdominal aortic aneurysm, BMI – body mass index. Results are shown as median (inter quartile range) and number (percentage of patients). Differences between groups were analysed using Pearsons Chi-Squared (categorical data) and Student T-test (continuous data).

Table 3. Mutant Allele frequency and Results of Hardy-Weinberg Equilibrium for the population genotyping of TAFI Thr325Ile, α2AP Arg6Trp, α2AP Arg407Lys and tPA 7351C→T

	Mutant allele frequency	Total Population	
		X^2	P
Arg6Trp	0.20	0.3459	0.5564
Arg407Lys	0.17	2.49	0.1143
tPA 7351C→T	0.36	0.3860	0.5343
Thr325Ile	0.30	0.0080	0.9285
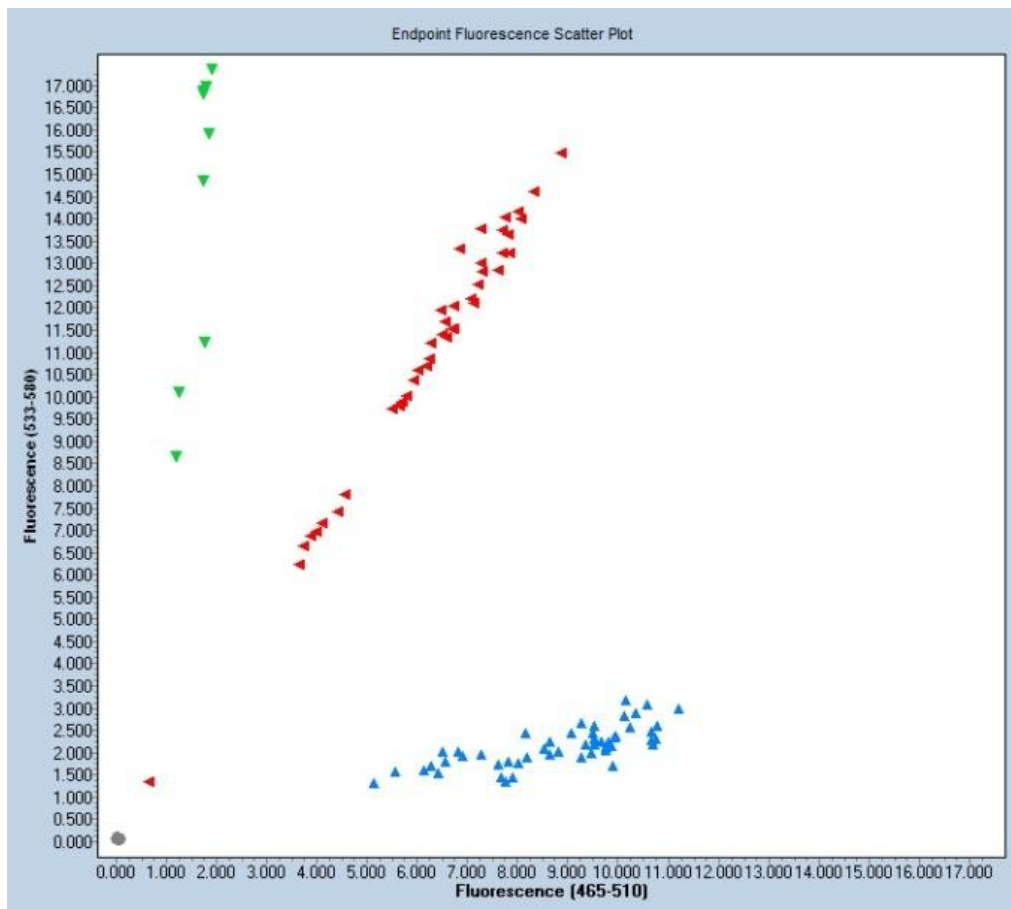
Figure 23. Example output of end-point genotyping using TaqMan Genotyping assays and real-time PCR

Genotyping was carried out using TaqMan genotyping assays. In these assays, allelic discrimination is based on the 5' to 3' exonuclease activity of the Taq DNA polymerase. For each SNP, two different TaqMan probes are used, one specific to the wild type (WT) and one specific to the variant allele. A 5' reporter dye and a 3' quencher dye are covalently linked to each of the two probes. When the probes are intact, the reporter dye (fluorescence dye) is not observed because it is in close proximity to the quencher dye. However, during PCR amplification, the reporter and quencher dyes are released due to the 5' nuclease activity of the Taq DNA polymerase. This results in increased fluorescence of the relevant probe's reporter dye (WT or variant). At the end of the PCR reaction, the relative amount of the two reporter dyes can be used to determine the genotype. This can be plotted on a graph as shown above. The example genotyping result shown is that of TAFI Thr325Ile; blue triangles represent Thr/Thr homozygotes, red triangles represent Thr/Ile heterozygotes, and green triangles represent Ile/Ile homozygotes.

3.3.1 TAFI Thr325Ile

The Thr325Ile polymorphism is positioned in the thermoregulatory centre of the TAFI gene. This coding polymorphism results in a TAFIa molecule that has a longer half-life at 37°C and a higher anti-fibrinolytic potential. The possession of this polymorphism has previously been linked with the incidence of some cardiovascular diseases (Shi et al., 2014). In the case of AAA, there was no association between possession of an isoleucine allele and AAA, with 52.3% of AAA patients and 49.2% of controls being in possession of at least one isoleucine allele, OR 1.13 (0.89-1.44), p=0.31 (Figure 24, Panel A).

3.3.2 Alpha-2-Antiplasmin Arg6Trp and Arg407Lys

There was a strong positive correlation between possession of a tryptophan allele at position 6 and possession of a lysine allele at position 407, suggesting that in α2AP, these alleles are in linkage disequilibrium (see Table 4, Pearson Chi-Squared p<0.0001). In the case of AAA, we found that there was a significant reduction in the number of patients in possession of a lysine allele at position 407 compared with control subjects (29.1% vs. 34.9%, OR 0.77 (0.53-0.98), p=0.04, see Figure 24, panel C). Despite the strong association between these two polymorphisms, there was no significant difference in the genotype distribution of the Arg6Trp polymorphism and AAA (Figure 24, panel B).

Table 4. The association between the Arg6Trp and Arg407Lys polymorphisms of α2AP

		Arg407Lys			
p<0.0001		*Arg/Arg*	*Arg/Lys*	*Lys/Lys*	**Total**
Arg6Trp	*Arg/Arg*	662	46	1	709
	Arg/Trp	78	256	4	338
	Trp/Trp	6	20	19	45
Total		746	322	24	1092

3.3.3 tPA 7351C→T

The tPA 7351C→T polymorphism lies in the enhancer region of the tPA gene, and results in a decrease in the rate of tPA release from the vascular endothelium. It has previously been associated with MI (Ladenvall et al., 2002). There was no association between this polymorphism and AAA, with the percentage of patients in possession of a T allele being similar between the two groups (57.5% of patients with AAA and 59.2% of control subjects, OR 0.93 (0.73-1.19) p=0.57, Figure 24, panel D).

All of these genotyping results are summarised in Table 5.
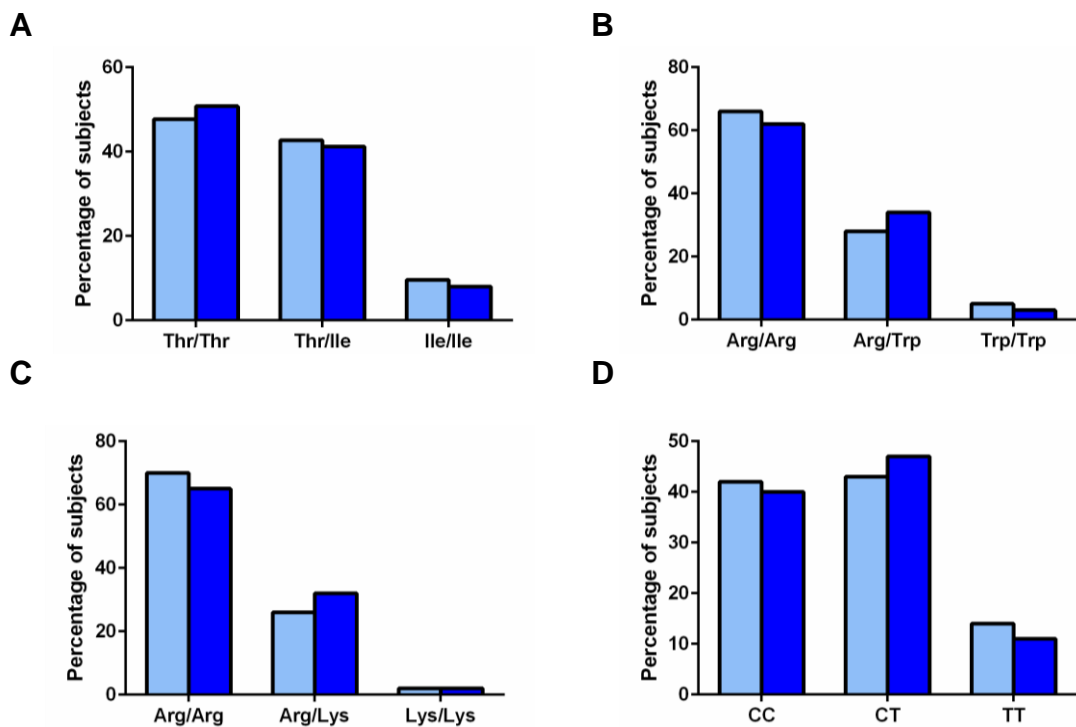
Figure 24. The genotype distributions of α2AP Arg407Lysand Arg6Trp, TAFI Thr325Ile and tPA 7351C→T. The Arg407Lys polymorphism of α2AP is negatively associated with AAA. α2AP Arg6Trp, TAFI Thr325Ile and tPA 7351C→T are not associated with the presence of AAA.

The genotype distribution of α2AP Arg407Lys (Panel C) and Arg6Trp (Panel B), TAFI Thr325Ile (Panel A) and tPA 7351C→T (Panel D) were studied in patients with AAA (n=602) and control subjects (n=490) using DNA extracted from whole blood, Taqman genotyping assays and PCR. Results are presented as the percentage of each group (patient or control) being in possession of each genotype. Patients with AAA are represented by the light blue bars, control subjects are shown as the dark blue bars. Only the Arg407Lys polymorphism was associated with AAA, with patients being less likely to be in possession of the rare Lys allele compared with control subjects (29.1% vs. 34.9%, p=0.04, panel C).

Table 5. The Arg407Lys polymorphism of α2AP is negatively associated with AAA

		Aneurysm n=602 (%)	Control n=490 (%)	OR (95% CI)	Chi-squared
α2AP	No Trp (Arg/Arg)	403 (66.9)	306 (62.4)	1	
Arg6Trp	Trp	199 (33.1)	184 (37.6)	0.82 (0.64-1.05)	P=0.12
	Arg/Trp	169 (28.1)	169 (34.5)		
	Trp/Trp	30 (5.0)	15 (3.1)		
α2AP	No Lys (Arg/Arg)	427 (70.9)	319 (65.1)	1	
Arg407Lys	Lys	175 (29.1)	171 (34.9)	**0.77 (0.59-0.98)**	**P=0.04***
	Arg/Lys	161 (26.7)	161 (32.9)		
	Lys/Lys	14 (2.3)	10 (2.0)		
tPA	No T (C/C)	256 (42.5)	200 (40.8)	1	
7351C→T	T	346 (57.5)	290 (59.2)	0.93 (0.73-1.19)	P=0.57
	C/T	260 (43.2)	232 (47.3)		
	T/T	86 (14.3)	58 (11.8)		
TAFI	No Ile(Thr/Thr)	287 (47.7)	249 (50.8)	1	
Thr325Ile	Ile	314 (52.3)	241 (49.2)	1.13 (0.89-1.44)	P= 0.31
	Thr/Ile	257 (42.7)	202 (41.2)		
	Ile/Ile	58 (9.6)	39 (8.0)		

DM - Diabetes Mellitus, MI - Myocardial Infarction, CVA/TIA - Cerebrovascular Accident or Transient Ischaemic Attack, ↑BP – Hypertension, AAA – abdominal aortic aneurysm, BMI – body mass index. Results are shown as median (interquartile range) and number (percentage of patients).

3.4 **TAFI Thr325Ile is associated with TAFI activity levels *in-vitro***

The correlation between the Thr325Ile genotype and TAFI activity levels *in-vitro* was investigated in a total of 391 study participants (both AAA and controls). In this population, the relative genotype frequencies were 49.4% Thr/Thr (n=193), 42.2% Thr/Ile (n=165) and 8.4% Ile/Ile (n=33). It has previously been shown that, when activated, TAFI 325Ile has a longer half-life and a higher anti-fibrinolytic activity *in-vitro* compared with the more common TAFI 325Thr. In the population under study, possession of an isoleucine allele was positively associated with increasing TAFI activity, determined using the modified clot lysis assay. Activity increased in a stepwise fashion with possession of an isoleucine allele, with Ile/Ile homozygotes having the longest TAFI-related retardation times (Thr/Thr 46.0 (33.5-55.5) mins, Thr/Ile 48.0 (37.6-60.3) mins, p=0.04, and Ile/Ile 56.5 (38.3-68.9) mins, p=0.004, panel A), and the highest TAFI-specific anti-fibrinolytic activity (Thr/Thr 3.1 (2.5-4.2), Thr/Ile 3.9 (2.7-5.1), p=0.002, and Ile/Ile 4.6 (3.6-6.3), p<0.0001, panel B).

A

B
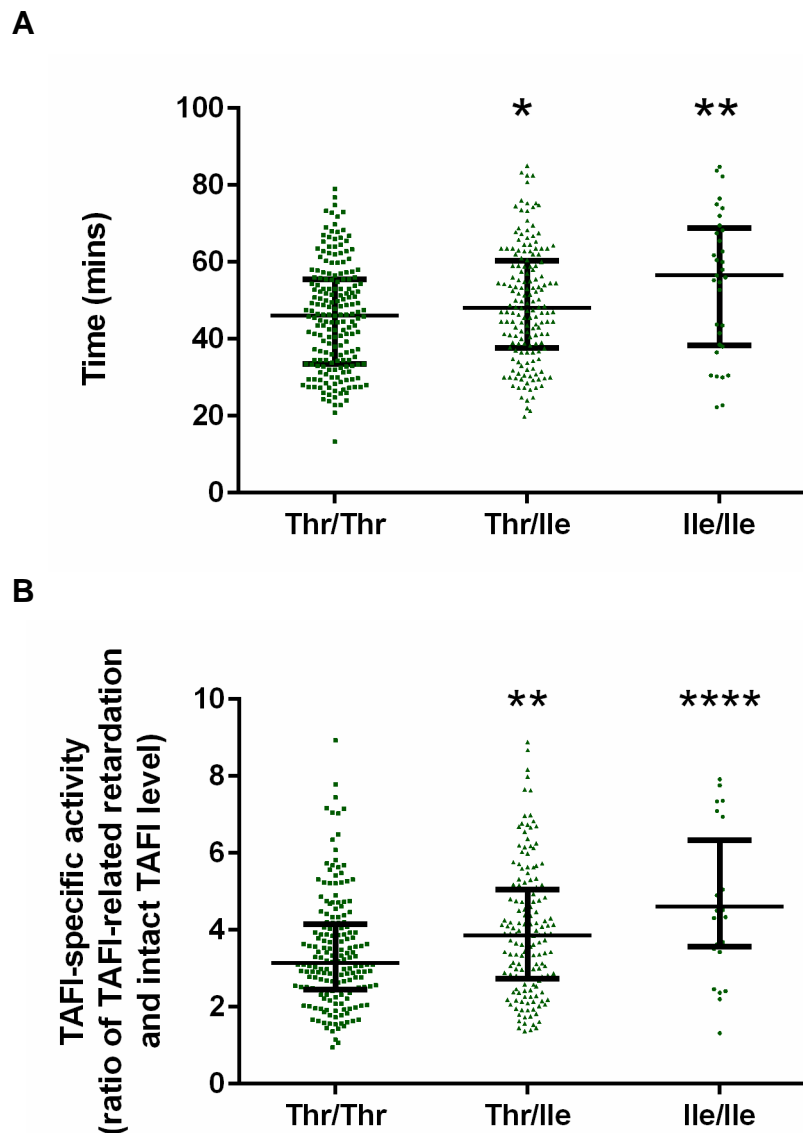
Figure 25. TAFI- related retardation to lysis time and TAFI-specific anti-fibrinolytic activity is increased with possession of the TAFI 325Ile polymorphism

Using a modified clot lysis assay, the TAFI-related retardation to lysis was measured in the plasma of 391 study participants of known TAFI Thr325Ile genotype (Thr/Thr n=193, Thr/Ile n=165 and Ile/Ile n=33). Results are presented as median (IQR). The TAFI-related retardation to lysis time was increased in those with an isoleucine allele, and increased in a stepwise fashion for Thr/Ile heterozygotes and Ile/Ile homozygotes (Thr/Thr 46.0 (33.5-55.5) mins, Thr/Ile 48.0 (37.6-60.3) mins, p=0.04, and Ile/Ile 56.5 (38.3-68.9) mins, p=0.004, panel A). By comparing these results with the levels of intact TAFI (as measured by ELISA), the TAFI-specific anti-fibrinolytic activity was calculated. The TAFI-specific anti-fibrinolytic activity was increased in a stepwise fashion with possession of an isoleucine allele (Thr/Thr 3.1 (2.5-4.2), Thr/Ile 3.9 (2.7-5.1), p=0.002, and Ile/Ile 4.6 (3.6-6.3), p<0.0001, Panel B).

3.5 The delay in clot lysis in patients with AAA may be attributed to TAFI

It has previously been shown that clot lysis time is delayed in fibrin clots produced *ex-vivo* using plasma samples of patients with AAA, compared with control subjects (Scott et al., 2011).

Plasma samples from patients with large AAA (n=32) were compared with plasma samples from control subjects (n=32). The basic demographics of these two groups are shown in Table 6. Turbidity-lysis experiments, using thrombin as the initiator of clot formation, confirmed a delay in the lysis time of patients with AAA compared with controls (AAA 34.28 ± 19.68 mins vs. controls 27.79 ± 6.173 mins, Figure 26, panel A). Through analysis of these lysis curves, it is clear that the delay in lysis in patients with AAA occurs late, after lysis has already been initiated. Diabetes is known to result in a prolongation of clot lysis (Bailey et al., 2015). Thus, due to the significant difference in the number of people with a diagnosis of diabetes between the patient and control groups, the data was analysed again, but with any diabetic participants excluded. Even once these patients had been excluded, there was still a difference, albeit slightly smaller, in the time to half lysis between patients and controls (32.35 ± 3.35 mins vs. 28.08 ± 1.09 mins, p=0.19), indicating that the delay in lysis time seen was not attributable only to the increase in diabetes in the patient population. The addition of excess TAFI (15 µg/ml final concentration in excess of plasma TAFI levels) to control plasma samples resulted in a delay in the lysis time that replicated the pattern seen in patients with AAA (lysis time increased from 27.79 ± 6.173 mins without additional TAFI to 29.08 ± 24.77 mins with

additional TAFI, p<0.0001, Figure 26, panel E). The addition of PCI (final concentration 30 µg/ml) to plasma samples of patients with AAA resulted in a decrease in lysis time back to the levels seen in controls (lysis times decreased from 34.28 ± 19.68 mins without PCI to 28.12 ± 7.195 mins with PCI, p=0.04, Figure 26, panel C). When thrombomodulin (final concentration 0.6 units/ml) was added to the activation mix (in order to ensure that all of the TAFI within the samples were activated), there was still a delay in the lysis time in patients with AAA compared with controls (AAA 123.5 ± 39.35 mins vs. controls 100.5 ± 17.14 mins, p=0.002, Figure 26, panel B). However, adding additional TAFI to the control samples did not result in any delay to lysis in these conditions (104.7 ± 15.05 mins without additional TAFI vs. 100.5 ± 17.14 mins with additional TAFI, Figure 26, panel F). As would be expected, addition of PCI to the samples of patients with AAA resulted in a decrease in the time to half-lysis (123.5 ± 39.35 mins without PCI to 41.48 ± 25.76 mins, p<0.0001, Figure 26, panel D).

Table 6. The demographics of patients with AAA and control subjects used for the study of the effect of TAFI on turbidity-lysis in patients with AAA

		Patients (AAA) n=32	Controls (no AAA) n=32	p-value
Aorta size (cm)		6.0 (5.1-7.0)	2.0 (1.9-2.5)	<0.0001
BMI (weight kg/height m^2)		25.7 (24.4-27.8)	26.4 (24.0-29.9)	0.26
Age (years)		72.5 (69.3-77.0)	72.5 (71.0-78.0)	0.40
Male n(%)		32 (100%)	31 (96.9%)	1.0
Drug history n(%)	Aspirin	23 (71.9%)	16 (50%)	0.12
	Statin	23 (71.9%)	13 (40.6%)	0.02
Past Medical History n(%)	DM	8 (25.0%)	1 (3.1%)	0.02
	MI	7 (21.9%)	3 (9.4%)	0.30
	Angina	6 (18.8%)	5 (15.6%)	1.0
	CVA/TIA	11 (34.4%)	2 (6.3%)	0.01
	↑BP	22 (68.8%)	10 (31.3%)	0.006
Smoking history n(%)		30 (93.9%)`	22 (68.8%)	0.02

DM - Diabetes Mellitus, MI - Myocardial Infarction, CVA/TIA - Cerebrovascular Accident or Transient Ischaemic Attack, ↑BP – Hypertension, AAA – abdominal aortic aneurysm, BMI – body mass index. Results are shown as median (interquartile range) and number (percentage of patients).
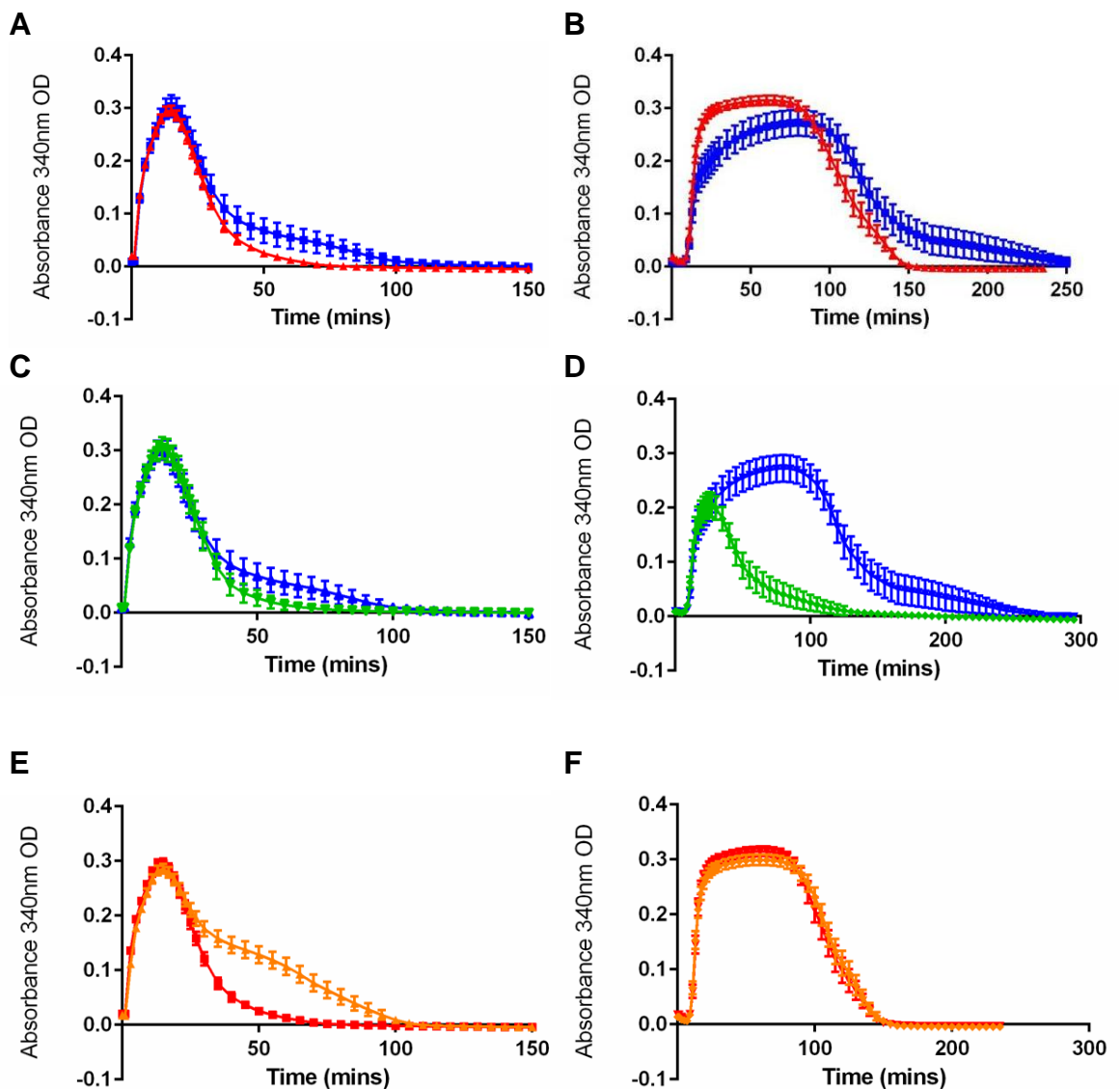
Figure 26. Turbidity-Lysis curves in the plasma of patients with AAA show a late delay in lysis which can be attributable to TAFI

Turbidity-lysis curves were generated using plasma samples from patients with large AAA (n=32) and control subjects (n=32). Using an activation mix containing thrombin, calcium and tPA, the lysis times of patients with AAA (blue line) were increased compared with control subjects (red line); this was due to a late delay in lysis (Panel A). A delay in lysis was also seen when thrombomodulin was included in the activation mix (Panel B). When potato carboxypeptidase inhibitor (PCI) was added to the plasma of patients with AAA (green line) and clotting was activated using thrombin and calcium, the secondary delay in lysis was abolished (Panel C). In the presence of thrombomodulin, addition of PCI had a marked effect, as all TAFI activity in the sample was inhibited (Panel D). When exogenous TAFI was added to the plasma of control subjects (orange line), a secondary delay in lysis, akin to that seen in patients with AAA was seen (Panel E). There was no effect of adding additional TAFI in the presence of thrombomodulin (Panel F).

Due to the non-uniform pattern of lysis in these samples, the area under the curve (AUC) was calculated in order to better represent the difference in the lysis profiles. These results demonstrated the same patterns, with a reduction in AUC in control subjects compared with AAA patients, which could be corrected with the addition of PCI to the AAA samples (AAA 656.5 ± 695.2, control 399.9 ± 134.3, AAA and PCI 542.8 ± 519.1).

Together, these results show that TAFI does appear to play a role in the delay in lysis that has previously been demonstrated in the plasma of patients with AAA, and that this delay in lysis time can be corrected through the inhibition of TAFI. This is summarised in Figure 27.
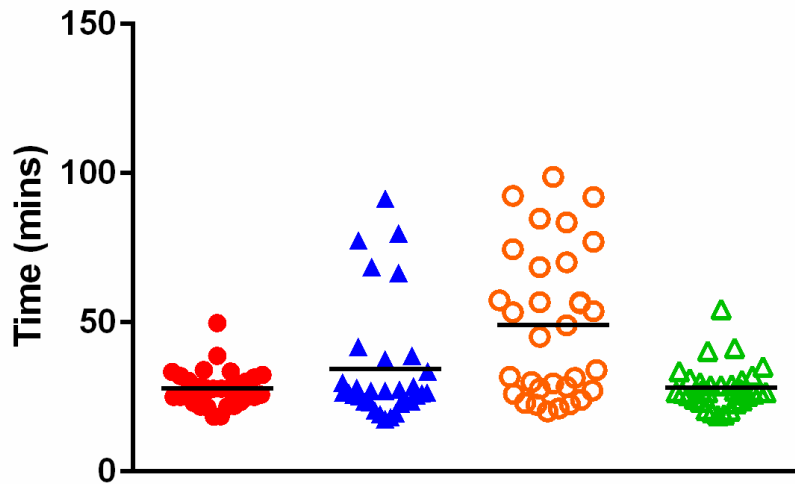
Figure 27. There is a delay in the fibrinolysis time in patients with AAA, and this can be corrected through the inhibition of TAFI

Turbidity-lysis profiles were generated using plasma samples from patients with AAA (n=32) and control subjects (n=32). An activation mix containing thrombin, calcium and tPA was used to initiate the reaction. There was a delay in the time to half lysis of patients with AAA (blue triangles) compared with control subjects (red circles), 34.28 ± 19.68 mins vs. 27.79 ± 6.173 mins. The addition of exogenous TAFI at 15 µg/ml to the plasma of control subjects (orange circles) resulted in a delay in lysis time (27.79 ± 6.173 mins to 29.08 ± 24.77 mins, p<0.0001). The inhibition of TAFI in the plasma of patients with AAA using potato carboxypeptidase inhibitor (green triangles) resulted in a decrease in the time to half lysis (34.28 ± 19.68 mins to 41.48 ± 25.76 mins, p=0.04).

3.6 The histological examination of the intra-luminal thrombus reveals an organised fibrin network

Samples of intra-luminal thrombus (ILT) taken from patients with AAA undergoing an open operation were collected, processed and paraffin embedded. Transverse sections of 5 µm were taken through the samples of ILT in order to be able to histologically examine its structure from luminal surface through to the clot adjacent to the aortic wall. Samples were stained using Martius Scarlet Blue, which stains fibrin of different ages in different colours, with mature fibrin appearing red, newly laid fibrin and erythrocytes appearing yellow and old fibrin staining blue (Lendrum et al., 1962). In all samples, the layers of the ILT could be clearly delineated, and, seemed to be divisible into the three layers that have been previously described; the luminal layer, the medial layer, and the abluminal layer. The luminal layer in all cases showed an abundance of both erythrocytes (yellow) and fibrin (red). In the medial layer, there is still some mature fibrin (red), but this is starting to degrade (blue), and there are fewer erythrocytes (yellow). In the abluminal layer, there is little/no mature fibrin (red), and in fact, all that can be seen is degraded fibrin (blue) with very few erythrocytes. Example sections from two different patients undergoing open AAA repair are shown in Figure 28. Both are taken at 4x magnification, with Panel A showing the sections through a relatively thick ILT, compared to Panel B which shows the sections through a much thinner ILT. In both, the same three layers can be appreciated, implying that even in patients with a relatively low ILT volume, the process of ILT deposition is likely to have been as chronic as in those patients with a greater ILT burden.

A

Lumen

Aortic Wall

B

Lumen

Aortic Wall
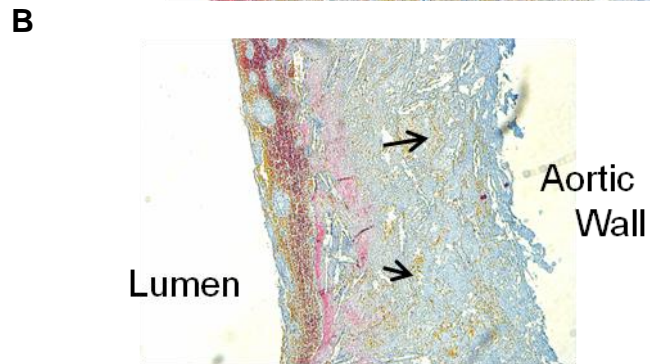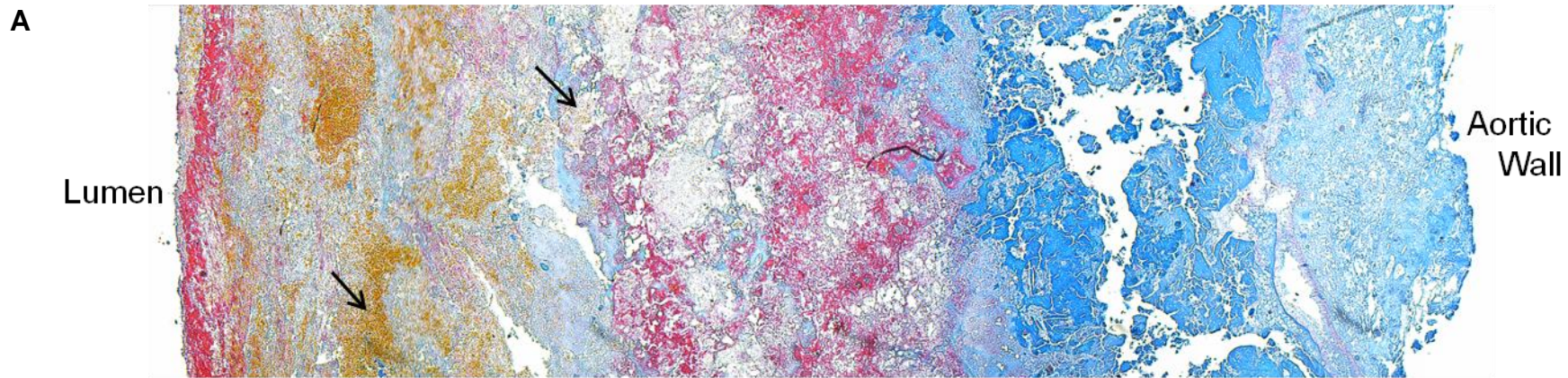
Sections of 5µm were taken through samples of human ILT, and were stained for fibrin using Martius Scarlet Blue. Nuclei were stained using Weigerts Iron Haematoxylin, Martius yellow was used to stain erythrocytes yellow, Crystal Ponceau was used to stain fibrin red and methyl blue was used to stain collagen, and old, degraded fibrin, blue. Shown here at 4x magnification, histologically, the layers of the ILT can be well differentiated, with the luminal layer being rich in fibrin and erythrocytes, whilst at the surface adjacent to the aortic wall (the abluminal surface) there is no fresh fibrin, and only scattered erythrocytes (marked with a black arrow). Panel A shows a sample from a patient with a high volume of ILT, with Panel B showing a sample from a patient with low volume ILT.

Red – fibrin, Yellow – new fibrin and erythrocytes, Blue – old fibrin

Figure 28. Histological sections through Intra-Luminal Thrombus (ILT), showing the layers of fibrin within the ILT

3.7 Discussion - The Role of TAFI in human AAA

The aim of this chapter was to investigate the role of TAFI in human AAA. In particular, the aim was to investigate TAFI levels, TAFI activity and TAFI polymorphisms in patients with AAA in relation to AAA size, growth and clinical outcome.

Patients were recruited for this study through the Leeds Aneurysm Development Study, a large prospective cohort study of patients with AAA and control subjects based in the north of England. All patients with AAA presenting to the Leeds Teaching Hospitals Trust were invited to take part. Control subjects were recruited from other surgical outpatient departments, as well as through volunteers from the general public. The large size of this cohort gives a unique opportunity to study multiple factors relating to patients with AAA. However, due to the nature of recruitment of such a cohort, the control population is not as well matched for age and gender as may be desirable. The control population tends to be younger, and there are more women in this population. However, when looking at a large population from this cohort, as in the gene polymorphism studies, it can be seen that after binary logistic regression analysis, the only factor that remains statistically significantly different between the two populations is the size of the abdominal aorta. Where smaller populations were used from within the cohort, as with the lysis experiments, patients were selected from the population at random, but extreme outliers of age were removed in order to better match the two groups under study. Originally, the aim had been to study TAFI in relation to AAA growth and clinical outcome. AAA growth is complex, with a number of different models

suggested for AAA growth, none of which fit every patient with the disease (Bailey et al., 2013a). Despite having access to scan data for a number of AAA patients over a period of up to 10 years, due to the heterogeneous nature of AAA growth, many thousands of participants with multiple scans over the study period would be needed in order to make meaningful conclusions relating to TAFI levels and AAA growth. In addition, the time of recruitment to the study (and the collection of the blood samples used for analysis) was varied across the study population, with some patients being recruited early, with small AAA, and others being recruited once their AAA had already grown to a size large enough to warrant elective intervention. This is a reflection of the silent nature of AAA disease; often patients are not aware that they have an AAA until it is identified incidentally. Whilst this allows us to compare the levels in patients with differing sizes of AAA, it does not allow us to collect growth data for all of the patients in the study; for some, a blood sample was only collected shortly before their large AAA underwent repair. This also makes it difficult to draw conclusions in relation to clinical outcome. The time point at which patients are recruited in relation to their disease progression is not fixed (as would be the case for a patient with an acute myocardial infarction, for example), and so drawing conclusions relating to plasma TAFI levels and survival would be misinformed. Due to the nature of this prospective cohort, the only data we have in relation to mortality is usually the date of death. Cause of death data, should it be available, may be more useful, as conclusions could be drawn in relation to TAFI levels and an increased risk of, for example, cardiovascular death.

Another slight issue with the study relates to the control group. The ideal control group for such a study would be an age-matched population without any

presence of cardiovascular disease, but finding a significant number of entirely healthy people of the correct age group to compare to patients with AAA is extremely difficult. Indeed, allowing for the fact that a number of the 'healthy' controls did actually have a history of cardiovascular disease, it may be that the differences detected in TAFI activity between patients and controls would have been even more pronounced had the control population not had any history of disease.

All plasma samples were collected from patients without the use of a tourniquet in order to allow collection of a free-flowing venous sample. Samples were taken onto cold citrate, and kept on ice, before being centrifuged at 4°C. This protocol was followed closely, and such conditions for collection are important in order to ensure that any TAFI within the sample remains stable (Foley et al., 2013). Samples were stored at -40°C until analysis. The maximum length of time that plasma samples were stored for was 10 years, and evidence suggests that this time frame allows for the preservation of plasma fibrinolytic proteins (Hernestal-Boman et al., 2010).

TAFI levels in the plasma of patients with AAA were studied using a number of different sandwich ELISA systems. Historically, there has been some difficulty in measuring TAFI using ELISAs, as a number of antibodies were specific only for one of the two alleles of the Thr325Ile polymorphisms (Willemse and Hendriks, 2006). In addition, the difference in half-life between these two forms of TAFI may have lead to falsely high or low results in patients who are homozygotes of either form of TAFI. The ELISAs used for this study were not specific for either isoform, and so, should have detected both forms with equal specificity. Although the half-life of the two forms is different, by measuring TAFIa/TAFIai in

combination, any TAFI already inactivated in the sample should have still been detected in equal measure, thus correcting for the difference in half-life between the two forms. Using the intact TAFI and TAFI-AP ELISAs from KU Leuven, the protocols of which I learnt by spending time in the laboratory of Professor Ann Gils, proved the most effective way to measure plasma TAFI levels. For this thesis, I had previously tried using commercially available ELISAs, but the results were extremely variable and inconsistent both within and across different plates (data not included). Despite this, there was still some drift in the detection of the Leuven ELISAs over time (see Figure 20). In order to counteract for any variability between plates, an even number of patient and control samples were measured on every plate, and any plates where the plasma levels of the standards fell outside two standard deviations from the mean were repeated. As per standard Leuven protocol, all plates were repeated at least twice, so that each plasma level recorded is the average of at least eight different wells (four dilutions on at least two different plates). This was done in order to identify outliers or errors in measurement, and allow for them to be repeated as necessary. Whilst there was no difference between the levels of the TAFI zymogen between patients with AAA and controls (13.1 (11.0-15.7) µg/ml vs. 12.7 (10.6-15.2) µg/ml), the levels of TAFIa/TAFIai and the TAFI activation peptide were significantly higher in patients with AAA compared with control subjects (TAFIa/TAFIai 20.5 (14.5-33.4) ng/ml vs. 14.2 (10.9-19.2) ng/ml and TAFI AP 318.7 (195.4-483.3) ng/ml vs. 248.6 (193.4-325.0) ng/ml). These data indicate an increased TAFI turnover in patients with AAA compared with control subjects. AAA is a chronic disease, and so, due to the continuous plasmin generation within the ILT and the aortic wall (Carrell et al., 2006), it is likely that

increased TAFI turnover will have been present for a number of years before the plasma sample used for this study was collected. Whilst initially an increase in TAFI turnover may have lead to a decrease in the plasma levels of the TAFI zymogen (as more zymogen was activated), over time, it is likely that, through homeostatic mechanisms, the production of TAFI is increased, and this could keep levels of the TAFI zymogen within normal limits. The activation of TAFI being increased would have resulted in higher levels of TAFIa/TAFIai and the TAFI-AP. Although the TAFIa/TAFIai ELISA aims to measure both active and inactive TAFI in the plasma samples, it is likely that due to the very short half-life of active TAFI, the majority of what is measured by this ELISA is actually inactivated TAFI.

In line with the plasma levels of the total TAFI zymogen, the anticipation was that the TAFI activity, as measured using a clot lysis assay, in which all of the TAFI in the sample is activated with exogenous thrombin and thrombomodulin, would be similar in patients with AAA compared with control subjects. However, the levels of TAFI activity (as measured by the TAFI-related retardation to lysis) were lower in patients with AAA compared with control subjects (44.3 (34.3-54.3) mins vs. 52.4 (39.1-63.3) mins). This is, however, in keeping with the findings of a much smaller study of 32 patients and 45 controls, which, using a commercially available kit (American Diagnostica, USA), which is based on a chromogenic assay, demonstrated a decrease in TAFI activity in patients compared with controls (Dubis et al., 2014). There were several limitations to this previous study, including the handling of the blood samples, and the failure to correlate activity with genotype for the Thr325Ile polymorphism, but despite this, it seems to corroborate the findings of the work in this thesis, as despite

much larger numbers and an alternative method, the conclusion is the same. The explanation for the decreased TAFI activity when measured *in-vitro*, but no change in the levels of TAFI zymogen between patients with AAA and controls, is not clear. One hypothesis may be that the compensatory overproduction of TAFI *in-vivo* in patients with AAA (which keeps total zymogen levels within normal limits) may result in the production of TAFI which is less functional, and therefore cannot be activated *in-vitro*. Another explanation may be that in the AAA patients, other plasma proteins may be up or down regulated which interfere in the TAFI activity assay, and this does not occur for the controls. Finally, this may also be a reflection of the importance of careful handling of plasma samples. Despite our samples being taken on the correct anticoagulant (sodium citrate), and kept cold (collected on ice and centrifuged at 4°C), it may be that there was some unavoidable activation of TAFI in the plasma samples *ex-vivo* during processing, that resulted in higher levels of TAFIai and TAFI-AP, and a reduction in the *in-vitro* activity. However, as all of our samples from both patients and controls were collected and stored in the same manner, any errors relating to sample collection should be consistent across the whole study.

The validity of this activity assay itself, however, does seem to be corroborated by an increase in activity in subjects with the isoleucine allele at position 325, a polymorphism that has previously been shown to result in a TAFI with a longer half-life and an increased anti-fibrinolytic activity (Schneider et al., 2002). Whilst, as would be expected, possession of an isoleucine allele at position 325 of TAFI was associated with an increase in TAFI activity, there was no association between this polymorphism and AAA.

In order to identify other proteins that may be involved in the changes in fibrin clot structure and fibrinolysis that have previously been demonstrated in patients with AAA, functional polymorphisms of alpha-2-antiplasmin and tPA were also investigated in this study population. This was not done to identify candidate genes for AAA development; for the identification of candidate genes, Genome Wide Association Studies (GWAS) and validation studies should be carried out in much larger numbers of patients. Indeed, GWAS studies of patients with AAA have already identified a number of candidate genes (Bown, 2014, Bown et al., 2011, Jones et al., 2013), however, no single polymorphism is able to entirely account for the incidence of AAA, with hypertension, smoking, and male gender remaining the most significant risk factors for AAA. Two functional polymorphisms of α2AP were investigated in this group of AAA patients and controls. The allele frequencies identified (Arg6Trp 0.80/0.20 and Arg407Lys 0.83/0.17) are in agreement with previous, albeit much smaller published studies related to these polymorphisms (Christiansen et al., 2007, Lind and Thorsen, 1999, Uitte de Willige et al., 2011). A strong correlation is evident between these two polymorphisms, which confirms the findings of a much smaller study of 30 healthy controls (Lind and Thorsen, 1999). There was a significant association between the Arg407lys polymorphism and AAA, with patients with AAA statistically less likely to have the rarer lysine allele compared with control subjects. The exact function of this polymorphism is not yet established. Arg407Lys is positioned in the extended C-terminus of α2AP, which is the region of the molecule that is involved in competitive binding to plasmin(ogen). The extended C-region of α2AP is proteolytically cleaved in healthy subjects, with only 65% of α2AP circulating in human plasma having an

intact C-terminus (Sasaki et al., 1986, Sugiyama et al., 1988, Uitte de Willige et al., 2011). C-terminally cleaved α2AP remains an active inhibitor of plasmin, but reacts more slowly, as it is unable to competitively bind to the lysine residues of plasminogen (Clemmensen et al., 1981). It may be that the Arg407Lys polymorphism has an effect on the cleavage of the C-terminus and thus the activity of the α2AP. Previous studies have not found an association between Arg407Lys and the level of C-terminal cleavage (Uitte de Willige et al., 2011), but further studies may be needed to elucidate the exact functionality of this polymorphism. Arg6Trp is positioned in the N-terminus of the α2AP molecule. The N-terminus is important because it contains Gln14, which is the site of cross-linking into fibrin by activated FXIII. Like the C-terminus, the N-terminus is also physiologically cleaved in plasma. Twelve amino acids are removed from the full length form (with Methionine at its amino terminus, Met-α2AP) to produce a shortened, more physiologically active form, with Asparagine at its amino terminus (Asn-α2AP). *In-vitro*, Asn-α2AP is cross-linked into fibrin 13 times faster than the full length Met-α2AP, and, as a result, clot-lysis time increases as the proportion of Asn-α2AP increases (Lee et al., 2004). The Arg6Trp polymorphism has been shown to affect the rate of N-terminal cleavage, with the proportion of the more active Asn-α2AP decreasing in the presence of the rare Trp allele (Christiansen et al., 2007). The expectation therefore was that there would be a negative association between this polymorphism and AAA, with patients being more likely to be in possession of the Arg allele, resulting in a greater proportion of the more active Asn-α2AP, which would contribute to the pathological fibrin clot structure and resistance to lysis that has previously been demonstrated in this disease (Scott et al., 2011).

There was, however, no association found between this polymorphism and AAA.

Finally, a polymorphism of tPA, 7351C→T, was investigated. The tPA 7351C→T polymorphism lies in the enhancer region of the tPA gene, and has been shown to be associated with the rate of tPA release from the vascular endothelium (Ladenvall et al., 2000), with rates of release decreased in the presence of the T-allele. Possession of a T-allele has previously been associated with MI (Ladenvall et al., 2002), although there was no association between this polymorphism and AAA.

Further studies to more specifically investigate plasma levels of α2AP in relation to AAA are already underway; clearly, the changes seen in the fibrin clot structure and fibrinolysis in patients with AAA are the result of a complex multi-faceted disease process, and are likely to be the result of the interaction between a number of different proteins.

Finally, this study has demonstrated a secondary delay in the lysis in patients with AAA, which can be attributed to TAFI. When additional TAFI was added to the plasma samples of control subjects, a delay in lysis matching the pattern seen in patients with AAA resulted. In corroboration with this, the addition of PCI, a potent inhibitor of TAFI, resulted in a decrease in the lysis time in patients with AAA back to the levels observed in controls. This secondary delay in lysis time appears to correspond with the plasmin-mediated activation of TAFI (Leurs et al., 2003), although further investigation is required to confirm this. This work was done in a plasma system, not a purified system, and so, although no exogenous plasmin(ogen) was added, there will have been some

plasmin(ogen) present in the plasma samples which would have been available to activate TAFI once fibrinolysis had been initiated. The addition of thrombin and thrombomodulin to the plasma demonstrated a maximal effect on the prolongation of lysis time, and thus seems to have activated all of the TAFI in the plasma samples. However, when just thrombin was added, a delay in lysis in patients with AAA (and in control samples with additional TAFI), only became evident once lysis had already begun. This seems to suggest that in patients with AAA, plasmin-mediated TAFI activation may play some role. It is known that the levels of D-dimer (the plasmin mediated fibrin breakdown product) are higher in patients with AAA compared with control subjects (Parry et al., 2009). Also, high levels of D-dimer and plasmin-antiplasmin complexes have been shown within the ILT of patients with AAA (Houard et al., 2007). Further investigation into, for example, levels of TAFI and activated TAFI within the ILT of patients with AAA may help to shed some more light onto the complex relationship between the proteins of fibrinolysis and their specific roles in the pathophysiology of this disease.

Chapter 4   Results – TAFI Inhibition in a Murine Model of AAA

The role of TAFI in AAA was further investigated using the murine Angiotensin II model of AAA. In this model, hyperlipidaemic (ApoE-/-) mice are continuously infused with Angiotensin II, which results in dissection of their aortas at points of maximal atherosclerosis (typically in the supra-renal region of the aorta), followed by thrombus formation, infiltration of inflammatory cells into the aortic wall, elastin breakdown within the wall, and AAA formation. This method was first described by Daugherty et al. (Daugherty et al., 2000). The aim of my study was to investigate the importance of TAFI activity in the different stages of AAA development, using TAFI inhibitors in combination with Angiotensin II infusion.

4.1 AAA formation in the Angiotensin II model of AAA

Mice were implanted with Alzet mini-osmotic pumps delivering 750 ng/kg/min Angiotensin II (Ang II) or NaCl 0.9% (control) for 28 days. In total, for all of the experiments used for this study, 35 mice were treated with Ang II alone, and 23 were treated with NaCl 0.9% mini-osmotic pumps (control). Both sets of mice had no noticeable changes in phenotype throughout the course of the 28 day experiment, appeared healthy and active, and gained weight as would be expected. Overall, the mortality related to Ang II infusion was 40%, which is within the limits acceptable for this model. This includes those mice who were terminated using Schedule 1 approved methods if they appeared unwell in the 'at-risk' period following initiation of Ang II infusion. Of mice surviving to 28 days after Ang II infusion, 52.4% developed AAA. After 28 days, macroscopic measurements of the aortas of mice treated with Ang II and controls were taken as described in Chapter 2.20. Results are presented as mean ± standard deviation.

Infusion with Ang II resulted in a mild dilatation of the entire aorta. The size of the thoracic aorta in mice treated with NaCl 0.9% was 0.62 ± 0.08 mm. This increased to 0.81 ± 0.09 mm in mice receiving Ang II who did not have a macroscopic AAA after 28 days, and 1.07 ± 0.25 mm in mice receiving Ang II who developed AAA (p <0.0001, see Figure 29, panel A). There was a more dramatic increase in the size of the supra-renal abdominal aorta, as is expected in this model of AAA. In mice treated with NaCl 0.9%, the abdominal aorta was 0.65 ± 0.10 mm. This increased slightly in mice receiving Ang II who did not develop macroscopic AAA (in line with the over dilatation of their aortas) to 0.99 ± 0.18 mm (p<0.0001). In mice developing macroscopic AAA, the size of the abdominal aortas increased to 2.60 ± 0.83 mm (p<0.0001). This can be seen in Figure 29, panel B. In human disease, in order to allow for individual variations in the size of the 'normal' segment of aorta, AAA are usually assessed not only by size, but also as a ratio of the surrounding 'normal' aorta. A ratio of abdominal aortic size divided by thoracic aortic size of more than 1.5 is considered aneurysmal. In this model, there was a slight increase in ratio in mice treated with Ang II that did not develop macroscopic AAA when compared with saline controls (1.22 ± 0.19 vs. 1.04 ± 0.12, p=0.005), despite the ratio being below the threshold for being considered an aneurysm. In mice with macroscopic AAA, there was a large increase in the ratio up to 2.59 ± 1.03, p<0.0001, Figure 29, panel C.
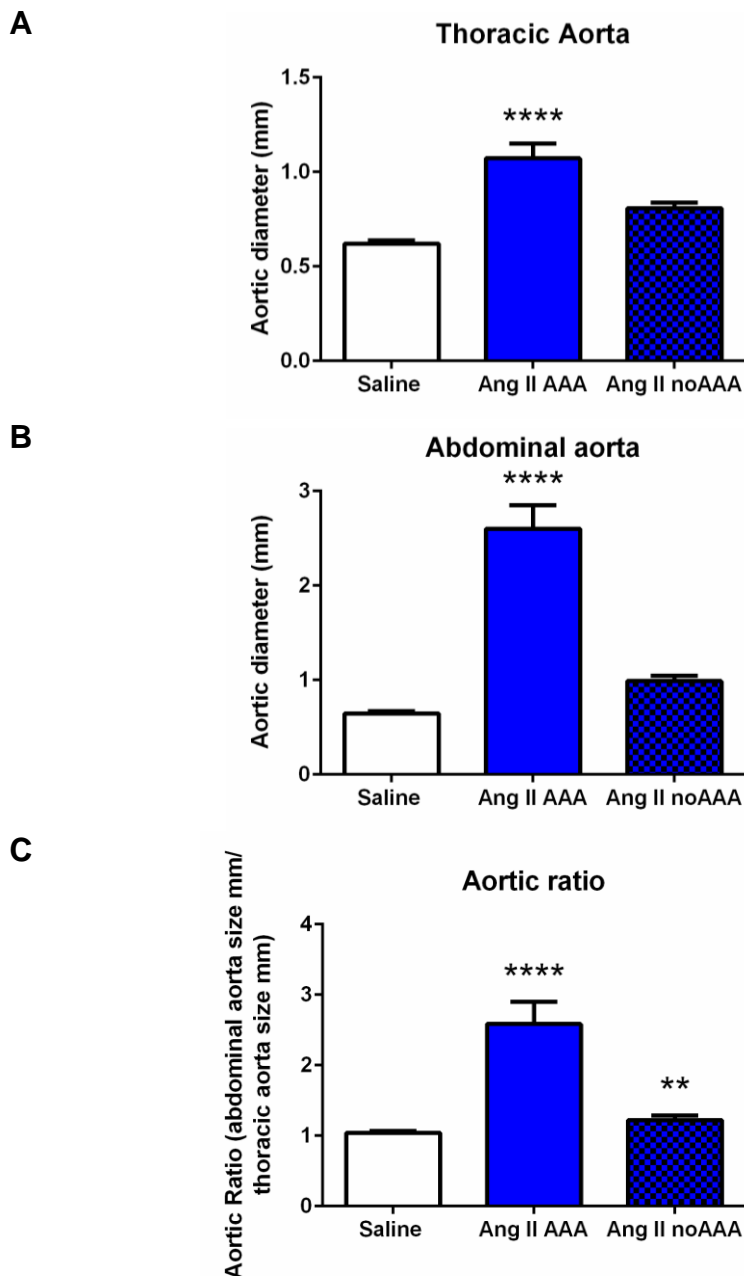
Figure 29. Aortic dilatation in the Angiotensin II model of AAA

ApoE-/- mice were treated with Ang II 750 ng/kg/min or NaCl 0.9% (controls) via subcutaneous mini-osmotic pump for 28 days. In mice surviving to 28 days (Ang II n=21 and NaCl 0.9% n=23) images were taken of the aorta, and measurements taken to determine the size of both the thoracic aorta and the supra-renal abdominal aorta. Based on the measurements taken, mice were classified as AAA (n=11) or no AAA (n=10), depending on whether or not a macroscopic AAA was present (aortic ratio >1.5). There was an increase in the size of the thoracic aorta in all mice treated with Ang II; NaCl 0.9% 0.62 ± 0.08 mm, Ang II no AAA 0.81 ± 0.09 mm (p<0.0001), Ang II AAA 1.07 ± 0.25 mm (p<0.0001), Panel A. The size of the abdominal aorta also increased in all mice treated with Ang II, with a more dramatic increase in those with a macroscopic AAA; NaCl 0.9% 0.65 ± 0.10 mm, Ang II no AAA 0.99 ± 0.18 mm (p<0.0001), Ang II AAA 2.60 ± 0.83 mm (p<0.0001), Panel B. The aortic ratio in mice developing AAA was 2.59 ± 1.04, whilst in control mice it was 1.04 ± 0.12 (p<0.0001), Panel C.

4.2 Fibrin Clot Structure is not altered in the Angiotensin II model of AAA

Plasma samples from mice treated with Ang II that developed AAA (n=7) were compared to samples from mice infused with NaCl 0.9% (controls, n=8). Turbidity and turbidity lysis was performed as described in section 2.13. The maximum optical density (max OD), an indicator of fibre thickness and clot density, was not different between the AAA and control groups (0.10 ± 0.07 vs. 0.09 ± 0.02 p=0.72, Figure 30, panel B). The time taken for the samples to reach the max OD was also not significantly different between the groups (11.11 ± 2.57 mins vs. 13.46 ± 2.32 mins, p=0.08, Figure 30, panel A), indicating that the clotting process proceeded in a normal fashion in both groups. The time to half lysis, that is, the time it takes for the optical density of the clot to fall to half of its maximum after *in-vitro* addition of tPA, which is an indicator of the ease of which a clot can be lysed, was also not significantly different between AAA and controls (50.59 ± 23.31 mins vs. 41.21 ± 14.93 mins, p=0.36, Figure 30, panel C). Using confocal microscopy, the microstructure of the fibrin clots was studied. The fibre density within the fibrin clot (measured as the number of fibres per 100 μm^2) was also not significantly different between the two groups (286.3 ± 260.4 vs. 361.3 ± 217.2, p=0.634, Figure 30, panel D and E). In unison, these data show that AAA development in the Angiotensin II model of AAA, unlike in the human disease state (Scott et al., 2011), does not have an effect on the fibrin clot structure. Further to these structural results, levels of D-dimer (a fibrin degradation product), as measured by a specific murine ELISA, were not significantly different between AAA and controls (885.8 ± 354.0 ng/ml vs. 842.1 ± 176.0 ng/ml, p=0.76, Figure 31), indicating that there was not a gross overall change in the rates of fibrinolysis in this model.
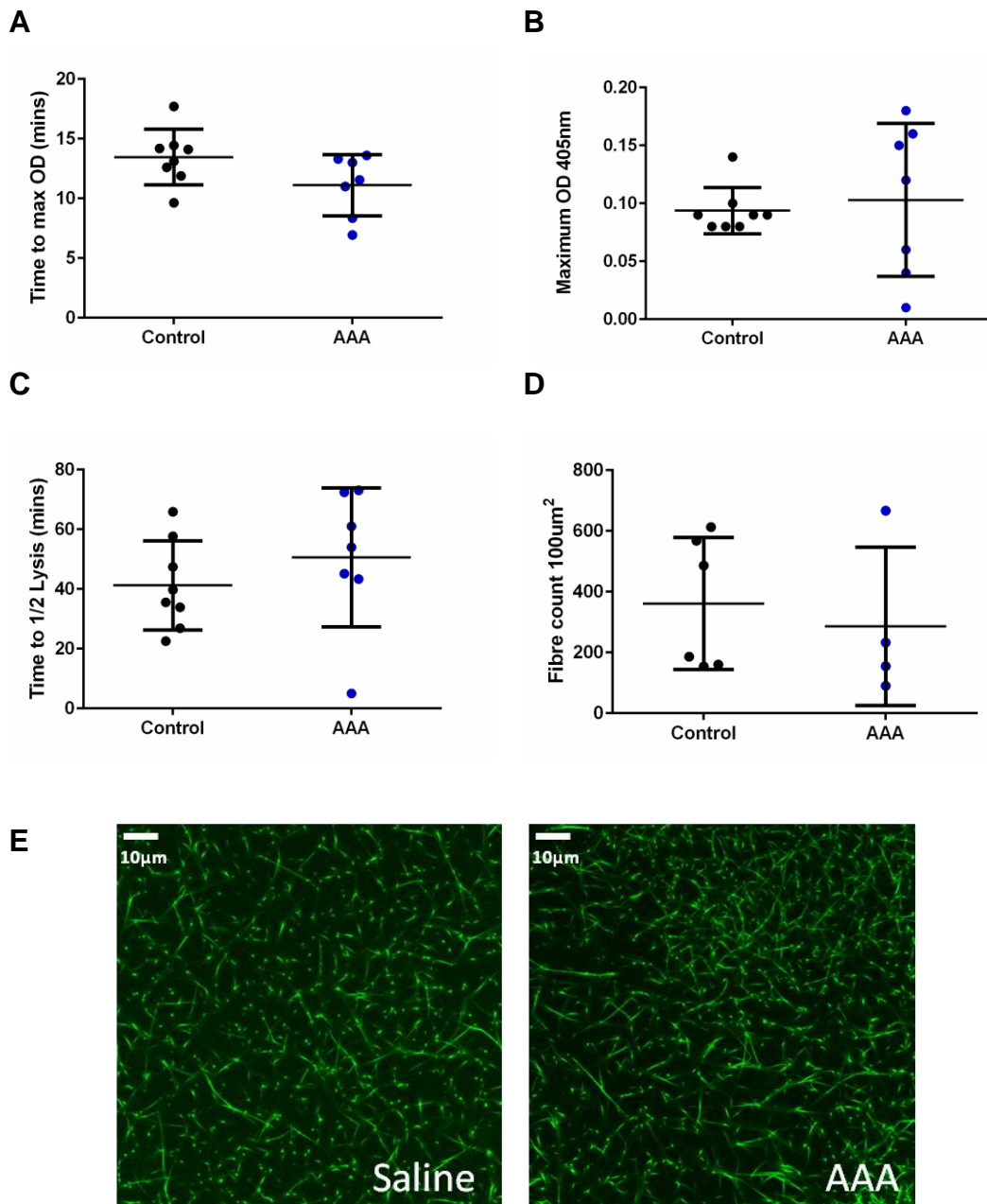
Figure 30. Fibrin clot structure is not altered as a result of Abdominal Aortic Aneurysm formation in the Angiotensin II model of AAA

Turbidity and turbidity lysis was performed using plasma from mice with AAA (n=7) and mice treated with saline control (n=8). There was no difference in the time taken to reach the maximum OD (11.11 ± 2.57 mins vs. 13.46 ± 2.32 mins, panel A), the maximum OD (0.10 ± 0.07 vs. 0.09 ± 0.02, panel B) or the time to half lysis (50.59 ± 23.31 mins vs. 41.21 ± 14.93 mins, panel C). Fibrin clots were formed which incorporated FITC-labelled fibrinogen (control n=6, AAA n=4), in order to allow for the microscopic study of the fibrin clot structure. This confirmed that there was no difference in the fibrin fibre density in mice treated with Ang II developing AAA and controls (885.8 ± 354.0 ng/ml vs. 842.1 ± 176.0 ng/ml, panel D). Sample images of the fibrin clot structure are shown in Panel E. Whiskers represent mean and standard deviation of the mean.
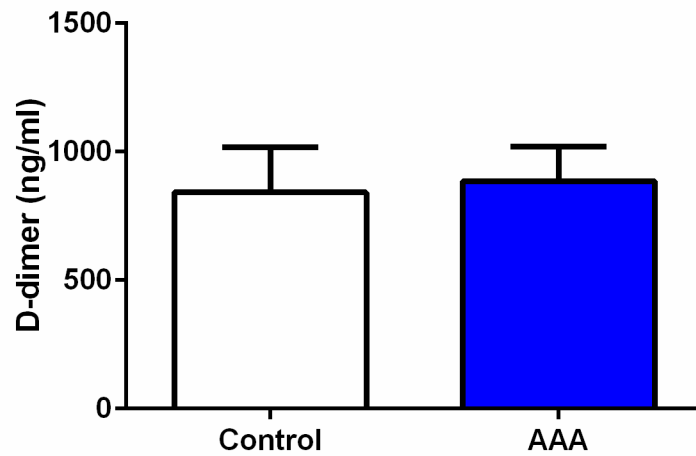
Figure 31. Levels of plasma D-dimer in mice developing AAA compared with control saline treated mice

Levels of D-dimer were measured in the plasma of mice treated with Ang II developing AAA and control (saline treated) mice, using a commercially available ELISA kit (Cat No. 024579, US Biological Life Sciences). There was no significant difference in the levels of D-dimer in plasma of mice with AAA compared with controls (885.8 ± 354.0 ng/ml vs. 842.1 ± 176.0 ng/ml).

4.3 The levels of TAFI within the aortic wall are not altered with AAA formation

TAFI levels within the wall of the abdominal aorta were measured in mice with AAA (n=8) and compared with controls (n=8). The protein was extracted from the wall using a homogenisation and lysis protocol as described in Section 2.21. The total levels of protein in the homogenate were measured using a BCA assay (Pierce™ BCA Protein Assay kit, Thermo Fisher Scientific Inc.). The total TAFI levels were measured using ELISA. The amount of total protein was higher in the homogenate of the aortas of mice with AAA compared with controls (9.3 ± 4.7 mg/ml vs. 5.3 ± 2.1 mg/ml, p=0.04), although this is likely a reflection of the dilated nature of the vessel resulting in a larger amount of tissue being collected. The total TAFI levels, as measured by ELISA, were also slightly higher in the aortas of mice with AAA compared with controls, although this did not reach statistical significance (344.8 ± 251.0 ng/ml vs. 167.4 ± 28.5 ng/ml, p=0.07), and again this may simply be a reflection of the homogenate containing protein from a larger amount of tissue. The TAFI levels in the tissue as a percentage of the total amount of protein was calculated, and described as a percentage. The amount of TAFI in the aortas of mice with AAA was comparable to that of control mice (0.00347 ± 0.0008% vs. 0.00341 ± 0.0008%, p=0.89). These results are summarised in Figure 32.
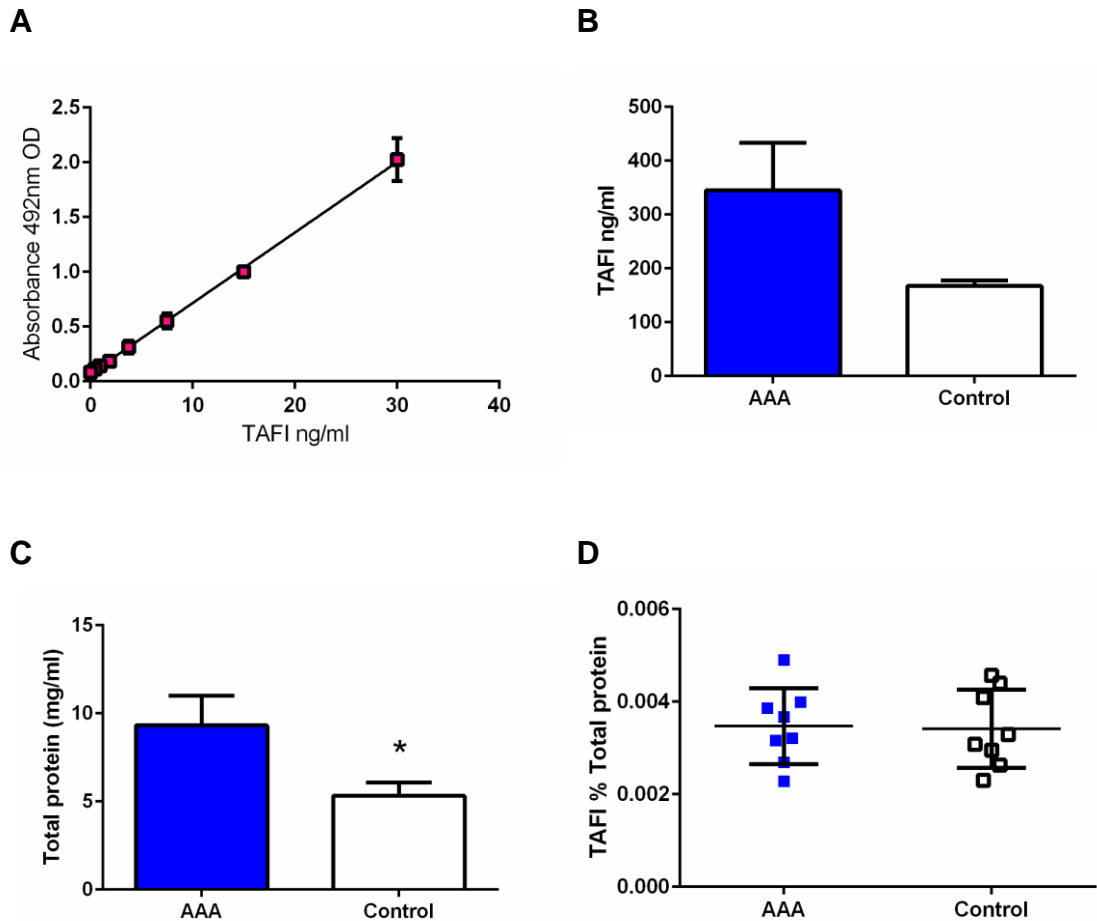
Figure 32. TAFI levels in the aortic tissue of mice with AAA is not increased compared with controls

Fresh aortas were harvested from mice receiving Ang II for 28 days and developing AAA (n=8) and control mice treated with saline (n=8). The tissue was homogenised and the protein extracted using a lysis buffer. Total protein levels were measured using a BCA assay. TAFI levels were measured using ELISA. A standard curve as produced using purified murine TAFI (panel A). The levels of TAFI and total protein were higher in the aortas from mice with AAA (TAFI: 344.8 ± 251.0 ng/ml vs. 167.4 ± 28.5ng/ml, p=0.07, Total: 9.3 ± 4.7 mg/ml vs. 5.3 ± 2.1 mg/ml, p=0.04) panel B and C, but the levels of TAFI as a percentage of the total protein was not different between the groups (0.00347 ± 0.0008% vs. 0.00341 ± 0.0008%, p=0.89), panel D.

4.4 Histological examination of the aortas of mice treated with Angiotensin II reveals loss of elastin and evidence of extensive intramural thrombus

After infusion of either Ang II (750 ng/kg/min) or NaCl 0.9% via a subcutaneous mini-pump for 28 days, the suprarenal aortas of mice from both groups were harvested, fixed in 4% paraformaldehyde and embedded in paraffin. Sections (5 µm) were taken through the cross-section of the aorta, and were stained for elastin, collagen and fibrin. Images were taken using an Olympus BX Dual View Microscope and Image ProPlus 8.0 at 10x magnification (normal aorta) and 4x magnification (AAA). In sections taken from mice with AAA, there was loss of elastin within the internal elastic lamella compared with control samples, as well as evidence of intramural thrombus. Using a stain for fibrin and erythrocytes, there was evidence of both erythrocytes (seen in yellow) and mature fibrin (stained red) within the medial layer of the aortic wall. Sample images compared with the macroscopic appearances are shown in Figure 33.
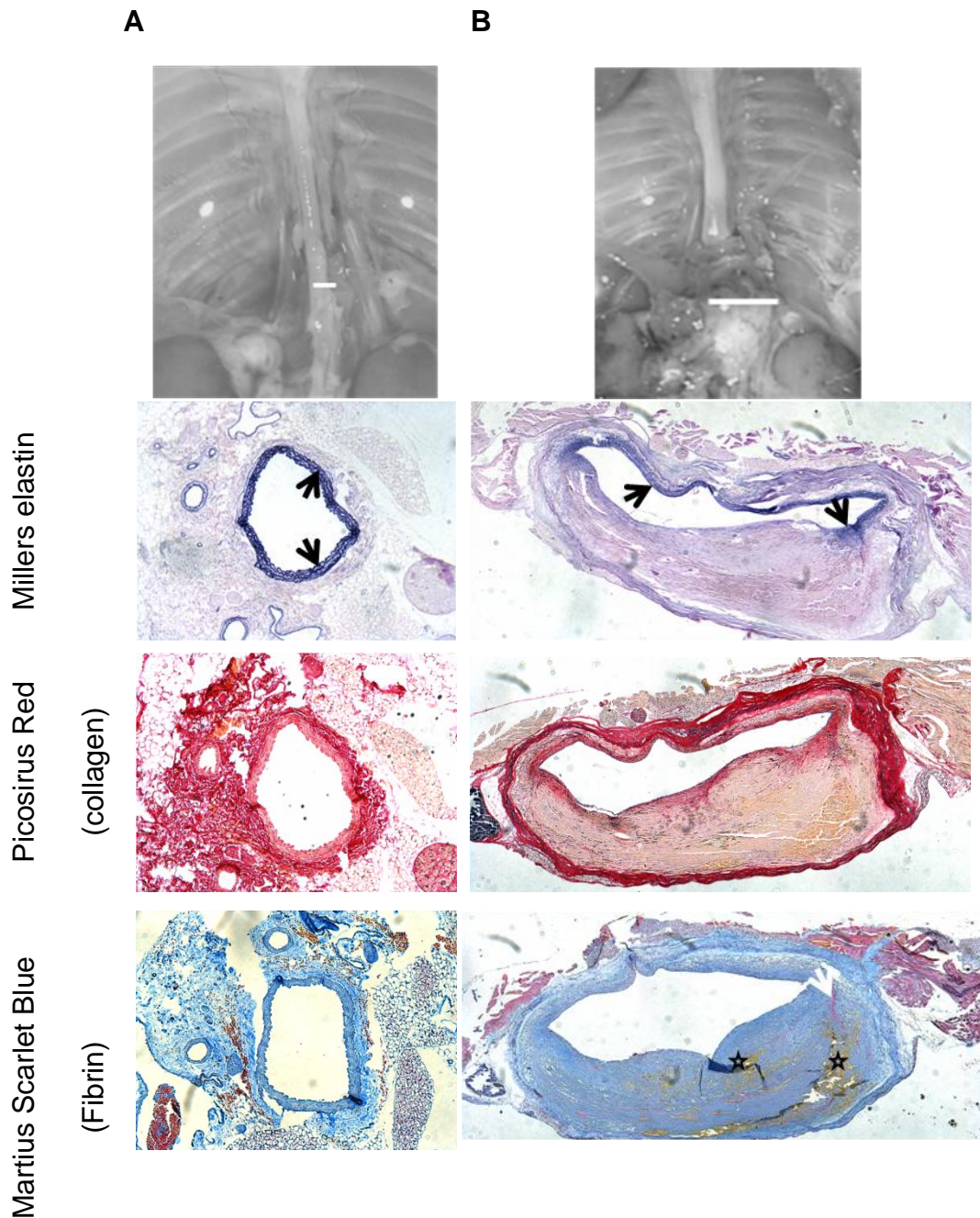
Figure 33. Histological examination of the aortas from mice treated with Angiotensin II reveals structural changes typical of AAA

Mice were infused with Angiotensin II or Normal Saline (control) for 28 days using subcutaneous mini-osmotic pumps. The supra-renal aortas were harvested, fixed using 4% paraformaldehyde and paraffin embedded. 5 µm sections were taken and stained for elastin, collagen and fibrin (n=10 in each group). They were then imaged using an Olympus BX40 Dual View Microscope. In mice treated with Ang II (Column B), changes characteristic of AAA are demonstrated. There is loss of elastin and collagen from within the structural matrix of the aortic wall (elastin is shown using a black arrow), and evidence of intra-mural thrombus, with erythrocyte deposition (black star) and fibrin formation (white arrow) present in the expanded medial layer. Column A shows images from a mouse treated with NaCl 0.9% (control).

4.5 Inhibition of TAFI in the Angiotensin II model of AAA results in a decrease in whole blood lysis time

ROTEM was performed on whole blood taken from mice treated for 28 days with either MA-TCK26D6 (n=2) or UK-396082 (n=1), and compared with samples from control mice treated with NaCl 0.9% (n=3). Even after 28 days, there was still a reduction in the lysis time in the blood of mice treated with either one of the inhibitors compared with the control (5478.6 secs vs. >7200 secs, supplemented with tPA, final concentration 20nM, see Figure 34). Mice treated with MA-TCK26D6 were also sacrificed after 7 and 10 days (n=3 in each group), and their blood used for ROTEM analysis. The maximum clot firmness, that is the maximal amplitude of the thromboelastogram, which represents the absolute strength of the fibrin and platelet clot, was not altered in mice receiving either of the two inhibitors compared with controls. Similarly, in mice receiving Ang II, addition of either of the two TAFI inhibitors did not alter the maximum clot firmness (control 54.7 ± 10.5 mm, MA-TCK26D6 7 days 59.7 ± 2.3 mm, 10 days 58.3 ± 10.0 mm, 28 days 52.5 ± 0.7 mm, UK-396082 28 days 57.0 mm, Ang II 53.5 ± 7.7 mm, Ang II and MA-TCK26D6 59.7 ± 3.1mm, 59.3 ± 3.6 mm, see Figure 34). The LI60, an index of the proportion of the clot remaining after 60 minutes, and thus an indicator of the lysis of a clot, showed a trend towards being decreased in mice receiving MA-TCK26D6 compared with control at 7 days (30.0 ± 26.5% vs. 31.7 ± 38.6%) and 10 days (25.0 ± 21.4% vs. 31.7 ± 38.6%), but this effect had resolved by 28 days (Figure 34). There were no spontaneous episodes of bleeding in mice receiving either of the two inhibitors.

A
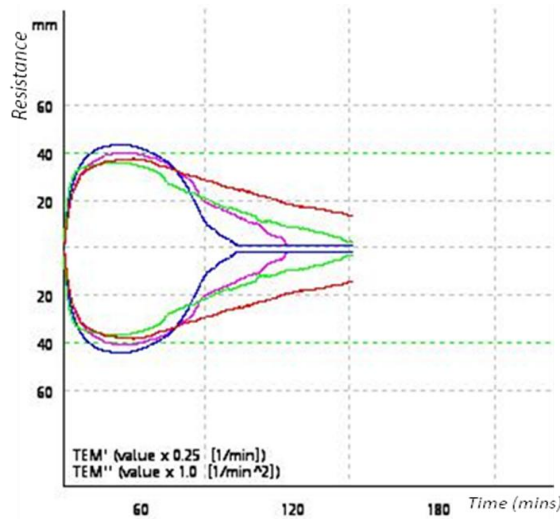
B
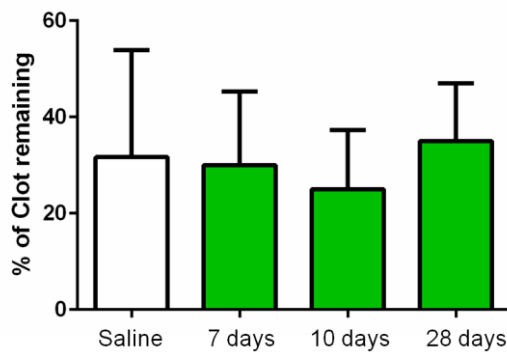
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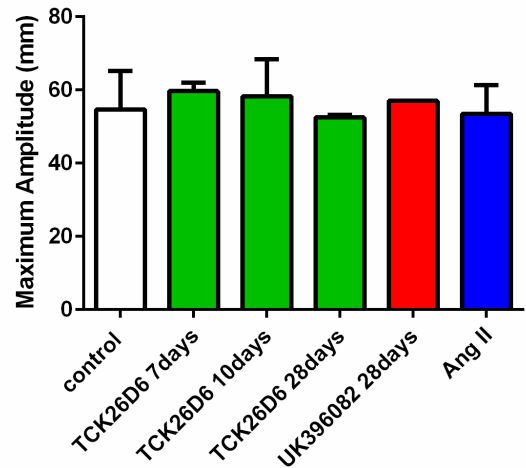
Figure 34. Whole blood lysis rates are reduced in mice treated with inhibitors of TAFI

Mice were treated with UK-396082 (n=1) or MA-TCK26D6 (n=2) in order to establish the safety of long-term administration of the inhibitors *in-vivo*. At 28 days, blood was taken from the inferior vena cava (IVC) onto 0.1M sodium citrate (blood:citrate ratio 9:1). Whole blood was used for rotational thromboelastogram (ROTEM) analysis, and confirmed that even after 28 days, there was still a reduction in the lysis time in mice treated with either MA-TCK26D6 (blue and pink lines) or UK-396082 (green line) compared with control (red line), Panel A.

The LI60 was lower in mice receiving MA-TCK26D6 compared with control at 7 days (n=3, 30.0 ± 26.5% vs. 31.7 ± 38.6%) and 10 days (n=3, 25.0 ± 21.4% vs. 31.7 ± 38.6%), but this effect had resolved by 28 days, n=3 (Panel B).

The Maximum Clot Firmness (MCF) is an indicator of the absolute strength of the clot, and this was not altered with the use of any of the TAFI inhibitors or Ang II infusion; control 54.7 ± 10.5 mm, MA-TCK26D6 7 days 59.7 ± 2.3 mm, 10 days 58.3 ± 10.0 mm, 28 days 52.5 ± 0.7 mm, UK-396082 28 days 57.0 mm, Ang II 53.5 ± 7.7 mm (Panel C).

4.6 Inhibition of TAFI *in-vivo* does not affect heart rate or blood pressure in the Angiotensin II model of AAA

In the second week post-implantation of Alzet® mini-osmotic pumps, non-invasive measurements of heart rate (HR) and blood pressure (BP) were taken in all groups of mice using the CODA non-invasive HR and BP device (Kent Scientific). Serial measurements were taken over two days (following an initial acclimatisation session where no readings were recorded) of diastolic blood pressure (DBP), systolic blood pressure (SBP) and mean arterial pressure (MAP). Blood pressure results were consistent across all the groups of mice, and are shown as mean ± standard deviation: NaCl 0.9% control DBP 76.9 ± 14.6 mmHg, SBP 106.6 ± 18.9 mmHg, MAP 86.6 ± 15.9 mmHg; Ang II DBP 81.3 ± 25.7 mmHg, SBP 110.9 ± 36.07 mmHg, MAP 89.7 ± 30.4 mmHg; Ang II and MA-TCK26D6 DBP 94.4 ± 22.6 mmHg, SBP 121.4 ± 21.2 mmHg, MAP 103.2 ± 23.3 mmHg; Ang II and UK-396082 DBP 91.1 ± 23.9 mmHg, SBP 121.3 ± 28.2 mmHg, MAP 100.6 ± 25.2 mmHg. These results are illustrated in Figure 35. Non-invasive measurement of HR also showed no significant differences between the four treatment groups, with the results for all of the four groups falling within normal limits. Results are given as the mean number of beats per minute (bpm) ± standard deviation: Saline 670.8 ± 92.5 bpm, Ang II 697.4 ± 66.19 bpm, Ang II and MA-TCK26D6 690.6 ± 65.19 bpm, Ang II and UK-396082 695.8 ± 78.41 bpm (see Figure 35). Mice in all four groups also showed normal weight gain across the study period (Figure 36).
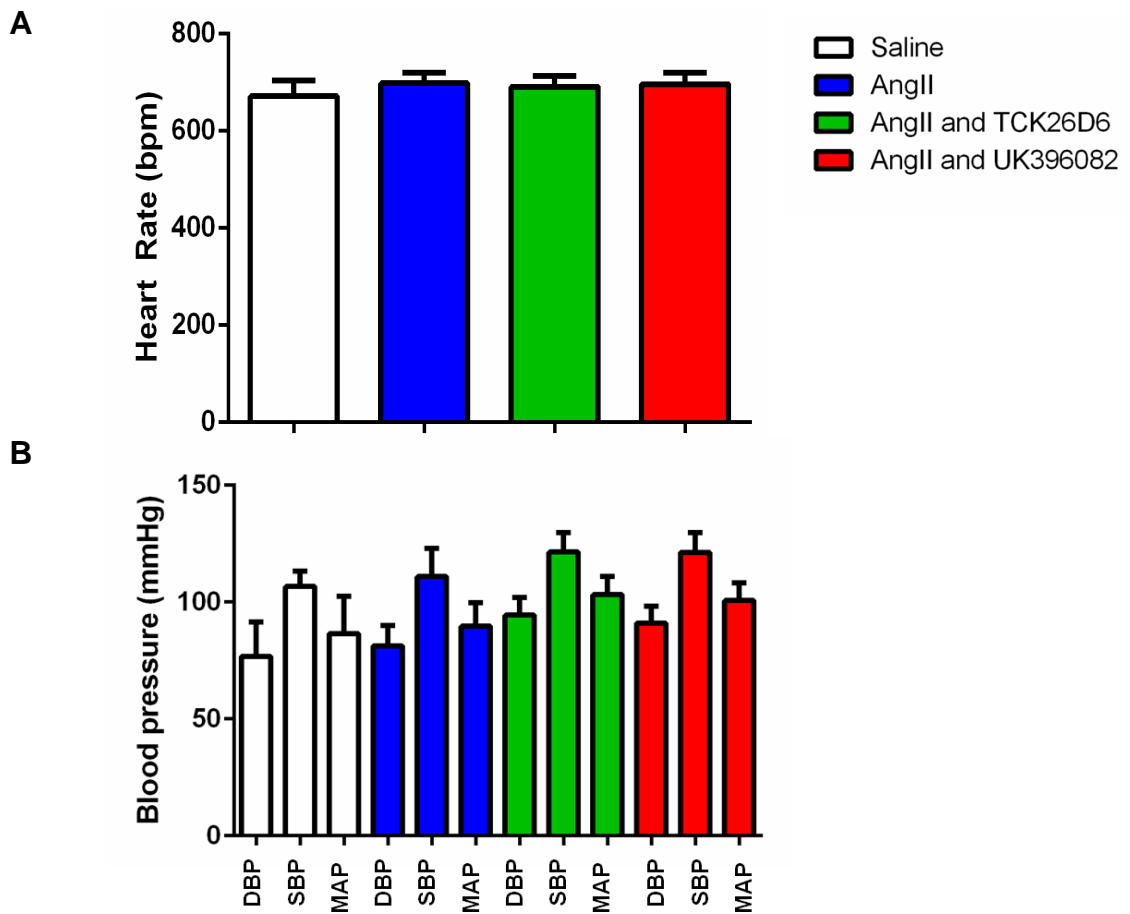
Figure 35. Angiotensin II infusion and TAFI-inhibition has no effect on heart rate and blood pressure in an *in-vivo* model of AAA

Blood pressure (BP) and heart rate (HR) measurements were taken in all groups of mice (Saline n=8, Ang II n=9, Ang II and MA-TCK26D6 n=9, Ang II and UK-396082 n=11) in the second week of the experimental period using the CODA non-invasive BP device. HRs were within normal limits in all four groups: Saline 670.8 ± 92.5 bpm, Ang II 697.4 ± 66.19 bpm, Ang II and MA-TCK26D6 690.6 ± 65.19 bpm, Ang II and UK-396082 695.8 ± 78.41 bpm. BP measurements were taken on two separate occasions, with at least 10 readings being measured on each occasion. These results were averaged for each mouse. BP readings were also within normal limits for all four groups: Saline 107/77, Ang II 111/81, Ang II and MA-TCK26D6 121/94 and Ang II and UK-396082 121/91.

A

B
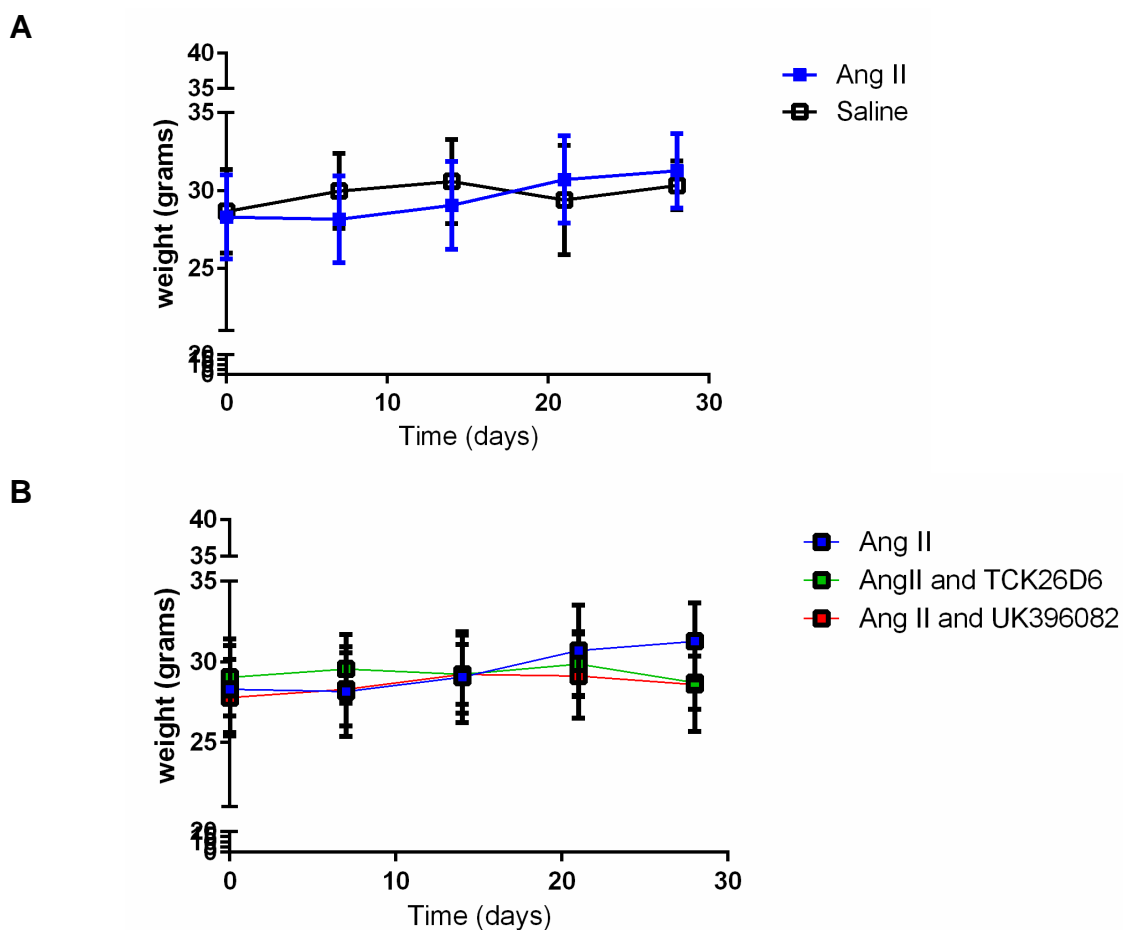
Figure 36. Angiotensin II infusion and TAFI inhibition in-vivo does not result in change in weight in ApoE-/- mice

Mice were weighed at least every 7 days throughout the course of the experiment. Infusion with Ang II did not have an effect on weight gain, with mice maintaining a normal weight throughout (Panel A). Treatment with MA-TCK26D6 or UK-396082 in addition to Ang II also did not result in a significant change in weight across the 28 days of the experiment (Panel B).

4.7 Inhibition of TAFI *in-vivo* results in a decrease in mortality in the Angiotensin II model of AAA

Mice were treated with Ang II in combination with either MA-TCK26D6 (n=12) or UK-396082 (n=12). Compared with mice receiving only Ang II (n=35), the mortality related to early rupture of the aorta was reduced in the presence of a TAFI inhibitor. Mortality fell from 40.0% to 16.6% for mice receiving MA-TCK26D6 (Log-rank Mantel-Cox test p=0.16) and from 40.0% to 8.3% for mice receiving UK-396082 (Log-rank Mantel-Cox test p=0.05). Saline control mice had a 0% mortality rate. These results are illustrated in Figure 37. In all groups, as is typical for the model, the majority of deaths related to the Ang II infusion occurred early post-operatively (prior to Day 8). Post-mortem examinations were carried out to confirm the cause of death, and in all cases were found to be due to aortic rupture (thoracic or abdominal).

A
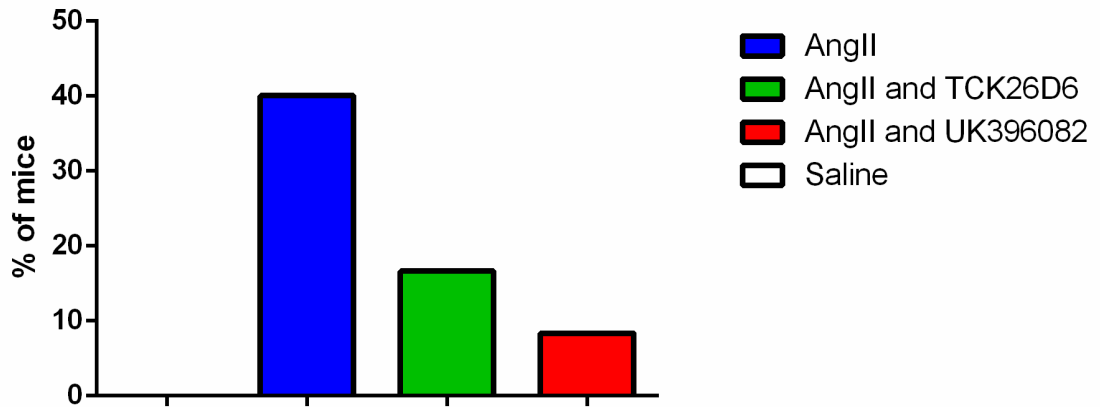
B
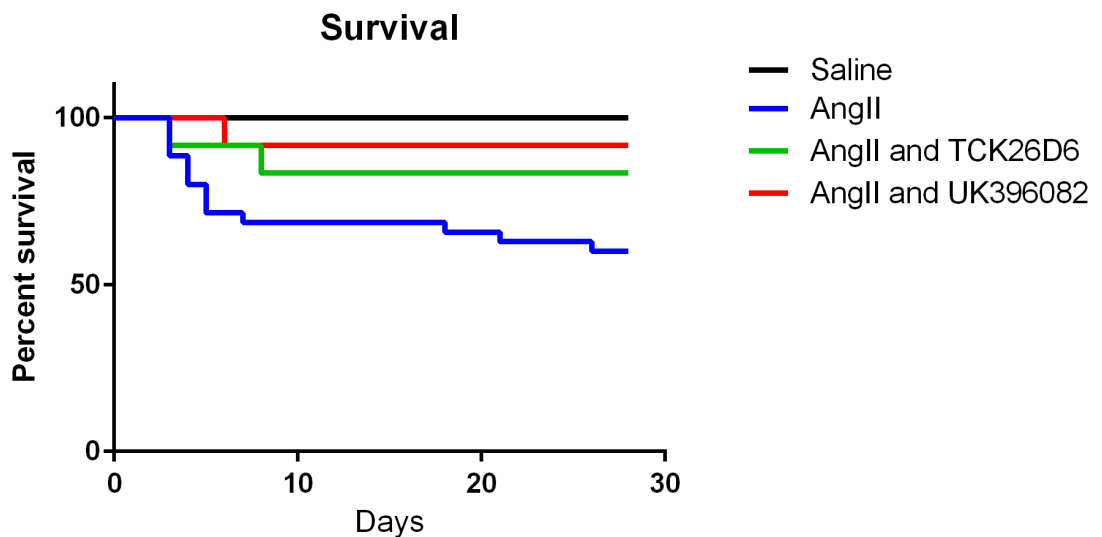
Figure 37. Inhibition of TAFI results in a decrease in mortality in the Angiotensin II model of AAA

The mortality of mice treated with Ang II 750 ng/kg/min via a subcutaneous mini pump (n=35) was 40.0%. Mortality in the NaCl 0.9%control group (n=23) was 0%. When an inhibitor of the plasmin-mediated activation of TAFI (MA-TCK26D6) was given in combination with Ang II (n=12), mortality rates fell to 16.6%. When UK-396082, a small molecule inhibitor of active TAFI was delivered in combination with Ang II (n=12), mortality rates fell to 8.3% (Panel A). Mortality in this model of AAA typically occurred early (Days 0-8 post initiation of Ang II infusion), see Panel B, and, in all groups, was the result of aortic rupture (as established by post mortem examinations).

4.8 Inhibition of TAFI *in-vivo* affects the development of Abdominal Aortic Aneurysms

Mice were treated with Ang II in combination with either MA-TCK26D6 (n=12) or UK-396082 (n=12). Unlike mortality, which fell regardless of which of the two TAFI inhibitors was used, the rates of AAA formation were affected in opposite ways by the two inhibitors. When UK-396082 was used, there was an increase in the rate of AAA formation, with 81.8% of surviving mice having a macroscopic AAA at 28 days compared with 52.4% of mice receiving Ang II alone (see Figure 38, panel A). When MA-TCK26D6 was used, however, there was a decrease in the incidence of AAA in surviving mice, with 30.0% having a macroscopic AAA at 28 days (see Figure 38, panel A). Mice that did not survive the experiment died due to aortic dissection and rupture as a result of the Ang II infusion. Thus, if all 'responders' to Ang II (that is rupture or the presence of AAA at 28 days) were analysed together, the level of response was almost equivalent between the Ang II and the Ang II with UK-396082 groups (71% vs. 83%), compared to the mice receiving Ang II and MA-TCK26D6, where the total response rate of all mice was only 42%. When an AAA did occur, there was no difference in the size of the AAA between the different groups; aortic ratios were Ang II 2.6 ± 1.0, Ang II and UK-396082 2.7 ± 0.8, Ang II and MA-TCK26D6 2.5 ± 0.4 (Figure 38, Panel B). Sample images of typical findings at 28 days for each group are shown in Figure 38, panel C-F. Images were inverted in Image J to allow for better visualisation of the margins of the aorta.
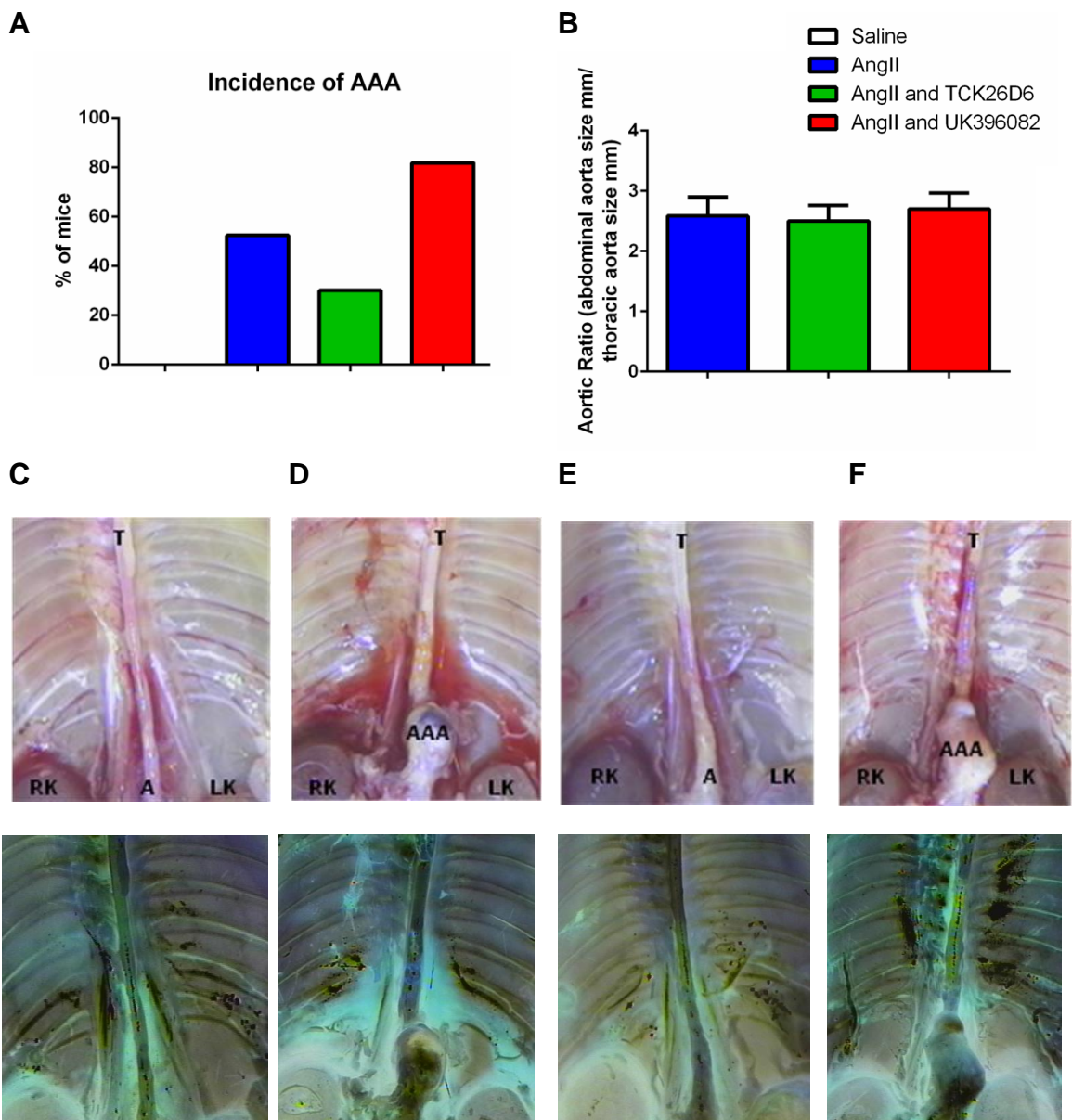
Figure 38. AAA formation is altered in the presence of a TAFI-inhibitor, with rates increased in the presence of UK-396082 and decreased in the presence of MA-TCK26D6

Mice were treated with saline control, NaCl 0.9%, (n=23), Ang II (n=35), Ang II and MA-TCK26D6 (n=12) or Ang II and UK-396082 (n=12) for 28 days. At 28 days, the incidence of AAA in surviving mice (NaCl 0.9% n=23, Ang II n=21, Ang II and MA-TCK26D6 n=10, Ang II and UK-396082 n=11) was established by direct imaging of the abdominal aorta.The incidence of AAA was increased in the mice treated with UK-396082 compared with Ang II alone (81.9% vs. 52.4%), but decreased in mice treated with MA-TCK26D6 (30.0% vs. 52.4%), Panel A. When AAA did occur, there was no significant difference in the size of AAA in the different groups (Ang II 2.6 ± 1.0 vs. Ang II and MA-TCK26D6 2.5 ± 0.4 vs. Ang II and UK-396082 2.7 ± 0.8), Panel B. Sample images of typical aortas at 28 days are shown in panels C-F. Panel C – Saline, Panel D – Ang II, Panel E – Ang II and MA-TCK26D6, Panel F – Ang II and UK-396082. **T** – thoracic aorta, **A** – abdominal aorta, **RK** – right kidney, **LK** – left kidney, **AAA** – abdominal aortic aneurysm.

4.9 Histological examination of murine aortas following TAFI inhibition

After 28 days, aortas were removed en-bloc from mice treated with NaCl 0.9% (n=10), Ang II (n=10), Ang II and MA-TCK26D6 (n=10) and Ang II and UK-396082 (n=10) to allow for histological examination. Aortas were incubated in 4% paraformaldehyde for at least 24 hours before being processed and paraffin embedded. Transverse sections (5 µm thick) were taken using a Leica RM2125 microtome (Leica Microsystems), and collected onto slides. Sections of the aorta were stained using H+E, PSR, MVG and MSB to allow for visualisation of collagen, elastin and fibrin within the samples. They were imaged using an Olympus BX40 Dual View Microscope and Image Pro-Plus 8.0 at 4-20x magnifications.

Compared to sections from mice treated with Ang II alone, there was evidence of increased intra-luminal clot formation, increased loss of elastin and disordered deposition of collagen in sections taken from mice treated with Ang II and UK-396082, but no evidence of intra-mural thrombus was seen on any sections. Figure 39 shows representative images from two mice treated with Ang II and UK-396082. By staining with Miller's stain, the composition of elastin in the internal elastic lamina can be seen; in some aortas, there was evidence of just gross dilatation with a thinning of elastin (Panel A), whilst in others there was evidence of a discrete rupture in the elastin (Panel B). By staining using Martius Scarlet Blue, the composition of any thrombus could be analysed. In some sections, there was evidence of a well established ILT (see Panel A), with old fibrin (stained in blue) being present within the thrombus. Unlike in human AAA, the laying down of the ILT does not seem to be in a sedimentary fashion

beginning at the aortic wall. In all cases where clot was seen, it lay centrally within the lumen.

The majority of mice treated with Ang II 750 ng/kg/min and MA-TCK26D6 did not develop AAA after 28 days. The histological appearance of these aortas was akin to that of control treated animals (see Figure 33). When AAA did occur, there was evidence of aortic dissection and disruption of the internal elastic lamina. Example results are shown in Figure 40.

A

| H+E | Millers elastin | Picosirus Red | Martius Scarlet Blue |

B
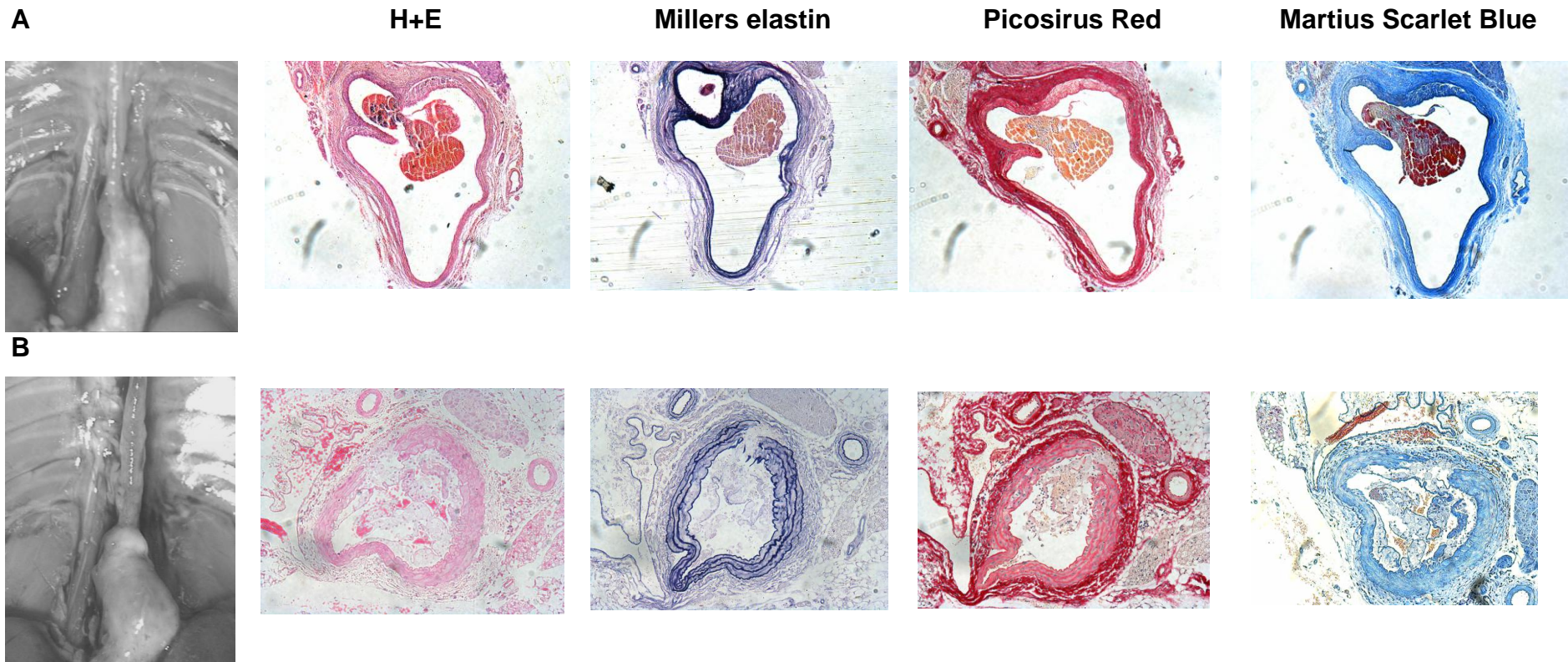
Figure 39. Histological Examination of the abdominal aorta of mice treated with Angiotensin II and UK-396082

In mice treated with Ang II 750 ng/kg/min in combination with UK-396082 30mg/kg/min, over 80% of mice had developed AAA at 28 days. There was gross heterogeneity in the macroscopic and microscopic appearance of these AAA. Unlike in the aortas from mice treated with Ang II alone, there was evidence of intra-luminal thrombus in a number of sections. Some mice displayed evidence of complete elastin breaks/dissections through the internal elastic lamina (as is seen in Panel B), whilst others showed elastin thinning and gross aortic dilatation without an area of specific elastin breakage (Panel A).

A   **Millers elastin**   **Picosirus Red**   **Martius Scarlet Blue**

B
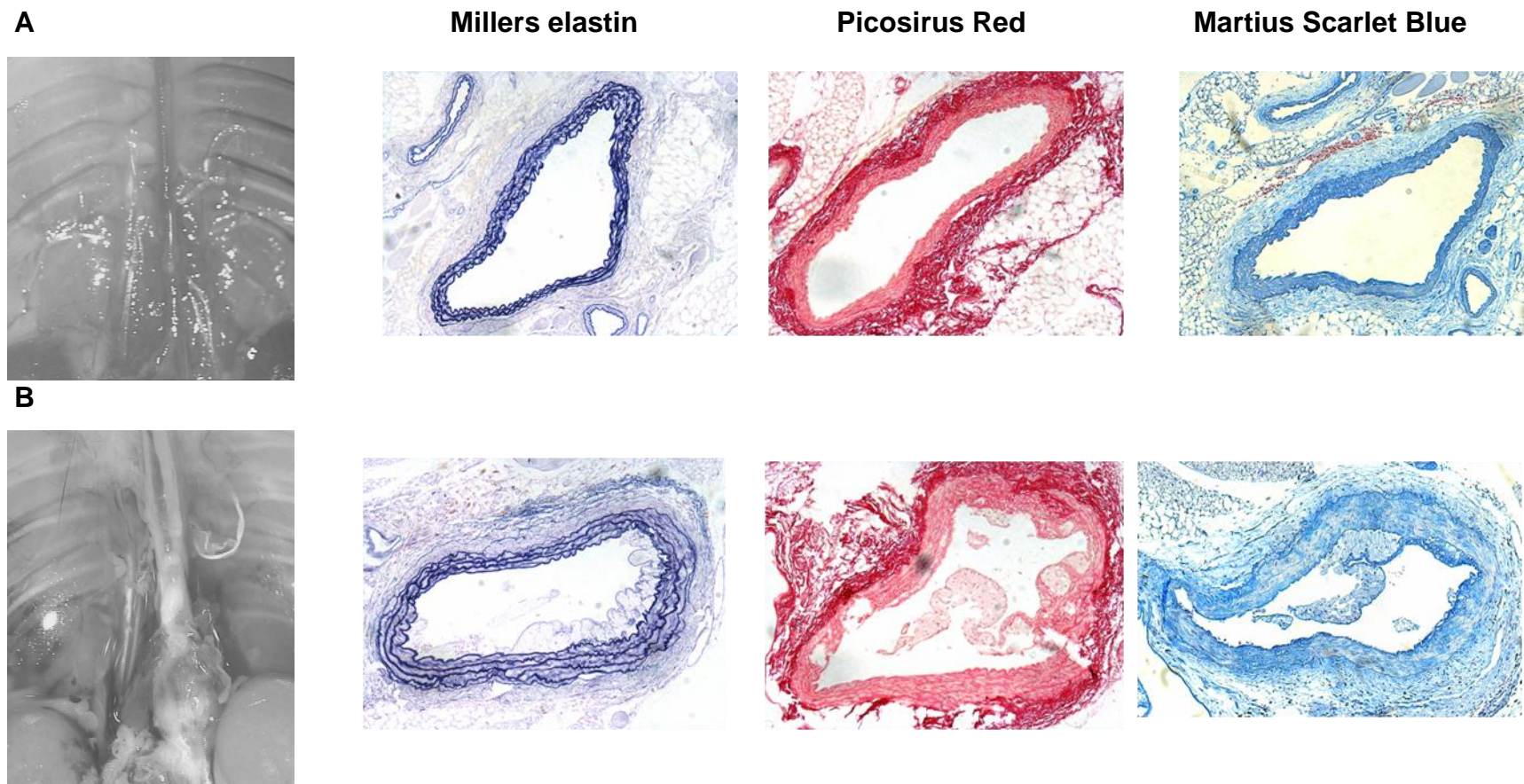
Figure 40. Histological Examination of the abdominal aorta of mice treated with Angiotensin II and MA-TCK26D6

In mice treated with Ang II 750 ng/kg/min in combination with MA-TCK26D6, there was a reduction in the incidence of AAA from 52.4% to 30% after 28 days. Those mice that did not develop aneurysms had, on histological examination, aortas that resembled those of saline controls (see Figure 33). This can be seen in Panel A. In those mice that did develop AAA, there was no evidence of intra-luminal thrombus, but there was evidence of dissection of the aortic wall and deregulated elastin and collagen within the internal elastic lamina and media (Panel B).

4.10 **Plasma levels of PAP, but not CRP or TAFI, are affected by TAFI inhibition *in-vivo***

Samples of free-flowing blood were taken from the inferior vena cava of mice after 28 days of treatment with NaCl 0.9%, Ang II, Ang II and MA-TCK26D6 and Ang II and UK-396082. Blood samples were centrifuged to give platelet poor plasma as described in section 2.9.4. Using ELISAs, the plasma levels of plasmin-antiplasmin complex (PAP), C-reactive protein (CRP) and TAFI were measured in mice from each group (n=10 per group).

Standard curves were created for each of the three ELISAs using known concentrations of PAP, CRP and TAFI. These are shown in Figure 41.

Plasma levels of PAP showed a slight decrease in mice treated with UK-396082 compared with Ang II alone; Ang II 82.5 ± 19.5 ng/ml, Ang II and MA-TCK26D6 82.4 ± 14.4 ng/ml, Ang II and UK-396082 64.2 ± 26.3 ng/ml (p=0.09), and a significant decrease compared with mice treated with saline alone (NaCl 0.9% 89.5 ± 21.1 ng/ml vs. Ang II and UK-396082 64.2 ± 26.3 ng/ml, p=0.02), Figure 41, panel A.

Plasma levels of TAFI zymogen were not significantly different between the groups, with all mice, regardless of TAFI-inhibitor treatment, having levels in the normal plasma range (NaCl 0.9% 3.5 ± 1.4 µg/ml, Ang II 3.4 ± 2.3 µg/ml, Ang II and MA-TCK26D6 3.5 ± 1.0 µg/ml, Ang II and UK-396082 3.6 ± 2.4 µg/ml, see Figure 41, panel B).

Plasma levels of CRP were significantly lower in mice receiving only Ang II, compared with saline or Ang II in combination with MA-TCK26D6 or UK-396082

(NaCl 0.9% 4.1 ± 3.2 ng/ml, Ang II 1.7 ± 0.6 ng/ml, Ang II and MA-TCK26D6 3.5 ± 1.7 ng/ml, Ang II and UK-396082 4.0 ± 2.4 ng/ml, p=0.03), Figure 41, panel C. All of these results still fall within the normal range for plasma CRP levels (in inflammatory conditions, murine plasma levels can reach 2 µg/ml (Black et al., 2004)).
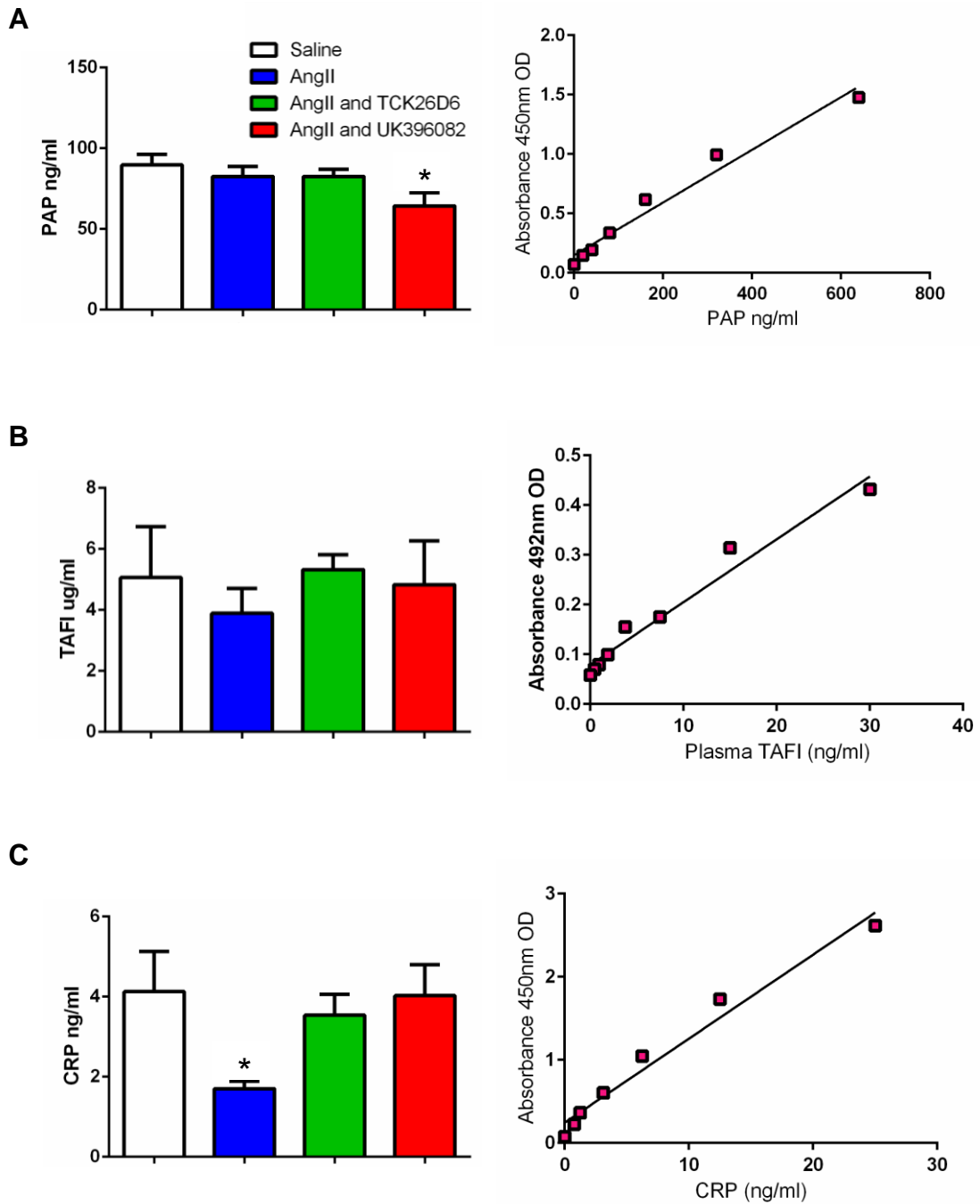
Figure 41. Plasma levels of PAP, TAFI and CRP in response to TAFI inhibition *in-vivo*

Plasma levels of PAP, TAFI and CRP were measured in mice receiving saline (n=10), Ang II (n=10), Ang II and MA-TCK26D6 (n=10) and Ang II and UK-396082 (n=10) using ELISAs. Typical standard curves for each ELISA are shown on the right hand side of the figure. Plasma levels of PAP were lowest in mice treated with UK-396082 in addition to Ang II (64.2 ± 26.3 ng/ml vs. saline 89.5 ± 21.1 ng/ml and Ang II 82.5 ± 19.5 ng/ml, p=0.02). Plasma levels of TAFI were not significantly different between the groups (Saline 3.5 ± 1.4 µg/ml, Ang II 3.4 ± 2.3 µg/ml, Ang II and MA-TCK26D6 3.5 ± 1.0 µg/ml, Ang II and UK-396082 3.6 ± 2.4 µg/ml). Plasma levels of CRP were slightly reduced in mice treated with Ang II alone, although CRP levels were not elevated outside of the normal range in any of the four groups.

4.11 The changes characteristic of the Angiotensin II model can be demonstrated *in-vivo* using the Vevo 2100 Ultrasound scanner

The growth of AAA during the 28 day experimental period was monitored using the Vevo pre-clinical USS system (FUJIFILM VisualSonics Inc.) and a MS550D MicroScan™ transducer at 22-55 mHz. Figure 42 shows the typical development of AAA over time in this model as seen on ultrasound scan. At baseline, the aorta can be observed both in cross-section and longitudinal section, and is uniform in size and shape. After 1 week, there is evidence of gross dilatation of the abdominal aorta, with evidence of dissection through the intimal layer of the vessel wall. By week 3, the aneurysm becomes well established, with a large amount of thrombus between the intimal and medial layers, and evidence of flow remaining within the lumen of the aorta.

Using colour Doppler imaging, it was possible to visualise flow within the aorta, and to differentiate between the main lumen and dissections in the early stages of the Angiotensin II model of AAA. This can be seen in Figure 43.

	Cross section	Longitudinal section	Colour Doppler

Day 0

Day 7

Day 21
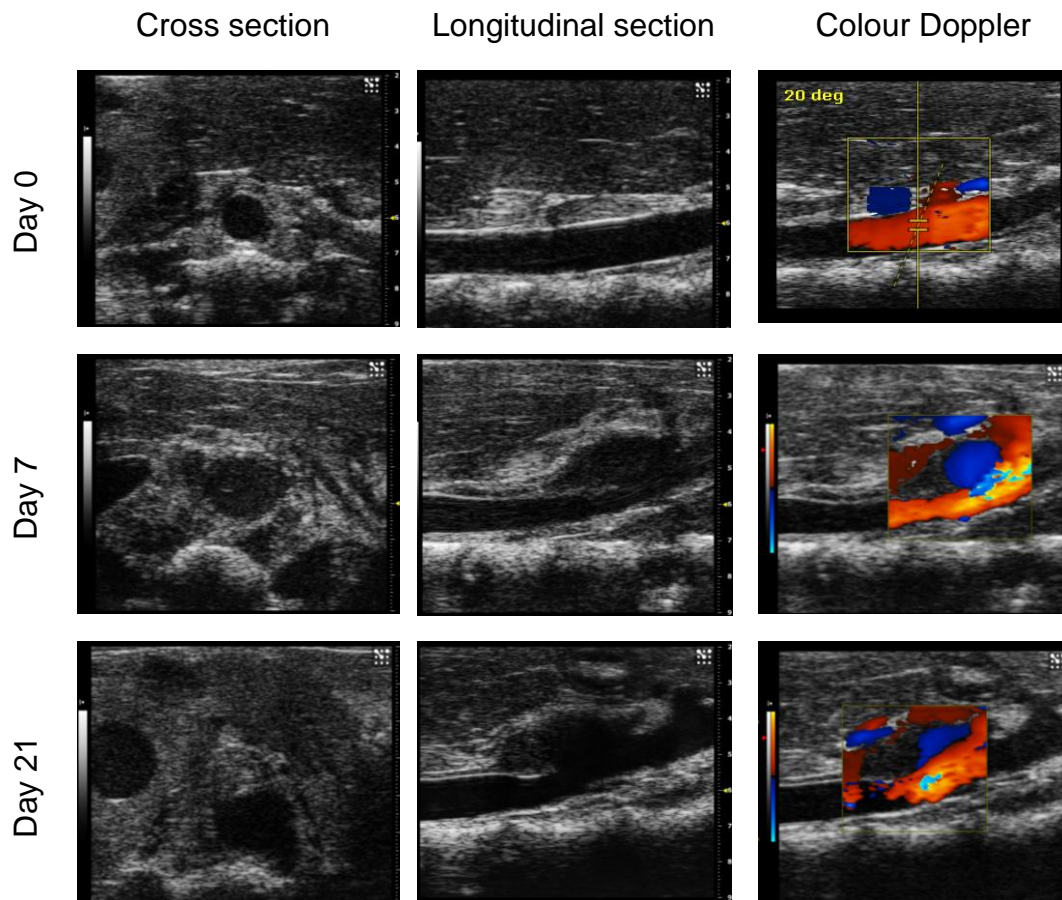
Figure 42. Development of AAA in the Angiotensin II model of AAA as visualised using ultrasound imaging

The abdominal aorta of mice treated with Ang II was visualised using a Vevo2100 pre-clinical ultrasound scanner (n=7). At day 0, the aorta is uniform in shape, with a normal diameter and arterial blood flow in seen within the lumen (red area). After 7 days of treatment with Ang II, the aorta is dilated, there is evidence of inflammation and turbulent flow can be seen within an area away from the main lumen (shown in blue). After 21 days, there is an area of dense thrombus within the medial/intimal layer, with reduced flow compared with at day 7, and an established AAA.
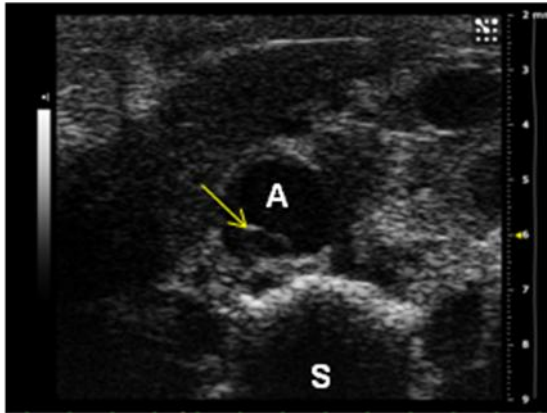
A   **B**
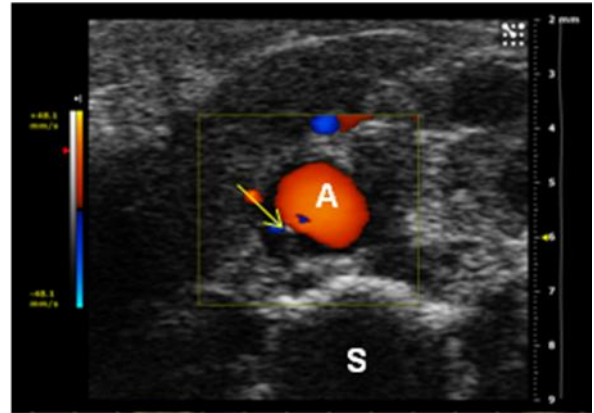
Figure 43. There is evidence of aortic dissection in the early stages of the Angiotensin II model of AAA

Aortic dissection is typical of the early stages of the Angiotensin II model of AAA, and this was clearly detected in some mice (n=2) using *in-vivo* abdominal aorta ultrasound scanning at 1 week post initiation of Angiotensin II infusion. Panel A shows a cross sectional image of the abdominal aorta with a large dissection flap. Panel B shows a colour Doppler of the dissection, with arterial flow demonstrated in the main lumen (red), but only a small amount of slow flow in the dissection (blue). A=aortic lumen, S=spine, yellow arrow=dissection flap.

4.12 TAFI inhibition after AAA formation has no effect on AAA progression *in-vivo*

Inhibition of plasmin-mediated TAFI activation with MA-TCK26D6 prevented AAA formation in the Angiotensin II model of AAA. Therefore, the effect of giving MA-TCK26D6 to mice once AAA had been established was investigated. In order to do this, mice were subjected to an abdominal ultrasound scan (USS) prior to any treatment in order to measure the baseline size of the aorta. Mice were then implanted with a pump delivering Ang II at a dose of 750 ng/kg/min as described in section 2.9.1. One week after implant, all surviving mice underwent a second scan, before being treated with either MA-TCK26D6 (n=11) or a saline control injection (n=7) of the same volume. Mice were scanned again at 21 days in order to establish the effect of the treatment. They were then sacrificed at day 28 and their aortas measured in-situ.

Mice were weighed every 7 days in order to monitor for any negative health effects of either inhibitor. Mice in all groups gained weight normally (see Figure 44), appeared healthy, and showed no episodes of spontaneous bleeding. Although there was some mortality prior to day 7, there was no subsequent mortality after administration of either the MA-TCK26D6 or control injections.
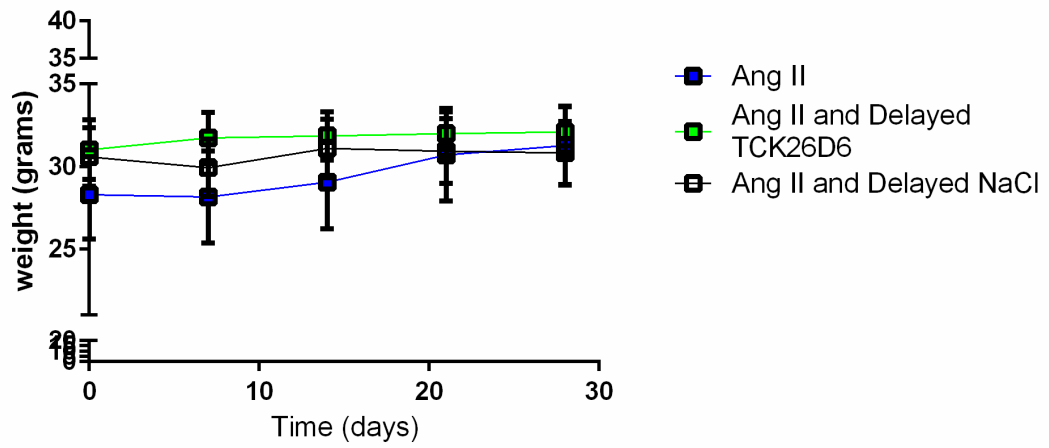
Figure 44. Late treatment with TAFI-inhibitors after AAA formation did not affect weight gain in the Angiotensin II model of AAA

Mice treated with Ang II alone (n=23), Ang II then MA-TCK26D6 at 7 days (n=11) and Ang II then NaCl 0.9% at 7 days (n=7) gained weight normally across the study period.

4.12.1 Aortic cross-sectional diameter

Using images taken from scans at baseline, 7 days and 21 days, AAA progression in the presence and absence of MA-TCK26D6 was measured. The aortic diameter was measured at a fixed point (5 mm above the right renal artery) in all mice. The inner to inner luminal diameter was used, as this was better defined in the USS images, and also is the current gold standard in human AAA imaging. The aortic diameter at all time points was equivalent in mice treated with MA-TCK26D6 at day 7 and mice treated with a saline (NaCl 0.9%) control injection. Results are shown as mean ± standard deviation. Day 0: MA-TCK26D6 1.19 ± 0.14 mm vs. NaCl 1.12 ± 0.08 mm, Day 7: MA-TCK26D6 1.56 ± 0.47 mm vs. NaCl 1.60 ± 0.42 mm, Day 21: MA-TCK26D6 1.93 ± 0.62 mm vs. NaCl 2.02 ± 0.69 mm, Figure 45, panel A and B). There was no difference in the change in aortic diameter over time between the two groups (MA-TCK26D6 increased by 0.7 ± 0.2 mm, NaCl increased by 0.9 ± 0.3mm, p=0.65, Figure 45, panel C).
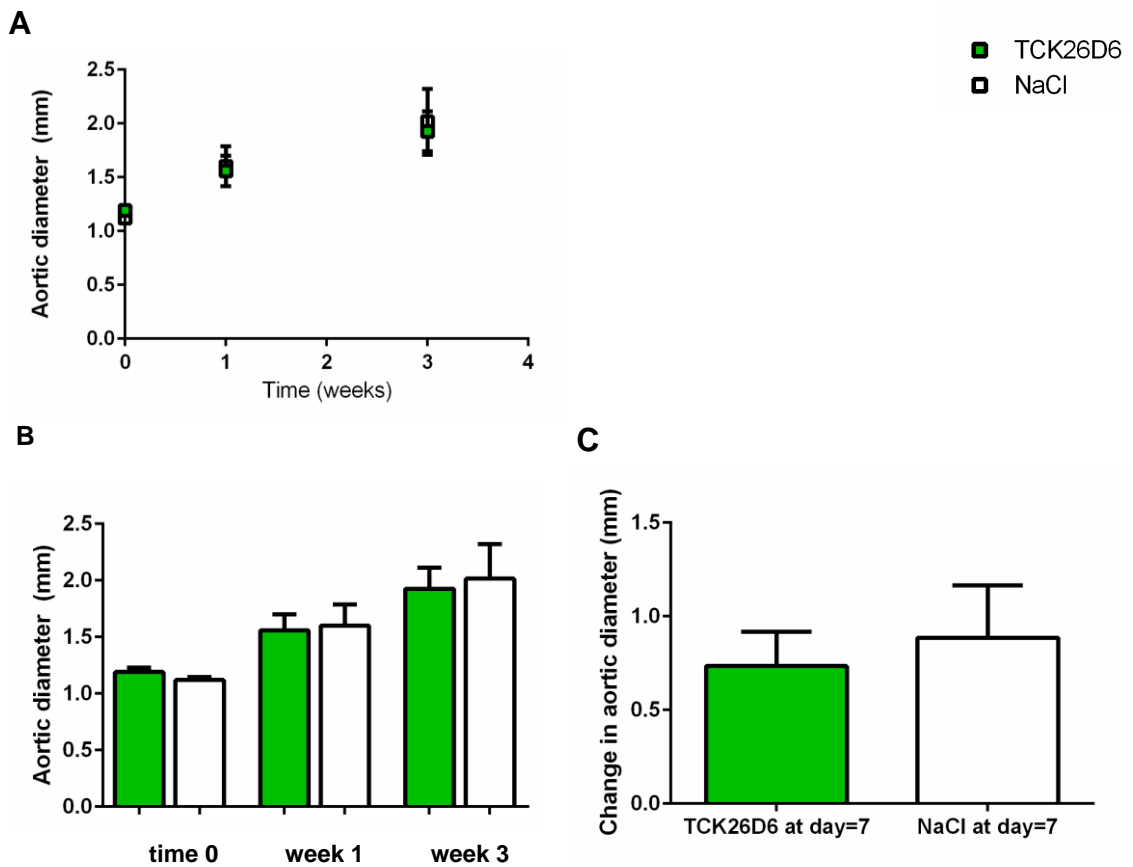
Figure 45. Delayed TAFI inhibition has no effect on the progression of AAA, as measured by cross-sectional diameter of AAA, in the Angiotensin II model of AAA

AAA were induced in hyperlipidaemic mice by infusion of Ang II 750 ng/kg/min. After 7 days, mice were treated with a single injection of either MA-TCK26D6 (n=11) or NaCl 0.9% control (n=7). AAA progression in both groups was monitored using Vevo2100 pre-clinical ultrasound scanning. After two weeks, there was no difference in the cross-sectional diameter of AAA in mice receiving the treatment versus those given a control injection (MA-TCK26D6 1.93 ± 0.62 mm vs. NaCl 2.02 ± 0.69 mm, panel A and B). There was no difference in the change in aortic diameter over the whole course of the experiment in mice receiving MA-TCK26D6 compared to controls (MA-TCK26D6 increased by 0.7 ± 0.2 mm, NaCl increased by 0.9 ± 0.3 mm, p=0.65, panel C).

4.12.2 Aorta 3D reconstructions

In order to gain a better representation of the aneurysmal aortic segment (rather than a single diameter measurement), the 10.5 mm of aorta lying proximal to the right renal artery was scanned in cross-section every 0.1 mm, and a 3D reconstruction of this segment of the aorta was produced. This was created by mapping the aorta on multiple images, and then removing all of the background in order to just isolate the aorta on the images. A sample of this can be seen in Figure 46. The volume of this segment was then calculated using VevoVasc software. Samples of reconstructions from each treatment group can be seen in Figure 47. Although there was a slight difference in the aortic volumes between the two groups at baseline (MA-TCK26D6 10.36 ± 2.89 mm^3 vs. NaCl 8.09 ± 0.71 mm^3, p=0.02), by day 7 when the treatment was given, there was no difference in the volume of the aorta between the groups (MA-TCK26D6 19.99 ± 11.02 mm^3 vs. NaCl 16.44 ± 7.44 mm^3), and, following treatment, the aortic volumes in each group had increased by the same amount (MA-TCK26D6 increased by 4.45 mm^3 following treatment, vs. NaCl which increased by 3.00 mm^3). See Figure 47.
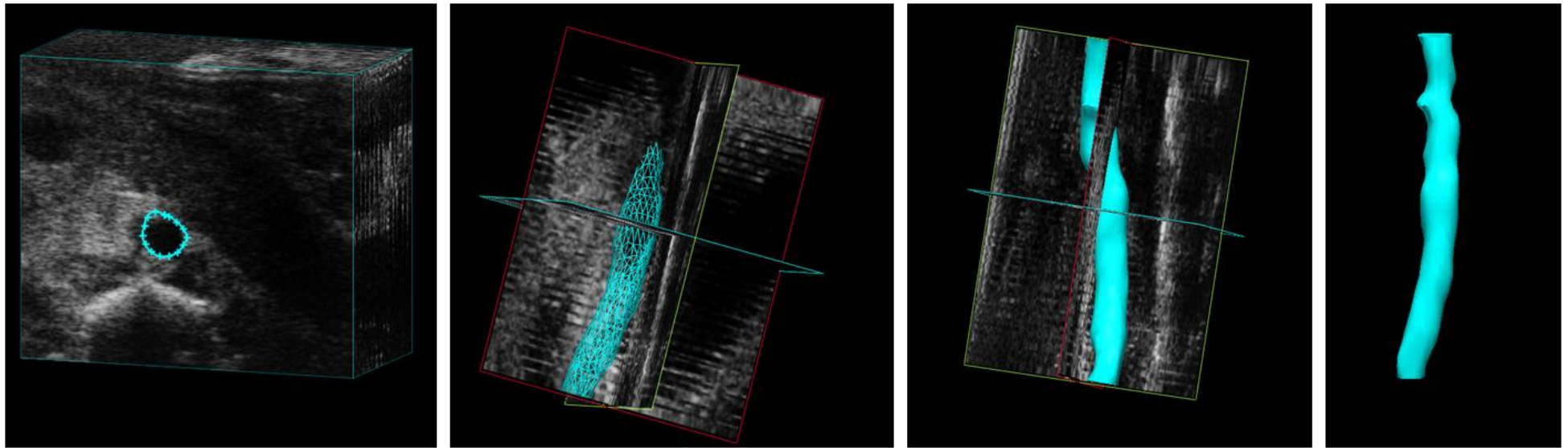
Figure 46. 3-Dimensional reconstruction of the Abdominal Aorta in the Angiotensin II model of AAA

Using the Vevo 2100 pre-clinical ultrasound scanner, serial cross sectional images were taken of the region 10.5 mm proximal to the right renal artery. The aortic diameter was mapped in multiple sections, and a 3D reconstruction of the segment of the aorta at risk of aneurysm formation created. The volume of this section was then calculated using VevoVasc software.
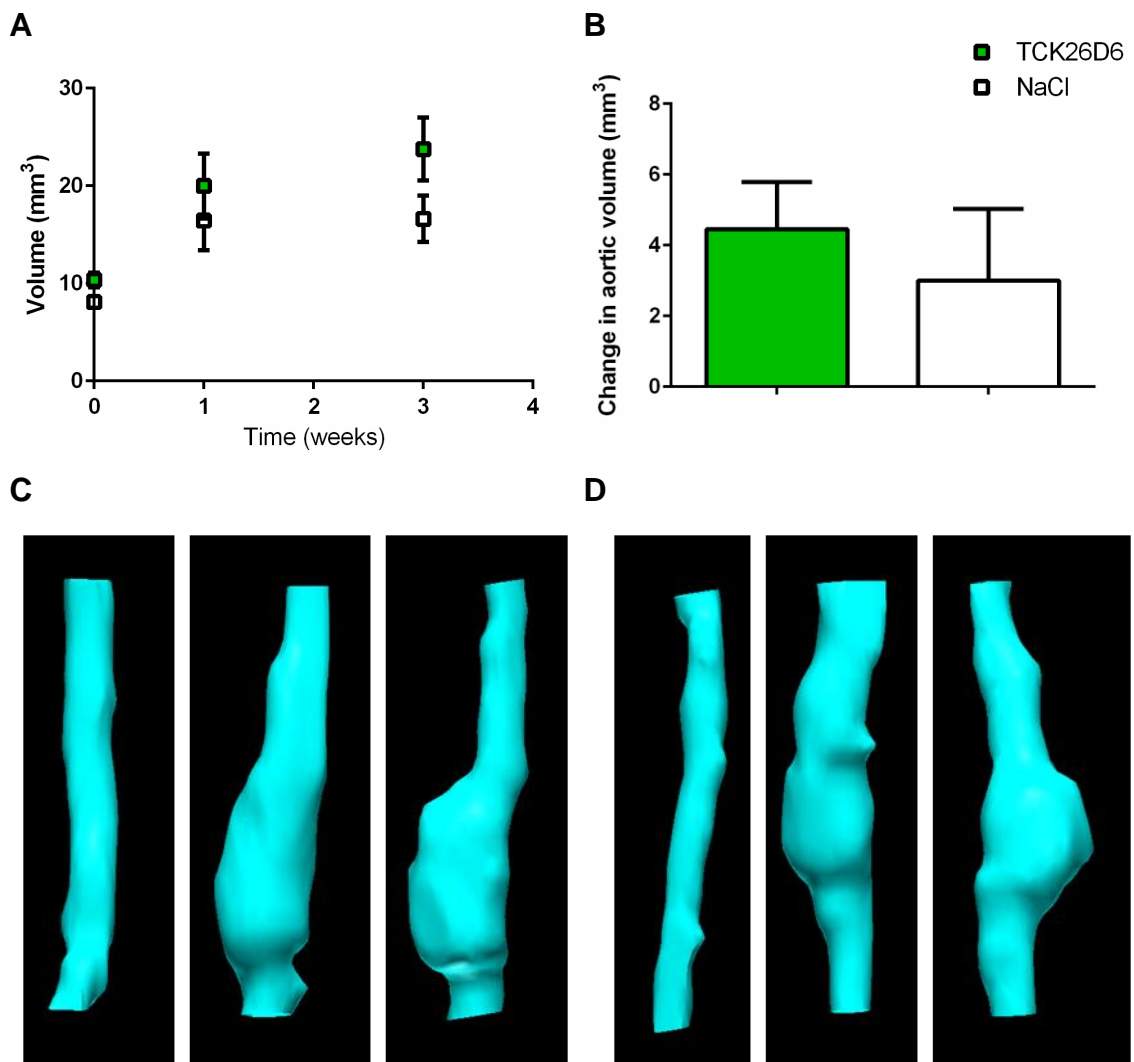
Figure 47. Delayed TAFI inhibition has no effect on the progression of AAA, as measured by the volume of AAA, in the Angiotensin II model

AAA were induced in hyperlipidaemic mice by infusion of Ang II 750 ng/kg/min. After 7 days, mice were treated with a single injection of either MA-TCK26D6 (n=11) or NaCl control (n=7). AAA progression in both groups was monitored using Vevo2100 pre-clinical ultrasound scanning, and 3D reconstructions of the aortic segment at risk of AAA formation were created. There was no difference in the change in aortic volume over time between mice receiving MA-TCK26D6 and controls (MA-TCK26D6 increased by 4.45 mm^3 following treatment, vs. NaCl which increased by 3.00 mm^3), panel A and B. Example 3D constructions showing AAA progression over time are shown in panel C (MA-TCK26D6 treated) and panel D (NaCl control).

4.12.3 Aortic distensibility

Using an ECG-gated ultrasound image of the abdominal aorta (taken in longitudinal section), the distensibility of the aortic wall (defined as the amount it moves with each beat of the cardiac cycle) was measured using VevoVasc software. As would be expected, the overall distensibility across all groups decreased over time as AAA developed, and wall became less elastic (Baseline 91.92 ± 29.08 1/Mpa falling to 53.11 ± 29.03 1/Mpa, p=0.0001, Figure 48, panel A). There was no difference in the change of distensibility over time in those mice receiving treatment with MA-TCK26D6 compared with NaCl 0.9% controls (MA-TCK26D6 decreased by 37.01 ± 30.19 1/Mpa vs. NaCl 0.9% decreased by 58.88 ± 37.31 1/Mpa, Figure 48, panel C).
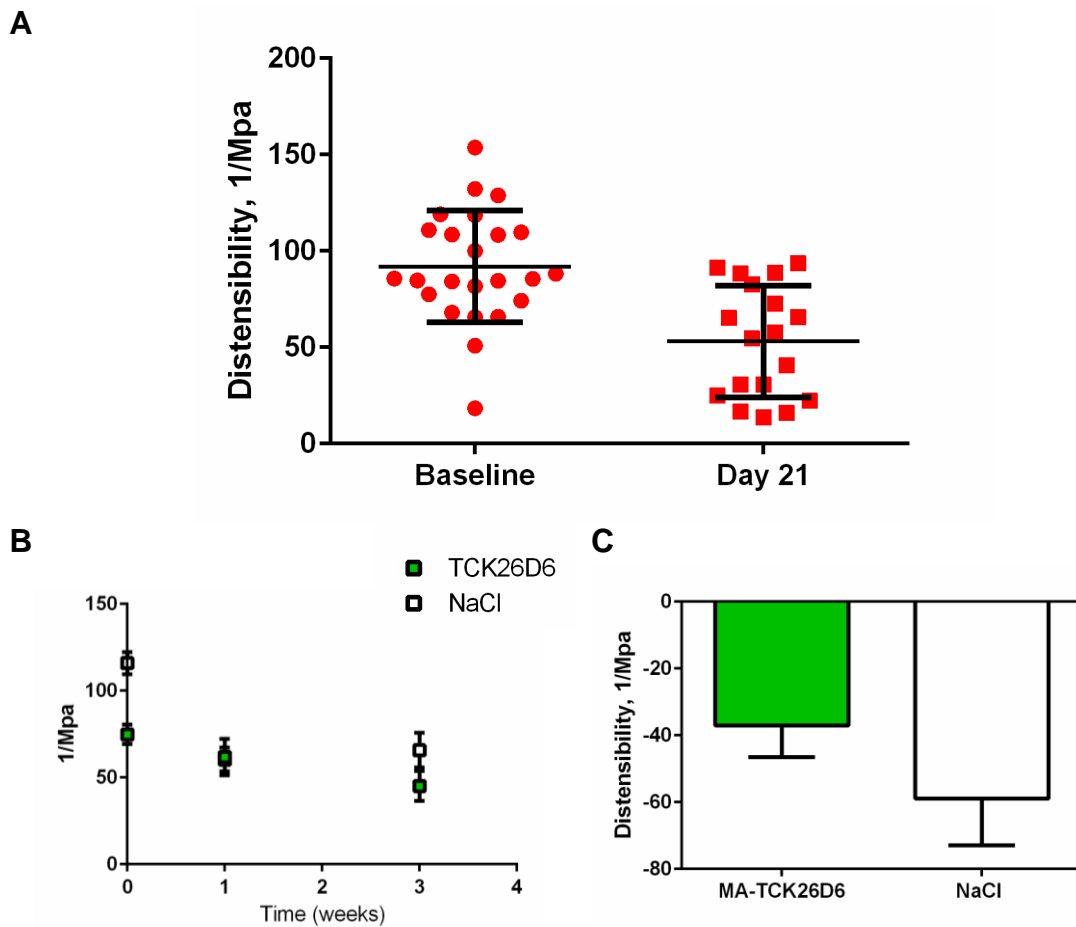
Figure 48. Aortic distensibility decreases with AAA formation, but is not affected by TAFI-inhibition, in the Angiotensin II model of AAA

Using an ECG-gated ultrasound image of the abdominal aorta (taken in longitudinal section), the distensibility of the aortic wall was measured using VevoVasc software. The overall distensibility across all groups decreased over time as AAA developed (n=18) Baseline 91.92 ± 29.08 1/Mpa falling to 53.11 ± 29.03 1/Mpa, p=0.0001, panel A). There was no difference in the change of distensibility over time in those mice receiving treatment with MA-TCK26D6 (n=11) compared with controls (n=7) (MA-TCK26D6 decreased by 37.01 ± 30.19 1/Mpa vs. NaCl decreased by 58.88 ± 37.31 1/Mpa, panels B and C).

4.13 Discussion – TAFI-inhibition in the Angiotensin II model of AAA

The aim of this chapter was to study the role of TAFI and TAFI inhibition in an *in-vivo* murine AAA model, and investigate proteins involved in fibrinolysis in plasma and aortic sections from this model.

A previous study examined the effect of TAFI on AAA development *in-vivo* by using the porcine pancreatic elastase infusion model (PPE model) in TAFI-/- mice (Schultz et al., 2010). They showed that TAFI offered some protection against AAA formation, as TAFI-/- mice developed larger AAA which were more prone to rupture than the wild type. Furthermore, this group provided evidence for the role of plasmin in AAA development and rupture, as when TAFI-/- mice were given tranexamic acid (a potent inhibitor of plasmin formation from plasminogen), AAA formation was reduced and rupture was completely abolished. The Angiotensin II model was chosen for this study. There are advantages and disadvantages to all three of the current murine models, as no one single model accurately represents the human disease process. The PPE model results in a rapid, chemically induced degradation of elastin within the aortic wall. Whilst elastin degradation is an integral part of the human disease process, it does not appear to be the trigger to AAA formation. The calcium chloride model again relies on topical chemical treatment to the aortic wall. Unlike the PPE model, where the insult to the aortic wall occurs from the luminal surface, in the calcium chloride model, the insult is applied to the adventitial surface, and the lumen is relatively protected (apparently the opposite of the human disease process). This model again causes degradation of elastin, likely through an intense inflammatory process related to the application of calcium

chloride. Unlike the PPE model and the Angiotensin II model, however, the calcium chloride model only results in a very modest dilatation of the aorta, which makes appreciating changes in AAA size as the result of pharmacological intervention difficult at a macroscopic level. The Angiotensin II model was chosen because, in relation to the fibrinolysis effects seen in AAA, it seemed to better model the human disease. In the majority of cases of classical AAA associated with atherosclerotic disease, there is not an acute dissection which results in AAA formation. However, there is evidence of thrombus formation resulting in inflammation and expansion in the human disease, and, as this thesis aimed to study a protein related to fibrinolysis, this part of the AAA mechanism seemed one of the most important to be able to replicate. In addition to this, the Angiotensin II model of AAA results in gross dilatation of the aorta which is appreciable on *in-vivo* ultrasound imaging, and this allowed for the *in-vivo* monitoring of AAA development at serial time points without the need for animal sacrifice. The ultrasound imaging of the AAA *in-vivo* allowed the confirmation of the likeness of this model to the human disease. Using measures of aortic distensibility, I was able to show that, in line with human AAA, the wall of the aorta becomes stiffer as the AAA develops. Whilst there is a mortality associated with this model that is higher than in the calcium chloride model, the ability to see gross aortic dilatation using USS and thus the ability to monitor this *in-vivo* without the need to sacrifice animals at sequential time points meant that overall, animal numbers were comparable to, or even lower than, those that would have been required using an alternative model. Finally, this model is the only one which demonstrates the sex diamorphism seen in the development of AAA in humans, and thus one could argue carried the most

physiological similarities. However, if animal numbers were no obstacle, in order to properly draw conclusions regarding TAFI inhibition *in-vivo*, all three models should be studied in relation to TAFI. This would require the production of a TAFI/ApoE-/- for the Angiotensin II model, and a TAFI-/- for the calcium chloride and PPE models. The PPE model and the calcium chloride models would then be repeated in wild type mice treated with MA-TCK26D6. Clearly, such studies are beyond the scope of this thesis.

Previous work from our laboratory has shown that there is a difference in the clot structure and fibrinolysis in patients with AAA compared with control subjects (Scott et al., 2011). As a result, the fibrin clot structure of clots produced using plasma taken from mice developing AAA in the Angiotensin II model was studied in relation to plasma from controls. The fibrin clot structure in mice with AAA, however, was not different from controls, suggesting that the changes in fibrin clot structure seen in humans is the result of a more complex, chronic disease process. The mice used for this study were only treated with Angiotensin II for 28 days. As most AAAs in humans develop over a period of years, it may be that the more rapid expansion seen in this model did not allow for the development of the pathological clot structure that can be found in the human disease state.

The downstream effects of Angiotensin II include vasoconstriction, increased sympathetic nervous system activity and the increased secretion of aldosterone (Peach, 1977), all of which contribute to hypertension in mice treated with Angiotensin II. In this study, however, there was no effect of Angiotensin II infusion on blood pressure. Blood pressures were taken over several days beginning at day 8 following the commencement of Angiotensin II infusion.

Recent studies have shown a clear increase in the blood pressure (BP) of ApoE-/-mice treated with Angiotensin II, which proceeds over the first 7-10 days of the infusion, and is then maintained (i.e. the hypertension is not a transient effect) (Haggerty et al., 2015). There are several reasons why this effect may not have been detected in this study. Firstly, BPs were measured using a volume/pressure tail cuff system. Although this has its advantages, as it is simple and non-invasive, it can have limited accuracy (Feng et al., 2008, Kurtz et al., 2005, Whitesall et al., 2004). Another option would have been telemetry, which has a greater degree of accuracy, but requires an invasive procedure for implantation (which carries its own associated mortality), requires very precise placement, and is expensive. Although telemetry is more accurate for measuring mean arterial pressures and pulse pressures, systolic blood pressure measurements are generally consistent between the two techniques (Haggerty et al., 2015). Secondly, the work in this chapter used a dose of Angiotensin II of 750 ng/kg/min. In the literature, doses of Angiotensin II used in this model of AAA vary from 500-1000 ng/kg/min, with the majority of studies choosing 1000 ng/kg/min. The reason a slightly lower dose was chosen in this instance was to attempt to minimise mortality associated with aortic rupture. When a dose of 500 ng/kg/min is used, there is no effect on BP (this is seen to be a 'subpressor' dose), and AAA formation rates are around 50% (Cassis et al., 2009). In fact, it has been demonstrated that the AAA formation associated with Angiotensin II infusion occurs via a mechanism which is independent of BP, as AAA still occur even when anti-hypertensive medication is administered alongside Angiotensin II (Cassis et al., 2009). Ultimately, it may be that the dose of Angiotensin II used in this study (750 ng/kg/min) remains in the subpressor

range, and so no obvious increase in BP with Angiotensin II infusion could be detected.

For the purposes of this thesis, TAFI was inhibited *in-vivo* using two different inhibitors. The first, MA-TCK26D6 is a monoclonal antibody that specifically inhibits plasmin-mediated TAFI activation. As such, it is likely that this inhibitor will only be active in areas of high plasmin(ogen) concentrations, such as within any thrombus that is formed. The mice treated with this inhibitor would also have retained the anti-inflammatory properties of TAFI. The second inhibitor, UK-396082, has previously been extensively characterised *in-vivo* and is an inhibitor of all active TAFI. Whilst the dosing of both of these inhibitors was based upon previous studies, in comparing the effect of these two inhibitors *in-vivo* it is hard to know if they were given at equivalent pharmacologically effective concentrations. Two inhibitors of the same kind, given by the same route, but with different inhibitory targets (such as two monoclonal antibodies targeting different activities of the TAFI molecule), would probably have allowed for more accurate comparisons to be made between the two groups. ROTEM was used to test for the effects of the TAFI inhibitors *in-vivo.* Whilst this has been used in recent years to measure TAFI activity in whole blood samples from mice (Mishra et al., 2014), both this and studies of plasma lysis are not particularly sensitive to subtle changes in TAFI activity (Foley et al., 2013). Even using a commercially available substrate method to measure TAFI activity may not have proved more accurate due to the interference of the other plasma carboxypeptidase, carboxypeptidase N (Foley et al., 2013).

Despite these issues, there was clearly an effect of the TAFI inhibitors on this model of AAA. In both cases, the mortality associated with the Angiotensin II

model of AAA was decreased. This suggests that clot formation and resistance of this clot to fibrinolysis early in the Angiotensin II model somehow increases the likelihood of aortic rupture. It may be that the prevention of clot formation at the site of dissection results in a decrease in the inflammatory response usually seen at this early stage, and thus prevents further damage to the aortic wall. The difference in the development of AAA with each inhibitor implies that later in the disease process, the route by which TAFI is activated is important. Using MA-TCK26D6, plasmin-mediated activation of TAFI is inhibited. Mice treated with this inhibitor had a reduction in the incidence of AAA development compared to mice receiving Angiotensin II alone. However, mice receiving UK-396082, an inhibitor of all active TAFI, had an increase in the incidence of AAA development. This pattern is more akin to the results of the PPE model in the TAFI-/- mouse. Taken together, these results seem to suggest that whilst the prevention of clot formation early in the development of AAA seems to prevent rupture associated mortality, the later stages of AAA development in this model rely on an inflammatory response at the site of injury. When the anti-inflammatory properties of TAFI are intact, and clot formation is prevented using MA-TCK26D6, there was a reduction in mortality and a reduction in AAA formation. However, when a global TAFIa inhibitor was used, clot formation was prevented, but there would have been no TAFI-related inhibition in the inflammatory response at the site of dissection, and thus AAA formation proceeded even without the initial clot formation. This hypothesis is also supported by the results of the mice given a dose of MA-TCK26D6 after AAA formation. In this group, clot formation at the site of injury and subsequent inflammation would have progressed in a normal fashion. Once this process

had occurred, the addition of a TAFI inhibitor preventing further plasmin-mediated TAFI activation had no effect, because the inflammatory response resulting the AAA formation and expansion was already established. This theory is further supported by the data relating to the levels of TAFI in the aortic wall. The levels of TAFI within the aortic wall of mice developing AAA was extremely low, and no higher than that in controls. This suggests that whilst TAFI may be important in the initial stages of this model, when clot formation occurs, beyond this stage, it appears that inflammation, or other factors at play in the AAA wall, may be more important than plasmin-mediated processes.

Plasma levels of CRP were measured in the mice in an attempt to quantify the potentially increased inflammatory process that may have been at work in mice treated with Ang II in combination with UK-396082 but not in the mice treated with Ang II in combination with MA-TCK26D6. However, unlike in humans, murine CRP is not an acute phase reactant (Ku and Mortensen, 1993), and so could not necessarily be expected to increase in line with such a localised inflammatory process. Indeed, the measurement of more specific inflammatory substrates of TAFI, such as C3a or C5a, may have provided a more specific measure of the effect of TAFI inhibition on inflammation in this model.

5.1 General Discussion

The aim of this thesis was to explore TAFI and its relationship with AAA. AAA is just one disease in a spectrum of cardiovascular diseases that, despite ongoing research and tireless health campaigning, remain a major global health problem. AAA is often not an isolated disease, and patients with AAA are frequently affected by a number of other atherothrombotic cardiovascular diseases, such as coronary artery disease and stroke, and in fact, are at significant risk of cardiovascular death (Brady et al., 2001). A pathological change in fibrin clot structure, towards a denser clot with smaller pores that is more resistant to lysis, is evident in patients across the whole spectre of cardiovascular diseases, and is even evident in the healthy relatives of patients with disease (Mills et al., 2002, Bhasin et al., 2008). The aetiology of this remains poorly understood; in many cases, whether such changes are cause or effect is yet to be fully determined (Bridge et al., 2014b). In line with all cardiovascular disease, the fibrin clot structure is altered in patients with AAA, and they show denser *ex-vivo* clots, with smaller pores, and an increased resistance to lysis (Scott et al., 2011).

It is not fully understood what triggers the formation of AAA in humans. With the advent of the UK national screening programme, more patients will be diagnosed with AAA in its early stages. For this group of patients, an important target seems to be the development of a pharmacological therapy that would prevent, or at least slow, AAA expansion. By doing so, even if the incidence of AAA remains unchanged, the incidence of those reaching the threshold for

surgery would be dramatically reduced, and, in line with this, the incidence of AAA rupture would also be reduced.

TAFI is intricately involved in the prevention of fibrinolysis. Acting as an indirect inhibitor of plasmin, it cleaves C-terminal lysine residues from partially degraded fibrin molecules, thus removing the site of co-localisation of plasminogen and tPA, and preventing the production of plasmin. TAFI is therefore of interest when investigating the cause of the resistance to lysis that can be observed in patients with AAA. Previous research in this area is very limited, consisting of only two studies. Both were based on very small populations, and, in both, methodological oversights may result in some discussion as to the validity of the results. The first measured TAFI zymogen levels in patients with ruptured (n=10) and non-ruptured (n=17) AAA, and quoted extremely high TAFI levels in both groups (in some cases >50 µg/ml), which fall outside the reference range of the commercially available ELISA which they employed. Furthermore, they failed to include a control group, and so whether these values are a reflection of extremely high TAFI levels in patients with large AAA, or an error related to the assay, is unclear (Hobbs et al., 2007). The second study to date compared TAFI activity in plasma from patients with AAA (n=32) and control subjects (n=45), and, using a commercially available chromogenic assay, found a reduction in TAFI activity in AAA patients compared to controls (Dubis et al., 2014). Neither of these studies related TAFI levels or activity to the functional Thr325Ile polymorphism. The work in this thesis has provided more robust evidence for the potential role of TAFI in human AAA. By using a large study population with more than two hundred patients and controls, the study was adequately powered to detect even small differences between the two groups.

By measuring as many TAFI isoforms as possible by ELISA (intact TAFI zymogen, TAFIa/TAFIai and TAFI activation peptide), I was able to better appreciate the role of TAFI in human AAA. For instance, if TAFI zymogen levels alone had been measured, one might conclude that there was no role for TAFI, as plasma levels were comparable between AAA and controls. However, by including the ELISAs of both TAFIa/TAFIai and TAFI activation peptide, a significant role for TAFI became more apparent. There is currently no perfect method for the determination of TAFI activity in human plasma (Foley et al., 2013). In this thesis, a decrease in TAFI activity was demonstrated using a clot lysis assay, confirming the results of the only previous study, which employed a chromogenic substrate technique (Dubis et al., 2014). These results in combination may at first appear contradictory, with evidence of increased activity *in-vivo* through increased plasma levels of TAFIa/TAFIai and TAFI-AP, but a decrease in activity in the *in-vitro* assay despite normal TAFI zymogen levels. One hypothesis is that overproduction of TAFI *in-vivo* in patients with AAA (which keeps total zymogen levels within normal limits) may result in the production of some TAFI which is less functional, and therefore cannot be activated *in-vitro*. Another explanation is that in the AAA patients, other plasma proteins may be up or down regulated and lead to an interference in the TAFI activity assay, which does not occur for the controls.

AAA disease is clearly a complex and multi-faceted disease. It is therefore entirely plausible, and in fact, probable, that the alterations seen in fibrinolysis in the *ex-vivo* clots of patients with AAA are the result of the interaction of a number of different plasma proteins. In order to generate novel hypotheses and direct potential next avenues for study, polymorphisms of proteins involved in

the fibrinolysis pathway were investigated in patients with AAA and controls. Clearly, a study such as this is not designed to be able to identify candidate genes for AAA, but instead, this study was used to highlight which other proteins may be of potential interest for further study. The Arg407Lys polymorphism of α2AP was found to be negatively associated with AAA. Plasmin-antiplasmin complexes have previously been suggested as a potential biomarker for expansion and rupture in patients with AAA (Moris and Georgopoulos, 2013). Studies into the role of α2AP in AAA have now been commenced in the LEADS population.

In addition to the study of humans with AAA, this thesis also investigated the effect of TAFI inhibition on AAA development using an *in-vivo* murine model. A single previous study in this area had used a PPE model of AAA, and demonstrated an increase in the incidence of AAA and rate of rupture in TAFI-/- mice (Schultz et al., 2010). In order to be able to investigate, model, and manipulate human disease states, the scientific community is highly reliant on animal studies. For AAA, models have been created in a number of species. A canine model of AAA has been employed to investigate endovascular therapies. This model involves the balloon dilatation of a polytetrafluoroethylene (PTFE) graft *ex-vivo*, which is then implanted in place of the infra-renal aorta (Chaer et al., 2006). Early porcine models were created by mechanical disruption of the external aortic wall via a crushing injury (Zatina et al., 1984). These large animal models of AAA have proved particularly useful in evaluating the short term success of surgical interventions. The problem with creating an animal model of AAA is that the initial stages that result in the human disease are not fully understood, and so it is difficult to develop a model to represent them. Clearly,

these large animal models, based on mechanical injury to the aortic wall, whilst useful for evaluating the technical success of surgical treatments, do not provide the opportunity to study the mechanisms related to AAA development. To this extent, three murine models have been developed and are currently used to study AAA *in-vivo*. These are the calcium chloride model, the PPE model and the Angiotensin II model, and all three are described in detail in Chapter 1. The advantages of these models over larger animal models are that they provide the opportunity to study the early stages of AAA development. However, as the initial stages of human AAA development are not known, the debate remains as to which model best replicates the human pathology (Bruemmer et al., 2011).

There are advantages to using murine models in the study of human disease. The murine genome is remarkably similar to the human genome (Waterston et al., 2002), and can be easily manipulated to mimic human disease states. Mice are the smallest mammals available for laboratory study, have a short generation time, and an accelerated life span (with one mouse year being equivalent to over 30 human years), making them a cost effective and efficient model for study.

The initial results from the *in-vivo* part of this thesis proved interesting. TAFI was inhibited through two different mechanisms with conflicting results. Whilst both resulted in a decrease in mortality, it was only the inhibition of plasmin-mediated activation of TAFI that resulted in a decrease in AAA incidence. Using UK-396082, a global inhibitor of all active TAFI, proved consistent with the TAFI-/- mouse with regards to AAA formation, since the incidence of AAA was dramatically increased in mice receiving UK-396082. Both of these inhibitors

were given at the initiation of AAA in the model, and although this may offer insight into the importance of clot formation in the early stages of the disease, it cannot be directly applied to a therapeutic agent for AAA disease in humans, as any treatment would only be commenced once an AAA was established. In fact, as a result of the data relating to the delayed treatment with MA-TCK26D6, the relevance of a TAFI-inhibitor in the treatment of human AAA disease has to be brought into question. The results of this study showed that delayed treatment with MA-TCK26D6 once a AAA was established had no effect on AAA expansion. Even with the suggestion that there may be a number of the inhibitors of plasmin involved in the pathological clot structure observed in AAA, as TAFI-inhibition had no effect on expansion, it may be that in late AAA disease, one, or several, of the other mechanisms related to AAA development, beyond those involved thrombosis and fibrinolysis, may to be a better target for pharmacological intervention. This does not, however, rule out the importance of further investigation into thrombosis and fibrinolysis in this group of patients. Independent of all other risk factors, the incidence of myocardial infarction and cardiovascular related death in patients with AAA is increased compared with the general population (Brady et al., 2001). Whilst TAFI may not be a therapeutic target when it comes to preventing the expansion of AAA, the results described in Chapter 3, showing that there is evidence of increased TAFI turnover in patients with AAA, and that the delay in fibrinolysis in this patient group can be corrected using a TAFI inhibitor, suggest that TAFI may still be an important target in protecting this patient group against cardiovascular-related mortality.

5.2 General Conclusions

In summary, there are several main conclusions to be drawn from this thesis. Firstly, I have shown that there is evidence of an increase in TAFI turnover in patients with AAA. Secondly, the delay in lysis seen in *ex-vivo* clots of patients with AAA (Scott et al., 2011) can be corrected through inhibition of TAFI, implying that TAFI may have a role in the systemic changes in fibrinolysis observed in this patient group. Thirdly, the pathological fibrin clot structure seen in patients with AAA appears to be related to the chronicity of AAA disease, rather than simply being a result of the presence of AAA, as demonstrated by the normal *ex-vivo* fibrin clot structure of mice with AAA. Finally, I have shown that, in the Angiotensin II model of AAA, TAFI-inhibition protects mice from the mortality associated with the model. Further to this, specific plasmin-mediated TAFI inhibition *in-vivo* also prevents AAA formation during the initial stages of the Angiotensin II model of AAA, but has no effect on the expansion of an already established AAA.

5.3 Future Work

Until a pharmacological target for the prevention of AAA development and expansion is identified, there will continue to be active interest in research into AAA formation and development. This thesis has explored the potential role of TAFI in some detail, and has rejected TAFI as a suitable target for the prevention of AAA expansion in human disease. Based on the results of the α2AP polymorphism Arg407Lys, investigation into α2AP levels and cleavage rates in the plasma of patients with AAA is already underway. The pathological clot structure observed in this group of patients, however, is yet to be fully

investigated, and perhaps changes to the fibrin clot itself, rather than proteins involved in its regulation, might hold the key to 'normalising' the pathological clot structure. Whilst the *ex-vivo* clots of patients with AAA, and indeed patients with cardiovascular disease in general, show a denser, stiffer structure that is resistant to lysis, which of these two pathological features (i.e. stiffness or resistance to lysis) is most important in disease has not been elucidated. As such, further work to specifically investigate stiffness and/or resistance to lysis of fibrin clots in AAA is already underway. Several programmes of work are planned. The first will use a number of new transgenic murine models, focusing on three main areas; defective fibrin formation, reduced fibrin mechanical stability, through alterations of fibrinogen gamma chain cross-linking, and decreased resistance of fibrin to fibrinolysis. The second programme of work will consider related aspects of fibrin biology via a different approach. Rather than using a number of specific murine strains, this programme will employ the hydrodynamic gene transfer of mutant fibrinogen α-chain cDNA in the fibrinogen knock out mouse. The cDNA will be mutated to prevent firstly α-chain/α-chain cross-linking, and then α2AP cross-linking, and compare clot formation and lysis *in-vivo* with each of these alterations individually, and then in combination, before assessing the effect on AAA formation. These studies will aim to identify which structural and functional characteristics of the fibrin clot play key roles in aneurysmal disease. It will also help to identify whether or not targets altering fibrin formation and stability could be possible therapeutic targets for aneurysmal disease.

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Appendix 1 - Protocol

THE LEEDS ANEURYSM DEVELOPMENT STUDY

Background

An abdominal aortic aneurysm (AAA) is a focal dilatation of the abdominal aorta greater than three centimetres in maximal diameter. The condition spontaneously evolves towards rupture, which confers a mortality risk in the region of 80% despite emergency surgery. The current principles of management involve watchful waiting with targeted intervention once the annual risk of rupture outweighs the mortality risks of intervention. This treatment threshold is currently set at 5.5 cm in otherwise fit patients. Open aneurysm repair (OAR) or endovascular aneurysm repair (EVAR) are both suitable treatment modalities.

The clinical risk factors for AAA include male sex, smoking, high blood pressure and age; diabetes is thought to be protective. Although atherosclerosis clusters with AAA, it's precise role in aneurysm development remains unclear. The pathobiology of AAA incorporates transmural inflammation, loss of vascular smooth muscle from the arterial media, over production of matrix metalloproteinases which destroy collagen and elastin. Acting in concert these factors lead to aneurysm formation, but the precise event that precipitates this process remains unknown. Furthermore, the factors which influence the ongoing growth of AAA once established are debated. It is also apparent that patients with AAA are at increased cardiac risk, but how this cardiac risk is related to the presence of AAA is also unclear.

Aims

The overall aim of the LEADS project is to investigate AAA development, growth and outcomes, and thus identify novel treatments. In order to do this, a Leeds based AAA database will collect demographic and diagnostic information on AAA and control patients and store patients' blood, DNA, RNA, protein, plasma and AAA tissue.

History

The Leeds Aneurysm Development Study (LEADS) (Leeds East 03/142) was first established in 2003 as a small case-control study (AAA vs. Controls) focused on the differences in inflammatory and coagulation markers in male patients with AAA and controls. The important novel findings stemming from this work led to the continuation of the study and widening of the inclusion criteria. In 2005 women were invited to participate in the study and in 2006 the study minimum age limit was reduced from 65 years to 55 years. As the project grew it became apparent that links should be made between the blood results observed at recruitment, and aneurysm tissue and post-operative blood samples. Thus, following an amendment to the protocol in 2006, these samples were collected for further study.

As the study grew in size and the findings were disseminated locally, the opportunity arose to collaborate with other researchers within and outside of the faculty at The University of Leeds. These new members of the research team offered fresh hypotheses relating to the development and growth of AAA. As a result, and again following amendments to the protocol in 2008 – 2010 data collected now includes aneurysm imaging and growth, genetics, survival and arterial stiffness (pulse wave velocity), with an aim of recruiting a total of 1500 aneurysm and 1500 control patients. As specified in the consent form, all participants are followed prospectively from the time of recruitment. This offers extensive information as to the dynamics of aneurysm expansion (by analysing serial USS and CT scans), AAA prognosis and patient survival. In

addition, an application has been submitted to link to the Office of National Statistics data set in order to collect reliable mortality data for our cohort.

As a result of increasing public health awareness, particularly with regard to smoking and changes in medications (statins and ACE-inhibitors), the number of patients with AAA has declined over the last ten years. As a result of decreasing patient numbers, but more significantly as a consequence of huge advances in endovascular aneurysm repair, the number of people in the UK undergoing open AAA repair has vastly decreased. Hence, a collection of aneurysm tissue linked to a data set which includes peripheral blood samples, patient characteristics and imaging is undeniably of importance internationally as a resource for further research in this area. Thus even upon completion of recruitment for this study, we will have established a valuable resource for continued research into the cause of AAA disease, which can be utilized for future medical students, BSc, MD and PhD projects through the Light Laboratories and the University of Leeds.

Study Design and Methodology

This is a prospective observational study recruiting cases (patients with AAA) and control (patients with normal diameter aorta) from a variety of sources. We aim to recruit a minimum of 1500 AAA patients and 1500 age and sex matched controls in order to allow the use of multilevel mixed effects modelling as part of the growth analysis.

This target will also provide sufficient patients for the genetic polymorphism analysis, which also requires significant patient numbers. Depending on the frequency of the genetic mutation of interest, in order to achieve at least 80% power with 95% confidence, at least 1000 patients are typically required per analysis. In addition, we aim to compare the AAA patient group with a control group to assess the overall impact of the risk factors such as cardiovascular disease history or e.g. smoking on the development of AAA. We seek to recruit 1500 controls in order to allow effective use of propensity score methods when applied to AAA patient subgroups.

Recruitment of Subjects

AAA

Patients undergoing routine ultrasound surveillance for small AAA (3-5.4 cm AAA) and those admitted for elective/semi-urgent AAA intervention (>5.4cms) will be recruited from a variety of sources.

Inclusion criteria for AAA.

- Caucasian

- Age ≥ 55 years

- Aortic Diameter on Ultrasound (≥30mm)

- Able to provide informed consent.

Exclusion criteria for AAA

- Non Caucasian – very low incidence

- Age <55 years – very low incidence

<u>Control</u>

Controls will be recruited from a variety of sources comparable with the initial source of referral of the AAA. These will include Vascular Surgery Out-patients Department (OPD), General Surgery OPD, Urology OPD, Cardiology OPD, Neurology and Diabetes OPD and General Practitioners in addition to volunteers who come forward as a result of advertisements in Trust and local media. All controls will have imaging of their abdominal aorta (ultrasound or CT scan) to confirm an anterio-posterior diameter of less than 30 mm. From the results of previous ultrasound screening programmes, a normal aorta (<30mm) at age 65 ensures that the patient is most likely to be free of an aneurysmal dilatation for life. The exact scanning protocol for control subjects may be changed in order to match with guidelines laid out by the National Abdominal Aortic Aneurysm Screening Programme.

Inclusion criteria for controls

- Caucasian

- Age ≥ 55 years

- Normal Aortic Diameter on Ultrasound (<30mm)

- Able to provide informed consent.

Exclusion criteria for controls

- Non Caucasian – very low incidence

- Age <55 years

Consent

Participants will be welcomed to take part at any time. Typically, participants are invited to participate at an outpatient clinic and provided with the patient information leaflet (enclosed). They are then given at least 24 hours (but typically one week) to consider participation before being contacted by the senior research nurse to arrange recruitment. Written, informed consent will be obtained at recruitment (usually during a recruitment visit or occasionally on attendance for vascular surgery inpatient treatment) using a standardised written consent form (as enclosed). Consent will be taken by the Lead Research Nurse, by the Chief Investigator and by the Chief investigators research fellows, as he deems appropriate. All those taking consent will have undergone NIHR approved Informed Consent training, as well as Good Clinical Practice training.

Should an individual who has given consent to take part lose capacity during the study, they would be would be withdrawn from the follow up process. Identifiable data or tissue already collected with consent would be retained and used in the study. No further data or tissue would be collected or any other research procedures carried out on or in relation to the participant.

Demographics, Medical History, Anthropometrics, Haemodynamics, Doppler & Arterial Stiffness

At the recruitment visit, patients will complete a standard proforma (enclosed) documenting demographics, medical history, drug history, social history and family history. The questionnaire is completed via structured face to face interview with a member of the research team. Following this, a full patient assessment is undertaken. This comprises:

- Measurement of systolic and diastolic blood pressure with oscillometric cuff

- Measurement of height and weight, and calculation of Body Mass Index (BMI)

- Measurement of abdominal circumference, hip circumference and waist circumference

- Ultrasound scan of the abdominal aorta (see below)

- ECG

- Palpation of peripheral pulses, Doppler of peripheral pulses and calculation of ankle-brachial pressure index (ABI)

- Arterial Stiffness measurement using Vicorder (see below)

Blood Sampling & Analysis

Venous blood is taken from the antero-cubital vein from the non dominant arm. A tourniquet is applied to aid identification of the vein, and inflated to 40mmHg. Once the needle position is confirmed within the vein, the tourniquet is released, and the first 10mls of blood are discarded, as per the laboratory protocol for all clotting studies. 50mls of free flowing venous blood is collected.

As part of their routine clinical care, blood samples are sent to the Leeds Teaching Hospitals NHS Trust Pathology Lab for routine tests. These results are then fed back to the supervising clinician or general practitioner. The remainder is transferred within 30mins to the Laboratory for processing and storage prior to experiments. This consists of 11ml of blood on room temperature citrate, two 6ml samples on EDTA, 10ml of blood on cold citrate and 3ml of blood on cold heparin.

1.2ml of the room temperature blood is put through the ROTEM device in order to elicit an immediate measurement of coagulation through the intrinsic and extrinsic pathways. The remainder is then centrifuged; the two cold samples are spun at 4000rpm for 30mins at 4 °C and the room temperature sample at 4000rpm for 20mins at room temperature. The resultant plasma is aliquoted and flash frozen in liquid nitrogen, before being stored at -80°C.

This plasma is used for all coagulation studies and screens for biomarkers of AAA and cardiovascular disease.

The two EDTA tubes each containing 6mls of whole blood are stored at -20°C to be used for DNA and RNA extraction. A grade D research technician (Fraser Macrae, BSc) is responsible for the processing and initial analysis of blood samples.

For patients and controls who fulfill any of the following secondary exclusion criteria, blood is only taken for DNA, RNA and protein extraction and analysis, so as not to bias results of any coagulation studies. These criteria are:

- Recent Surgery (less than 3 months)

- Active chronic inflammatory conditions

- Recent Thrombotic (arterial and venous) event (less than 3 months)

- Anticoagulation treatments; Warfarin, Factor Xa inhibitors, Therapeutic Tinzaparin

- Active malignancy

<u>Vicorder</u>

The Vicorder device will be used to measure arterial compliance and elasticity. This is done by simultaneously recording a pulse wave from the carotid brachial and femoral and ankle sites by using an oscillometric method. First a neck pad is applied over the carotid artery to prevent compression of the trachea and compression of both carotid arteries at the same time. Next the cuff is placed around the patient's right thigh. Both carotid and femoral cuffs are inflated automatically to 65mmHg and the corresponding oscillometric signal from each cuff is digitally analysed to extract, in real time, the pulse delay. After acquiring several steady pulses the recording is frozen on the display and the transit time in milliseconds presented. The distance between the upper edge of the right femoral cuff and the sternal notch minus the distance between the lower edge of the carotid cuff and the sternal notch is recorded and entered into the computer and displayed as PWVcf (pulse wave velocity common femoral). The procedure is then repeated with the cuff at (i) brachial and femoral and (ii) brachial and ankle. If the pulse wave is poor on one side the cuffs will be switched to the opposite side. Further details can be seen at www.dopstudio.co.uk. The recording takes just over one hour and no patients have reported any adverse effects.

<u>Imaging</u>

All subjects (AAA and controls) will undergo ultrasound examination of the abdominal aorta (maximum anteroposterior (AP) diameter, position and area of ILT) at the university. The results of these scans are initially stored on the hard drive of the ultrasound machine in an anonymised format, before being transferred to a secure university server for long term storage. This ultrasound examination at recruitment will be separate to any AAA surveillance the patient undergoes at the Leeds Teaching Hospitals NHS Trust. We will ensure all patients with AAA are enrolled in a suitable surveillance programme if they wish as part of the National Abdominal Aortic Aneurysm Screening programme (NAAASP). Following recruitment in the study and initial USS examination at the University, subsequent aortic imaging (USS, Colour Doppler USS, CTA, MRA, Angiography) reports will be obtained from the Leeds Teaching Hospitals NHS Trust secured results server and added to the anonymised database held within the University.

As patients reach their intervention threshold all patients will undergo further imaging, usually CT, or if contraindicated, MRA, as part of the routine NHS care within the Leeds Teaching Hospitals NHS Trust. The images are stored on a secured, limited access PACS system. We will transfer anonymised CT images to CD to allow transfer of images to the University where they will be analysed in the department of Biomechanical Engineering using relevant software. This work involves the segmentation, mapping and ultimately geometric analysis of the AAA and the ILT in three dimensions. The aims of this work are: (1) compare the ultrasound and CT/MRI, (2) to study the force and stresses upon the wall, (3) to study the flow through the aorta and (4) to model the impact of a metal stent on the aorta. Non-identifiable patient

images will be stored on the encrypted University server only. The CDs containing these images will be stored in a limited access locked room in the University accessible to the lead research nurse and the PI.

All patients recruited as control subjects will also undergo USS at the University. They will be counselled as to the potential implications of finding an incidental AAA in the same manor as patients attending NAAASP visits at the Leeds Teaching Hospitals NHS Trust. Any control patients found to have an AAA will pass into the AAA group and will be enrolled in the Leeds Teaching Hospitals NHS Trust AAA surveillance protocol.

Growth modelling

It is well established that once an AAA has developed it tends to grow with time, however, the pattern of growth is much more unclear. AAA measurements obtained from the Leeds Teaching Hospitals NHS Trust server from patients enrolled in the study will be used for this analysis by a team of biostatisticians led by Dr Paul Baxter. The statistics team will only have remote access to blinded data (see below) for their analyses. Growth modelling work will use multilevel mixed effects models to characterise the growth of AAAs within patients. Linear mixed models with random slopes and intercepts can be considered a reasonable basic model. However, more complex non-linear mixed models of quadratic and logistic growth are also of interest. As an alternative, allowing for autocorrelation in residuals from a linear mixed model will be used as a simple strategy for modelling non-linearity in growth. Autocorrelation can be addressed by Box-Jenkins autoregressive moving average (ARMA) structures or contrasted with a spatial autocorrelation model that allows for unequal spacing of observations through time. Growth mixture modelling will provide an alternative, experimental strategy, for characterising growth. Modelling with propensity score matched controls is likely to use techniques including linear and generalised linear models with multilevel and latent variable techniques, where appropriate, to respect complex data structures.

Operative Samples

In those patients who require open surgery, the following samples will be taken (1) abdominal aortic wall, (2) Intra-luminal thrombus, and (3) blood from the AAA sac. All of these samples are normally discarded at the time of surgery.

In those patients who require endovascular stenting a blood sample will be obtained from (i) the sheath placed in the femoral artery and (ii) a catheter placed in the AAA sac. The sheath and passage of the catheter through the AAA are part of the normal procedure; blood is normally aspirated from these sheaths /catheters and then flushed with heparinised saline.

Follow-up

1. Control patients

There is no routine follow up for the purposes of this study.

2. Small AAA 30-54mm

In addition to the NHS follow up US scans (see above), those patients with a small AAA 30-54mm will be invited to return at yearly intervals (until the AAA exceeds 55mm) to update their demographic data and medications and to give a repeat blood sample.

3. Large AAA >55mm at recruitment in those suitable for elective repair

Those patients who undergo a successful AAA operation (Open or Endovascular) will be recalled back to the LIGHT, University of Leeds (if they are fit and well) or we can visit them at home after 3 and 12 months for a repeat blood sample and a repeat ultrasound examination of the AAA repair site. Previous large scale studies have demonstrated that patients return back to a normal quality of life after 3 months and as such the majority would be fit enough to travel. Post operative imaging undertaken as part of routine clinical care as outlined by LTHT protocols will be accessed electronically by the research team.

4. Large AAA >55mm in patients who have declined/been turned down for intervention

The medical results server held by the LTHT will be accessed annually for blood results (including Hb, WCC, Plts, Clotting, U&E, CRP and Creatinine and Lipid Profile) and/or any subsequent relevant imaging in order to track AAA growth. In addition, should they wish to return, these patients are also invited back on a yearly basis in order to update their demographic data and medications and to give a repeat blood sample.

At recruitment, consent is taken for all patients and controls to be followed up through Hospital Episode Statistics (HES), Office of National Statistics mortality data (ONS) and The Myocardial Infarction National Audit Project (MINAP) to allow collection of relevant information regarding patient outcomes, specifically relating to cardiovascular morbidity, and mortality.

Laboratory Methods

Plasma levels of TAT, F1+F2, D-dimer, TAFI, PAI-I AG, tPA Ag, plasminogen, alpha2-antiplamin, MMP's, insulin and proinsulin are be measured using ELISA kits as well as commercially available antibodies, and fibrinogen is assayed using the Clauss Method. Thrombin generation is measured using the Calibrated Automated Thrombogram (CAT). Other coagulation factors (FVII, FIX, vWF, FXI, FXII) may also be measured by ELISA.

Cardiovascular Disease (CVD) biomarkers would be analysed using relevant biochemical and immunological methods.

Measurement of plasma glucose and lipid sub-fractions are carried out in the routine laboratories of the Chemical Pathology department of the LGI.

Insulin resistance is assessed using the HOMA model with fasting insulin/glucose product, which we have found to be a subtle indicator of insulin resistance in relatives of type 2 diabetic subjects compared to controls. In addition there is evidence that proinsulin is inadequately processed in the presence of insulin resistance and that the insulin:proinsulin ratio is a good indicator of underlying insulin resistance. This will be investigated as a marker of insulin resistance in these subjects.

Factor XIII activity is assayed by a method which employs cadaverine incorporation as a surrogate for fibrin cross-linking and which has been developed in the Unit of Molecular Vascular Medicine in Leeds. Factor XIII A and B subunit antigens will be assayed by in house ELISA. These assays have a sensitivity of 0.4ng/ml of Factor XIII A and B subunits with an intra-assay coefficient of variation of 4% and 6% respectively.

Assessment of fibrin structure/function; Fibrin permeation characteristics

Measurement of the permeability of fully polymerised and cross linked fibrin provides structural data regarding fibre thickness and the mass-length ratio. This is done by assessing the permeation or Darcy constant Ks. The Ks represent the surface of the gel allowing flow through a network and thus provides information on the pore structure will be calculated using the following formula: $Ks = {}^{Q.L.\eta}/A\,t\,\Delta P$ where Q Is the volume of liquid (ml) with the viscosity η (10^{-2} poise) flowing through a clot with length L (1.3 cm) and a cross-sectional area A (0.049 cm^2) in time t (sec) under pressure ΔP (dyne/cm^2). Duplicate clots are formed for each plasma sample, using human thrombin and calcium. Samples are incubated in a moist atmosphere for 2 hours to allow for full clot formation and cross-linking. The fibrin clots will be washed with 0.05 mol/L Tris-Cl , pH 7.5, 0.1 mol/L NaCl, and permeated with the same buffer at a pressure drop of 4 cm. Flow rate of the buffer through the clots is analysed by measuring total buffer filtrate at 30minute intervals.

Fibrin polymerisation

Fibrin polymerisation will be studied by following generation of turbidity at 350 nm over the time course of clot formation. Turbidity analysis provides kinetic information regarding the lag period and rate of fibrin polymerisation, in addition to fibre thickness, which can be calculated from the maximum absorbency at complete clot formation. Clot formation of each sample will be triggered in duplicate with thrombin and calcium in microtiter plate. Immediately upon addition of thrombin/calcium, absorbency will be read every 7 seconds at 350 nm for 15 minutes using a Dynex MRX-TC2 plate reader in kinetic mode. Parameters such as lag phase before start of fibrin polymerisation slope of the polymerisation curve (A/min) and maximum absorbency at full polymerisation will be recorded. The addition of a fibrinolytic agent (tPA) allows for analysis of lysis of the fibrin clot, again reading the absorbency every 7seconds over a period of up to 8 hours until full lysis has occurred.

Visco-Elastic properties

Fibrin cross-linking by FXIIIa is a strong determinant of the elasticity of the fibrin clot. We have recently purchased ROTEM equipment. The ROTEM provides a fully automated method to measure visco-elastic properties of the fibrin clot in whole blood samples or in blood plasma. In collaboration with physics (Leeds University) we have also developed magnetic tweezers equipment which we may use to investigate visco-elastic properties in more detail.

Confocal Microscopy

Fibrin clot structure will be imaged using confocal microscopy. In brief, thrombin and FITC fibrinogen will be added to plasma samples, and placed into a 6 channel confocal microscopy plate. After an incubation period to ensure full clot formation and fibrin cross linking, the clots can be imaged using the confocal microscope. This provides both 2dimensional and 3 dimensional images. In addition to providing detailed pictorial representations of the clot, this will also allow accurate measurement of fibre thickness and pore size (by taking relevant measurements at known magnifications).

Scanning electron microscopy.

Fibrin clot structure will be further investigated by scanning electron microscopy. We will make use of electron microscopes (Camscan Series 4 and Camscan CS-44-EX) available in the microscopy suit of the Department of Material Sciences at the University of Leeds. Clots will be made as previously described. The clot will be extensively washed with 0.067 mol/l Na-cacodylate buffer Ph 7.2, fixed with 2 %

gluteraldehyde in cacodylate buffer overnight. Samples will be further processed dehydration using a stepwise acetone gradient, critical point drying and sputter coating with palladium-gold. Each sample will be observed in at least 6 different areas for digital image analysis. Overall clot structure will be analysed, in addition to measurement of fibre image analysis. Overall clot structure will be analysed, in addition to measurement of fibre thickness and number of branch points per area using Image software version 1.24 (Wayne Rasband, National Institutes of Health, USA).

Molecular Biology

DNA extraction

DNA is extracted from the whole blood samples taken at recruitment. Prior to extraction, samples are defrosted in a room temperature water bath.

The process uses a commercially available DNA extraction kit (QIAGEN maxi spin kit). In brief, protease and a lysis buffer are added to the whole blood samples, and then incubated at 75°C for 10minutes. 100% ethanol is added, and following a thorough mixing stage, the samples are decanted into the QIAGEN spin column. They are centrifuged at 3000rpm for 2minutes, and the filtrate discarded. The column is then washed in a two stage process, before being transferred into a clean centrifuge tube. The elution buffer is added, the column incubated to ensure DNA binding, and two final centrifugation stages are undertaken.

The concentration of the resulting DNA sample solution is elicited using the Nanodrop reader, at an absorbance of 260nm. Samples are diluted to a final concentration of 10ng/μL using DNAase and RNAase free TBS, and stored at 4°C. Long term storage of concentrated DNA for future work is at -20°C.

RNA Extraction

RNA will be extracted using a standardized TRIreagent protocol. Total RNA is extracted from VSMC using Tri-reagent (Sigma), bromocriptine and glycogen. RNA precipitates were re-suspended in water followed by DNase 1 turbo digestion at 37oC for one hour. RNA can be quantified using Ribogreen® (Molecular Probes, Invitrogen). 0.6ug of RNA is reverse transcribed to cDNA using the Applied Biosystems High Capacity RNA to cDNA kit (RT+). 0.6ug is also used for a genomic DNA control in a reaction without reverse transcriptase (RT-). Real time PCR is performed using SYBR Green I on a LightCycler (Roche). The specificity of PCR is confirmed by performing RT negative control experiments and melt curve analysis of PCR products. PCR cycle crossing points (Cp) are determined using a fit points methodology (Light Cycler Software 3.5). Relative abundance of target RNA is quantified as compared to the housekeeper gene beta actin.

Protein Extraction

Vascular smooth muscle cells are collected on ice cold phosphate buffered saline and pelleted at 4oC before lysis using Laemmli sample buffer supplemented with complete protease inhibitor (Roche). The lysate is centrifuged to remove particulate matter and equal ammounts of protein are separated by 8% sodium dodecyl sulphate polyacrylamide gel electrophoresis and transferred onto nitrocellulose membrane. Membranes are incubated with blocking buffer for two hours at room temperature followed by incubation with primary antibodies of interest overnight at 4oC. Membranes are washed and incubated with the secondary antibody of interest. Signals are detected using super signal west pico chemiluminescent.

Genotyping

Currently, DNA samples are genotyped for known polymorphisms of Fibrinogen, Factor XIII and TAFI. Commercially available probes are used (Taqman Genotyping Assays). Genetic polymorphisms in other coagulation and fibrinolysis related proteins will also be investigated.

In brief, the probe is added to TaqMan Genotyping MasterMix in a 20:1 ratio (henceforth referred to as MasterProbe). 2.7µL of MasterProbe and 2.2µL of the diluted DNA (10ng/µL) are added to each well of a 384 well plate. Two no template controls (containing only MasterProbe) are included on each plate. A LightCycler480 is used for programming the real time PCR. Each run consists of 50 amplifications, with end-point genotyping following cooling.

Operative Samples

Participants undergoing open aneurysm repair at Leeds Teaching Hospitals NHS Trust as part of their usual care will be given the opportunity to donate tissue samples as part of the study. All tissue taken as part of this process would usually be discarded during the operation.

Aortic Wall

A segment of anterior aortic wall will be biopsied at the time of surgery and divided into different transport media as follows: (1) Dulbecco's Modified Eagle Medium supplemented with 10% foetal calf serum and 1% penicillin/streptomycin transported on ice to Dr Porter's primary tissue culture lab and (2) sterile 0.9% saline, transported at room temperature to the lab for histological analysis.

Dr Porter and her team will undertake Smooth muscle cell isolation & culture. An explant technique will be used to acquire primary vascular smooth muscle cells (vSMC). The media will be isolated and dissected into small fragments ($1mm^3$) and transferred in 2ml of culture medium to tissue culture flasks and maintained at 37^oC in a humidified incubator in 5% CO_2 in air. After 1-2 weeks, vSMC will be observed to migrate out from the explants and passaged in order for experimentation. The primary cells will be confirmed as SMC by staining for intracellular smooth muscle α-actin and myosin heavy chain-2 fibres. Isolated vSMC can be used for DNA, mRNA and protein characterisation, in addition to functional and phenotypic analysis studies. Functional studies include proliferation, invasion, migration and apoptosis assays. Secretory function can be demonstrated with the use of zymography. Phenotypic studies will allow the identification of extracellular markers that will help characterise the vSMC.

Our early work suggests that vSMC isolated from AAA exhibit poor proliferation, reduced migration and we have observed a flatter more rhomboid phenotype compared to vSMC isolated from internal mammary artery in use in the lab. To further understand the driving forces behind this altered phenotype, the vSMC will be used for calcium signalling experiments in conjunction with Prof. Beech using a range of pharmacological agents, RNA interference strategies and antibodies. This work will involve isolation of mRNA/protein from the cultured vSMC in addition to functional assays as previously described.

Intraluminal Thrombus

The material will be fixed in formalin before sectioning on a microtome to allow immunohistochemical analysis of blood coagulation and fibrinolysis related proteins. Blood and tissue samples taken from the same patient will be appropriately coded to allow them to be matched during analysis.

Data Management

The LEADS database itself is managed by Thomas Fleming, Data Management Technology Group, University of Leeds. All information stored on this database is annonymised. The master database of information is stored on a password protected computer in an access-limited area of the university. Access to the original database is limited to the database manager, the lead research nurse, and the vascular research administrative assistant who works alongside her (David Watson). Access-only use is granted to members of the research team at the discretion of the principal investigator.

Identifiable patient information is held only as paper copies, and is kept in a secure, locked drawer in a limited access area of the University of Leeds. The lead research nurse (Mrs Anne Johnson) acts as the custodian for this information, and is the only one to have access to the identifiable data. Personal identifiable information will be destroyed five years after the end of the study.

Appendix 2 - Patient Information Sheet

THE LEEDS ANEURYSM
DEVELOPMENT STUDY

Version 1 March 2013

You are being invited to take part in a <u>RESEARCH</u> study. Before you decide, it is important for you to understand why the research is being done and what it will involve.

Please read the following information carefully and discuss it with friends, relatives and your GP if you wish. Ask us if there is anything that is not clear, or if you would like more information.

A leaflet is produced by INVOLVE (Promoting public involvement in NHS, public health and social care research) - www.invo.org.uk gives information about medical research and looks at some questions you may want to ask.

What is the purpose of the study?

Aortic aneurysm is a common condition where the major blood vessel (aorta) in the tummy dilates like a balloon. The vast majority of patients have no symptoms. There is some evidence that abdominal aortic aneurysms (AAA) run in families and it is unclear whether this risk is due to environmental factors (e.g. diet, smoking, high blood pressure etc) or whether a person's genes may contribute.

Given time, however, the aneurysm expands and may burst. Of all the cases of ruptured AAA only 10% survive emergency surgery compared to 95% who survive elective surgery. The wider availability of ultrasound scans as a simple non-invasive method to accurately assess the presence of aneurysms and the National Aortic Aneurysm Screening means that in future it is likely that we will identify large numbers of subjects with early disease.

At the present time patients with small Aortic Aneurysms (3.0 – 5.0cm) are followed up by routine ultrasound scans every 3-12 months depending upon their size. Once the Aortic Aneurysm exceeds 5.5cms patients are referred by their consultants for more specialised scanning to assess the suitability for treatment. This can be either a CT or MRI/MRA scan. A CT (Computerised Tomography) scan is a type of x-ray examination where a cross sectional picture of the area scanned is produced. Magnetic Resonance Scanning (known as MRI/MRA)) is a way of looking inside your body without the use of x-rays. Instead it uses a large magnet, radiowaves and a computer to scan your body and produce a detailed picture.

Surgery in the form of open or stenting (minimally invasive) is reserved for those patients in whom the abdominal aortic aneurysm exceeds 5.5cms in diameter.

The overall aim of the LEADS project is to investigate AAA development, growth and outcomes, and thus identify novel treatments.

Why   have   I   been   chosen?
You have been chosen because you have an Abdominal Aortic Aneurysm, so called "triple A"

Do I have to take part?

It is up to you to decide whether or not to take part. If you do decide to take part, you will be given this information sheet to keep and be asked to sign a consent form. If you do decide to take part, you are still free to withdraw at any time and without giving a reason. This will not affect the standard of care you receive.

What will happen if I take part?

If you are happy to help us with this research project this is what will happen:

You will have nothing to eat or drink after midnight before your visit.

You should try not to smoke during this period.

You will be asked some simple questions about your health.

You will have cuffs placed around your arm, leg and a pad on your neck which will be used to measure the stiffness of your blood vessels.

You will have a heart tracing.

You will give a sample of blood (50mls, about 4 tablespoons). These samples will allow us to study the levels of blood sugar, fat and factors in the blood, which cause it to clot. We will also look at the genes which are involved in the development of aortic aneurysms and the formation of new blood vessels and blood clotting.

You will have a simple ultrasound test (very similar to the scans that pregnant women have) to measure the size of the abdominal aorta. This is painless and takes 5-10 minutes. In those cases where it is difficult to see the aorta we will arrange a separate ultrasound scan at St James' Hospital, this will be undertaken by Dr Michael Weston, Consultant Radiologist.

The above tests will take place in the clinical research unit of the LIGHT laboratories of the University of Leeds. You will be asked to attend for an appointment with a research nurse, which will last just over one hour. We will contact you to confirm a suitable day and time for the assessment. We will send you details of how to get to the LIGHT building.

We will access your electronically held medical records annually. We will register your data with the NHS Information Centre and the NHS Central Register so that we can track your progress over the study time period. As such your data will be identifiable to us. With your consent, we will also access other nationally kept databases, such as including the Office of National Statistics (ONS), Hospital Episode Statistics (HES) and the National Myocardial Infarction Audit Project (MINAP), to track any other medical problems you may encounter over the period of the study.

We will write to you on a yearly basis to ask if you would be prepared to return for a further a repeat assessment.

If your aortic aneurysm is larger than 5.5cms your consultant will arrange for you to have either a CT scan or an MRI scan. This is part of the normal workup to assess how best to treat your aortic aneurysm. We would like to have your permission to store these anonymised images and use them at a later date to study (1) the comparison between ultrasound and CT/MRI, (2) the force and stresses upon the aortic wall, (3) the flow through the aorta and (4) the impact of a metal stent on the aorta. This work will be undertaken by Dr Peter Walker (Department of Mechanical Engineering, University of Leeds).

If you are having an operation for your aortic aneurysm we would ask permission to collect the following samples

    (1)   fluid between the aortic aneurysm wall and the blood clot

    (2)   a blood sample (20 mls) from the aneurysm sac,

    (3)   a sample of the wall of the aortic aneurysm and

    (4)   the blood clot within the aneurysm.

All of these tissues are usually discarded at the time of surgery

Post operation

You will be seen in the out-patient department at approximately six weeks following surgery.

At this stage we will ask if you would be happy for a further sample to be taken at 3 to 6 months post operatively. This could be arranged as a home visit.

You will have nothing to eat or drink after midnight before your visit.

You should try not to smoke during this period.

You will give a sample of blood (50mls, about 4 tablespoons). These samples will allow us to study the levels of blood sugar, fat and factors in the blood, which cause it to clot.

What are the potential risks from taking part?

Taking a blood sample is unlikely to cause any problems, although some people experience minor discomfort or bruising.

What are the potential benefits from taking part?

There are no direct benefits to you for taking part. In the future we hope that the information we get from this study may help us to improve the treatment of patients with abdominal aortic aneurysms. We will be able to assess whether life-style changes and or new drug treatments may help those patients with small abdominal aortic aneurysms and relatives at highest risk of developing abdominal aortic aneurysms.

What if new information becomes available?

Sometimes during the course of a research project, new information becomes available. In your case this may be an increase in the size of your abdominal aorta. If this happens, your research nurse will tell you about it and discuss with you whether you want to continue in the study. If you decide to withdraw,

arrangements will be made for your care to continue. If you decide to continue in the study you will be asked to sign an updated consent form and the necessary information passed onto your consultant surgeon and general practitioner.

What if something goes wrong?

If taking part in this research project harms you, there are no special compensation arrangements. If you are harmed due to someone's clinical negligence, then you may have grounds for a legal action but you may have to pay for legal advice. Regardless of this, if you wish to complain about any aspect of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms should be available to you.

Will my taking part in this study be kept confidential?

All information, which is collected, about you during the course of this research will be kept *strictly confidential.* Any information that leaves the laboratory will have your name and address removed so that you cannot be recognised from it.   The blood, fluid and tissue samples taken to look at the proteins and genes will be given a study number so that it is impossible in any way to identify you from them.

You should be aware that the samples of blood, fluid and tissue will be stored in a freezer and may be used for future studies at a later date.

Any abnormalities detected in your blood sugar or fat will be forwarded to you and your general practitioner.

What will happen to the results of the study?

The results of the study will be published in a variety of surgical journals and discussed at surgical meetings. Please note that you will not be identified in any report or publication.

Who is organising and funding the research?

The study is based in the Division of Cardiovascular and Diabetes Research at the Leeds Institute of Genetics, Therapeutics and Health at the University of Leeds. The study is currently funded by The Garfield Weston Trust, Circulation Foundation and the British Heart Foundation.

Who has reviewed the study?

Leeds (East) Research Ethics Committee has reviewed the study.

Contact for further information

Any further questions regarding the study can be easily answered by calling **Anne Johnson Vascular Research Nurse 0113 343 7702** or by writing to Professor Julian Scott, Leeds Vascular Institute, Leeds General Infirmary, Leeds LS1 3EX

Thank you for considering whether to take part in this research

Appendix 3 - Patient Consent Form

THE LEEDS ANEURYSM DEVELOPMENT STUDY

Subject Identification Number: _____ Name of researcher: Prof D J A Scott

Anne Johnson

1. I confirm that I have read and understand the study information sheet dated __/__/__ and have had the opportunity to discuss it with relatives and friends, and ask any questions. ☐

2. I understand and accept that if I take part in the study I may not gain any direct personal benefit from it. ☐

3. I understand that all information collected in the study will be held in confidence and that, if it is presented or published, all my personal details will be removed from it. ☐

4. I understand that any blood and tissue samples collected as part of the study are given freely as a gift. I consent to my samples being examined for genetic factors which are thought to influence the formation of aneurysms. I agree to transfer any property rights to the samples I provide to the University of Leeds on the understanding that it is only used for research. ☐

5. I confirm that I will be taking part in this study of my own free will. I understand that I may withdraw from it, at any time and for any reason, without my medical care or my legal rights being affected. I understand that if I later decide to withdraw from the study, any samples or information already collected from me may continue to be used for the purposes of the study, unless I specifically withdraw consent for this to happen. ☐

6. I agree to my electronically held medical records being accessed by the research nurses assigned to the study. ☐

7. I agree to information held about me by other registered databases, including the National Office of Statistics (ONS), Hospital Episode Statistics (HES) and the National Myocardial Infarction Audit Project (MINAP),being accessed by the study team for the purposes of the study. ☐

8. I agree to participate in this study. ☐

Name _____ Signature _____ Date _____

Appendix 4 - DNA extraction from whole blood using the QIAGEN Maxi-Column Kit

Before starting

1. Prepare a 70°C water bath

2. Buffer AW1 should be prepared (mixed with ethanol as indicated on the bottle)

    a. Stored at room temp

3. Buffer AW2 should be prepared (mixed with ethanol as indicated on the bottle)

    a. Stored at room temp

4. QIAGEN protease should be prepared (Add 5.5mls of distilled water)

5. Blood should be defrosted in a room temperature water bath

Procedure

1. Pipette 500µL QIAGEN protease into the bottom of a 50ml centrifuge tube (each vial of protease will be enough for 11 blood samples)

2. Add 6ml of blood (one full pink topped tube)

3. Add 4ml of PBS

4. Mix briefly

5. Add 12ml of Buffer AL (provided in kit), mix thoroughly by inverting the tube 15 times then shaking vigorously for a minute.

6. Incubate at 70°C for 10minutes (incubating for longer will not adversely affect the DNA yield)

7. Add 10ml of ethanol (96-100%) to each sample

8. Mix by inverting 10 times and then shaking vigorously again

9. Transfer half of the solution into the QIAamp maxi column within a 50ml centrifuge tube.

10. Ensure the rim is dry and close the cap

11. Centrifuge at 1850xg or 3000rpm for 3 minutes

12. Remove the maxi-column from the centrifuge tube, discard the filtrate, ensure the tube is dry and replace the column

13. Transfer the remaining solution into the maxi-column

14. Centrifuge again at 1850xg or 3000rpm for 3 minutes

15. Remove the maxi-column from the centrifuge tube, discard the filtrate, ensure the tube is dry and replace the column

16. Add 5ml of Buffer AW1 to the QIAamp maxi column. Pipette carefully, without moistening the rim. Pipette to the side of the column (not directly onto the membrane)

17. Centrifuge at 4500xg or 5000rpm for 1 minute.

18. Without discarding the filtrate, add 5ml of buffer AW2 to the QIAamp maxi column.

19. Centrifuge at 4500xg or 5000rpm for 15minutes

20. Place the QIAamp maxi column in a clean 50ml centrifuge tube.

21. Discard the collection tube containing the filtrate

22. Pipette 1ml of Buffer AE directly onto the membrane of the QIAamp maxi-column.

23. Incubate at room temperature for 5 minutes.

24. Centrifuge at 4500xg or 5000rpm for 2minutes.

25. Two final steps:

a. For maximum concentration of DNA – reload the eluate containing the DNA onto the maxi column. Incubate for 5 mins at room temperature. Centrifuge at 4500xg or 5000rpm for 5minutes.

b. For maximum yield of DNA – Pipette 1 ml of fresh AE buffer onto the membrane of the QIAamp maxi column. Incubate for 5 mins at room temperature. Centrifuge at 4500xg or 5000rpm for 5minutes.

Appendix 5 - Angiotensin II Dosing Schedule

1004 pump	Ang II (750 ng/kg/min)		to make 120 µl - stock 25 µg/µl	
Mouse Weight (g)	Concentration for pump (µg/µl)	angII (µg) needed to make 120 (µl) volume	Stock required µl	water required µl
20	6.43	771.43	30.86	89.14
21	6.75	810.00	32.40	87.60
22	7.07	848.57	33.94	86.06
23	7.39	887.14	35.49	84.51
24	7.71	925.71	37.03	82.97
25	8.04	964.29	38.57	81.43
26	8.36	1002.86	40.11	79.89
27	8.68	1041.43	41.66	78.34
28	9.00	1080.00	43.20	76.80
29	9.32	1118.57	44.74	75.26
30	9.64	1157.14	46.29	73.71
31	9.96	1195.71	47.83	72.17
32	10.29	1234.29	49.37	70.63
33	10.61	1272.86	50.91	69.09
34	10.93	1311.43	52.46	67.54
35	11.25	1350.00	54.00	66.00
36	11.57	1388.57	55.54	64.46
37	11.89	1427.14	57.09	62.91
38	12.21	1465.71	58.63	61.37
39	12.54	1504.29	60.17	59.83
40	12.86	1542.86	61.71	58.29

Appendix 6 - Mouse Identification Code

Mice within the CEU at the University of Leeds undergo ear notching to allow for identification of individual subjects housed within the same cage/undergoing the same experimental protocol at the same time.

● Represents a site of ear notching
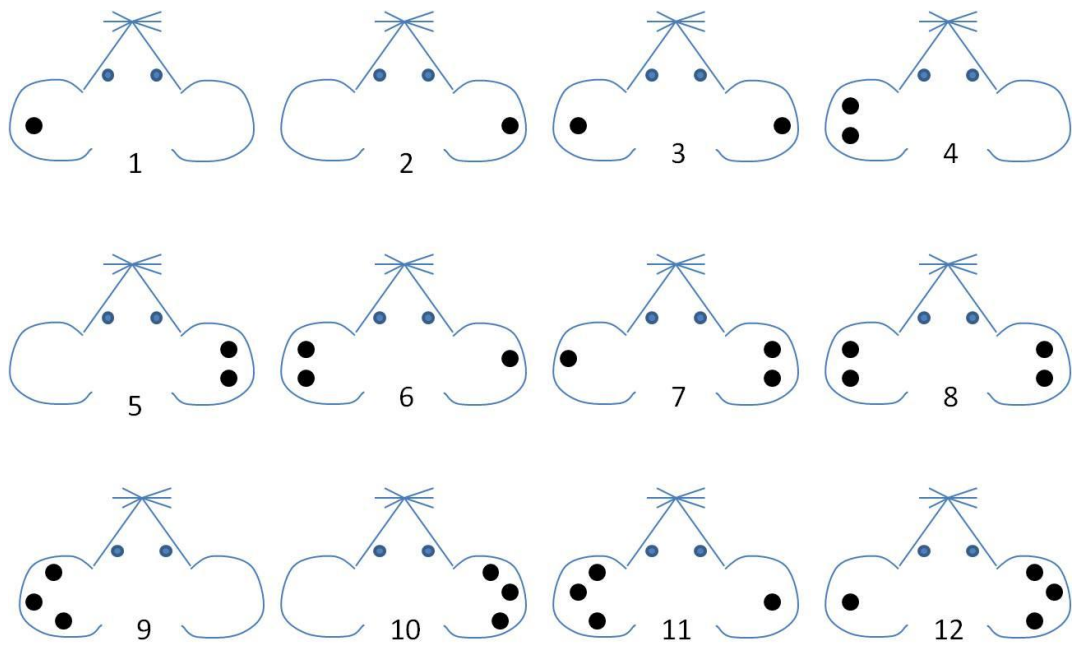
Appendix 7 - Histology Processing Protocol

17 hour processing programme for the dehydration of samples for histology

1	70% ethanol	30minutes
2	70% ethanol	30minutes
3	70% ethanol	30minutes
4	90% ethanol	1 hour
5	100% ethanol	2 hours
6	100% ethanol	2 hours
7	100% ethanol	2 hours 30minutes
8	Histoclear	30minutes
9	Histoclear	1 hour 30 minutes
10	Histoclear	1 hour 30 minutes
11	Paraffin	1 hour 30 minutes
12	Paraffin	2 hours