Mapping the Interaction of VWF and Factor XIIa and its Potential as a New Anticoagulant Target

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Doctor of Philosophy in the Faculty of Medicine

by

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Abstract

Von Willebrand Factor and Factor XII have well established roles in haemostasis and coagulation. While VWF is a mediator of platelet capture to sites of vascular injury under high shear stress, FXII initiates the intrinsic pathway of coagulation. Interestingly, multiple studies have shown that absence or inhibition of FXII does not impair haemostatic function but has a thrombo-protective effect, rendering it a potentially safe anticoagulant target.

In this study, I investigated a novel binding interaction between VWF and FXII. Using substrate-based activation assays, I showed that VWF is a potent and previously unrecognised activator of FXII. Additionally, I demonstrated that both inactive FXII and activated FXII bind to VWF but not to VWF lacking the A1 domain suggesting that the FXII binding site lies within the A1 domain. By carrying out further binding studies using static binding assays and surface plasmon resonance with isolated and full-length VWF A1 domain mutants, I identified amino acids E1452 and D1459 on the α-6 helix of the A1 domain as the FXII binding site in VWF.

The physiological relevance of the VWF/FXII interaction was examined using plate-based thrombolysis as well as perfusion assays investigating the formation of platelet rich blood clots under conditions of flow. Using adapted thrombolysis assays, I showed that clot lysis times were significantly reduced when either VWF, FXIIa or activity of both proteins was blocked. Furthermore, I demonstrated that the interaction between VWF and FXIIa facilitates VWF mediated platelet capture under conditions of high shear and that interference with FXII/VWF binding has a prohibitive effect on overall thrombus formation.

Based on these results, I hypothesise binding of VWF to FXII is an important component of thrombus formation and inhibition of this interaction might be a novel and safe way to prevent thrombosis without increasing the risk of bleeding side effects.

Declaration of Originality

I, Patricia Henne, hereby declare that the work presented in this thesis is my own. All work and data analysis of the results were performed by myself unless otherwise specified.

Patricia Henne

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Abbreviations

VWF	Von Willebrand Factor
TF	Tissue Factor
TFPI	Tissue Factor Pathway Inhibitor
F	Factor
FVIII	Coagulation Factor VIII
GP	Glycoprotein
s^{-1}	Inverse second
TXA2	Thromboxane A2
ADP	Adenosine Diphosphate
FV	Coagulation Factor V
FXII	Coagulation Factor XII
FX	Coagulation Factor X
FVII	Coagulation Factor VII
FXI	Coagulation Factor XI
FIX	Coagulation Factor IX
HMWK	High-molecular-weight Kininogen
PK	Pre-kallikrein
FII(a)	(activated) Thrombin
FXIII(a)	(activated) Coagulation Factor XIII
tPA	tissue-type Plasminogen acti
uPa	urokinase-type Plasminogen activator
AT	Antithrombin
TM	Thrombomodulin
EPCR	Endothelial Protein C Receptor
APC	Activated Protein C
PAI1	Plasminogen Activator Inhibitor-1
PAI2	Plasminogen Activator Inhibitor-2
TAFIa	Thrombin-activable Fibrinolysis Inhibitor
TM	Thrombomodulin
DVT	Deep Vein Thrombosis
NET`s	Neutrophil Extracellular Traps
DNA	Deoxyribonucleic Acid
B2GP1	β2 Glycoprotein 1
Apo-H	Apolipoprotein H
TTP	thrombotic thrombocytopenic purpura
ADAMTS13	A Disintegrin And Metalloproteinase with a Thrombospondin type 1 motif member 13
COX-1	Cyclooxygenases 1
COX-2	Cyclooxygenases 2
NSAID	Nonsteroidal Anti-inflammatory Drugs
VWD	Von Willebrand Disease
ASPGR	Asialoglycoprotein receptor

MGL	Macrophage galactose type-lectin
SIGLEC-5	Sialic-acid binding immunoglobulin-like lectin 5
CLEC4M	C-type lectin domain 4 member M
LRP-1	Low-density lipoprotein receptor-related protein 1
SE-A1	Scavenger receptor class A member
ITP	Immune Thrombocytopenia
HIV	Human Immunodeficient Virus
PEG	Polyethylene Glycol
siRNA	small interfering Ribonucleic Acid
AAV	Adeno-associated Virus
CK	Cysteine Knot
TGF-β	Transforming Growth Factor β
ER	Endoplasmatic Reticulum
kDa	Kilodaltons
PDI	Thioredoxin-like Protein Isomerase
WPBs	Weibel Palade Bodies
VEGF	Vascular Endothelial Growth Factor
cAMP	cyclic Adenosine Monophosphate
ADAM28	A Disintegrin And Metalloproteinase domain-containing protein 28
TSP-1	Thrombospondin-1
LPR-1	Lipoprotein Receptor-related Protein 1
ASGPR	Asialoglycoprotein
CLEC4M	C-type Lectin Domain family 4 member
WHO	World Health Organisation
IL	Interleukin
EGF	Epidermal Growth Factor
C1INH	C1 esterase Inhibitor
ATIII	Antithrombin III
RA	Rheumatoid Arthritis
HEA	Hereditary Angioedema
CD	Cluster of Differentiation
ERK	Extracellular signal-regulated Kinase
GAG	Glycosaminoglycans
HUVEC	Human Umbilical Vein Endothelial Cells
Th17	Effector memory T-helper cells
TNF-α	Tumor Necrosis Factor α
LRP1	Lipoprotein receptor-related protein
C1	Complement Component 1
PPT	Partial Thromboplastin Time
CTI	Corn Trypsin Inhibitor
SPR	Surface Plasmon Resonance
C	Celsius
MEM	Minimal Essential Medium
FBS	Fetal Bovine Serum

%	Percent
mM	Millimole
U	Unit
ml	Millilitre
mg	Milligram
DMSO	Dimethyl Sulfoxide
HEK293T	Human Embryonic Kidney 293T
PBS	Phosphate Buffered Saline
MCS	Multiple Cloning Site
CMV	Cytomegalovirus
PCR	Polymerase Chain Reaction
µM	Micromolar
µl	Microlitre
ng	Nanogram
EDTA	Tris-borate-ethylenediaminetetraacetic Acid
RT	Room Temperature
w/v	Percent weight/Volume
V	Volt
UV	Ultraviolet
Rpm	Rounds per minute
LB	Laura-Bertani
cm^2	Square centimetre
Dr.	Doctor
Δ	Delta
µg	Microgram
T	Tumor
PEI	Polyethylenimine¨
M	Mol
g	G-force
TFF	Tangential Flow Filtration
IMAC	Immobilised Metal Ion Chromatography
Ni^{2+}	Nickel Ion
NaCl	Sodium Chloride
IEC	Ion Exchange Chromatography
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
v/v	Percent volume/volume
PBST	Phosphate Buffered Saline containing 0.1% Tween
HRP	Horseradish Peroxidase
Ab	Antibody
BCA	Bicinchoninic Acid
Cu^{+1}	Cuprous Ion
BSA	Bovine Serum Albumin
ELISA	Enzyme-linked Immunosorbent Assay
ddH_2O	Double-distilled water
H_2SO_4	Sulphuric acid

g	Gram
ACD	Sodium Citrate
PRP	Platelet Rich Plasma
mU	Milliunit
PGE1	Prostaglandin E1
KCl	Kalium Chloride
$MgCl_2$	Magnesium Chloride
$CaCl_2$	Calcium Chloride
$DiOC_6$	3,3'-Dihexyloxacarbocyanine Iodide
NaH_2PO_4	Monosodium Phosphate
$NaHCO_3$	Sodium Bicarbonate
K_D	Dissociation Constant
COOH	Carboxylic Acid
EDC	1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide
NHS	N-hydroxysuccinimide
$ZnCl_2$	Zinc Chloride
RU	Response Unit
m^2/s	Metre squared over second
R_{MAX}	Maximal Retention Values
pM	Picomolar
mAu	Milli Absorbance unit

1. Introduction

1.1. Haemostasis

Haemostasis is defined as the physiological response to vessel wall injury and is characterised by an interplay between pro- and anticoagulant factors which aim to restrict the extravasation of blood after vascular injury[1,2]. Classically, the haemostatic response is regarded to consist of several mechanisms including constriction of the damaged vessel, formation of a platelet plug, initiation of the blood coagulation cascade via the intrinsic and extrinsic coagulation pathway and fibrinolysis[3,4].

General principles of haemostatic reactions had already been described in 1904 by Paul Morawitz who introduced the hypothesis that proteins on the platelet surface (thrombokinases) are able to convert a certain type of zymogen (prothrombin) present in the blood plasma into an active enzyme (thrombin)[5]. Furthermore, Morawitz postulated that this protein subsequently is able to cleave a fibrous protein (fibrinogen) to insoluble protein fibres (fibrin) which in his eyes were the main components of the coagulant system[5,6,7]. Over the next six decades, analysis of patients with bleeding tendencies as well as different animal experiments led to the discovery of a multitude of proteins involved in coagulation that later were termed coagulation factors[8,9]. Based on these findings, two independent research groups led by Earl Davie and Robert Gwyn Macfarlane respectively published a first model of the coagulation cascade with the name "intrinsic pathway of coagulation" in 1964[10,11]. Aside from formulating a first plausible clotting model, both groups also recognised the importance of phospholipid rich surfaces as well as the influence of platelets on the haemostatic system and divided the processes involved in haemostasis into primary and secondary haemostasis[4,12]. Over the last five decades, the general model of haemostasis and especially the coagulation cascade has been further refined and now includes a second and seemingly more relevant pathway of coagulation[13]. Furthermore, the new model recognises the intricate interplay between platelets, the different coagulation factors and the vascular system[1].

1.1.1. Primary Haemostasis

Primary haemostasis generally refers to the formation of a platelet plug and is based on the interaction of platelets with the vascular and to a certain extent the coagulation system (Figure 1.1)[14]. Upon vessel wall injury, subendothelial matrix proteins which usually are covered by a smooth endothelial cell layer, come in contact with platelets circulating within the blood stream.

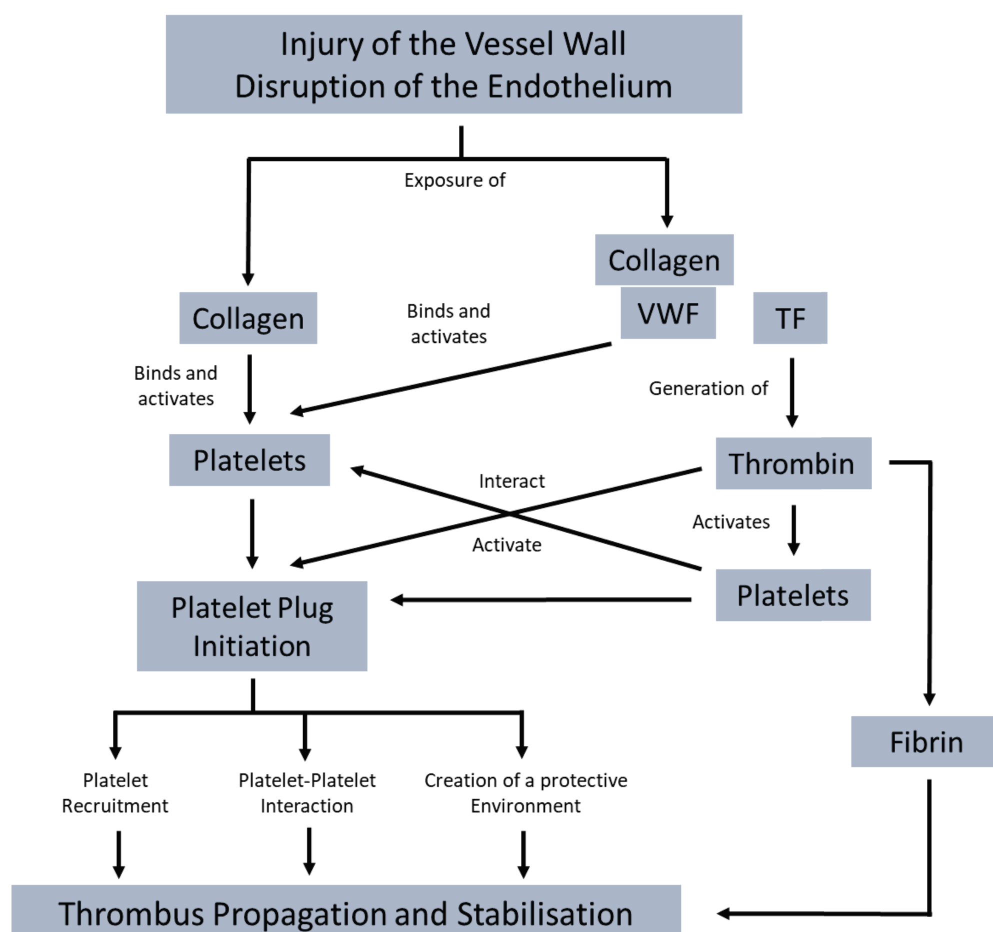

Figure 1.1: Overview of the platelet side of primary haemostasis. Upon vessel wall injury, collagen and collagen bound VWF are exposed to the blood stream. Due to interaction of platelet GPIb-V-IX with the A1 domain of VWF and platelet GPVI with collagen, platelets are arrested at sites of vascular damage and become activated. Small amounts of thrombin formed via the extrinsic pathway of coagulation support platelet activation and together with platelet-platelet interactions allow the formation of an initial platelet plug. By creation of a protective environment and recruitment of additional platelets, platelet plug growth is propagated. Simultaneously, fibrin which has been generated during secondary haemostasis interacts and cross-links the loosely packed platelets forming a dense and stable thrombus (based on[1]). TF = Tissue Factor.

Due to several protein-protein interactions, platelets subsequently adhere to subendothelial matrix bound Von Willebrand Factor (VWF), become activated and ultimately aggregate resulting in the formation of a platelet plug serving to prevent further blood loss[2,4].

1.1.1.1. The Vascular System

The vascular system is an intricate network of blood vessels allowing the circulation of blood and distribution of nutrients throughout the body. Apart from its function as a transport system, the vascular network also plays a delicate role in the maintenance of blood fluidity and a haemostatic balance by secretion of anticoagulant factors such as thrombin inhibitors[15,3]. In order ensure appropriate procoagulant function, different cell types present in the subendothelium are capable of expressing proteins which are able to initiate clotting reactions as for example tissue factor (TF). Under physiological conditions, interaction between TF and its primary binding partner coagulation factor FVIIa present in the flowing blood is prevented by a thin layer of endothelial cells lining the inner wall of every blood vessel. In cases of a vascular breach however, TF is capable of directly binding FVIIa initiating the extrinsic pathway of coagulation which eventually results in the generation of a blood clot and seal of the vascular injury[16].

The majority of blood vessels are composed of three cell layers. While the outer and middle layer mainly consists of connective tissue, the inner layer is made up of a mix of endothelial cells, a carbohydrate-rich layer called glycocalyx and connective tissues which is supported by a collagen-rich basement membrane[17,18,19]. Especially the inner vessel wall lining as well as the basement membrane play an important role in the prevention of unwanted coagulant reactions or initiation of localised clotting events. The endothelium hereby either employs physical changes as for example a widening of certain blood vessels in a mechanism called vasodilation or directly interacts with molecules involved in the different haemostatic processes[19,20]. The glycocalyx for example repels circulating blood cells and platelets through its negative charge hence circumventing inappropriate formation of small platelet aggregates or thrombi[21]. In addition, endothelial cells express and secrete a multitude of anti-coagulant molecules such as tissue factor pathway inhibitor (TFPI) and thrombomodulin which inhibit certain enzymes or cofactors of the coagulation cascade[1,3,15].

While maintaining an anti-coagulant milieu in healthy vessels, disruption of the vessel wall has to be counteracted in a rapid manner in order to prevent excessive blood loss. Vascular injury initially results in a narrowing of the blood vessels termed vasoconstriction which aims at a localised reduction of blood flow and diversion of the blood circulation[18,22]. Similar to the anti-coagulant molecules described above, endothelial cells also are known to express, secrete and store a variety of pro-coagulant factors[23]. The most important pro-coagulant molecules derived from endothelial cells are Von Willebrand factor (VWF) which is involved in the initial recruitment of platelets to sites of vascular injury, coagulation factor VIII (FVIII) which plays an important role in the intrinsic pathway of coagulation and TF which is the initiator of the extrinsic pathway of coagulation[1,1724]. Furthermore, recent studies have suggested a role of the endothelium as a biological surface for the assembly of the prothrombinase complex and therefore an involvement in secondary haemostasis which so far had only been ascribed to activated platelets[17].

1.1.1.2. Platelets

Platelets or thrombocytes are considered the essential cellular components of primary haemostasis. They commonly are described as small cell fragments and origin from megakaryocytes residing in the bone marrow[12]. Platelets circulate in the blood in a resting state and show neither aggregation with other platelets nor interaction with healthy vessel walls[1,25,26]. Upon disruption of the vessel wall or damage of the endothelium however, platelets are able to bind to subendothelial matrix bound VWF, change their shape, become activated, degranulate and finally aggregate (see Figure 1.2) forming an unstable platelet plug[3,23,27].

Adhesion of platelets to a site of vascular damage primarily is dependent on VWF and is further supported by subendothelial collagen[1,25,28]. Observation of platelet plug formation has shown that especially interaction between VWF and the glycoprotein Ib-V-IX (GPIb-V-IX) present on the surface of resting platelets is necessary in order to initiate tethering and arrest of circulating platelets[1]. Unlike most interactions, this initial arrest is more efficient at high shear rates due to the fact that VWF adopts a globular form in which its platelet binding sites are shielded at shear rates lower than $>500s^{-1}$ effectively preventing unwanted interaction with platelets[29]. In case of exposure of the subendothelial matrix, globular VWF is able to bind

to collagen and undergoes a conformational change exposing binding sites for platelet GPIb-V-IX[30–32]. While collagen is known to facilitate the interaction between VWF and platelets, the protein itself is able to bind to platelets via the signalling collagen receptor glycoprotein VI (GPVI)[25,26]. Binding of GPVI to collagen is supported by several integrins such as $\alpha_2\beta_1$ and $\alpha_{IIb}\beta_3$, receptor dimerization events as well as interaction of VWF to GPIb-V-IX[1,33].

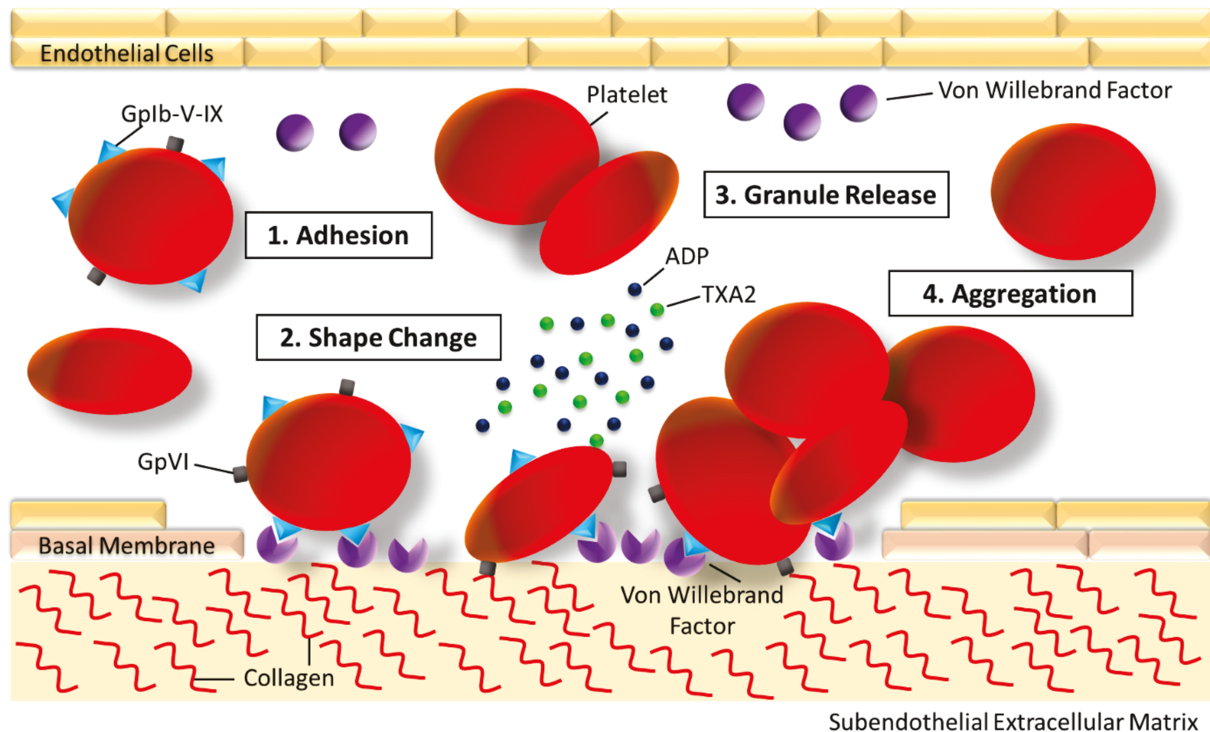

Figure 1.2: Overview of platelet activation. Resting platelets circulate in healthy blood. Vascular injury exposes subendothelial collagen which subsequently binds circulating VWF. Bound VWF undergoes a conformational change exposing its binding site for GPIb-V-IX present on the platelet surface. Tethering and adhesion of platelets to the subendothelial matrix is further supported by direct interaction between collagen and platelet surface receptor GPVI. Platelet binding results in a shape change due to a signalling cascade resulting in the rearrangement of the thrombocytic cytoskeleton which additionally promotes platelet degranulation. Contents of the released granules subsequently act on local platelets leading to platelet aggregation and ultimately platelet plug formation [adapted from[1]]. GP = Glycoprotein; ADP = adenosine diphosphate; TXA2 = Thromboxane A2.

Binding of both collagen and VWF to their respective platelet ligands results in platelet activation which is characterised by conformational changes of different receptor molecules on the platelet surface[25]. One example of such receptors are integrins which are a family of transmembrane receptors residing in the membranes of platelets in an inactive state. Upon activation, they support stable adhesion of platelets to the subendothelial matrix as well as platelet aggregation[1,3,34,35]. The most important integrin is $\alpha II_b\beta_3$ (GPIIbIIIa) which becomes activated after interaction of the platelets with either soluble platelet activators such as

thromboxane A2 (TXA2), adenosine diphosphate (ADP) or thrombin (generated during secondary haemostasis), structural platelet activators such as collagen or even mechanical activators as for example shear stress[1,26,36]. Activation of the integrin family does not only result in an increase of the adhesive potential of platelets and platelet aggregation via binding of fibrinogen but also drives certain aspects of extended platelet activation such as expression and secretion of TXA2, secretion of ADP, a change of the intracellular calcium concentration as well as a rearrangement of the cytoskeleton and therefore a change in platelet shape and surface properties[1,23,35].

Increase of internal calcium concentration as well as reorganisation of the cytoskeleton leads to an event referred to as degranulation during which platelets release their internal storage organelles[1,26]. Two of these organelles, the α granules and the dense bodies, contain a multitude of coagulation factors and proteins involved in the haemostatic system; α granules for example contain the coagulation factors V (FV) and fibrinogen while platelet dense bodies store ADP and calcium necessary for further platelet activation[25]. Apart from secretion of coagulation factors, merging of the α granules as well as the dense bodies with the outer platelet membrane additionally results in the exposure of phosphatidylserine which leads to the formation of large negatively charged areas on the platelet surface[37]. These negatively charged patches subsequently serve as a platform for localised initiation and propagation of the coagulation cascade resulting in the generation of fibrin which cross-links the loosely packed platelets leading to the formation of a tightly packed thrombus[1,3,17].

1.1.2. Secondary Haemostasis

Secondary haemostasis is characterised by the generation of a thrombin burst and the formation of a stable thrombus held together by cross linked fibrin[38]. Although termed ‘secondary’ it occurs more or less simultaneously with primary haemostasis and involves the sequential activation of a series of coagulation factors acting as serine proteases and their respective cofactors which ultimately culminates in the conversation of the protein fibrinogen to insoluble fibrin fibres which cross link and therefore stabilises the platelet plug formed during primary haemostasis[35,38].

1.1.2.1. The Coagulation Cascade

As previously mentioned, first models of the coagulation cascade were introduced more than 50 years ago and have been further developed ever since. The cascade itself is divided into two pathways (extrinsic and intrinsic) that can be distinguished based on their mechanism of initiation. While the extrinsic pathway is reliant on the exposure of TF at sites of vascular injury, the intrinsic pathway is triggered by the interaction of coagulation factor XII (FXII) with negatively charged surfaces[4]. After activation of the serine protease factor X (FX), both pathways converge into a third pathway referred to as the common pathway leading to the formation of thrombin and fibrin fibres (Figure 1.3)[1,38].

1.1.2.1.1. The Waterfall of Coagulation

TF is a cell surface protein present on subendothelial cells as for example smooth muscle cells lining the inner layer of the vessel wall and the initiator of the extrinsic pathway of coagulation[15]. Upon vascular injury, TF becomes exposed to flowing blood and binds to its primary ligand factor VII (FVII). Interaction of the two proteins results in the activation of FVII and the formation of an enzymatically reactive complex referred to as the extrinsic tenase complex. The tenase complex subsequently converts inactive FX into its active form FXa[35,39].

The initial step of the common pathway is the formation of another enzyme complex consisting of FXa, its cofactor activated factor V (FVa) and prothrombin[38,39]. This so called prothrombinase complex subsequently cleaves and therefore activates prothrombin to thrombin. Thrombin itself is considered to be the most important enzyme of the coagulation cascade and is responsible for the conversion of fibrinogen to fibrin which upon interaction with platelets present in the primary platelets plug supports the formation of a stable thrombus[2,38,39].

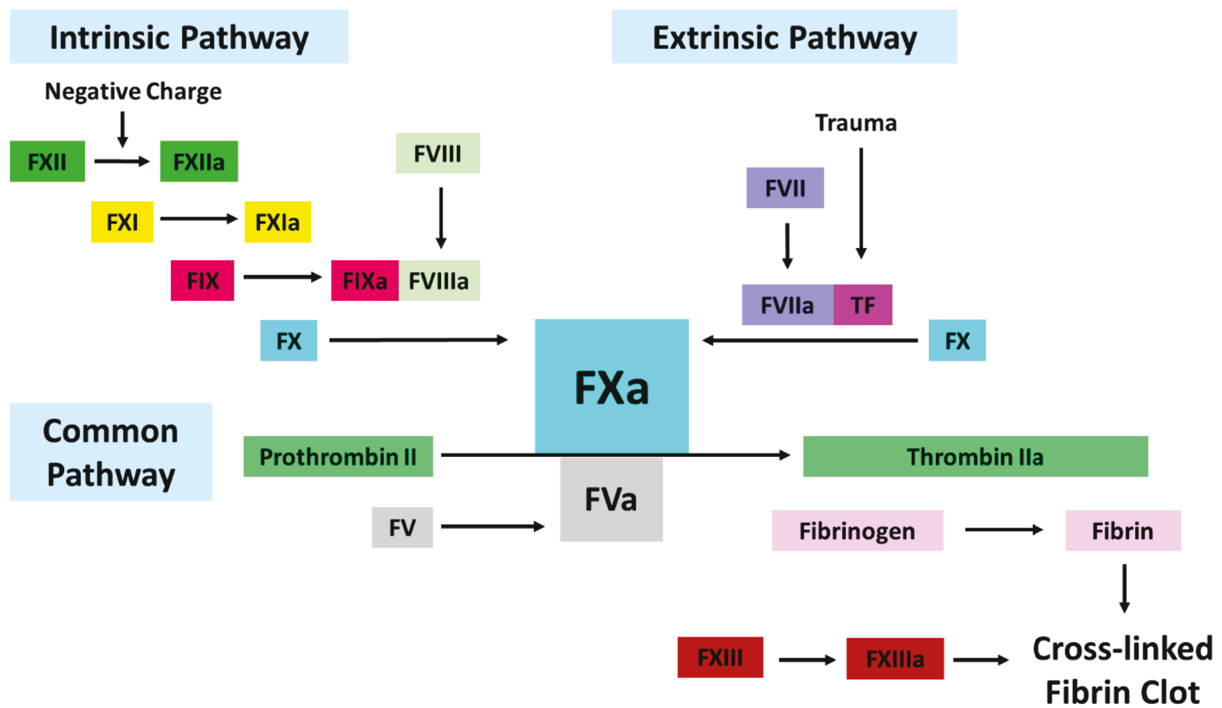

Figure 1.3: Waterfall model of the coagulation cascade. Secondary haemostasis is divided into the intrinsic and extrinsic pathway of coagulation. The intrinsic pathway is characterised by contact of FXII to negatively charged surfaces and subsequent sequential activation of several serine proteases. The extrinsic pathway is reliant on the exposure of TF after vascular injury and involves formation of a TF/FVIIa complex. Reactions occurring in both sides of the coagulation cascade lead to the cleavage of inactive FX to FXa which marks conversion of the intrinsic and extrinsic into the common pathway. Predominant aim of the common pathway is the cleavage of soluble fibrinogen to insoluble fibrin fibres which interact with platelets and therefore stabilise the platelet plug formed during primary haemostasis [adapted from[1]]. F = Factor; TF = Tissue Factor; a = activated.

1.1.2.1.2. The Cell Based Model of Coagulation

While postulation of a general model of the coagulation cascade proved extremely useful in the further understanding of haemostatic mechanism and allowed for the development of clinical tests indicating deficiencies in either of the two pathways, some clinical presentations of spontaneous or recurrent bleeding seemed to stand in stark discrepancy to the model formulated[2,1]. One of the clinical observations which seemed to contradict the commonly accepted coagulation model was based on fact that patients suffering from deficiencies in either FVIII or FIX (haemophilia A or B respectively) presented abnormal and inadequate clot formation. Since both these factors are involved in the same pathway of coagulation, their absence completely disrupts generation of FXa via the intrinsic route[40]. Considering the concept of the waterfall cascade model however, FX still should get sufficiently activated by

the extrinsic tenase consisting of TF and FVIIa[38,41]. In addition, normal FIX and FVIII levels in combination with reduced or even absent expression of FXII, high-molecular-weight kininogen (HMWK) as well as pre-kallikrein (PK) which all are involved in the initiation of the intrinsic pathway of coagulation does not result in the presentation of a bleeding disorder further questioning the structural accuracy of the waterfall model[4,38,41].

In 1992, a new and more elaborate model of coagulation was introduced by Kenneth Mann who postulated that coagulation is not initiated entirely by either the intrinsic or extrinsic pathway alone but relies on an intricate interplay of both pathways[1,38]. Mann furthermore emphasised the role of cells as active providers of surfaces on which the different coagulation factors are able to assemble, be activated and execute their specific functions[1,2,42]. By doing so, he combined several important haematological discoveries; for example the observation that FXI can be directly activated by thrombin in the presence of charged surfaces such as activated platelets while simultaneously providing an answer to previously unaddressed questions such as the fact that absence of the several of the intrinsic pathway coagulation factors does not seem to have any clinically relevant implications[1,2,4,41]. In general, the cell-based model of coagulation can be divided into three overlapping phases (initiation, amplification and propagation phase) establishing a concept of localised pro- as well as anti-coagulant areas during both primary and secondary haemostasis[1].

1.1.2.1.2.1. The Initiation Phase

In the cell-based model, TF is considered as the primary activator of coagulation and initiation of clotting reactions is mainly dependent on cells presenting TF on their cell surface[12,42,43]. After vascular injury and exposure of subendothelial cells such as smooth muscle cells, fibroblasts and pericytes, FVII circulating in the blood stream binds to exposed TF and subsequently becomes activated[35,39]. The TF/FVIIa complex then converts FX and FIX into their active forms FXa and FIXa which partly dissociate from the surface of the endothelial cells[42,43]. Cell-bound FXa together with FVa however is able to form a prothrombinase complex resulting in the production of small amounts of thrombin (FIIa)[43,1,39]. FIXa dissociating from the surface of the endothelial cells eventually binds to receptors present on the surface of activated platelets supporting the formation of the intrinsic tenase complex (Figure 4)[1,39,42,43]. It is important to notice that during this initiation phase, association of the coagulation factors

with the endothelial cell surface provides a protective environment allowing FXa to cleave its substrates without interference of any anti-coagulant signals[1,42,43]. Dissociation of especially FXa from the cell surface on the other hand rapidly results in the inactivation of the enzyme by different anti-coagulant molecules that will be discussed in more detail in chapter 1.1.3.[1,2,4].

1.1.2.1.2.2. The Amplification Phase

Amplification of coagulation is heavily influenced by events of the primary haemostasis. As already described, breaches of the endothelial cell layer result in the interaction of platelets with VWF and collagen bound by or present in the subendothelial matrix of the vessel wall bringing the platelets in close contact to TF bearing endothelial cells[1,27,39]. The small amounts of thrombin generated during the initiation phase subsequently act on the bound platelets increasing their adhesive potential and support further activation of the platelets itself but also of coagulation factors FV, FVIII and FXI[1,13,43].

Activation of the platelets in this phase additionally results in their degranulation and release of FV which subsequently can be activated by platelet bound FXa[1,42,43]. In addition, thrombin activates VWF associated FVIII resulting in its release from the VWF-collagen complex present in the subendothelial matrix. After its activation, FVIIa stays bound to the platelet surface associating with FIXa in order to form the intrinsic tenase complex (Figure 1.4)[1,42,43].

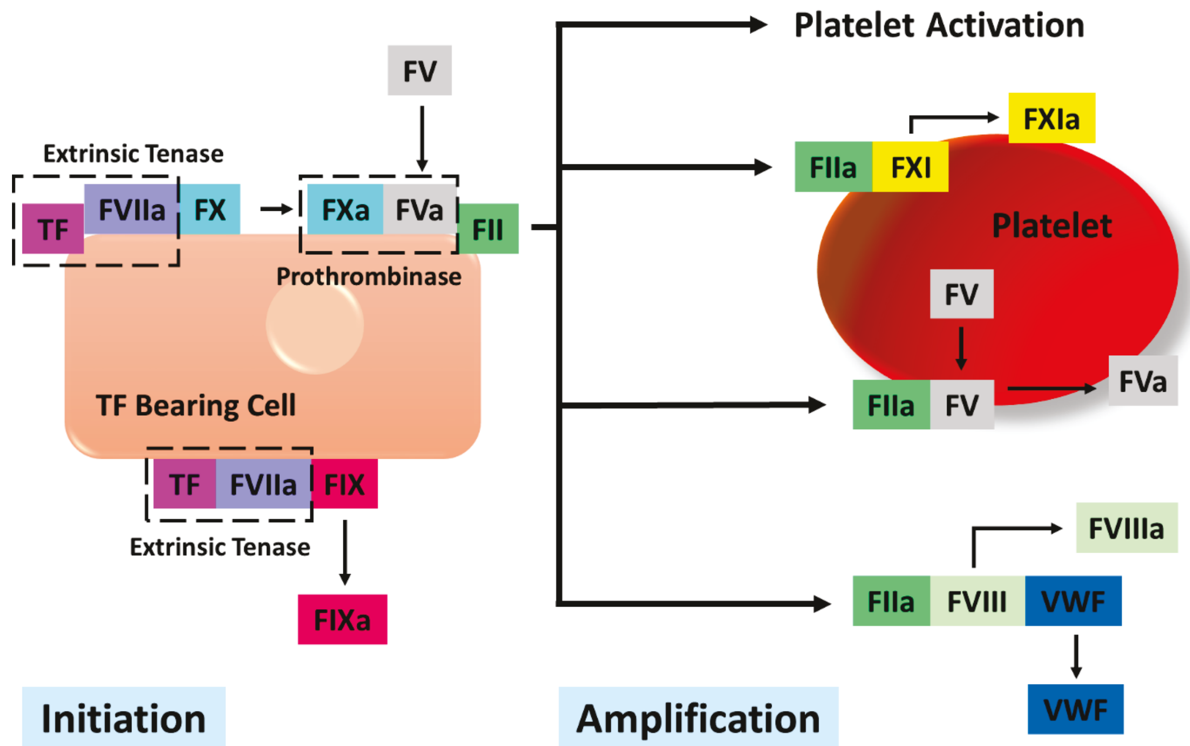

Figure 1.4: Initiation and amplification phase of coagulation. Vascular injury leads to the exposure of TF in the subendothelial tissue. TF subsequently binds circulating FVII which becomes activated and together with TF forms the extrinsic tenase complex. After formation, the complex binds plasma FIX resulting in its conversion to FIXa which subsequently dissociates from the endothelial cell surface in order to bind to specific receptors present on activated platelets. Simultaneously, the tenase complex converts FX to FXa. FXa together with FVa then forms a prothrombinase complex which converts prothrombin (FII) to thrombin (FIIa). Active thrombin is involved in the activation of platelet associated FXI, FV as well as in the release and activation of FVIII from collagen bound VWF (based on[1,42,43]). F = Factor; FII = Thrombin; TF = Tissue Factor; VWF = Von Willebrand Factor; a = activated.

1.1.2.1.2.3. The Propagation Phase

With completion of the amplification phase, platelets present activated FVa, FVIIIa, FXIa as well as FXa on their cell surface allowing for the formation of multiple FIX/VIIIa intrinsic tenase and FXa/FVa prothrombinase complexes which marks the beginning of the propagation phase[1,42,43] (Figure 1.5). Assembly of the intrinsic tenase complex hereby is characterised by transition of FIXa from its site of activation on endothelial cells to the platelet surface or the direct conversation of plasma FIX to FIXa by platelet associated FXIa[1,2,42,43].

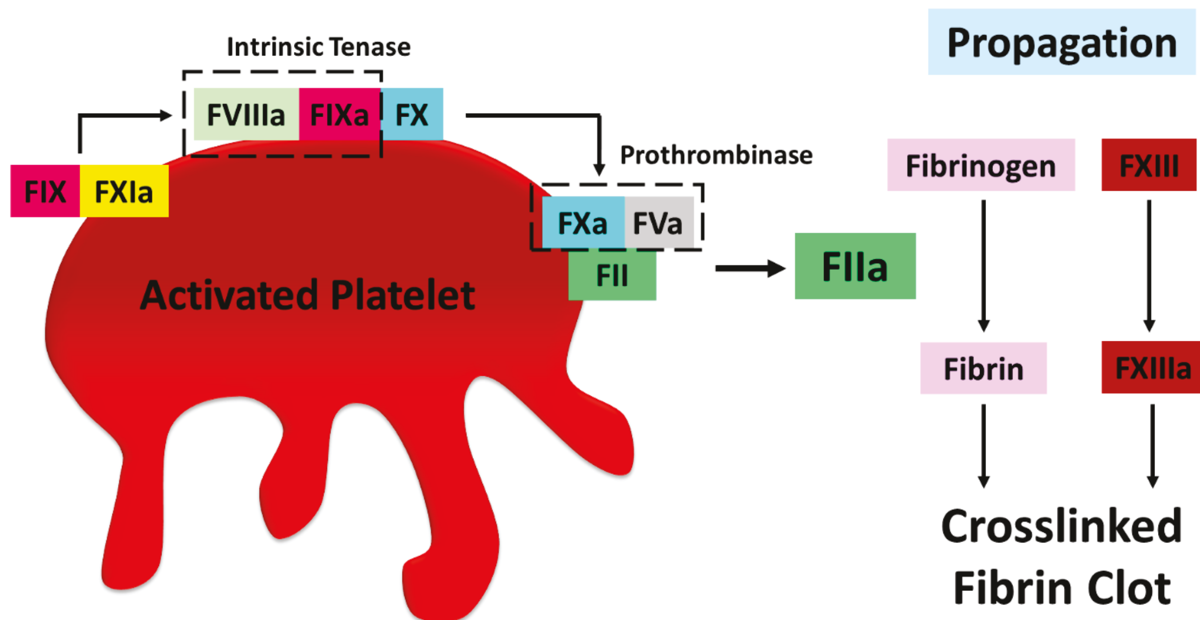

Figure 1.5: Propagation phase of coagulation. The propagation phase is characterised by the transition of FIXa from endothelial cells to the cells surface of activated platelets. Alternatively, FIX also can be directly activated by FXIa present on the cell surface of activated platelets. Alternatively, FIX also can be directly activated by FXIa present on the cell surface of activated platelets. In both cases, FIXa together with FVIIIa form the intrinsic tenase complex which subsequently converts FX to FXa. FXa then binds to FVa presented on the cell surface of the activated platelets forming a prothrombinase complex. The prothrombinase complex subsequently converts prothrombin to thrombin which in turn cleaves fibrinogen to fibrin. Together with FXIIIa, fibrin then crosslinks the platelets and thereby stabilised the primary platelet plug (based on[1,42,43]). F = Factor; FII = Thrombin; a = activated.

All these mechanisms ultimately serve the purpose of FX activation and formation of the FXa/FVa prothrombinase complex which produces large amounts of thrombin and therefore is responsible for the conversion of fibrinogen to fibrin which together with activated factor XIII (FXIIIa) stabilises the primary platelet plug (Figure 5)[1,42,43]. Association of the coagulation factors as well as the creation of a protective environment on the surface of the platelets ensures localised production of fibrin and restricts clotting reactions to the specific site of a vascular injury[1,2,13,42,43].

1.1.2.2. The Fibrinolytic System

After formation of a stable thrombus and prevention of further blood loss, the vascular tissue enters the process of regeneration and wound healing[1,38]. One part of this process comprises the disintegration of formed blood clots by the fibrinolytic system[1,44]. Aside from dissolving

already formed blood clots, the fibrinolytic system also supports the prevention of thrombus formation in healthy blood vessels[44,45].

The fibrinolytic system consists of predominantly three serine proteases called plasmin, tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA)[1,46]. The central enzyme displaying fibrinolytic activity is plasmin. Plasmin is expressed and secreted by hepatocytes as a zymogen referred to as plasminogen[45,46]. Even though plasminogen is not capable of cleaving fibrin, it has binding ability towards fibrin fibres formed during primary and secondary haemostasis and therefore is incorporated into the growing thrombus during clot formation[1,45,46]. While both tPA as well as uPA efficiently cleave plasminogen, conversion to plasmin usually is catalysed by tPa which is expressed and secreted by endothelial cells[1,38]. Interestingly, fibrin has been reported to act as a cofactor for tPA therefore promoting its own degradation[1,35]. Simultaneous binding of tPA to both plasminogen and fibrin accelerates plasminogen conversion resulting in an approximately 500-fold increased generation of plasmin compared to plasmin activation in solution[1,46]. The second known plasminogen activator is uPA which is expressed by kidney cells[1,45,46]. Upon activation, plasminogen is converted to plasmin which is able cleave fibrin fibres into small fibrin degradation products that subsequently are cleared from the circulation by phagocytosis[1,44–47].

1.1.3. Control of Coagulation

Blood coagulation is a fine balance between pro- and anti-coagulant stimuli and has to be tightly regulated (Figure 1.6 and 1.7) in order to prevent unwanted clot formation in healthy vessels.

1.1.3.1. Tissue Factor Pathway Inhibitor

TFPI primarily aims to inactivate or down-regulate the proteolytic activity of FXa[1,48]. It is expressed by platelets and endothelial cells and belongs to the group of protease inhibitors. The peptide is either released into the blood stream at sites of vascular injury by the degranulation of platelets and up-regulation of TFPI expression by endothelial cells or stays associated to the respective cell surfaces after both events[1,49]. While cell surface bound TFPI

is able to bind FXa present in the FXa/FVa prothrombinase complex, plasma TFPI interacts with circulating FXa directly[1,35,49]. Inactivation of FXa by TFPI is enhanced by association with its cofactor protein S which greatly increases the affinity of TFPI to FXa as well as overall TFPI stability[35,49,50]. After formation, the TFPI/factor S complex acts as a pseudo-substrate for FXa effectively prohibiting conversion of prothrombin to thrombin, fibrin fibres formation and therefore thrombus stabilisation[1,48,49].

1.1.3.2. Antithrombin

Similar to TFPI, antithrombin (AT) belongs to the serpin protease inhibitor family and is characterised by its inhibitory effect on the coagulation factors FIXa, FXa, FXIIa and thrombin[1,50]. Due to its widespread functionality, it is considered as one of the most important regulators of coagulation[1,50,51]. Antithrombin is expressed by liver cells and circulates the blood as a small protein without enzymatic activity but acts as a pseudo substrate for the serine proteases involved in coagulation[1,50,51]. For example, AT engages with the reactive site loop of thrombin resulting in the formation of a covalent complex rendering thrombin inactive. The inhibitory effect of antithrombin is enhanced by simultaneous binding of heparin or heparans on the vessel wall, which promotes extrusion of the reactive site loop[1,50–52].

1.1.3.3. Protein C/Protein S

The protein C/protein S pathway targets the tenase and prothrombinase complexes built during secondary haemostasis by proteolytic inactivation of FVa and FVIIIa[1]. The pathway is dependent on a multitude of proteins as well as receptors and involves several signalling and sequential activation steps. The main molecules involved in the protein C/protein S pathway are the serine protease protein C, the glycoprotein protein S, the serine protease thrombin, the endothelial membrane protein thrombomodulin (TM) as well as endothelial protein C receptors (EPCRs)[1,35,53]. Similar to most other proteases involved in coagulation, protein C circulates the blood in an inactive state and has to be converted into activated protein C (APC) in order to become proteolytically active. Activation of protein C is catalysed by thrombin bound to its cofactor thrombomodulin on the surface of endothelial cells.

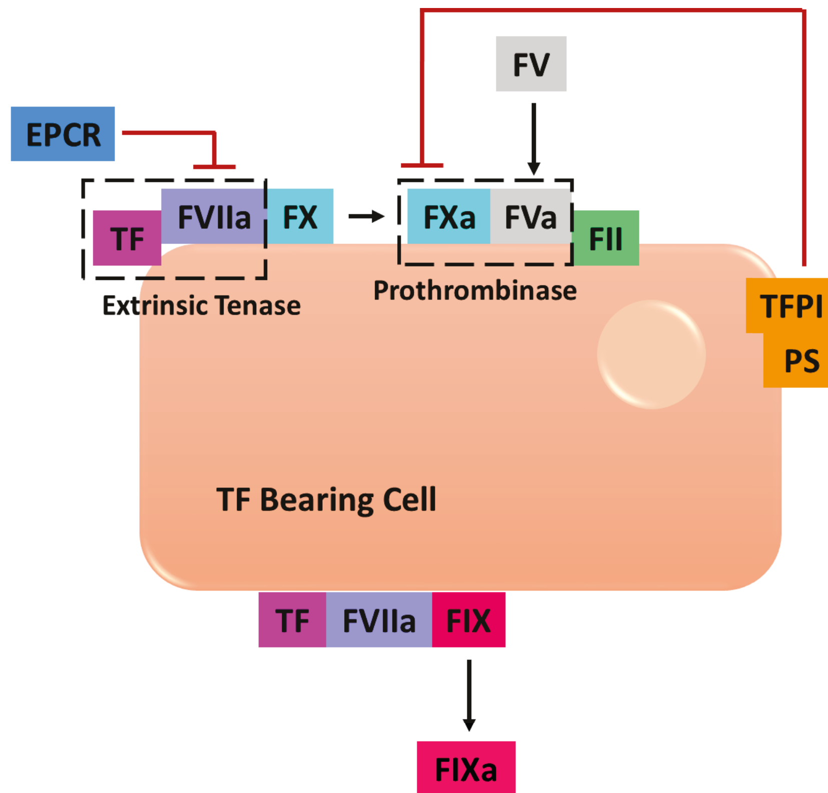

Figure 1.6: Regulation of cell coagulation during the initiation phase. In order to prevent unwanted clot formation events, the mechanism of coagulation is tightly regulated. During the initiation phase, FXa is inhibited by TFPI and its cofactor protein S interfering with the formation of the prothrombinase complex. Cofactor function of FVIIa within the extrinsic tenase complex is down-regulated by EPCRs. Upon binding to the receptor, FVIIa is internalised and therefore cleared from circulation prohibiting formation of the tenase (based on [1,42,43]). F = Factor; PS = Protein S; TFPI = Tissue factor pathway inhibitor; EPCR = Endothelial protein C receptor; TF = Tissue Factor; a = activated.

After formation, the thrombin/thrombomodulin complex catalyses activation of protein C bound to nearby EPCRs. APC subsequently associates with its cofactor protein S which greatly enhances its proteolytic potential[1,53,54]. Even though formed protein C/protein S complexes can either stay bound or dissociate from the endothelial cell surface, only surface bound complexes exhibit anticoagulant activities by blockage of FVa and FVIIIa activity. Inactivation of these two cofactors results in the down-regulating of the tenase and prothrombinase complexes and therefore limits fibrin formation[1,2,53,54].

1.1.3.4. Fibrinolytic System

Even though it is considered as the third phase of haemostasis, the fibrinolytic pathway displays a major regulatory function since degradation of fibrin fibres is mandatory for wound healing and has to occur not only within a thrombus but throughout the circulatory system[1,55]. The fibrinolytic system generally exhibits anti-coagulant functions by degradation of fibrin fibres but also can be inhibited which results in a pro-coagulant response[1,12,44].

Conversion of plasminogen to plasmin for example can be controlled by inhibition of both uPA and tPA by the two serpins plasminogen activator inhibitor-1 and 2 (PAI1 and PAI2)[1,50]. Furthermore, plasmin activity itself can be inhibited by α-2 antiplasmin and α-2 macroglobulin rendering the enzyme incapable of fibrin degradation[1,2,35]. In addition, the thrombomodulin/thrombin complex has been shown to exhibit antifibrinolytic activities by binding and activating a plasma peptidase called thrombin-activable fibrinolysis inhibitor (TAFIa)[1,46]. TAFIa activity leads to the modification of fibrin fibres by removing the lysine residues to which plasminogen binds and therefore reduces plasmin generation[56].

1.1.3.5. Protease Inhibitors

Aside from TFPI and antithrombin several other protease inhibitors actively regulating the process of coagulation have been reported.

Heparin cofactor II for example effectively inhibits activation of thrombin and therefore directly prohibits conversion of fibrinogen to fibrin fibres as well as propagation of the coagulant response[1,57].

The plasma protein α2 macroglobulin is another protein which has been observed to display inhibitory function on a large variety of enzymes, as for example thrombin which is responsible for the formation of fibrin fibres and kallikrein which is involved in the activation of FXII. Similar to antithrombin, interaction of α2 macroglobulin with a catalytically active enzyme results in a conformational change of both proteins resulting in the shielding and therefore inaccessibility of the active domain for potential substrates[1,58].

Another well-known protease inhibitor is the C1 inhibitor whose main function lies in the regulation of the complement system. Nonetheless, C1 inhibitor also is considered to be the main physiological regulator of kallikrein activity and furthermore is able to down-regulate

activity of both FXIa and FXIIa effectively interfering with the formation of the intrinsic tenase complex[1,59].

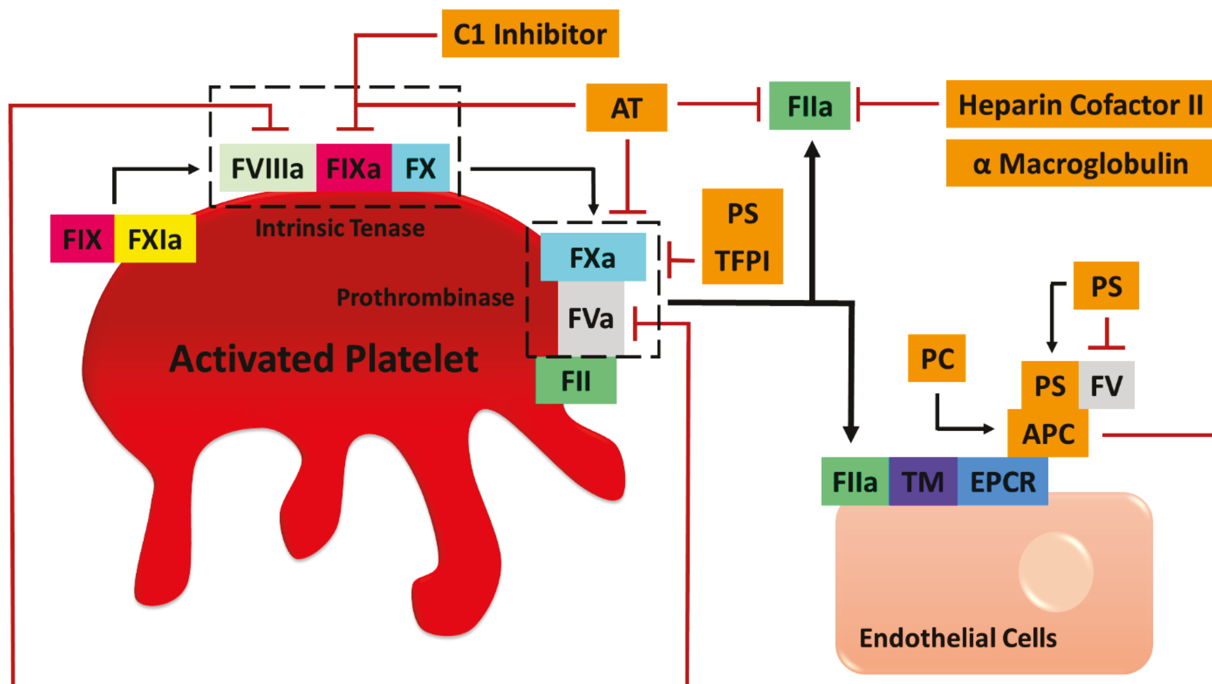

Figure 1.7: Regulation of cell coagulation during the propagation phase. In order to prevent unwanted clot formation events, the mechanism of coagulation is tightly regulated. During the propagation phase, several inhibitory pathways can be active. AT inhibits FIXa, FXa as well as FIIa preventing formation of the intrinsic tenase complex, the prothrombinase complex as well conversion of fibrinogen to fibrin fibres. Activity of the intrinsic tenase additionally is down-regulated by the protein C/protein S complex as well as the C1 inhibitor which act on FVIIIa and FIXa respectively. Aside from antithrombin, the prothrombinase complex moreover is inhibited by the TFPI/protein S as well as the protein C/protein S complex. Fibrin fibres formation is regulated by inhibition of thrombin (FIIa) by either AT, heparin cofactor II and α macroglobulin. Formation of the activated protein C/protein S complex can be regulated by inactive FV which competes with protein C for binding to thrombomodulin (based on [1,42,43]). F = Factor; PS = Protein S; TFPI = Tissue Factor Pathway Inhibitor; AT = Antithrombin; PC = Protein C; EPCR = Endothelial Protein C Receptor; FII = Thrombin; TM = Thrombomodulin.

1.1.3.6. Cofactors

Even though some of the enzymatic reactions within the process of coagulation are catalysed by a single protease, amplification of protein cleavage and clotting factor activation often is associated with the formation of protein complexes[60]. In these events, designated cofactors bind to the different proteases significantly increasing their catalytic potential. For example, interaction of FXa with FVa within the prothrombinase complex accelerates the conversion of prothrombin to active thrombin more than 50 times compared to FXa alone[1,60,61]. Aside from

their supportive function, some cofactors also have been observed to display anti-coagulant functions and therefore can be classified as negative regulators of coagulation as well.

Most of the proteins involved in the formation of the protein C/protein S complex for example have been observed to exhibit anti-coagulant activities independent from the complex they usually are associated with[1,53,60]. For example, FV itself is capable of acting as an additional cofactor for APC supporting the inactivation of FVa, FVIa and FVIIIa and therefore down-regulation of tenase and prothrombinase complexes[1,60]. Another protein displaying anti-coagulant activities is protein S. In the absence of APC, protein S for instance is capable of inhibiting FXa function as well as acting as a direct competitor of thrombomodulin for FVa binding. Both functions interfere with regular prothrombinase activity and therefore disrupt fibrin formation[1,60].

Even though the EPCR is not considered as classical cofactors, the receptor seems to play a role in the restriction of coagulation beyond its original function as a protein C binder within the protein C/protein S pathway[1,53,62]. Aside from protein C, EPCRs also show binding affinity towards plasma FVIIa. Interaction of the two molecules results in the internalisation of FVIIa clearing it from the circulation therefore preventing the formation of tenase complexes[1,62].

1.1.3.7. Localisation of Anti-Coagulant Activity

As already discussed in chapter 1.1.2.1.2., locally restricted activation of coagulation factors or formation of enzymatically active complexes plays an important role in the regulation of haemostatic processes[1,2,42].

Recent studies for example showed that the protein C/protein S complex only inactivates FVa if both the complex as well as FVa are membrane bound[1,63]. The same studies furthermore showed that the surface necessary for initial thrombin generation leading to the conversion of protein C to APC has to be provided by endothelial cells but not platelets[1,43,64]. Even though biological properties and expression patterns of different receptors displaying an affinity for most coagulation factors are very similar in both membranes, platelets seem to be able to create a protective environment interfering with protein C/protein S complex activity[1,42,43]. Anti-coagulant function of the complex therefore is restricted to the surface of endothelial cells at sites of vascular breaches preventing clotting reactions in healthy vessels.

Another example underlining the importance of localised activation of certain coagulation factors involves formation of the tenase complex. FX activation relies on the assembly of distinct complexes consisting of either TF/VIIa or FIXa/FVIIIa. While the TF/FVIIa tenase complex is formed on the cell surface of endothelial cells, FIXa/FVIIIa assembly occurs on the surface of activated platelets. The majority of FXa built by the endothelial TF/FVIIa tenase subsequently stays membrane bound and together with FVa forms a prothrombinase complex which is characterised by the generation of small amounts of thrombin. However, efficient initiation of large thrombin bursts and subsequent conversion of fibrinogen to fibrin fibres requires assembly of a prothrombinase complex on the surface of activated platelets which only can be achieved by association of FIXa with FVIIIa[1,2,4,12,42,43]. Despite the fact that both tenase complexes result in the generation of FXa, amplification reactions and therefore efficient and reliable formation of a stable platelet plug heavily relies on the location of initial tenase formation[1,42,43].

1.2. Haemostatic Disorders

Haemostasis is defined as the intricate interplay between procoagulant and anticoagulant factors in the blood and has evolved to ensure local restriction of blood loss after vascular damage while maintaining blood fluidity and circulation in order to supply organs with oxygen and other nutrients[38,52]. As discussed in chapter 1.1, haemostasis consists of a complex series of sequential protein activation and conversion steps culminating in the generation of fibrin and the formation of blood clots[1]. The processes involved in coagulation are tightly regulated by a range of anticoagulant molecules as well as the fibrinolytic system which subsequently promotes clots lysis[35].

The majority of clotting factors are secreted by hepatocytes residing in the liver. In addition, the liver also is involved in the clearance of activated clotting factors and fibrinolytic products. Consequently, hepatic disorders or diseases affecting normal liver function can lead to an in- or decrease of clotting factors or members of the anticoagulant system in the blood plasma resulting in either bleeding or thrombosis[41,65].

Coagulation pathologies arise from an imbalance in haemostasis most frequently due to abnormally high or low levels of the proteins involved. Coagulopathies are characterised by

either excessive blood loss (bleeding) or the formation of unwanted blood clots (thrombosis)[1,38,41]. There are multiple reasons for the fluctuation of clotting and fibrinolytic factors which can range from natural variation of protein expression levels related to age, autoimmune diseases targeting coagulation or anticoagulant factors, medication for diseases unrelated to haemostasis to genetic defects in genes encoding for haemostatic proteins[1,38,41,52].

1.2.1. Thrombosis

While haemostasis is characterised by a fine balance between pro- and anticoagulant factors that ensures appropriate blood clotting reactions in wound healing, thrombosis is defined as the pathological formation of a blood clot inside a blood vessel[1,12,41]. Even though there are multiple reasons that can lead to such events, three distinct risk factors are commonly accepted as the main contributors for thrombosis (Figure 1.8)[1,12,41,66].

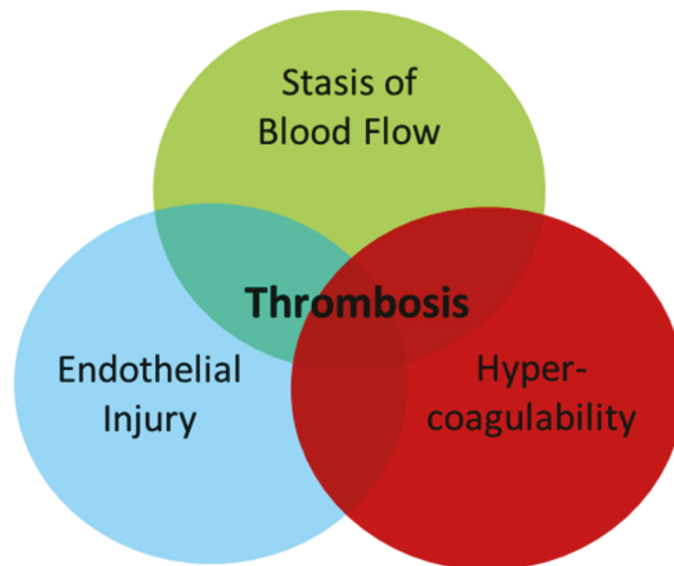

Figure 1.8: Risk factors for the development of thrombosis. Commonly acknowledged risk factors contributing to the development of thrombosis are stasis of blood flow after prolonged immobility, endothelial injury caused by for example trauma, hypertension or shear stress and a hyper-coagulant state of the blood resulting from abnormalities in for example blood vessel wall formation, blood flow or coagulation factor levels (based on [1,12]).

Firstly, alterations in normal blood flow caused by for example prolonged immobility after surgery or during long distance flights lead to a stasis of blood flow which facilitates the

accumulation of activated coagulation factors and formation of thrombi. Secondly, endothelial injury or inflammation after trauma, contact with procoagulant surfaces such as medical devices or bacteria as well as hypertension or high shear stress within the blood vessels all reduce endothelial anticoagulant functions and therefore increase the risk thrombus formation. The third commonly acknowledged risk factor for the development of thrombosis is related to abnormalities in the process of blood coagulation itself. These abnormalities can be induced by either inflammation, infection, irregular formed vessel walls, abnormal levels of coagulations factors or coagulation factor mutations such as FV Leiden[1,12,41,66].

1.2.1.1. Forms of Thrombosis

Thrombosis can be divided into venous and arterial thrombosis according to the type of blood vessel affected by the inappropriate formation of blood clots[67]. Venous thrombosis describes the development of a blood clot in one of the veins[68]. Due to the fact that the veins are responsible for the transportation of blood towards the heart, embolism of a venous thrombus will result in the at least partial occlusion of the pulmonary arterial circulation; an event referred to as a pulmonary embolism. The most common site of venous thrombosis is within the deep veins of the leg or pelvis and is referred to as deep vein thrombosis (DVT)[68,69]. Thrombi may also develop in other venous circulations included the upper limbs, the cerebral sinuses and the splanchnic circulation[1,67]. In general, stasis of the blood leads to the activation of the endothelium which attracts platelets and neutrophils to form initial aggregates. Subsequent release of neutrophil extracellular traps (NET`s) consisting of a network of DNA and proteins enhances further platelet capture and therefore initiates both primary as well as secondary haemostasis and blood clot formation[67,70].

The second type of thrombosis is arterial thrombosis[67]. Arterial thrombosis arises in the arteries which compared to the veins operate at higher pressure and flow and conduct the blood flow away from the heart to distribute oxygen and other nutrients throughout the body. Unlike venous thrombosis, formation of an arterial thrombus is usually secondary to the development of atherosclerotic plaques which expose large amounts of tissue factor after their rupture[71]. In addition, the high arterial shear produced by atheromatous narrowing will

also lead to binding and capture of VWF and platelets to the vessel wall contributing to the generation of pathological thrombi[12,71].

Even though arterial thrombosis is considered as the more complex disease, both forms are characterised by either abnormal platelet aggregation or the excessive generation of thrombin which can lead to thromboembolism, hypertension, necrosis, organ damage, myocardial infarction, limb ischemia and stroke[12,41,67]. For a long time, it was assumed that both arterial as well as venous thrombosis were caused by different mechanisms and therefore could be associated with varying risk factors. Better understanding of the development of the disease however revealed that both forms share many common risk factors such as high cholesterol, obesity as well as additional genetic and environmental risk factors which will be discussed in more detail in the subsequent chapter[12,66].

1.2.1.2. Causes of Thrombotic Events

Thrombophilia generally describes abnormalities within the blood coagulation system leading to increased formation of blood clots[12,72,73]. Analysis of thrombotic events occurring in patients showed that more than 50% of all thrombotic episodes have some identifiable contribution from abnormalities affecting the different stages of normal clot formation[1,67,72]. Even though thrombosis is a multifaceted disease, some of the underlying causes usually can be distinguished and are commonly divided into hereditary (genetic) or acquired factors with the later arising from environmental influences manifesting themselves over time[41,67,74].

1.2.1.2.1. Hereditary Thrombotic Disorders

The best characterised hereditary thrombophilic traits affect coagulant and anticoagulant factors[41]. Factor V Leiden is the most common hereditary risk factor for thrombotic events in the European population and arises from a single point mutation in the gene encoding for coagulation factor V. FV Leiden is inherited in an autosomal dominant fashion and has been reported to increase the risk of thrombosis between four to seven times for heterozygous and 12 to 26 times for homozygous carriers. The mutation itself results in the expression of a form of FV relatively resistant to inactivation by activated protein C which is one of the major naturally occurring regulators of coagulation. FV Leiden therefore leads to a prolonged

formation of the prothrombinase complex and thus to an augmented generation of thrombin, fibrin and formation of blood clots[41,67,75,76].

A second well characterised mutation consists of a single point mutation at position 20210 in the gene encoding prothrombin[73,75]. Mutation of the gene is inherited in an autosomal dominant manner and leads to an overexpression of prothrombin and therefore elevated thrombin levels which in turn results in an increase in the conversion of fibrinogen to fibrin and formation of blood clots in the vascular system. Genetic studies have shown that heterozygous carriers of the gene have a two to three times and homozygous carriers a more than 10 times increased risk of developing thrombosis[1,73,75,77].

Another genetic disorder increasing the risk of thrombosis is antithrombin deficiency which is a rare autosomal dominant disorder characterised by reduced antithrombin plasma levels and recurring deep vein thrombosis[1,78]. Antithrombin plays an important role in the coagulation cascade by inhibiting activity of both FXa and thrombin resulting in the disruption of regular fibrin formation. Antithrombin deficiency therefore leads to an unregulated and continuous formation of fibrin and excessive blood clot formation[1,78,79].

One of the major regulators of the blood coagulation cascade is activated protein C which degrades the coagulation cofactors FVIIIa and FVa by proteolytic cleavage, thus reducing formation of the intrinsic tenase and the prothrombinase complex[53,64]. Disruption of the assembly of these two complexes results in suppression of thrombin formation and therefore impedes the conversion of fibrinogen to fibrin and the formation of stable blood clots[35,47,53,80]. Due to its important role as a regulator of blood clot formation, deficiency of protein C which is classified as a rare autosomal dominant disorder is strongly associated with thrombosis[1,75,80].

Activated protein C function also can be affected by genetic mutations in the gene encoding for glycoprotein protein S which serves as the cofactor for protein C. Protein S deficiency is associated with increased risk of thrombosis and again is a rare autosomal dominant disorder[1,81]. Protein S and Protein C are usually encountered in an heterozygous state[80,81] and complete absence of Protein S or Protein C which can be observed in homozygous carriers results in the development of a life-threatening clotting disorder called purpura fulminans

which is characterised by systemic formation of small blood clots throughout the body and widespread tissue necrosis shortly after birth[80–82].

In addition to the currently known hereditary risk factors, several other molecules, genes and mutations thereof that yet have to be fully characterised have been suggested to play a role in the uncontrolled formation of blood clots[1,12,75]. Elevated serum levels of coagulation factors VII, IX and XII which have a less well defined genetic basis have been identified as frequent contributors to increased thrombotic risk[4,73,83,84]. Furthermore, platelets have been shown to exhibit an elevated aggregation potential termed as sticky platelet syndrome in certain patient samples. Based on genetic tests, the syndrome is thought to be inherited in an autosomal dominant fashion but clear association of the disorder with a specific gene has not been successful so far[1,85]. In addition, most hereditary thrombotic disorders such as both protein C and S deficiency seem to be based not only on their genetic components but also show variability in their severity during the lifespan of a patient indicating a complicated interplay of inherited and acquired components[1,41,67].

1.2.1.2.2. Acquired Thrombotic Disorders

The best characterised acquired thrombotic disorder presents itself in form of an autoimmune disease called antiphospholipid antibody syndrome[73,86]. The syndrome is characterised by the abnormal expression of antibodies directed against plasma proteins interacting with phospholipids present in the membrane of cells[86]. Different antibodies exhibiting binding affinities towards several proteins directly involved in the coagulation cascade have been identified. The most common target of antiphospholipid antibodies is β2 glycoprotein 1 (β2GP1). Upon binding of the antibodies to apolipoprotein H (Apo-H), the complex is capable of inhibiting protein C activity which together with some yet poorly understood mechanisms results in the occurrence of occasional thrombotic events[86,87]. Aside from β2GP1, antiphospholipid antibodies binding to protein S have been reported as well. Interaction between the antibody and protein S effectively prevents the protein to execute its function as protein C cofactor thus decreasing overall protein C activity and therefore regulation of tenase and prothrombinase assembly. Another binding partner of antiphospholipid antibodies is prothrombin[88]. Binding of the antibodies to prothrombin leads to an increased conversion of prothrombin to thrombin which in turn accelerates the cleavage of fibrinogen to fibrin[1,73,88].

In each case, binding of the antibodies to their respective target molecules results in uncontrolled thrombin generation and therefore excessive clot formation.

A second autoimmune disorder associated with increased risks of thrombosis is called thrombotic thrombocytopenic purpura (TTP)[1,12,41]. TTP is characterised by excessive formation of small blood clots due to reduced levels of an enzyme called a disintegrin and metalloproteinase with a thrombospondin type 1 motif member 13 (ADAMTS13)[1,67,89]. Under normal circumstances, ADAMTS13 cleaves large VWF multimers into smaller units, effectively reducing VWF`s ability to exhibit its physiological function of capturing platelets at sites of vascular injury[89,90]. In acquired TTP however, patients have elevated levels of autoantibodies inhibiting the enzymatic activity of the metalloprotease and accelerating its clearance which results in augmented levels of large and ultra-large VWF multimers in the circulation. This results in spontaneous interaction between platelets and VWF and platelet capture in areas with high shear forces within the blood vessels or at sites of endothelial injury. The resulting microthrombi give rise to a microangiopathic haemolytic anaemia and microthrombi causing organ dysfunction[89–91].

Fluctuation in plasma protein levels throughout different stages of life and in response to different environmental influences is a commonly acknowledged phenomenon and has been observed to affect several proteins involved in the coagulation cascade[1,92]. Especially elevated levels of coagulation factors VIII, IX, XI as well as other proteins involved in blood clotting as for example thrombin, fibrinogen or VWF are associated with an increased risk for the development of thrombosis[1,41,67,13,42,84]. The reason for variability in protein plasma concentrations are widespread and comprise genetic variants leading to increased expression of the respective protein, age related elevated protein expression, intercurrent diseases, pregnancy and environmental factors such as the use of hormonal contraception, obesity, smoking and stress[1,41,67,73,92]. Independent of the underlying cause, elevated plasma protein concentrations of a selection of coagulation factors often lead to an enhancement of coagulative or inhibition of anticoagulant events and therefore increase the formation of blood clots throughout the vascular system[1,41,66,67].

Aside from changes in the coagulation system itself, external factors such as surgery and usage of artificial devices as for example heart valves and pacemakers can increase the risk of unwanted local blood clot formation. Even though excessive research has been invested in

finding biocompatible materials, most commonly used materials have been reported to promote protein absorption, adhesion of platelets and offer a suitable surface for the assembly of coagulation complexes allowing the generation of thrombin and therefore the formation of blood clots in the absence of vascular injury[1,73,93].

1.2.1.3. Treatment of Thrombotic Events

Current treatment strategies for thrombotic events can be distinguished based on whether the medical intervention aims to dissolve an already formed blood clot or serves to prevent occurrence of thrombosis in the future[1,66,74]. The first of these utilises the administration of thrombolytic agents such as tPA[1,94]. As already described in chapter 1.1.3.2., tPA is an essential protease of the fibrinolytic system and catalyses the conversion of plasminogen to plasmin which in turn is responsible for the proteolytic cleavage of blood clots[47]. In life threatening situations when treatment with a fibrinolytic agent might be too time consuming or too dangerous, thrombi can also be broken down using ultrasound and mechanical devices or can be surgically removed in order to reinstate normal blood flow[95].

Treatments to prevent the formation of thrombi differ depending primarily on whether the expected thrombi are arterial or venous. While arterial thrombotic clots consist of mainly aggregated platelets, thrombi formed in the veins usually are composed of a densely packed mix of fibrin and red blood cells[1,12,66]. Based on the molecular composition of the formed blood clot and clinical trial experience, thromboprophylactic treatment therefore either targets platelet aggregation (antiplatelet therapy) or focuses on the inhibition of proteases involved in secondary haemostasis (anticoagulants)[12,73]. One fundamental drawback of these treatment options is that all currently used anti-coagulant agents or antiplatelet therapies increase the risk of haemorrhage which limits their therapeutic application[1].

1.2.1.3.1. Antiplatelet Therapy

Antiplatelet medications generally aim at the prevention of excessive platelet aggregation and therefore serve to control unwanted formation of platelet rich thrombi characteristic for arterial thrombosis[1,67]. The group of antiplatelet agents comprises a multitude of

pharmaceutical drugs which target different processes occurring during the event of platelet activation in primary haemostasis[96].

The most commonly known antiplatelet drug is aspirin which inhibits the activity of cyclooxygenases 1 and 2 (COX-1, COX-2) in an irreversible manner. Cyclooxygenases are key enzymes necessary for the production of prostaglandins and especially COX-1 and COX-2 are involved in the synthesis of prostaglandin H2 which is an essential precursor molecule for TXA2 generation. As already mentioned in chapter 1.1.1.2, TXA2 is a potent platelet activator, stimulates platelet aggregation and interference in its generation, as for example with aspirin which predominantly inhibits COX-1, effectively prevents platelet aggregation and therefore thrombus formation[97,98].

Aside from cyclooxygenase inhibitors which target the synthesis of TXA2, function of the lipid also can be affected directly using thromboxane inhibitors such as Terutroban which is a small peptide and antagonist of the thromboxane receptor. Administration of the agent leads to blockage of the receptor and prevents binding of TXA2 resulting in disruption of TXA2 dependent platelet activation and aggregation[99–101].

A second class of antiplatelet drugs are ADP receptor inhibitors such as Clopidogrel and Prasugrel. Most members of this class of inhibitors target the P2Y12 receptor which is present on the cell surface of platelets and mediates their response to ADP. Binding of ADP to the receptor results in the activation and subsequent aggregation of platelets. Consequently, interaction of the inhibitors with the $P2Y_{12}$ receptor disrupts one of the most prevalent platelet activation processes and therefore reduces the formation of platelet rich blood clots[101–103].

Another way to effectively decrease platelet activation and aggregation focuses on the inhibition of the platelet receptor GPIIb/IIIa. This integrin receptor which is present on the cell surface allows platelets to bind both VWF and fibrinogen resulting in the arrest of platelets at sites of vascular injury and incorporation of fibrinogen into the developing blood clot. Administration of the most commonly used GPIIb/IIIa receptor inhibitors abciximab, eptifibatide and tirofiban leads to a significant decrease in the formation of platelet-based thrombi and is one of the most potent forms of antiplatelet therapy[96,103,104].

1.2.1.3.2. Anticoagulants

Different to anti-platelet therapies, anticoagulant strategies do not interfere with the function of platelets but directly target different proteins and molecules involved in secondary hemostasis[67,105]. Currently used anticoagulants can be distinguished based on their mode of action and mainly aim at the prevention or partial inhibition of blood clot formation or serve to significantly prologue clotting times[106].

The oldest group of anticoagulants comprises the vitamin K agonists which mediate their effect by inhibiting an enzyme called vitamin K epoxide reductase[106,107]. The most widely used vitamin K agonist is warfarin which is a competitive inhibitor of the reductase preventing recycling of inactive vitamin K into its active form[108,109]. Active vitamin K is required for the complete synthesis of several serine proteases involved in coagulation and deficiency of vitamin K can lead to serious bleeding complications such as haemorrhagic disease of the newborn. Consequently, blockage of the catalytic function of the reductase results in a reduction of clotting factors II, VII IX and X and therefore can be used for the prophylactic prevention of thrombosis[105,107,108].

A second member of the traditional group of anticoagulants is heparin which belongs to the family of glycosaminoglycans and naturally exists as long unbranched polysaccharides[105,109]. Heparin can be administered in either an unfractionated form consisting of polysaccharide chains of variable length or a low molecular weight form which contains shorter length polysaccharide chains. In both cases, heparin is able to potentiate the action of antithrombin which is a potent inhibitor of thrombin, FXa and FIXa. It therefore inhibits the formation of the intrinsic tenase and the prothrombinase complexes and interferes with the conversion of fibrinogen to fibrin. Mechanistic studies have shown that interaction of heparin and antithrombin increases the inhibitory function of antithrombin by more than 500-fold effectively decreasing coagulative events during thrombotic episodes[57,106].

A second and consistently expanding class of anticoagulants comprises several small molecule inhibitors that directly target the proteases necessary for normal blood clotting. Due to their central and critical role in the coagulative system, both FXa as well as thrombin were identified as the logical choice during the initial developmental phases of small molecule inhibitors[105,106,110]. Inhibition of both molecules showed good results with fast activation and favourable safety profiles in initial clinical trial and are currently further developed[110–112].

Since the ultimate goal of anticoagulant strategies is the attenuation of thrombosis without affecting haemostasis, inhibition of proteases involved in the intrinsic pathway of coagulation as for example FXI and FXII might be a suitable alternative to the traditional anticoagulants. Epidemiological studies have suggested that both FXI and FXII play a role in the development of thrombosis while simultaneously having no or very little contribution to haemostasis itself[113]. Strategies targeting both FXI as well as FXII include antisense oligonucleotides which reduce hepatic protein expression, monoclonal antibodies that block activation or activity of the proteases and small molecules that either block the catalytic domain of the enzymes or induce allosteric modulation and have shown promising results in clinical trials[110,113,114,115].

1.2.2. Bleeding

As previously mentioned, the main focus of haemostasis lays in the preservation of an intact circulatory vascular system which is tightly regulated by pro- and anticoagulant factors. In some instances however this balance can be subject to certain changes leading to for example bleeding events which also are referred to as haemorrhage[1,41]. In general, haemorrhage can occur due to internal or external factors and comprises a wide range of symptoms based on the severity of blood loss[8].

The most common reason for a haemorrhage is physical traumatic injuries which can occur after accidents, internal organ damage or premeditated interventions as for example surgery[116]. Under normal conditions, blood loss due to trauma is counteracted by blood clotting events occurring during primary and secondary haemostasis. Cases of excessive blood loss for example following trauma can be complicated by significantly reduced levels of clotting factors due to dilutional coagulopathy[116,117]. The primary action in cases of substantial blood loss is the direct transfusions of red blood cells which reinstates the original blood volume ensuring maintenance of a constant blood pressure and adequate supply of the organs with oxygen. Due to prior processing blood transfusions frequently consist of red blood cells only and lack clotting factors, fibrinogen or platelets. Hence administration of some 'blood' preparations leads to a dilution of the plasma level of clotting factor further increasing the inability of the haemostatic system to initiate adequate clotting reactions[116–118].

In addition to trauma, administration of medications unrelated to the regulation of haemostasis are capable of prolonging bleeding times and therefore may cause bleeding[1,41]. For example, nonsteroidal anti-inflammatory drugs (NSAID) such as aspirin and ibuprofen which frequently are prescribed for the reduction of pain or inflammation can increase the risk of spontaneous bleeding due to their mechanism of action[119]. NSAIDs interfere with the synthesis of prostaglandins which exhibit hormone like functions and are involved in a multitude of biologically processes. By inhibition of COX-1 and COX-2, the drugs prohibit the generation of TXA2 which is essential for the activation of platelets during primary haemostasis. In general, use of antiplatelet and anticoagulant drugs as for example during the treatment of thrombosis should be strictly monitored in order to prevent the opposite effect of provoking bleeding[41,98,119].

Apart from environmental factors and liver disease[120], bleeding events also can be related to medical conditions. For example, different autoimmune diseases in which autoantibodies target and inhibit molecules involved in blood clotting have been associated with spontaneous bleeding episodes[1,41]. Furthermore, genetic components play an important role in bleeding disorders and mutations in the respective genes can lead to deficiencies in or impaired function of clotting factors[1,2,41].

1.2.2.1. Treatment of Bleeding Disorders

Fast and appropriate treatment of any kind of bleeding disorder is essential in order to prevent the excessive loss of blood. For a long time, bleeding disorders were managed by the transfusion of whole blood but a deeper understanding of haemostasis acquired over the last five decades allowed for the development of alternative and more specific treatment strategies[1,41,118].

The current standard form of treatment for a variety of bleeding disorders including haemophilia A and B as well as fibrinogen deficiency is called replacement therapy[121,122]. Replacement therapy entails the intravenous infusion of molecules involved in normal haemostasis which are missing in the respective disease. After initial introduction of this treatment approach, relevant coagulation factors such as FVIII and FIX were extracted from donated human plasma preparations and subsequently administered to the patient[6].

Unfortunately, transfusion of plasma preparations carries a relatively high risk of contamination and widespread incidences in which bleeding patients were infected with for example the human immunodeficiency virus (HIV) were reported[122]. In order to circumvent these complications, extensive research was focused on the development of recombinant expression techniques for both FVIII and FIX. Consequently, the majority of haemophilic patients in the developed world are nowadays treated with high dose and ultra-pure FVIII and FIX concentrate which shows good efficacy and safety profiles[1,40,122,123].

Another common drawback of FVIII and FIX replacement therapy consists in their relatively short half-life which lies between eight and 12 hours for FVIII and 18 to 24 hours for FIX and necessitates frequent transfusion of FVIII and FIX preparations[123]. Typically, haemophiliacs have to be treated every other day making the appropriate management of the disorders more difficult. In the past several years, different strategies in order to prolong the half-life of both clotting factors have emerged. One option is an increase of the hydrodynamic size of a protein by for example PEGylating[124]. During PEGylating, a polyether called polyethylene glycol (PEG) is attached to the surface of the protein leading to an increase of the overall molecular size which subsequently prevents early clearance of the complex from the circulation by the or macrophage receptors[124]. Another approach entails the generation of a fusion protein; for example FVIII fused to the D'D3 portion of VWF[125]. Since VWF is known to bind to FVIII under physiological conditions and therefore naturally prolongs the plasma half-life of the clotting factor, fusion products of these two molecules were expected to exhibit a significantly extended plasma half-life. Clearance studies of the FVIII-VWFD'D3 complex showed an increase in plasma half-life from eight to 12 to more than 37 hours reducing transfusion intervals from every other day to once or twice a week[125,126].

Aside from direct administration of the clotting factors, conversion of FX to FXa also can be achieved using bispecific molecules as for example Emicizumab[127]. Emicizumab is a humanised antibody capable of simultaneously binding FIX(a) and FX(a) and therefore mimics FVIIIa function. Proof of concept studies have shown that administration of the antibody resulted in an accelerated conversion of FX to FXa and enhanced thrombin generation and importantly reduced bleeding in patients with haemophilia A[127,128].

A different approach in the prevention of bleeding complications strongly resembles treatment strategies applied during thrombosis and is associated with the inhibition of the antithrombotic pathway. While inhibition of antithrombotic mechanisms has no direct influence on for example low plasma levels of clotting factors, it decelerates events leading to the inactivation of said factors or clot lysis and therefore allows more time for the primary and secondary haemostasis to unfold. Inhibition of the antithrombotic pathway for example can be accomplished by using small interfering ribonucleic acid (siRNA) directed against antithrombin[129]. siRNA targeting antithrombin effectively disrupts the overall expression of the molecule by binding to the antithrombin messenger RNA and targets it for destruction[129]. Another way to intervene with the antithrombotic pathway is blockage of activated protein C activity. As already discussed in chapter 1.1.3.3., activated protein C is the natural inhibitor of the FVa and FVIIIa and therefore disrupts formation of the intrinsic tenase and the prothrombinase complexes[130]. Several monoclonal antibodies specifically targeting activated protein C activity and a novel protein C inhibitor serpin have been developed and proof of concept studies showed significantly reduced bleeding times in the presence of the antibodies which could open up a new treatment strategy for bleeding patients[53,131].

Even though the current treatment options for patients suffering from bleeding episodes have significantly increased their overall life expectancy, they merely focus on the pathological phenotype but are not able to correct the underlying cause of the disorder. An attractive alternative to these temporarily acting solutions is gene therapy. In 2011, Nathwani et al published a study in which patients suffering from Haemophilia B were treated with adeno-associated viral (AAV) vectors containing the gene for wild-type FIX under the control of a liver specific promoter. Results of this study showed modest and continuous expression levels of FIX in hepatocytes allowing a majority of the patients involved to discontinue their otherwise mandatory prophylactic treatment consisting of the transfusion of the clotting factor several times per week[132]. In 2017, two further gene therapy studies analysing the approach of gene therapy in order to achieve expression of normal plasma levels of clotting factors FVIII (Haemophilia A) and FIX (Haemophilia B) were published[133,134]. Similar to Nathwani`s approach, patients involved in these studies were treated with AAV vectors carrying the gene for the respective clotting factor and again results showed consistent expression levels of both proteins rendering further prophylactic treatment of the patients unnecessary while simultaneously exhibiting good safety profiles[133–135].

1.3. Von Willebrand Factor

Von Willebrand Factor is a large multimeric plasma glycoprotein circulating in the blood and plays an essential role in normal haemostasis[136,137]. VWF predominantly is involved in processes of the primary haemostasis, which as already discussed in chapter 1.1.1., is dependent on the arrest of platelets at sites of vascular injury as well as subsequent platelet activation and aggregation[23]. In zones in which the velocity of the blood flow is low, arrest of platelets can be partially mediated by interaction of the platelet receptor GPVI with collagen which is exposed by the subendothelium after occurrence of a vascular breach[35]. Under conditions of higher shear stress however, binding affinity of GPVI to collagen is not strong enough in order to result in a stable adhesion of the platelets to the vessel wall but is dependent on the presence of VWF[52,29]. In addition to its binding ability to both collagen fibrils on exposed connective tissue as well as the glycoproteins present in the cell surface of platelets, VWF also is known to form a complex with coagulation factor FVIII. Interaction of VWF with FVIII is essential for the stabilisation of the clotting factor and serves to maintain FVIII in circulation thereby prolonging its plasma half-life[137].

1.3.1. Expression of VWF

VWF is encoded by an approximately 178 kilobase gene located on the short arm of chromosome 12p13.31 (OMIM entry Number 613160)[137]. The VWF genes consists of 52 exons which can be translated into a 2813 amino acid pre-pro VWF protein serving as a precursor molecule for the mature VWF form[136].

Even though VWF expression can be observed in all tissues, the vasculature as well as in adjacent endothelial cells, expression profiles of the glycoprotein are heterogeneous indicating expression to different extents throughout the body[138]. Since VWF synthesis is exclusive to endothelial cells and megakaryocytes, high VWF protein levels can be observed in the blood plasma as well as α granules residing in platelets[139].

Apart from varying transcription and translation rates, VWF expression and overall plasma levels also can be influenced by extracellular stimuli such as physical exercise and pathological

conditions as for example bacterial infections, exposure of the immune system to endotoxins or the occurrence of necrosis[31,32,139].

1.3.2. Structure of VWF

VWF is one of the largest and most complex proteins reported and consists of a 22 amino acid signal peptide, a 741 residue pro-peptide and a mature subunit consisting of 2050 amino acids (Figure 1.9)[30,137]. The VWF propeptide as well as the mature subunits are composed of four repeated domains resulting in the following protein structure: D1-D2-D`D3-A1-A2-A3-D4-C1-C2-C3-C4-C5-C6-CK[137,139]. Most of the individual VWF domains show homology to other haemostatically unrelated proteins[140]. While VWF A domains for example share common motifs with complement factors B and protein C2, collagen and leukocytes adhesion receptors, VWF C domains have shown to have homologous sequences to thrombospondin[141]. In addition, the C-terminal cysteine knot (CK) which is necessary for the formation of inter-subunit disulphide bonds and therefore dimerization of VWF during protein synthesis is a `mucin-like` domain homologous to the transforming growth factor-β (TGFβ) [30,136,141].

Multimerisation is essential for correct function of VWF and provides multiple binding sites for proteins residing in the subendothelium and molecules circulating in the blood[142]. As already previously mentioned surface-bound VWF is capable of binding to flowing platelets while arrest of platelets at sites of vascular injury is initiated by VWF`s ability to bind to collagen[137]. The major collagen binding sites resides in the VWF A3 domain and are responsible for the interaction of VWF with collagen type I and III which are one of the main components of the subendothelial matrix, connective tissues as well cartilage[143]. Additional binding sites for fibrillar collagen IV and collagen VI have been attributed to the A1 domain of VWF. Apart from collagen, the VWF A1 domain also has been observed to be involved in the interaction between VWF and heparin[137,144]. After binding to collagen, VWF is capable of interacting with platelets via its A1 domain which recognises GPIbα present on the platelet cell surface[145]. Subsequent platelet aggregation and stable arrest of platelets at sites of vascular injury is facilitated by an RGD sequence consisting of the amino acids arginine-glycine-asparagine present in the C4 domain of VWF which is able to bind to the integrin GPIIb/IIIα on activated platelets[29,137].

Aside from platelet capture, VWF also serves as the carrier molecule for clotting factor FVIII. In order to protect FVIII from proteolytic degradation, VWF binds to FVIII via its D`D3 region increasing the overall molecular size of the clotting factor which effectively prevents FVIII degradation[137,144,146].

Regulation of VWF function is dependent on its multimeric size with larger multimers being considered as the more physiologically active form of the glycoprotein[137,142]. Cleavage of ultra-large VWF multimers into smaller protein components therefore is mandatory for appropriate haemostatic function and is predominantly mediated by ADAMTS13[147,148]. The cleavage site of ADAMTS13, allowing processing of VWF by the metalloprotease consists of the central scissile bond Tyrosine^{1605}-Methionine^{1606} and lies in the VWF A2 domain[149]. Aside from ADAMTS13, plasmin has been reported as an alternative or in some cases backup enzyme responsible for the degradation of VWF[150]. Different to ADAMTS13, degradation of VWF by plasmin occurs within the linker region connecting the VWF A1 and VWF A2 domain effectively cleaving ultralarge VWF multimers into smaller VWF multimers that exhibit appropriate haemostatic function [150,151]. Another mechanism involved in the regulation of VWF consists of the clearance (discussed in chapter 1.3.6.) of the glycoprotein from circulation which serves to reduce overall VWF availability and therefore its overall haemostatic capacity.

In order for VWF monomers to dimerise and form large multimers, the different VWF subunits have to be able to build disulphide bonds between each other[30]. In general, disulphide bonds are formed by oxidation of the amino acid cysteine which is abundantly present in the individual VWF monomers. In fact, VWF monomers contain 4 times more cysteines compared to other proteins emphasizing how dependent correct VWF function is on appropriate secondary structure, including dimerisation and multimerisation[30,137,152].

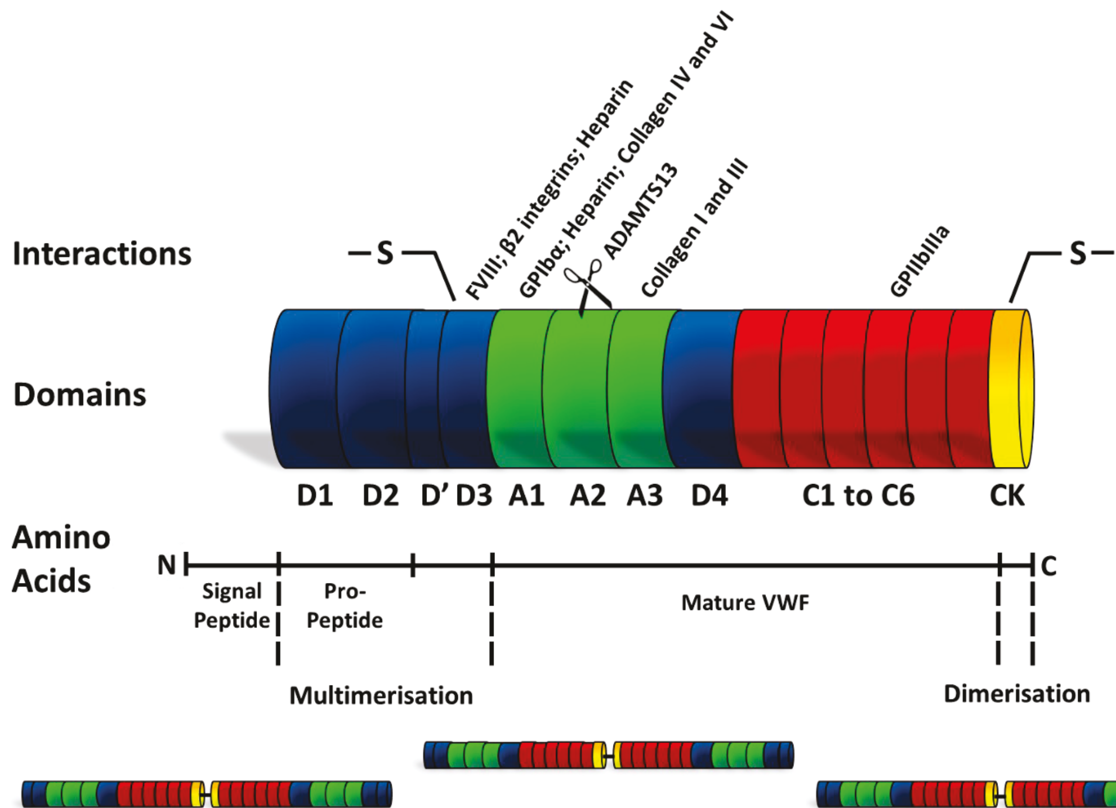

Figure 1.9: Schematic representation of the Von Willebrand Factor (VWF) structure. VWF consists of four different repeated protein domains. Dimerisation of VWF monomers is catalysed by the cysteine knot (CK). Multimerisation of VWF is facilitated by the formation of N-terminal disulphide bonds between VWF dimers (not depicted). The main binding sites important for the haemostatic function of VWF are indicated together with the ADAMTS13 cleavage site (adapted from [137,139]). F = Factor; GP = Glycoprotein; CK = Cysteine Knot.

1.3.3. VWF Biosynthesis

Posttranslational modifications subsequent to protein transcription and translation are essential for overall protein function. The majority of posttranslational protein alterations occur within the Golgi apparatus to which the proteins are transported using the endoplasmatic reticulum (ER)[144]. Unfortunately, passage of extremely large proteins through the extensive network of the ER is difficult. Due to the fact that VWF can reach multimeric sizes of more than 200000 kilodaltons (kDa) and therefore cannot easily be carried through the ER, formation of VWF multimers occurs in a two-stage process consisting of initial dimerisation of VWF monomers in the ER followed by multimerisation in the Golgi[30,137,144].

1.3.3.1. Dimerisation in the Endoplasmic Reticulum

After translation, translocation of the VWF precursor protein into the ER is mediated by a series of amino acids present at the N-terminal end of the peptide. Translocation of the precursor protein also is accompanied by the addition of N-linked glycans to the molecule (Figure 10)[153]. Once inside the ER, the monomeric VWF subunits subsequently arrange tail to tail in order to form dimers which become disulphide linked via cysteines present in the CK[137,140]. In general, the ER is the primary site for the formation of disulphide bonds since it provides an oxidising environment and contains a protein called thioredoxin—like protein isomerase (PDI) which is responsible for the exchange of dithiol-disulphide and correct protein folding[154]. Dimerisation of VWF monomers is dependent on three specific amino acids located in the CK at positions 2771, 2773 and 2811 and mutations in either of the amino acids have been associated with loss of large VWF multimers and pathological bleeding due to impaired haemostatic function of VWF[30,154].

Aside from initial dimerisation, the ER also plays an important role in the posttranslational glycosylation of VWF which is essential for VWF maturation. Using β-glycosidic bonds, high-mannose N-linked carbohydrates are attached to asparagine residues throughout the VWF polypetide[137,155]. Interestingly, inhibition of VWF glycosylation does not interfere with monomer dimerisation but causes the precursor molecule to be retained inside the ER interrupting and therefore preventing subsequent procession of VWF and formation of large VWF multimers[156].

After glycosylation and dimerisation of single VWF monomers, the protein is transported through the ER to the Golgi apparatus. Prior to the final translocation of the VWF dimers from the ER to the Golgi, the signal peptide is cleaved off representing another step in the maturation cascade of VWF[137,140].

1.3.3.2. Multimerisation in the Golgi Apparatus

Even though the Golgi apparatus mainly serves as a packing station, conditions inside the Golgi are favourable for further processing of VWF into larger multimers (Figure 1.10). Due to the acidic pH and elevated Ca^{2+} concentrations inside the Golgi, the VWF dimers assemble in a structure referred to as dimeric bouquet in which the VWF C1-CK domains serve as stem and

globular A2, A3 and D4 domains as flowers[30,31,140]. Apart from the environmental conditions encountered inside the ER, multimerisation of VWF is additionally supported by the VWF domains D1 and D2 (propetide) which act as endogenous chaperone correctly aligning the VWF dimers[31]. In addition to its chaperone function, the propetide also has an isomerase activity which catalyses the cross-linkage of individual VWF dimers via cysteine residues in their D3 domain[153].

Similar to dimerisation, VWF mulitmerisation is accompanied by further glycosylation consisting of modification of the N-linked glycans added in the ER and addition of 10 O-linked oligosaccharides at specific conserved sites[155]. Addition of N-linked glycans is associated with correct protein folding and disulphide bond formation, prevention of faulty multimer assembly and increase of the overall protein stability[157]. As already mentioned, inhibition of N-linked glycosylation prohibits secretion of the VWF dimers from the ER but also affects release of VWF multimers from their storage vesicles at later stages of the proteins life cycle and has been shown to modulate proteolytic cleavage of VWF by ADAMTS13[157]. Similar to the addition of N-linked glycans, O-linked glycosylation of VWF results in a stabilisation of the protein due to the introduction of steric hindrance of flexible VWF regions which decreases the overall susceptibility of the protein to protease degradation[158]. Furthermore, O-linked glycans have been observed to function as extracellular receptors involved in cell adhesion and intracellular trafficking. Hence, mutations affecting O-linked glycosylation which can lead to premature unfolding of the VWF multimer under high shear conditions have been associated with increased VWF mediated platelet adhesion to subendothelial collagen as well as elevated proteolysis of VWF multimers by ADAMTS13[159]. On the termini of the N- and O-linked glycans, VWF is further modified by the attachment of galactose and sialic acid leading to a glycosylation pattern that can recognised by ADAMTS13 and simultaneously prohibits degradation of the multimers by other proteases[160]. The final glycan modification is the addition of ABO(H) sugars to a small proportion (15%) of N – linked glycans and to an even smaller proportion of O-linked glycans[137,157].

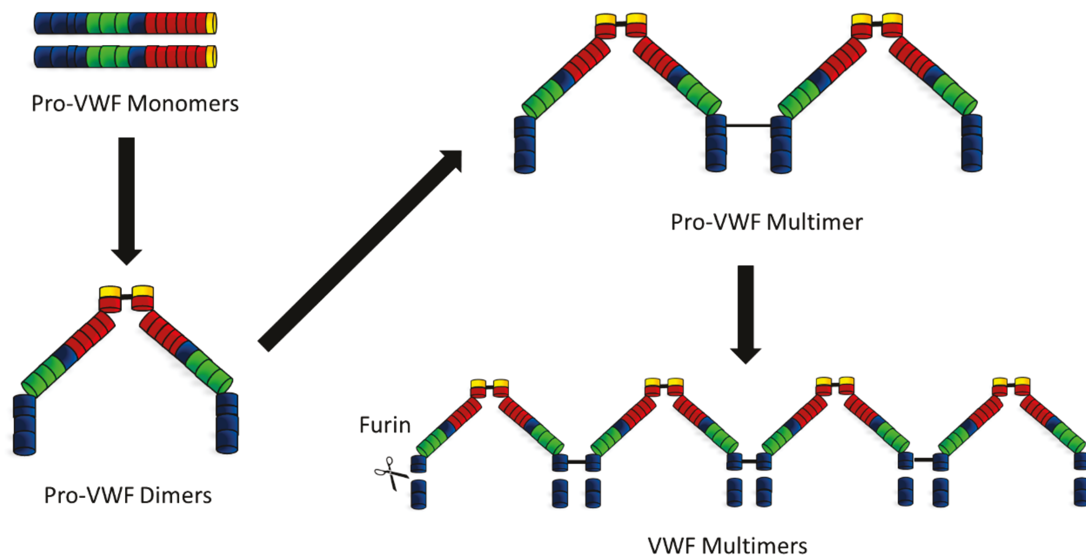

Figure 1.10: Maturation and assembly process of VWF multimers. VWF is synthesized as pre-pro-VWF that comprises a signal peptide, a pro-peptide and the mature subunit. After removal of the SP, pro-VWF subunits associate in the endoplasmatic reticulum into dimers by the formation of disulphide bonds between the cysteine-rich carboxyl-terminal CK domains. Subsequently, dimers further multimerize by forming additional disulphide bonds between the amino-terminal cysteine-rich D3 domains in the Golgi. Pro-VWF multimers subsequently are subjected to Furin cleavage removing the N-terminal propeptide resulting in matured VWF multimers that either directly secreted into the blood or stored in Weibel Palade Bodies or endothelial α-granules (adapted from [136,137]). VWF = Von Willebrand Factor.

Subsequent to multimerisation and glycosylation, the propetide is cleaved off the mature VWF subunit by an enzyme called furin[161]. Aside from ensuring correct multimerization, the propetide also plays an important role in the storage process of VWF in either Weibel Palade bodies in endothelial cells or α granules in megakaryocytes[30,137,153]. After cleavage, the propeptide has been observed to stay associated with the D3 domain and only dissociated from the VWF multimer after its final release from the respective storage organelle[137,140,161].

1.3.3.3. Intracellular Storage and Secretion

While the majority of newly assembled VWF multimers will be packaged into Weibel Palade bodies present in endothelial cells or α-granules formed by megakaryocytes, a small amount may be directly secreted, luminally or abluminally, from endothelial cells[137,152]. Stored VWF subsequently can be released upon stimulation of the respective cells in case of for example vascular injury or inflammation[139].

Formation of Weibel Palade bodies (WPBs) occurs within the Golgi apparatus and as already previously mentioned is dependent on the VWF propeptide[140,156]. Additional factors necessary for the formation of these storage vesicles are an acidic pH and an adapter protein called AP1/clathrin coat[162]. Due to their distinctive shape and especially their length, interaction of AP1/clathrin coat with VWF multimers leads to an arrangement of VWF in a helical structure effectively compacting the protein by a 100-fold without which storage of the multimers would not be feasible[30,162,163]. Due to the limited space inside the WPBs, VWF multimers are co-localised with other proteins such as P-selectin, interleukin-8, angiopoietin-2 and in some cases FVIII indicating a role of the vesicles in not only haemostasis but also inflammation and angiogenesis[30,162,163].

The majority of basal secretion of VWF from WPBs occurs in the absence of a stimulant[30,152]. The secretory events however are stimulated by a range of molecules including histamine, prothrombin, vascular endothelial growth factor (VEGF) and certain complement factors which predominantly are released upon vascular injury[152,162]. Therapeutically, the release of VWF can be achieved using desmopressin, a vasopressin analogue operating via endothelia V2 receptors[126]. The general mode of action of these secretagogues consists of an increase in the Ca^{2+} concentration within either the endothelial cell or the megakaryocyte which triggers fusion of the storage vesicles with the respective cell surface[31]. Aside from calcium, an increase of intracellular cyclic adenosine monophosphate (cAMP) levels also is able to initiate release of VWF multimers by rearrangement of the cytoskeletal structure[30,163].

1.3.4. Function of VWF

As for a multitude of other proteins, functional activity of VWF is dependent on its tertiary conformation. Under normal conditions, VWF circulates the blood in an inactive globular form in which most of its platelet binding sites are shielded therefore preventing unwanted interaction with for example platelets that could result in pathological thrombus formation[137,164]. In case of vascular injury or other pathological conditions involving the vascular system however, VWF is capable of binding to collagen present in the exposed subendothelial matrix[30,31]. While binding of VWF to collagen type IV and VI is mediated by the VWF A1 domain, the binding site for collagen type I and III lies in the VWF A3 domain which is thought to be more important for normal haemostasis[137,140]. Interaction of VWF with collagen

results in an arrest of the multimer from circulation increasing the hydrodynamic shear forces acting upon the protein which ultimately leads to a conformational change of VWF from a globular to a string-like shape[137,164]. Conformational change hereby is accompanied by exposure of the remaining binding sites of VWF allowing the interaction with platelets[144]. Shear rates necessary in order to induce the conformational change in VWF usually range from $1000s^{-1}$ to $5000s^{-1}$ and can be observed for example in arterioles or under pathological conditions when arteriosclerotic plaques minimise the overall dimeter of the vessel[164,165]. As a consequence of VWFs sensitivity towards shear forces, the thrombogenic activity of VWF multimers increases proportionally to the shear stress it encounters[136,165].

While conformational change of the multimers is predominantly observed after binding of VWF to collagen and subsequent exposure to shear stress, it also can be initiated by a combination of shear forces and drastic changes in the pH[166]. Endogenous high molecular VWF multimers that have been stored within endothelial cells for example, undergo a conformational change upon release from the Weibel Palade bodies due to a shift of the pH from an acidic milieu and simultaneous encounter of shear from the circulation[136,139,166]. Both influences result in an elongation of the VWF multimers exposing the GPIbα platelet binding site within the VWF A1 domain followed by the integrin αIIbβ3 binding site present in the VWF C4 domain[165].

As already mentioned in chapter 1.1.1.2., GPIbα as part of the the GPIb-IX-V complex as well as αIIbβ3 are responsible for the interaction between VWF and platelets and binding of platelets to VWF multimers is highly dependent on shear stress[136,144]. In areas with low shear, VWF is present in its globular inactive form and platelet binding sites are shielded inhibiting any form of interaction[137]. In zones with increased shear forces however VWF becomes activated and subsequently is able to arrest platelets from the circulation[164]. The binding process of platelets to VWF can be divided into two phases in which platelets are either transiently captured from flowing blood or firmly attach to sites of vascular injury[161,166]. Flow-based platelet capture experiments showed that the initial arrest of platelets is mediated by the A1 domain of VWF[137]. Furthermore, primary interaction of VWF with platelets is a transient process characterised by a continuous translocation of the platelets along the vessel surface which is referred to as rolling platelet aggregates[166]. Interestingly, even temporary binding to VWF induces activation of the platelets which is accompanied by multiple

intracellular signalling processes and a rearrangement of the cytoskeleton leading to the exposure of integrin αIIbβ3 on the platelet surface[26,36]. Presentation of the active integrin subsequently facilitates complete arrest of the platelets from the circulating blood based on the interaction of αIIbβ3 with the VWF C4 domain[29,145]. Despite the high affinity of integrin αIIbβ3 to VWF, kinetics of the interaction between the two molecules are too slow in order to permanently bind platelets under conditions of high shear stress. The slowing of the initial rolling platelets accompanied by the activation of arrested platelets therefore is mandatory for the formation of a stable platelet plug and interaction with secondary haemostasis[29,144,145,148].

1.3.5. Regulation of VWF Activity

As previously mentioned, haemostatic activity of VWF increases proportionally to the size of the multimers formed because larger multimers contain more collagen as well as platelet binding sites compared to VWF dimers or a single VWF monomer[137,142]. While circulating VWF usually is comprised of several monomers, VWF secreted by endothelial cells or megakaryocytes can form large structures consisting of high numbers of monomers which are referred to as ultra large VWF[30]. Unless their release from the individual storage organelles is specifically stimulated by for example therapeutics such as vasopressin, ultra large VWF multimers are seldomly detected in healthy individuals due to the tight regulation of VWF activity and size[137,144]. Three distinct proteins have been observed to be responsible for the regulation of the haemostatic activity of VWF.

As already briefly mentioned in chapter 1.2.1.2., the predominant regulator of VWF is ADAMTS13 which is a metalloproteinase circulating the blood. ADAMTS13 is capable of cleaving a scissile bond formed between the two amino acids tyrosine and methionine at position 1605 and 1606 within the VWF A2 domain[31,137]. Dissolution of this peptidyl bond results in the disintegration of the VWF multimers into two monomers with a size of 176kDa and 140kDa which are haemostatically inactive[147,167]. An additional ADAMTS13 cleavage side which is assumed to facilitate initial binding of the protease to VWF hence ensuring effective degradation of VWF is suspected to be present in the VWF D4 domain[168]. Proteolysis of VWF can only occur after VWF has changed its conformation from a globular to its string like form due to either shear forces *in vivo* or the presence of denaturing agents such as urea *in*

vitro[137,149]. Consequently, only active VWF involved in the capture of platelets is attacked by proteolytic degradation while circulating VWF is protected from ADAMTS13 activity[30,165].

Based on the generally accepted role of ADAMTS13 as the main regulator of VWF activity, absence of the protease as for example observed in thrombotic thrombocytopenic purpura (TTP) leads to uncontrolled formation of small thrombi due to enhanced VWF activity. Interestingly, a complete deficiency of ADAMTS13 however has not been shown to result in continuous formation of small blood clots in all patients suggesting the presence of an alternative enzyme capable of regulating the haemostatic capacity of VWF[169,170]. Several *in vitro* as well as *in vivo* studies identified plasmin which usually is responsible for the degradation of fibrin in the context of fibrinolysis as a second regulator of VWF activity[151,171]. Similar to ADAMTS13, cleavage of VWF by plasmin requires a conformational change of the glycoprotein from its globular to string-like form in order to allow access of the protease to its designated cleavage site[151]. Detailed analysis of the interaction between VWF and plasmin showed that cleavage of VWF by the protease occurs at the peptide bond at position Lysine^{1491}-Arginine^{1492} within the linker region between the VWF A1 and VWF A2 domain effectively reducing overall VWF size and therefore regulating its haemostatic activity[150].

Aside from ADAMTS13 and plasmin, VWF also serves as a substrate for the a disintegrin and metalloproteinase domain-containing protein 28 (ADAM28) which has been shown to cleave VWF into a 200kDa and 180kDa fragment in *in vitro* experiments[31,172]. Different to ADAMTS13, ADAM28 seems to be capable of cleaving VWF in both its globular and unfolded form and has been reported to have two distinct cleavage sides in the glycoprotein. The first cleavage site for ADAM28 consists of the scissile bond formed between the two amino acids glycine at position 1242 in the VWF D3 domain and leucine at position 1243 in the VWF A1 domain[173]. Furthermore, ADAM28 has been observed to attack the bond formed between the two leucine residues at position 1482 in the VWF A1 domain and 1483 in the VWF A2 domain[173]. The exact physiological role of the interaction between ADAM28 and VWF has not been elucidated yet but is hypothesised to play an accentuated role in ADAM28 associated pathological conditions such as cancer[174].

Another protein involved in the regulation of VWF is thrombospondin-1 (TSP-1). Different to the previously described proteases, TSP-1 decreases the rate of proteolytic VWF cleavage. TSP-1 is secreted by activated platelets and directly competes with ADAMTS13 for VWF

binding acting as a negative regulator of the metalloprotease and therefore ensures the integrity of VWF multimers and their function in circulation[175].

Apart from proteins that take an active role in the regulation of VWF activity, molecules which indirectly influence cleavage and therefore degradation of VWF have been reported. The best characterised protein of this class is P-selectin which is better known as a mediator of cell adhesion on activated endothelial cells[176]. Interestingly, recent studies have shown that P-selecting which is stored in Weibel Palade bodies alongside VWF is capable of binding the glycoprotein subsequent to the release of both proteins from the endothelial storage organelles[177]. Closer analysis of the binding interaction revealed that P-selectin binds to the D`-D3 region of VWF and showed that this protein-protein interaction results in VWF being anchored to the surface of the endothelial cells it was released from[178,179]. Attachment of VWF to the cell surface subsequently results in the elongation of the glycoproteins due to shear forces exhibited by the circulation resulting in the presentation of the ADAMTS13 binding site present in the VWF A2 domain[177,180]. Binding of P-selectin to VWF therefore indirectly supports proteolytic cleavage and therefore regulation of the haemostatic activity of VWF.

1.3.6. VWF Clearance

After proteolytic degradation, cleaved VWF fragments dissociate from the endothelium and either readopt a folded conformation or are cleared from the circulation. VWF fragments can be subjected to endocytosis and subsequently are further proteolytically decomposed by different proteases[30,31]. Different studies have shown that clearance of VWF and VWF fragments is mediated mainly by macrophages derived from the liver and the spleen but also hepatocytes as well as endothelial cells have demonstrated an ability to influence overall VWF plasma levels[181].

The average plasma half-life of VWF is approximately 16 hours but different factors including genetics, age and physical exercise that can either mark VWF for accelerated degradation or protect the protein from proteolytic cleavage have been identified[31,137,181].

The predominant factor influencing clearance of VWF from circulation are the glycosylation patterns of the glycoprotein[182]. As already discussed in chapter 1.3.3., VWF is heavily glycosylated and posttranslational modification of the protein is essential for its overall

function. A big proportion of the N- and O-glycan chains (added to VWF during its procession through the endoplasmatic reticulum and the Golgi apparatus) are modified by addition of sialic acid residues which serve to protect VWF against premature proteolysis[183,184]. Consequently, reduced sialylation based on for example absence of the enzyme sialyltransferase ST3Gal-IV has been associated with increased clearance rates and therefore a reduced VWF plasma half-life in both mice and humans[185,186]. Aside from sialylation, presence of the ABO blood group determinants has been associated with VWF clearance as well. For example, individuals displaying determinant O show 20-30% lower VWF plasma levels compared to individuals of the blood groups A and B suggesting different VWF clearance rates depending on the determinant presented[187,188].

While glycosylation is an important modulator of VWF clearance on a protein level, removal of VWF from circulation by for example macrophages or endothelial cells is facilitated by different types of receptors which mainly recognise the different sugars attached to the glycoprotein[182,189]. Several *in vitro* as well as *in vivo* studies in mice led to the identification of two types of receptor families that have shown to play an active role in VWF clearance and therefore regulation of the overall VWF plasma concentrations. The first group of receptors associated with VWF clearance consists of lectins. Over the last decade multiple receptors derived from this family as for example asialoglycoprotein receptor (ASPGR)[190], macrophage galactose type lectin (MGL)[191], sialic-acid binding immunoglobulin-like lectin 5 (SIGLEC-5)[192], C-type lectin domain family 4 member M (CLEC4M)[193] as well as galectin-1 and galectin-3[194] have been characterised in their function as promoter for the clearance of VWF multimers from circulation. The second class of receptors affiliated with the regulation of VWF activity belongs to the family of scavenger receptors present on the cell surface of macrophages. So far binding of VWF to low-density lipoprotein receptor-related protein-1 (LRP1)[195] and scavenger receptor class A member I (SR-A1)[196] has been observed in *in vitro* studies and is thought to promote clearance of VWF by macrophage mediated endocytosis.

Apart from glycosylation and sugar dependent binding of VWF to different receptors on macrophages, hepatocytes or endothelial cells, clearance of VWF also can be modulated by the presence of mutations withing the VWF protein. So far, more than 30 different point mutations, predominantly located in the D3 domain of the glycoprotein, have been linked to an enhanced VWF clearance[197,198] The first mutation that was identified in relation to

enhanced VWF clearance occurs at position 1205 and is called VWF Vinzenca[199]. Substitution of an arginine residue with histidine at this position is characteristic for type 1 VWD which will be discussed in more detail in a subsequent chapter. Aside from R1205H, two additional mutations with an amino acid to change to either cysteine (R1205C) or serine (R1205S) which similar to the Vincenza mutation result in an increased clearance of VWF from the circulation have been found[200]. In addition to position 1205, single-point mutations associated with elevated VWF clearance rates predominantly mediated by macrophages have been traced back to position 1130 (cysteine to phenylalanine), W1144G (tryptophan to glycine) and C1149R (cysteine to arginine) within the A3 domain of VWF[198]. Deeper genomic analysis carried out over the past several years furthermore implicated a role of different mutations (I1416N, R1306Q and V1316M found in the VWF A1 domain, S2179F present in the D4 domain and C2671Y located in the CK, both present) outside the VWF A3 domain in accelerated clearance rates of VWF[197,198,201].

1.3.7. Role of VWF in Disease

Due to its essential role in haemostasis, defects in protein expression, structure and function or inhibition thereof by for example antibodies but also failure of adequate regulation of VWF activity can lead to pathological conditions characterised by either a bleeding phenotype termed Von Willebrand Disease (VWD) or enhanced clot formation[137,165].

One of the best characterised diseases involving VWF is Thrombotic Thrombocytopenic Purpura (TTP). TTP is characterised by abnormal formation of platelet rich clots in the microvasculature leading to obstruction of small blood vessels which can have fatal effects when for example the circulatory system of the brain or heart is affected[89]. Increased occurrence of such microthrombi is cause by a functional defect of ADAMTS13 which is responsible for the regulation of VWF by cleaving high molecular weight VWF multimers thereby reducing or neutralising their haemostatic activity[90,91]. Failure of the protease to process the VWF multimers leads to persistence of ultra-large VWF multimers with increased interaction between VWF and platelets in the circulation and enhanced activation of the platelets ultimately leading to platelet aggregation and clot formation in small vessels[90]. The deficiency of ADAMTS13 giving rise to TTP can be either inherited in an autosomal recessive fashion or acquired as a form of autoimmune disease[89,137]. While hereditary TTP is caused by

genetic mutations affecting the synthesis or proteolytic capacity of ADAMTS13, acquired TTP is based on the generation of autoantibodies targeting and therefore reducing ADAMTS13 activity[89]. The current treatment strategies for patients suffering from either acquired form of TTP involve both the removal of the antibodies directed against ADAMTS13 as well as infusion of the proteolytically active protease in a process referred to as plasma exchange. Alternatively, the hereditary form of TTP might be treated by infusion of recombinant ADAMTS13 which recently has been developed[89,91,202,203].

Aside from TTP, increased VWF activity also has been shown to be involved in a multitude of additional diseases such as myocardial infarcts, ischaemic stroke and arteriosclerosis. One common denominator in all those diseases is an overall inflammation of the vasculature which stimulates an increased secretion of VWF multimers and therefore local recruitment of platelets resulting in thrombotic occlusions and stenosis in microvessels[165,166].

1.3.7.1. Von Willebrand Disease (VWD)

Von Willebrand disease is the most common inherited bleeding disorder affecting 0.01- 1% of the overall world population depending on the definition used and is characterised by either qualitative or quantitative defects of VWF expression or the protein itself[137,153]. Based on the extent of the defect in VWF function or expression, symptoms accompanying VWD vary in their severity and include easy bruising, mucosal haemorrhage from nasal or oral cavities, prolonged bleeding from wounds and in more severe cases gastrointestinal and joint bleeding[204].

In general, VWD is divided into three types that can be distinguished by the pattern of deficiency. Type 1 VWD is defined as a simple quantitative deficiency leading to reduced VWF levels even though the circulating protein is relatively normal in function. Type 3 is defined as a complete deficiency with a complete absence of protein production. In contrast, VWD type 2 is characterised by functionally impaired VWF proteins and often is linked to more severe bleeding events compared to type 1. Based on the structural or functional defects of the proteins, type 2 is further divided into several subcategories referred to as 2A, 2B, 2M and 2N[31,137,204–207].

1.3.7.1.1. Quantitative Defects in VWF Levels

As already mentioned, VWD type 1 is caused by a partial VWF deficiency and is considered as the most common form of VWD affecting between 65 and 75% of all VWD patients[204]. Sequence analysis of patient genes showed that VWD type 1 is caused by different mutations which have been found in every domain of VWF apart from the A1, A2 and A3 domain[206]. The most prevalent form of mutations consists of various missense or single nucleotide variants affecting either intracellular transport of proVWF dimers or enhanced clearance of VWF multimers from the circulation. One example of such a single point mutation occurs in VWD Vicenza which is characterised by an amino acid substitution (arginine to histidine) at position 1205 in the VWF D3 domain leading to an accelerated clearance of VWF from the circulatory system by macrophages[31,206].

While patients with VWD type 1 and 2 show slightly prolonged bleeding times and easy bruising, VWD type 3 represents the most severe form of the disease[166]. VWD type 3 often is accompanied by significant mucosal and musculoskeletal bleeding episodes which partly are due to the FVIII deficiency secondary to virtually undetectable VWF plasma levels[205–207]. VWD type 3 is inherited in a recessive fashion and even though partial gene deletions, different gene splice variants and gene conversions have been observed, the predominant cause for the disease is based on frameshift and nonsense mutations within the VWF gene[137,206]. Different to VWD type 1 in which the genetic alterations mainly affect intracellular trafficking and VWF clearance, mutations occurring in type 3 have been shown to have an impact on every stage of VWF synthesis and assembly of the mature VWF multimers[31,137,205,206].

1.3.7.1.2. Qualitative Defects in the VWF Protein

In contrast to both type 1 and type 3, VWD type 2 is not primarily the result of reduced VWF expression but the overall structure and functionality of the protein[137,208]. For example, VWD type 2A is characterised by different mutations affecting mainly the VWF A2 domain resulting in a conformational change of the overall protein structure. In addition, the mutations also have been observed to interfere with either correct multimer formation or increased susceptibility to ADAMTS13 cleavage leading to reduced high molecular weight VWF multimer formation and therefore reduced VWF mediated haemostatic functions[137,204,208,209]. Aside from the A2 domain, type 2A missense mutations affecting the D`D3 as well as the CK have

been reported to lead to impaired VWF dimerisation and therefore reduced VWF multimer levels in circulation[137,206,208].

Another form of VWD type 2, termed VWF type 2B, is characterised by an enhanced interaction between VWF and platelets due to gain of function mutations within the VWF A1 domain. The increased affinity of the VWF A1 domain to GPIb present on the surface of platelets has been shown to facilitate interaction between VWF and platelets even in the absence of high shear or injury within the vasculature. As a result, type 2B can result in a bleeding phenotype due to increased proteolytic clearance of VWF multimers by ADAMTS13, feedback platelet binding inhibition as well as abnormal formation of platelet aggregates[153,204,206].

Similar to type 2A, VWD type 2M is characterised by a loss of function of HMWM. While the overall formation of VWF multimers is not affected in type 2M, different mutations within the VWF A1 and A3 domain prevent normal interaction of the VWF multimers with platelet GPIbα leading to impaired platelet capture at sites of vascular injury[31,137,206,207].

Identification of VWD type 2N initially was rather difficult since the symptoms characteristic for the disease strongly resemble pathological symptoms observed in Haemophilia A. VWD type 2N is caused by the inheritance of various homozygous and compound heterozygous allele variations affecting either the VWF D`D3 domain or the furin cleavage site present in the mature VWF multimers[153,206]. As a result, formed multimers are not able to exhibit their normal function as the carrier molecule for blood clotting factor FVIII leading to impaired haemostatic functions and a potentially severe bleeding phenotype[205,206,208].

1.3.7.1.3. Treatment of VWD

Due to the broad variability in symptoms and severity of the disease, there currently is no universal treatment strategy for all VWD subtypes and patients have to be medicated based on the symptoms they present with. The majority of bleeding phenotypes can be reversed or at least kept under control using VWF replacement strategies by either administration of purified plasma derived VWF, recombinant VWF or desmopressin[207,209,210].

Desmopressin is a synthetic protein that triggers secretion of VWF stored in the Weibel Palade Bodies present in endothelial cells leading to a three to five-fold increase in VWF

concentration approximately one hour after administration[210]. While treatment of VWD patients with desmopressin is characterised by the release of functionally active VWF multimers, the short half-life of VWF of around 12 hours only results a temporary elevation of VWF plasma levels and therefore requires repeated treatment cycles[205]. Unfortunately, long-term administration of desmopressin has been linked to a decreased responsiveness towards the medication over time making the need for an alternative treatment strategy necessary when prolonged treatment is required[207,210]. Due to the cost-effective production and multiple options of application, desmopressin and analogues thereof are considered to belong to the category of most used and most effective medicines currently in use by the World Health Organisation (WHO)[207,208,210]. Based on its mechanism of action, desmopressin or desmopressin analogues mainly are used for patients suffering from VWD type 1 since the drug only acts upon release of functionally intact VWF from WPBs but cannot counteract impaired VWF function or lack of protein expression observed in VWD type 2 and 3 respectively[208,210].

Aside from triggering VWF multimer release from storage organelles, VWD also can be treated by the infusion of purified plasma derived VWF. The most commonly used VWF preparations are HumateP® and Voncento® which consists of a mixture of plasma purified VWF and blood clotting FVIII effectively restoring both VWF and FXIII to physiological levels[210,211]. Similar to desmopressin, administration of HumateP® only has a transient effect and often is used prior to surgery in order to prevent bleeding complications during surgical operations. As opposed to desmopressin, manufacture of VWF/FVIII concentrates is a cost intensive process and additionally carries a risk of transmission of blood borne pathogens since it is purified from pooled human plasma[204,210,211]. Despite its disadvantages however, FVIII/VWF concentrates currently are also used in the prophylactic treatment of patients presenting severe forms of VWD and have been found to be effective in the prohibition or minimisation of mucosal and joint haemorrhage[210,211].

Aside from human plasma purified VWF, patients suffering from VWD alternatively can be treated using VWF which has been recombinantly expressed in mammalian cell expression systems. These modern recombinant expression technologies hereby ensure high expression levels of functional active VWF proteins forming the full range of multimers and additionally circumvent the risk of potential contamination of the protein preparation. So far, recombinant

VWF has been shown to decrease bleeding times, stabilise FVIII levels and has been successfully used for the treatment of VWD type 1 and 3[139,207,210].

In addition to the two treatments strategies described above, recombinant interleukin-1 (IL-1) which usually is used in thrombocytopenia has been observed to elevate VWF and FVIII plasma levels in both mice and human[210]. Even though the exact mechanism of action has not been fully elucidated yet, IL-1 is thought to induce transcription of the VWF gene leading to increased VWF expression. An advantage of this treatment strategy especially for patients being unresponsive to desmopressin lies in the fact that administration of interleukin results in in a slow but sustained increase of VWF levels circumventing the risk of spontaneous bleeding episodes[209,212].

Even though VWD treatment strategies are capable of at least temporarily elevating VWF levels and allow the management of the bleeding phenotypes characteristic for this disease, none of the drugs currently in use have a curative effect. Similar to the treatment of patients suffering from Haemophilia B, a gene therapy based approach ensuring constant expression of physiological levels of functionally intact VWF multimers would be an interesting solution especially for patients suffering from VWD type 3[31,207]. Over the last decade, several studies aiming at VWF based gene therapy have been completed and initial proof of principle experiments showed promising protein expression as well as adequate safety profiles[139,213].

1.4. Blood Clotting Factor XII (FXII)

FXII which also is known as Hagemann factor is a serine protease involved in the initiation of the coagulation system, inflammation as well as several cellular signalling responses[39]. The protease was first described in 1955 when blood samples of the 37-year-old John Hagemann showed abnormalities in the formation of blood clots in routine tests prior to surgical intervention[214]. Even though Hagemann never experienced any form of bleeding complications, coagulation tests of his blood samples showed significantly prolonged clotting times leading to Hagemann’s referral to the haematologist Oscar Ratnoff[214,215]. Deeper analysis of both Hagemann’s as well as his family’s history led to the identification of a deficiency of a previously unknown clotting factor which subsequently was named Hagemann factor or FXII[215,216].

Similar to many other clotting factors, FXII is predominantly expressed by hepatic cells and circulates the plasma in a globular inactive state referred to as a zymogen[214]. Upon activation by a multitude of different exogenous as well as endogenous activators which will be discussed in more detail in chapter 1.4.2., FXII is converted into FXIIa capable of exhibiting its proteolytic activity[217,218]. Interestingly, characterisation of the physiological function of FXII(a) has been difficult since deficiencies in protease expression or function are very rare and do not seem to have any pathological implications even though they have been associated with protective effects in relation to thrombosis and inflammation in animal models[214,216,219,220].

1.4.1. Structure of FXII

Human FXII is encoded by the *F12* gene located on chromosome 5 in humans. The gene consists of 13 exons translating into the expression of a 615 amino acid single-chain protein[220,221]. FXII is highly conserved between different species and undergoes several post-translational modifications in its maturation process resulting in the addition of two N-linked and five O-linked glycans as well as the formation of 20 disulphide bonds[1,219,221]. Subsequent to its translation and glycosylation, the N-terminal signal peptide consisting of 19 amino acids is cleaved off and the mature FXII protein is secreted into the circulation[214,216,219,222].

Full-length FXII circulates the blood as a single chain β-globulin comprising 596 amino acids. Upon cleavage by kallikrein, the protease dissociates into a heavy and light chain exhibiting different physiological functions. The heavy chain of FXII is composed of six partly repeated structural domains and contains a fibronectin type II, an epidermal growth factor-like, a fibronectin type I, a second epidermal growth factor-like, a kringle as well as a proline rich domain (Figure 1.11)[214,216,219].

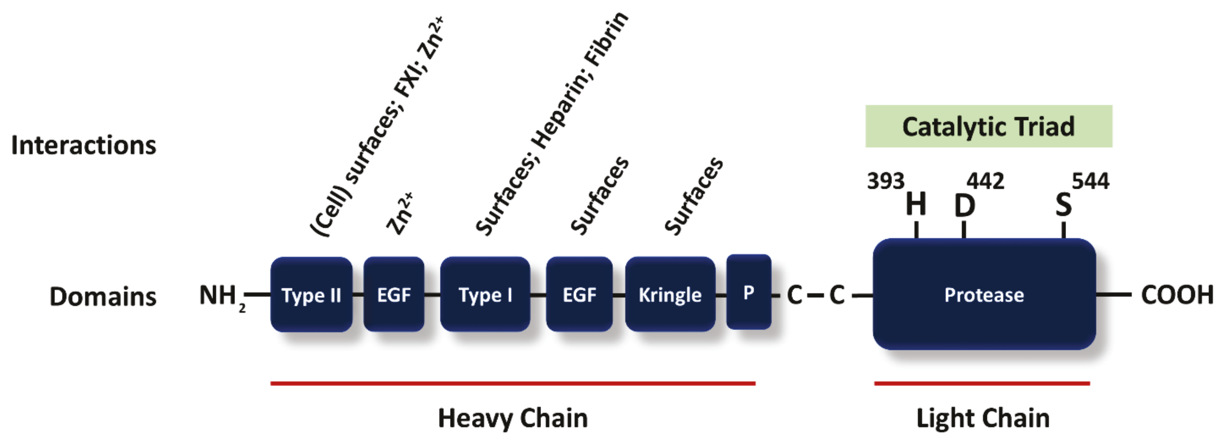

Figure 1.11: Schematic representation of full-length FXIIa. FXIIa consists of a heavy and a light chain connected by a single disulphide bond. The heavy chain is composed of several, partly repetitive structural domains: a fibronectin type II domain (Type II), an epidermal growth factor-like domain (EGF), a fibronectin type I domain (Type I), a second epidermal growth factor-like domain (EGF), a kringle as well as a proline rich domain (P). The light chain of FXIIa contains the catalytic triad His^{393}-Asp^{442}-Ser^{544} responsible for the protease function of the protein. The main binding sites important for the interaction with different binding partners are indicated (based on [223]). EGF = Epidermal growth factor; P = Proline rich domain.

Even though the overall composition of FXII has been the subject of different structural studies for years, the exact functions of the different domains have still not been fully characterised. Different protein-protein and protein-surface experiments allowed for the identification of surface binding sites in both fibronectin, the kringle as well as the second epidermal growth factor-like domain. Furthermore, Zn^{2+} binding sequences which have been shown to mediate binding of FXII to anionic surfaces and FXII autoactivation have been allocated to the first epidermal growth factor-like as well as the fibronectin type II domain[214,219,222]. In addition, the fibronectin type II domain seems to play an important role in the interaction of FXII with endothelial cells and neutrophils while binding of the protease to heparin and fibrin is assumed to be facilitated by the fibronectin type I domain[216,222]. Different to the heavy chain, the light chain of FXII is not involved in any binding interactions but exhibits enzymatic activity mediated by a His^{393}-Asp^{442}-Ser^{544} catalytic triad with protease function[217,222,224]. Upon conversion of inactive FXII to active FXIIa, the protease undergoes several conformational changes and cleavage reactions mediated by primarily kallikrein resulting in distinct protein fragmentation patterns and the formation of α-FXIIa and β-FXIIa[1,219,224]. While α-FXIIa has a molecular weight of 80kDa and consists of an n-terminal heavy chain (50kDa) and a c-terminal light-chain (30kDa), β-FXIIa possesses a distinctively smaller heavy chain of 2kDa[219,222,224].

1.4.2. Activation and Regulation of FXII

A surprisingly wide range of agents mediating the conversion of FXII zymogen to proteolytically active FXIIa has been identified over the last few years (Table 1.1.)[1,2,225]. In general, activation of FXII can be either temporary, based on a transient conformational change of the protease or permanent, which involves enzymatical processing of FXII by for example kallikrein[225,226].

Even though autoactivation is a commonly observed mechanism and is thought to be mandatory for the initiation of FXII activation, its exact mechanism has not yet been fully elucidated[1]. Screening of different activators suggested that autoactivation of the protease predominantly occurs after binding of the enzyme to a negatively charged surface via hydrophobic interactions and seems to involve a transient conformational change resulting in the exhibition of minimal proteolytic FXIIa activity. During the temporary conformational change, autoactivation of FXII is believed to result in a fluctuation of the protease between its zymogenic and active state while complete activation of FXII is thought to be dependent on irreversible FXII cleavage by predominantly kallikrein[217,226].

1.4.2.1. Exogenous Activator

The first officially described activators of FXII belong to the category of silica-based materials and comprise glass as well as the white clay minerals kaolin and celite which nowadays commonly are used as initiators of FXII activity in diagnostic tests evaluating the coagulation potential of patients[225,227,228]. Aside from silica-based materials, polyphenolic compounds predominantly extracted from plants have been reported to mediate FXII activation as well. Based on the presence of multiple phenol structures, members of the polyphenol class as for example ellagic acid are capable of directly interacting with FXII rendering the protease enzymatically active and similarly to kaolin are used in diagnostic tests for bleeding disorders arising from deficiency in clotting factors[214,229].

The use of medical devices in conjunction with unwanted activation of the coagulation system poses a frequently encountered problem and has been associated with the occurrence of thrombotic events as well as embolism and stroke[230]. Due to mostly hydrophilic interactions,

proteins are capable of absorbing to the surface of for example heart valves, pace makers, intravenous ports or catheters creating a thrombogenic surface. Direct binding of FXII to the surface of medical devices has been strongly associated with FXII autoactivation which has been shown to trigger initiation of coagulation[217,230,231].

1.4.2.2. Endogenous Activators

The main physiological activator of FXII is kallikrein which is capable of cleaving FXII at three distinct activation sites resulting in the generation of α-FXIIa as well as β-FXIIa[217,226]. Kallikrein is a serine protease circulating the blood in a zymogenic form referred to as prekallikrein. Interestingly, conversion of pre-kallikrein to kallikrein is mediated by FXIIa creating a feedback activation loop between the three proteins which ensures fast generation of both active FXII and kallikrein important for the intrinsic pathway of coagulation as well as the kallikrein-kininogen pathway involved in inflammatory responses respectively[217,226,232].

Over the last few years, activated platelets have gained a renewed interest as potential activators of FXII[233]. Studies have shown that integrins and receptors displayed on the surface of activated platelets are capable of directly binding to FXII thereby initiating coagulation via FXII activation[234,235]. In addition, FXII activation has been observed subsequent to the release of dense granules from activated platelets indicating storage of an activating agent within the platelets. Closer analysis of the granule content eventually lead to the identification of a class of inorganic polymers referred to as polyphosphates which are capable of mediating activation of FXII[233,236]. So far, the strong activation potential of predominantly long chain polyphosphates has been associated with the occurrence of FXII driven pulmonary embolism as well as FXII-bradykinin mediated edema making polyphosphates one of the most important physiological activators of FXII[233].

Aside from its role in primary haemostasis, collagen also has been reported to function as an activator of FXII[225]. The interaction between the protease and collagen was first described more than 40 years ago and was said to enhance FXII-driven thrombin generation as well as the overall coagulation potential[225]. Binding of FXII to collagen is based on the negative charge of the collagen fibrils leading to the conversion of FXII to FXIIa in an autoactivating manner[217,225].

Due to its preference to bind to negatively charged surfaces, generation of FXIIa also can occur after binding of the protease to negatively charged polymers such as polysaccharides incorporated in the surface of a wide variety of different cells types[237]. One of the best described polysaccharide FXII activators is dextran sulfate with high molecular weight dextran being a more potent activator compared to low molecular weight dextran[238,239]. Interestingly, incubation of FXII with dextran sulfate seems to result predominantly in the generation of bradykinin via the kallikrein-kinin pathway but shows minimal initiation of coagulation[223].

Activator	**Reference**
Non-physiological materials	
Silica based such as glass, kaolin or celite	Chatterjee et al.[240]; Kheirabadi et al.[241]
Phenolic compounds such as ellagic acid	Ratnoff et al.[242]
Plastic used in medical devices	Yau et al.[243]; Yau et al.[230]
Physiological activators	
Kallikrein	Cochrane et al.[244]; Rojkjaer et al.[245]
Autoactivation (after absorption to negatively charged surfaces)	Chen et al.[246]
Phospholipids	Faxälv et al.[236]
Platelet integrins	Piedras et al.[247]
Polyphosphates	Müller et al.[248]; Puy et al.[249]
Collagen	Wilner et al.[250]; Meijden et al.[251]
Polysaccharides such as dextran sulfate	Samuel et al.[252]
Misfolded or aggregated proteins	Maas et al.[253]; Bergamaschini et al.[254]
Nucleic acids such as RNA and DNA	Kannemeier et al.[255]
Heparin	Oschatz et al.[256]
Sulfatides	Røjkjær et al.[257]; Nakayama et al.[258]
Endotoxins	Morrison et al.[259]

Table 1.1.: Summary of published FXII activators. Non-physiological and physiological activators of FXII with corresponding references.

Misfolded or aggregated proteins have been observed to trigger FXII activation which would explain elevated FXIIa activity in for example Alzheimer disease or amyloidosis[216,253]. Blockage of FXII activation by the protein amyloid b which is characteristic in Alzheimer disease for example positively correlated with decreased cognitive impairments and reduced pathological inflammatory events in the brain[260]. In addition, aggregated immunoglobulins frequently

observed in certain forms of amyloidosis have been shown to initiate FXII activation resulting in systemic generation of bradykinin and widespread inflammation[216,220].

Aside from aggregated proteins, extracellular RNA as well as heparin are capable of activating FXII[261,256]. Interaction of both heparin as well as extracellular RNA with FXII has been observed in response to stimulation and degranulation of for example mast cells by allergens in cases of allergy or events occurring during cell lysis in for example sepsis[217,262].

1.4.2.3. Regulation of FXII Activity

Based on its diverse physiological functions, activity of FXIIa has to be tightly regulated in order to prevent uncoordinated initiation of coagulation events, local inflammatory reactions as well as untargeted cellular signalling responses. Three different molecules with regulatory function of FXIIa have been identified so far.

One of the major regulatory molecules responsible for the direction of proteolytic activity of the intrinsic pathway of coagulation is called C1 esterase inhibitor (C1INH). C1INH has been described to exhibit inhibitory function on a range of proteases with FXIIa being one of its main targets[214,217]. Aside from C1INH, antithrombin III (ATIII) and plasminogen activator inhibitor-1 (PAI-1) have been observed to inhibit proteolytic activity of FXIIa[219,263]. All three regulators belong to the group of serpins which comprises a multitude of proteins with inhibitory function on serine proteases. Their mode of action involves acting as a pseudo-substrate, resulting in irreversible binding to their target and induction of a major conformational change, effectively disrupting the catalytically active site of the respective protease[219,238,264].

1.4.2.4. Cleavage of FXII upon Activation

In order to convert the FXII zymogen (Figure 1.12) into its active form (α-or β-) FXIIa, the protein has to undergo conformational changes which are either achieved by autoactivation or cleavage by predominantly the serine protease kallikrein[217,226]. Even though autoactivation of FXII has been observed and seems to play an important role in the initiation of coagulation,

sequential processing of the protein by kallikrein is mandatory in order to achieve full activation. In order to enable cleavage of FXII by kallikrein, FXII contains three distinct arginine based kallikrein cleavage sites at position R334, R343 and R353[219,222,265].

While initial cleavage and subsequent fragmentation of FXII directs overall function of the clotting factor, cleavage of FXII at position R353 is considered to be essential in order to convert inactive FXII to proteolytically active full-length α-FXIIa[219,265]. In general, formation of α-FXIIa is considered as an on/off switch of the protease and cleavage of FXII at position R353 is thought to lock the protein in its active state[219,266]. The importance of initial FXII cleavage by kallikrein at position R353 additionally is supported by the observation that mutations in the factor XII gene at this exact position, as for example observed in so called FXII Locarno deficiencies, result in an inability of FXII to be activated[267].

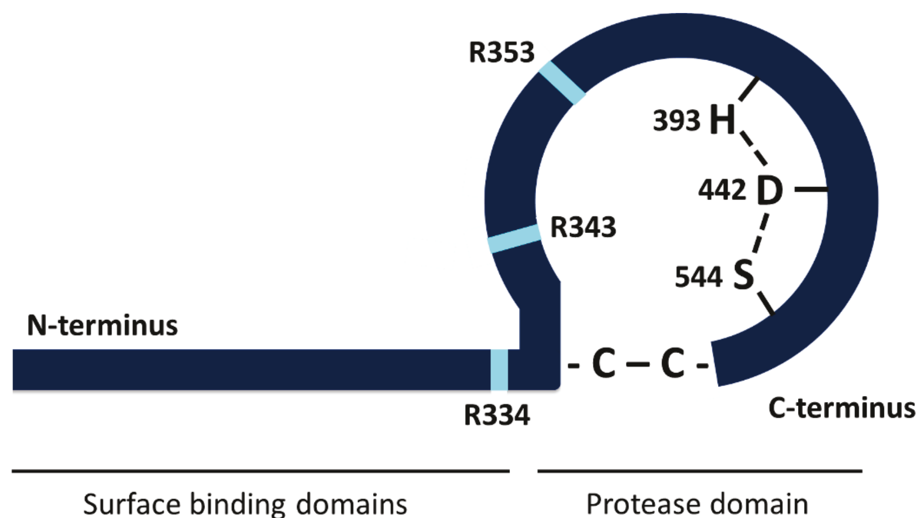

Figure 1.12: Schematic representation of the FXII zymogen. Known kallikrein cleavage sites are indicated in light blue. Amino acid residues are numbered according to the mature sequence of FXII. FXII circulates the blood in an inactive state in which the catalytic triad (H393-D442-S554) is shielded (based on [223]).

Even though the kallikrein cleavage site R343 is in close proximity to R353, cleavage at only this position does not result in constitutive activation of the protease[219,268]. Interestingly, experiments using a FXII peptide consisting of only the catalytic triad showed a lack of enzymatic activity of the isolated peptide which additionally seemed to have reverted back to a zymogen-like conformation[219,265]. These unexpected results led to the hypothesis that conformationally stable and proteolytically active FXIIa consists of a tethered peptide which

is responsible for correct formation of a mature active site and overall modulation of FXIIa activation. These findings lead to the conclusion that two potential forms of active α-FXIIa exist (see Figure 1.13) which both depend on initial cleavage of FXII at position R353 but can be followed by an additional proteolytic incision at position R343[219,222].

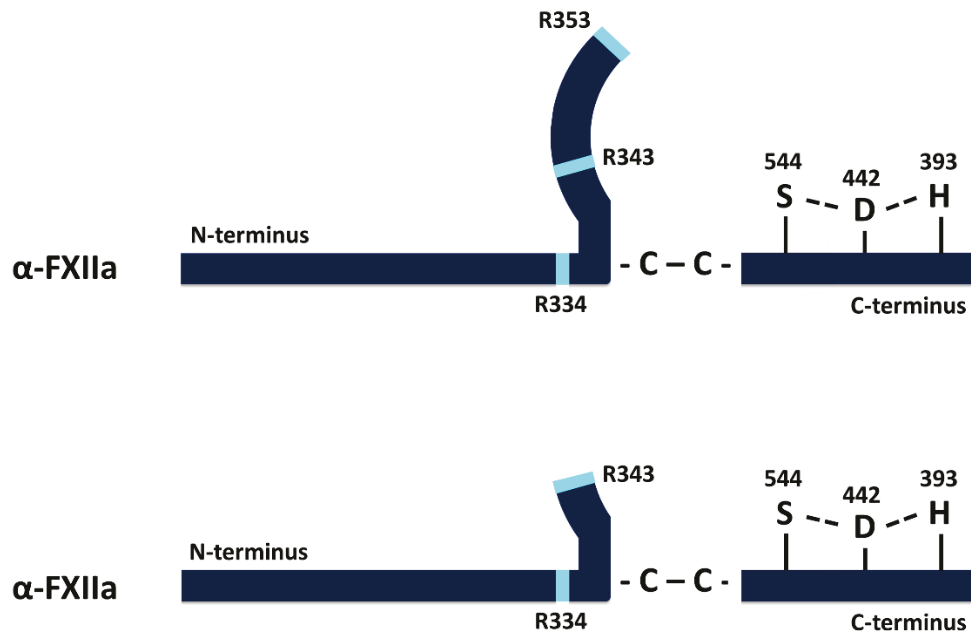

Figure 1.13: Schematic representation of α-FXIIa. Known kallikrein cleavage sites are indicated in light blue. Amino acid residues are numbered according to the mature sequence of FXII. In order to exhibit its proteolytic function, FXII has to undergo a conformational change leading to the conversion of FXII to α-FXIIa by kallikrein procession. Two versions of proteolytically active α-FXIIa have been described. Both forms depend on initial cleavage by kallikrein at position R353 resulting in a two-chain protein linked by a disulphide bridge. Additional cleavage at position R343 by kallikrein generates the second form of active α-FXIIa (based on [223]).

After generation of α-FXIIa, the protease can be cleaved an additional time by kallikrein at cleavage site R334 which results in the generation of an approximately 30kDa protein referred to as β-FXIIa or FXIIaf (Figure 1.14)[219]. FXII activation studies showed that cleavage of FXII at R334 alone is not capable of activating the protease but can severely impede the ability of the clotting factor to bind to different surfaces due to a separation of the surface binding domains from the protease domain[219,268]. Consequently, and distinct from α-FXIIa which still is in possession of the domain containing the FXI binding site, β-FXIIa has not been found to contribute to the initiation of coagulation by FXI cleavage. Due to the loss of its surface binding domains, β-FXIIa furthermore has been observed to dissociate from any given surface and is assumed to promote different aspects of coagulation in the fluid-phase[222,268].

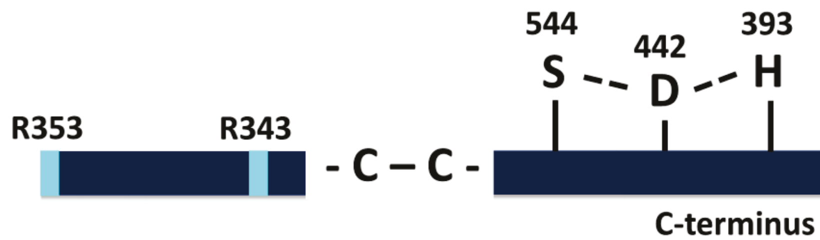

Figure 1.14: Schematic representation of β-FXIIa. Known kallikrein cleavage sites are indicated in light blue. Amino acid residues are numbered according to the mature sequence of FXII. After formation of α-FXIIa, the active protease can be additionally cleaved at position R334 by kallikrein resulting in the generation of a 30kDa fragment referred to as β-FXIIa. Due to the lack of the surface binding as well as FXI binding domains, β-FXII is has not been found to contribute to the initiation of coagulation but exhibits its function in fluid-phases (based on [223]).

While activation of FXII by kallikrein is characterised by protein fragmentation described above, western blot analysis of autoactivated FXIIa does not show the formation of multiple protein fragments but seems to be dependent on a reversible conformational change of full-length FXII leading to the exposure of the catalytically active domain of the protease[223,226]. Based on these observations, autoactivation of the protease is thought to be characterised by a continuous switch from inactive to active FXII until cleavage at site R535 stabilises FXII in its active state.

1.4.2.5. Differential Activity of FXIIa

In order to better understand the interaction profiles of FXII with its different activators, both the native conformation as well as the conformational changes of FXII upon activation have been the focus of several biochemistry related studies over the past few years. Similar to other enzymatically active proteins, inactive FXII is assumed to circulate the blood in a globular form in which the catalytically active core of the protease is shielded[223,269]. Consequently, FXII has to adopt a linear conformation in order to become active and exhibit its various functions (Figure 1.15).

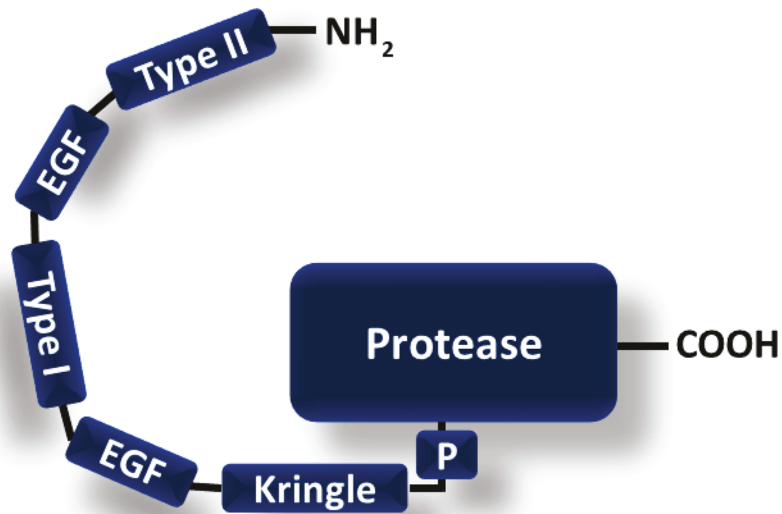

Figure 1.15: Hypothetical molecular model of plasma circulating FXII. FXII is assumed to circulate the blood in a globular form shielding its various cleavage sites. In this form, both prekallikrein and FXI interaction sites are encrypted preventing initiation of the kallikrein-kinin and coagulation pathway. In addition, the globular conformation protects FXII against kallikrein cleavage at site P353 leading to constitutive activation of the protease. P: Proline rich region; EGF = Epidermal growth factor-like domain; Type I = Fibronectin type I domain; Type II = Fibronectin type II domain (adapted from [223]).

It is generally believed that FXII activation and function are dependent on association of the protease with a surface via its multiple surface binding domains and that the degree of binding interaction directly influences the effector function of FXIIa[223,270]. Studies using truncated versions of FXII or inhibitory antibodies showed that surface binding ability of FXII is not mediated by a single designated binding domain but requires a combination of different domains depending on the nature of the target surface[222,223]. Interestingly, the same studies revealed a differentiation in FXII function depending on which surface binding domains were actively engaged in surface association and showed that binding of FXII to a surface does not necessarily result in the activation of the protease[222].

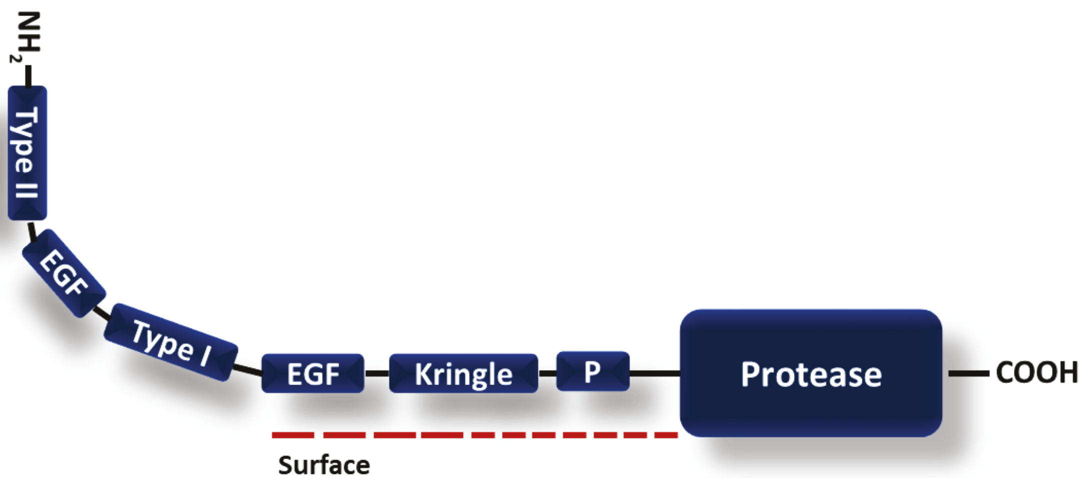

Figure 1.16: Hypothetical molecular model of partially extended FXIIa. Upon binding to a surface via its kringle and second epidermal growth factor like domain, FXII partially unfolds. Exposure of its prekallikrein and kallikrein binding sites result in activation of the protease by kallikrein cleavage at position R353 and initiation of the kallikrein-kinin pathway by proteolytic procession of prekallikrein. P = Proline rich region; EGF = Epidermal growth factor-like domain; Type I =Fibronectin type I domain; Type II = Fibronectin type II domain (adapted from [223]).

Based on these findings, a molecular model explaining the differences in FXIIa surface interaction and subsequent effector functions was formulated[223]. As already mentioned, association of FXII with a surface does not necessarily result in activation of the protease. Furthermore, surface binding of FXIIa has been shown to predominantly lead to conversion of prekallikrein to kallikrein and subsequent generation of bradykinin but less frequently results in the activation of clotting factor XI[220,222,269]. Contact of FXII with dextran sulfate or protein aggregates for example has been shown to initiate the kallikrein-kinin pathway but does not trigger blood clotting[223,270]. This apparent preference can partly be attributed to the significantly higher prekallikrein levels in plasma compared to FXI but cannot explain why binding of FXII to different surfaces does not consistently trigger coagulation responses[220]. Based on the molecular model suggested by Griffin as well as Maat and Maas, binding of FXII to a surface via its second EGF-like as well as the kringle domain is mandatory in order to initiate the kallikrein-kinin pathway[223,271]. In order to exhibit coagulation functions however, additional N-terminal-located domains have to be involved in the surface interaction resulting in a linearisation of FXIIa exposing its FXI binding site[224]. The model therefore proposes that partially unfolded FXIIa (Figure 1.16) is capable of activating prekallikrein but only fully extended FXIIa (Figure 1.17) is able to activate FXI[223].

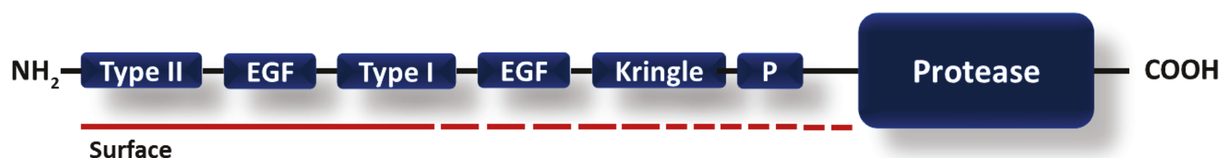

Figure 1.17: Hypothetical molecular model of fully extended FXIIa. Binding of FXII to a surface via all available binding domains results in adoption of a linear conformation. Exposure of the prekallikrein, kallikrein and FXI binding site leads to activation of FXII by kallikrein cleavage at position P353, proteolytic procession of prekallikrein and conversion of FXI to FXIa by activated FXII. Different to partial extended FXIIa, linearised FXIIa is capable of triggering both initiation of bradykinin generation via the kallikrein-kinin pathway as well as coagulation via FXI activation. P = Proline rich region; EGF = Epidermal growth factor-like domain; Type I = Fibronectin type I domain; Type II = Fibronectin type II domain (adapted from [223]).

1.4.3. Function of FXII and Pathological Implications

FXII function has been the subject of many debates over the past few years and its definitive physiological role has not been completely unveiled yet. Even though the protease was first identified as part of the coagulation system, it was soon discovered that FXIIa function extends far beyond this aspect and also drives mechanisms involved in inflammation, the complement system and cellular responses such as cellular growth and movement[216,220,224].

1.4.3.1. FXII mediated Bradykinin Formation

While a definitive physiological role of FXII in coagulation has been subjected to controversial debates, its ability to initiate the kallikrein-kinin system is well recognised[217,272]. The kallikrein-kinin system is a hormonal network consisting of a variety of different enzymes and peptides and is responsible for the generation of a small peptide called bradykinin which is a well characterised inflammatory mediator[273,274]. The main proteins involved in the formation of bradykinin are FXIIa, prekallikrein, kallikrein and high molecular weight kininogen (HMWK)[273]. Upon activation, FXIIa is responsible for the conversion of prekallikrein to kallikrein which in turns cleaves HMWK resulting in the release of bradykinin into the blood stream[232,272]. Bradykinin subsequently acts on the adrenergic G protein-coupled receptors B1 and B2 on endothelial cells resulting in a dilation of the blood vessels, an increase in cellular permeability, a drop in blood pressure, the recruitment of leukocytes during inflammation and elevated cytokine expression.

1.4.3.2. FXII in Sepsis

FXIIa mediated activation of the kallikrein-kinin system and generation of bradykinin has been associated with a multitude of different pathological conditions and has been suggested to play a finely balanced role in sepsis[262]. In general sepsis is an inflammatory immune response to infection caused predominantly by bacteria, virus or fungi. The severity of the condition is highly variable depending on the nature of the original infection and the host response and symptoms include fever, impaired blood clotting and blood stasis which can lead to organ failure as well as a decreased systemic vascular resistance resulting in extremely low blood pressure[275]. Sepsis is a multifaceted pathological condition characterised by excessive inflammatory reactions and impaired haemostasis and at least partially is influenced by activity of FXIIa[262,275,276]. As already discussed above, activation of the coagulation system via FXIIa ultimately leads to the formation of fibrin fibres which, in the context of sepsis, have been found to capture bacteria thus enhancing clearance of the pathogens by the immune system[275]. In addition, FXIIa mediated bradykinin generation has been shown to facilitate the production of inflammatory mediators which in turn supports recruitment of immunological cells to the site of infection further promoting elimination of the infecting agents[262,277].

Contrary to the antibacterial function of FXIIa described above, extensive generation of FXIIa mediated bradykinin formation however simultaneously carries the risk of excessive inflammatory and immunological responses which can have life-threatening consequences[262,278]. Different studies in baboons and mice have shown that blockage of FXIIa activity results in reduced hypotension, bacterial outgrowth and overall mortality after infection with normally lethal doses of *Escherichia coli* and *Klebsiella pneumoniae* respectively[262,275]. A beneficial role of FXIIa in sepsis therefore is assumed to be highly dependent on a tight regulation of the activation of the coagulation system as well as bradykinin formation[279].

1.4.3.3. FXII in Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a chronic and systemic inflammatory disease that predominantly occurs within the connective tissue of the joints[280]. Similar to sepsis, the course of the disease is highly variable depending on the degree of inflammation and can range from inflammation

of a single joint with minimal tissue damage to a state referred to as polyarthritis which is characterised by severe functional impairment of multiple joints and joint deformity[280]. Analysis of both tissue as well as synovial fluid, have shown that patients suffering from RA display increased enzymatic activity of FXIIa and kallikrein resulting in elevated bradykinin plasma levels[281]. Furthermore, levels of bradykinin present in the patient samples positively correlated with the degree of joint inflammation suggesting a causative role of the peptide but also FXIIa in the development of the disease[280–282].

1.4.3.4. FXII in Hereditary Angioedema

Another inflammatory disorder associated with dysregulated enzymatic activity of FXIIa is hereditary angioedema (HEA)[283]. In general, HEA can be divided into three separate types which are all characterised by episodes of acute swelling affecting multiple organs, including the upper airway carrying the risk of organ failure or asphyxiation respectively. While HEA type 1 and 2 are caused by a genetic defect in the C1INH gene resulting in increased FXIIa activity due to a lack of regulation by C1INH, HEA type 3 is associated with the presence of two separate amino acid substitutions at position 1302 (threonine to lysine or threonine to arginine) in the FXII gene. Exchange of the amino acid within the protein entails the loss of a glycosylation site leading to an increased potential of FXII to be activated[283,284,285]. Even though the genetic basis of the disease differs between type 1/2 and 3, the common focal point in all three types of the disorder lies in augmented FXIIa mediated bradykinin formation and therefore inflammation[283,286].

1.4.3.5. FXII in Anaphylaxis

In general, anaphylaxis is described as an immunological reaction in response to an encounter with an allergen and is characterised by a multitude of different symptoms such as rashes, shortness of breath, vascular leakage, swelling episodes and hypotension which in severe cases can lead to organ failure and death[287]. The most common allergens observed to trigger anaphylactic responses of different degrees include food such as nuts or dairy products, insect toxins or different components of medications[287]. The molecular mechanism of anaphylaxis is heavily based on the local recruitment of immunological cells such as mast cells and basophils which after activation secrete large quantities of cytokines causing extensive inflammation.

Interestingly, heparin which is released from mast cells alongside cytokines has been observed to initiate bradykinin generation by activation of FXII and the severity of the anaphylactic response positively correlated with overall mast cell degranulation as well as heparin, FXIIa and bradykinin levels found in plasma[288,289]. Consequently, inhibition of FXII activity has been found to significantly reduce vasodilation and therefore hypotension in anaphylactic patients and therefore could represent a novel treatment strategy to counteract abnormal mast cell activation[287,290].

1.4.3.6. FXII Mediated Cellular Responses

Even though its primary functions seem to be restricted to the initiation of coagulation and kallikrein-kinin driven bradykinin generation, FXII also has been found to directly interact with different cell types eliciting a variety of effector functions[232]. Different studies showed that the N-terminal region of the protease exhibits binding ability towards several receptors such as cluster of differentiation 39 (CD39) involved in cell-cell adhesion or urokinase-type plasminogen activator receptors (uPAR) mandatory for wound healing and cell movement[232,291,292]. Binding interactions have been observed to be highly dependent on elevated local Zn^{2+} concentrations which seem to facilitate the binding of FXII(a) to both endothelial cells as well as neutrophils[293]. In particular, binding of FXII to uPAR has raised scientific interest since interaction of the two molecules has been shown to trigger extracellular signal-regulated kinase (ERK) 1/2 phosphorylation in endothelial cells and neutrophils as well as accumulation of cyclic adenosine monophosphate (cAMP) in dendritic cells leading for example to the initiation of protein transcription or rearrangement of the cell cytoskeleton[294]. Furthermore, FXIIa has been found to be capable of interacting with glycosaminoglycans (GAGs) such as heparin sulphate present on the surface of most cells and therefore potentially is involved in the modulation of a wide range of biological functions including angiogenesis, blood coagulation and formation of metastases[295]. Interestingly, binding of FXIIa to heparin sulphate seems to be directed by the overall distribution and density of the polysaccharide on the cell surface allowing local regulation and sequestration of FXIIa mediated cellular responses. In addition, local composition of GAGs has been observed to be subjected to significant changes and reorganisation under pathological conditions which could explain differential activity of FXIIa under normal physiological conditions compared to different disease states[214,223,232,295].

1.4.3.7. Mitogenic Function of FXII

Deregulated formation of new blood vessels facilitating uncontrolled cell growth is considered as one of the main problems during the formation of metastatic tumours in different kind of cancers[296]. Even though the direct influence of FXII(a) on the development of pathological conditions dependent on cell proliferation has not been well examined, different studies have shown a link between abnormal cell growth and mitogenic activity of FXII[297]. For example, FXII and FXIIa both have been observed to induce proliferation of human umbilical vein endothelial cells (HUVEC) by binding of the protease to the uPAR and ERK 1/2 receptors resulting in the subsequent initiation of intracellular signalling events[298]. Furthermore, Matrigel tube formation assays showed outgrowth of new blood vessel after exposure of wild-type mouse endothelial cells to FXII but lack of vessel formation in cells extracted from uPAR deficient mice[298]. In addition, analysis of biopsies taken from FXII deficient mice revealed a significantly reduced number of blood vessels compared to wild-type mice indicating a potential role of FXII(a) in angiogenesis[232,298]. This is reinforced by the observation of elevated uPAR and CD39 receptor expression in tumour cells, increased FXII mRNA levels in epithelial ovarian cancer cells and an augmented invasive potential of ovarian cancer cells after their exposure to FXII[299]. Blockage of FXII function therefore might be a potential target for the treatment of cancer and should be further explored.

1.4.3.8. FXII as a Modulator of Inflammatory Responses

While the role of FXII in both coagulation and generation of bradykinin via the kallikrein-kinin pathway is entirely dependent on the activation of the protease, zymogen FXII has been observed to interact with a variety of different immunological cells[232,234]. So far, the subset of cells capable of being stimulated by FXII comprise neutrophils, monocytes, macrophages and dendritic cells[234]. Like the cells affected, the effector function of FXII(a) on the previously listed cell types is widespread and includes the induction of cell aggregation, release of different inflammatory mediators as well as morphological changes in order to facilitate cell movement (Figure 1.18)[232].

Other studies showed that exposure of neutrophils to FXII results in a rapid aggregation reaction followed by the release of lactoferrin and elastase which both exhibit protease

function and are involved in the host defence against bacterial and viral infection[300]. In addition, interaction of FXIIa with neutrophils has been observed to influence cellular movement including enhanced migratory potential and cell adhesion as well as the release of NETs involved in the neutralisation of invading pathogens[232,300].

Binding of FXII to monocytes has been found to lead to a significant reduction in Fc receptor expression diminishing the cells ability to interact with antibodies circulating the blood as well as an increase in the cytokine IL-1 generation characteristic for inflammatory processes. In addition, FXIIa stimulates the secretion of the cytokines interleukin 1, 4, 8 and 10 (IL-1, IL-2, IL-8 and IL-10) as well as TGF-β which is regulates cell growth and proliferation[232,301].

While binding of FXIIa to monocytes triggers the release of IL-1, interaction of the protease with dendritic cells results in augmented expression of interleukin 6 and 23 (IL-6 and IL-23) which both act as inflammatory mediators and additionally are thought to influence the differentiation of naïve T-helper cells to effector memory T-cells (Th17). Consequently, studies using FXII deficient mice showed that absence of FXII positively correlated with reduced Th17 cells numbers and Th17 mediated neuroinflammation[232,302].

FXIIa	**Macrophages**	Increased secretion of IL-4, IL-8, IL-10 and TGF-β
	Blood Monocytes	Increased secretion of IL-1, IL-4, IL-8, IL-10 and TGF-β
	Neutrophils	Enhanced adhesion, migration and NET release
FXII(a)	**Splenic Dendritic Cells**	Increased secretion of IL-6 and IL-23; reduced expression of IL-10, IL-12 and TGF-β
	Bone Marrow Driven Macrophages	Increased secretion of IL-6, IL-12 and TNF-α
FXII	**Neutrophils**	Cell aggregation and degranulation
	Blood Monocytes	Reduced Fc receptor expression; increased IL-1 expression

Figure 1.18: Regulatory functions of FXII and FXIIa on cells of the inflammatory system. Both FXII zymogen as well as the active protease FXIIa have been found to regulate properties of a variety of immunological cells. Depending on the target cell, effector function include increased production and secretion inflammatory regulators such as interleukins or growth factors and modulation of cell-cell as well as cell-extracellular matrix interaction. IL = interleukin; TGF = transforming growth factor; NET = neutrophil extracellular trap; TNF = tumor necrosis factor (adapted from [232]).

Similar to dendritic cells, contact of macrophages with FXII leads to the production of interleukin 12 (IL-12) and IL-6 as well as tumor necrosis factor α (TNF-α) which is another growth factor involved in cell growth and differentiation. Exposure of macrophages to FXIIa however results in the secretion of interleukin 4 (IL-4), IL-8, IL-10 and TGF-β which as discussed previously regulate inflammatory responses and cellular growth respectively[303,304].

1.4.3.9. FXII and Cell Movement

Movement of cells represents a complex interplay between cells and the extracellular matrix as well as localised proteolysis and extensive rearrangement of the cytoskeleton in order to facilitate cell migration[305]. As discussed earlier, FXIIa serves as ligand for uPAR which is associated with the coordination of several aspects important for overall cell movement. For example, uPAR has been observed to facilitate localised activation of the extracellular matrix degrading protease plasmin via urokinase interaction as well as the communication between cells and the extracellular matrix through binding towards different integrins subsequent to its activation by FXIIa[214,293,298]. In addition, a wide range of processes important for cell movement and migration are dependent on chemotactic signals exhibited by different cytokines such as IL-6, IL-8 or TGF-β. As mentioned above, both expression and secretion of these cytokines by immunological cells at least partly seems to be dependent on the interaction of FXII(a) with cells of the immune system indicating a secondary role of FXII in the process of cell movement[232,304].

1.4.3.10. FXII Sheddase Function

In general, sheddases are membrane associated enzymes capable of cleaving extracellular parts of proteins incorporated into the cell surface. Proteolytic cleavage of the transmembrane proteins usually results in either a conformational change of the cleaved protein initiating its activation or the release of small peptides which subsequently can unfold their agonistic or antagonistic function on other cells by binding to their respective receptors[306]. Recent findings indicate that FXIIa might exhibit sheddase function under certain circumstances as the protease has been found to enzymatically cleave the low-density lipoprotein receptor-related protein 1 (LRP1) which is involved in the catabolism of different proteases, lipoproteins and growth factors[307]. Enzymatic processing of LRP1 by FXIIa has been

observed on a variety of different cell surfaces and requires local activation of the FXII zymogen directly on the cell surface. Interestingly, elevated LRP1 shedding has been heavily associated with the development of different pathological conditions such as RA, atherosclerosis, Alzheimer's disease and cancer which all are characterised by abnormal plasma levels or deregulated function of either FXII or FXIIa further adding to the list of diseases that potentially are influenced by FXII activation and function[232,307].

1.4.3.11. FXII and the Immune System

Apart from supporting the activation and mediation of different response mechanism exhibited by a variety of immunological cell types, FXIIa also is capable of interacting directly with the immune system via the classical complement pathway[308]. In general, the complement system serves to support immunological cells in their ability to interact with antibodies, promote inflammatory responses and facilitate neutralisation and clearance of pathogens. The complement system comprises a number of small proteins which, similar to the proteases involved in coagulation, circulate the blood in an inactive state. Activation of complement can occur through three different pathways referred to as the alternative, the lectin or the classical pathway. Activation of the classical pathway normally is triggered by the interaction of an antigen-antibody pair with a protein complex called complement component 1 (C1) which subsequently triggers sequential activation of several proteases culminating in the formation of a membrane attack complex responsible for the lysis of pathogenic cells[309]. Interestingly, FXIIa has been found to be able to bind and activate C1 in a non-immunologic way and has been suggested to support pathogenic cell lysis at sites of infection after activation via bacterial lipopolysaccharides or polyphosphates[232,308,310].

1.4.3.12. FXII in Haemostasis and Thrombosis

Even though the overall role of FXII can be described as elusive, proteolytic activity of FXIIa in the process of coagulation is well recognised and builds the basis of one of the most important routine tests (partial thromboplastin time (PPT)) currently used for the detection of abnormalities in blood clotting[35,220,224]. In general, PPT tests measure the time blood needs in order to form blood clots and rely on the activation of the contact pathway which is initiated by the conversion of FXII to FXIIa. Subsequent to the generation of FXIIa, the protease cleaves

inactive FXI to active FXI which in turn activates FIX. FIXa then initiates FX activation ultimately leading to the generation of fibrin strands and the formation of stable blood clots[311,312].

Fibrinolysis is an important component of normal haemostasis and describes the degradation of previously formed fibrin fibres in order to facilitate wound healing. As already discussed in chapter 1.1.3.2., breakdown of fibrin is mediated by the protease plasmin which is incorporated into the growing blood clot in its zymogenic form plasminogen. In order to exhibit its degrading function, plasminogen has to be cleaved by tPA and subsequently undergoes a conformational change resulting in its activation[313]. Interestingly, FXIIa has been found to be involved in the regulation of the fibrinolytic pathway through either direct or indirect interaction with plasminogen[314,315]. Biochemical analysis of FXIIa showed that the protease has a high homology to tPa which is considered as the main activator of plasmin and is capable to directly convert plasminogen to plasmin resulting in the initiation of fibrin degradation[224,315]. In addition, activation of plasmin on the cells surface has been shown to be mediated by the release of tPA following stimulation of predominantly endothelial cells with bradykinin which in turn is regulated by the FXIIa activity via cleavage of kallikrein[232,272]. Aside from tPA, plasmin activation also can be mediated by proteolytic activity of kallikrein. As already discussed previously, kallikrein activity is heavily dependent on its interaction with FXIIa which is responsible for the cleavage of inactive prekallikrein to active kallikrein therefore indirectly driving activation of plasmin via kallikrein.

Despite the fact that FXIIa is capable of triggering coagulation events *in vitro*, its importance for physiologically occurring clotting mechanism has been questioned due to the fact that the generation of clots *in vivo* primarily seems to be dependent on the interaction of TF with FVIIa and subsequent activation of FX[35,220]. In addition, individuals with a FXII deficiency do not present a bleeding phenotype, further challenging the relevance of FXII in the process of coagulation[224,316]. Interestingly, several studies conducted over the last fifty years have given support to the idea that absence of FXII in fact might have a thromboprotective effect[317,318]. FXII deficient mice for example have been shown to develop blood clots with significantly reduced size and stability after mechanical, chemical or vascular injury and additionally were protected from pulmonary embolism indicating a severe defect in the generation of occlusive thrombi[114,319,320]. An additional study addressed the question to what extent FXIIa function must be inhibited in order to achieve protection against the formation of thrombotic clots

after vascular injury or blood flow restriction. Reconstitution experiments in heterozygous FXII deficient mouse models as well as studies using FXII antisense nucleotides with the aim to gradually silence the proteolytic function of the protease showed that a reduction in FXII plasma levels of at least 75% FXII is necessary in order to establish a protective effect against unwanted thrombus formation[321].

Based on these results, studies using FXII deficient mice in ischemic stroke models showed decreased lesion volumes due to reduced fibrin formation in the microvasculature of the ischemic tissue[322,323,114]. The fact that FXII predominantly seems to be involved in fibrin dependent clot formation raised the question whether the contribution of the protease to overall coagulation events might only be relevant in pathological settings[224,324,325]. Pharmacological studies inhibiting FXII activity using peptide-based inhibitors or antibodies further supported this assumption in experimental stroke mouse models in which reductions in FXIIa plasma levels positively correlated with an improved reconstitution of motoric and cognitive function[114,317,320,322,326–328]. Simultaneously, cumulative results of all studies examining the role of FXIIa in the formation of pathological thrombi showed a complete absence of bleeding events suggesting that targeting FXIIa function might be an attractive option in order to prevent or treat cases of pathological thrombosis without compromising normal haemostatic function[114,316,320,329].

1.5. Interaction between VWF and FXII

Even though primary and secondary haemostasis have differential roles in coagulation, both mechanisms aim at the maintenance of a functional circulatory system and the formation of stable blood clots preventing excessive blood loss in case of vascular injury[38]. While primary haemostasis concerns the formation of platelet rich haemostatic plugs, secondary haemostasis involves the sequential activation of a series of serine proteases culminating in the generation of fibrin stabilising the previously formed platelet plugs[23,35]. Over the last few decades, a multitude of molecules linking processes occurring in either primary or secondary haemostasis have been identified supporting the claim that a finely interwoven and co-dependent relationship of these two mechanisms is mandatory for normal haemostatic function[4,35].

Preliminary data obtained in our laboratories indicating a binding interaction between VWF and FXII establish yet another relationship between two distinct proteins involved in primary and secondary haemostasis.

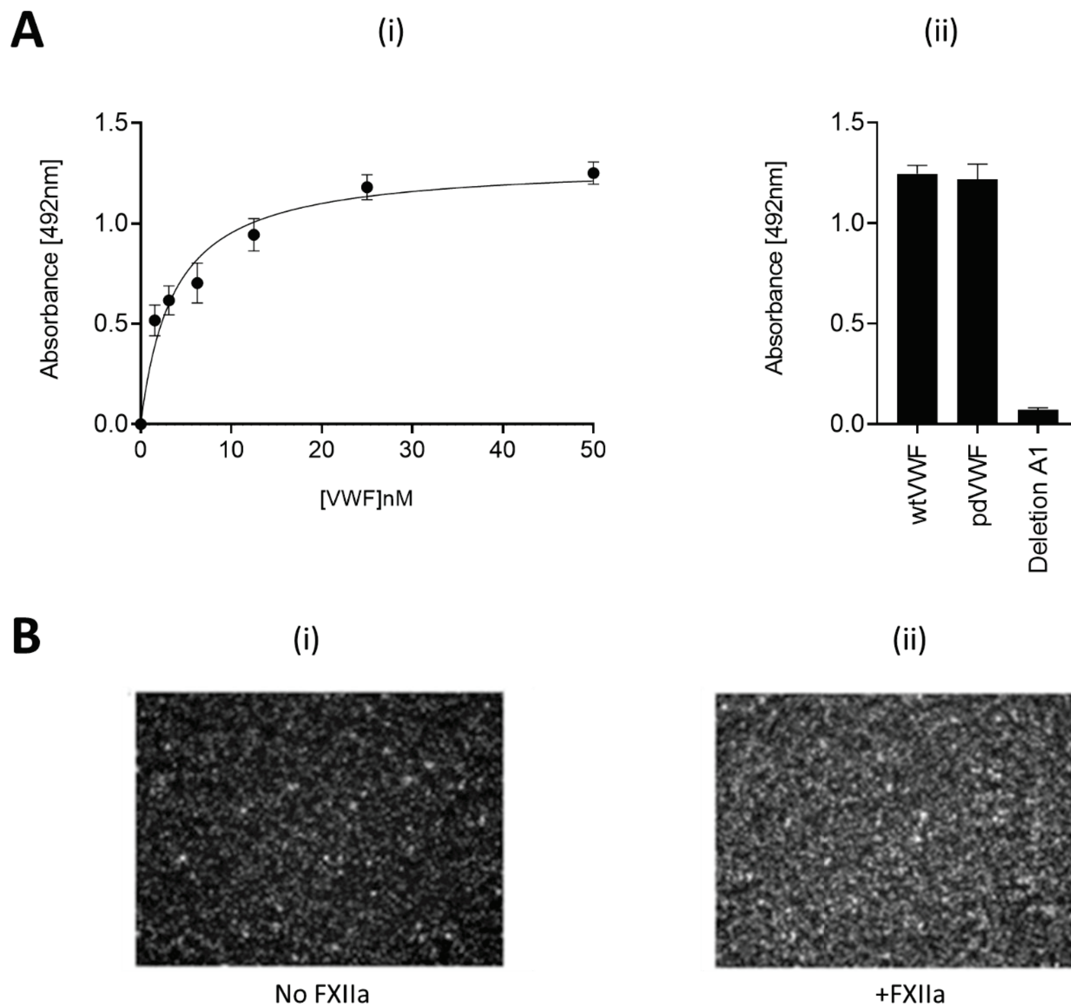

Figure 1.19: FXIIa binds to VWF and enhances platelet capture to VWF under shear stress. A) Static plate binding assay: (Ai) α FXIIa was immobilised on microtiter plates and incubated with increasing concentrations of purified plasma derived VWF. Bound VWF was detected with anti-VWF-HRP antibodies. Data were fitted to the one-site binding equation using Prism software showing that VWF binds to α-FXIIa with high affinity (KD,app 4nM). (Aii). Immobilised α-FXIIa was incubated with either purified wild-type recombinant VWF, plasma-derived VWF or VWF deletion A1 lacking the VWF A1 domain. Deletion of the A1 abolished binding to α-FXIIa. B) Flow assay: Plasma free blood containing labelled platelets were perfused over purified VWF immobilised on ibidi slides in the in the absence (B-i) or presence (B-ii) of 10μg/ml FXIIa. Evaluation of overall platelet capture showed increased VWF mediated platelet arrest in the presence of FXIIa. VWF = Von Willebrand Factor; F = Factor; pd = plasma derived; nM = nanomolar; Abs = absorbance: nm = nanometer; wt = wildtype. Data adapted from FXII grant proposal[330].

Static plate based binding assays showed direct binding of plasma derived VWF to immobilised α-FXIIa in a concentration dependent manner. Further analysis of the binding interaction with

a VWF mutant lacking the VWF A1 (VWFΔA1) domain suggested the existence of a FXII binding site within the VWF A1 domain. Flow based binding assays with immobilised VWF and plasma free blood in the absence and presence of FXIIa additionally revealed that interaction of the proteins positively correlates with augmented VWF driven platelet capture (Figure 1.19)[330]. In addition, perfusion assays using collagen coated microfluid chambers indicated direct binding and colocalisation of FXIIa to VWF fibres under pathological shear conditions, supporting the idea that FXIIa mediated fibrin generation can be initiated by the formation of a complex between VWF and FXII. Interestingly, fibrin formation was significantly reduced or absent when i) initial binding of VWF to collagen was disrupted using an anti-VWF antibody ii) FXIIa function was inhibited by addition of corn trypsin inhibitor (CTI) and iii) plasma VWF was substituted by VWFΔA1 lacking the proposed FXII binding site. Together these observations led to the hypothesis that the formation of thrombi might be driven by an interplay between FXII and VWF which could be of therapeutic relevance especially under pathological conditions[330].

1.6. Hypothesis and Aims of the Study

Even though FXII is a previously unknown binding partner of VWF, the two molecules have several points of overlap in their physiology (Figure 1.20). VWF is essential for the capture of platelets at sites of endothelial injury by binding to collagen present in the subendothelial matrix[32].

At the same time, collagen also has been reported to be an activator of FXII and has been observed to mediate conversion of FXII zymogen into proteolytically active FXIIa[225]. Upon binding to collagen and due to the shear forces to which VWF is subjected, the globular glycoprotein unravels revealing its platelet binding site present in the VWF A1 domain effectively initiating arrest of platelets from circulation[32]. Based on the data obtained in our lab, unravelling of VWF and exposure of the VWF A1 domain also would facilitate direct binding of FXII to the VWF fibres since the FXII binding site has been proposed to be located in the VWFA1 domain[330]. Arrest of platelets by VWF ultimately results in their activation representing another link between VWF and FXII since polyphosphates secreted by activated

platelets are considered as one of the most potent physiological activators of the protease[29,149,233].

Based on the literature review and the preliminary data proposing an interaction between VWF and FXII, I hypothesise that binding of VWF towards FXII is likely to be of significance for the formation of thrombosis as well as the initiation of coagulation and therefore provides a novel link between primary and secondary haemostasis. Taking into consideration that FXII itself seems to play a role in pathological thrombus formation and that a FXII deficiency does not seem to compromise haemostatic capabilities, the FXII/VWF complex might be a novel target for a combined anti-platelet and anti-coagulant therapy. Furthermore, modulation of this novel binding interaction additionally might present a new avenue for the prevention of thrombotic events for example observed after implantation of medical devices without increasing the risk of bleeding complications[330].

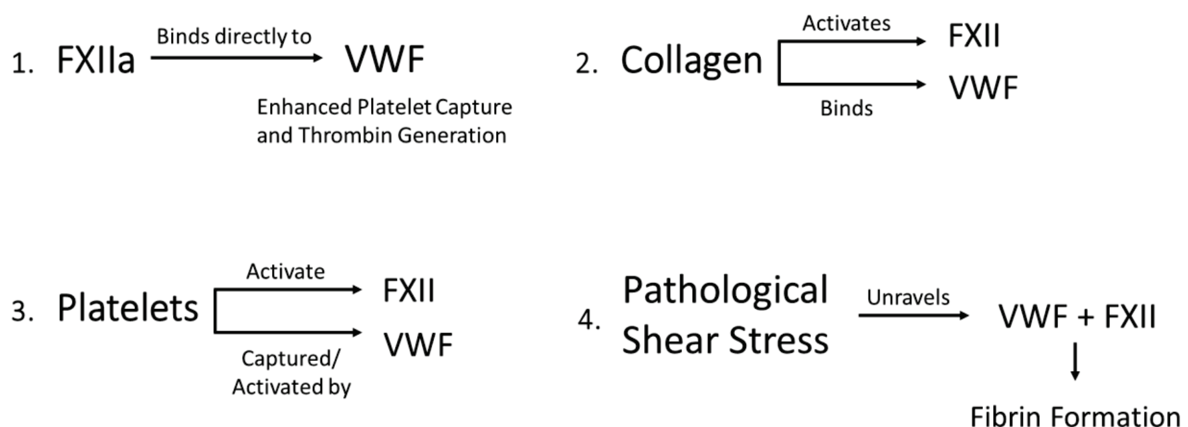

Figure 1.20: Overview of the relationship between VWF and FXII. 1) Preliminary data obtained in our laboratory showed that VWF is able to directly bind to FXIIa in static plate binding assays. Flow based binding assay using immobilised VWF and plasma free blood showed enhanced VWF mediated platelet capture in the presence of FXIIa. 2.) Binding of VWF to subendothelial matrix collagen is mandatory in order to facilitate VWF mediated arrest of platelets from the circulations. At the same time, collagen has been reported to be capable of initiating conversion of zymogen FXII to active FXIIa. 3.) Binding of platelets to VWF ultimately results in their activation. Activated platelets have been observed to secrete polyphosphates which are considered as one of the most potent physiological activators of FXII. 4.) Haemostatic function of VWF is dependent on a conformational change of the protein. Subjection of VWF to high shear as for example encountered under pathological conditions results in the linearisation of the glycoprotein exposing its platelet binding as well as its proposed FXIIa binding site present in the A1 domain, leading to fibrin formation. VWF = Von Willebrand Factor; F = Factor.

The aim of this project is the characterisation of the binding interaction between VWF and FXIIa and the functional effect of this interaction on both proteins. In addition, the project

intends to investigate how binding between VWF and FXIIa affects pathological thrombus formation and whether blockage of the protein complex formation could be translated into a new and safe anti-coagulant.

The project was designed in order to address the following research objectives using the experimental approach described below.

1) **Verification of a binding interaction between VWF and FXII**

   A general binding interaction between full-length VWF and FXIIa, FXII and FXII activated with its physiological activator kallikrein will be analysed in static plate binding assays. In addition, data summarised in the FXII-VWF grant proposal suggested the presence of a FXII binding site in the VWF A1 domain which will be further studied using static plate binding assays using VWF proteins lacking the A1 domain (VWFΔA1) or the isolated VWF A1 domain respectively.

2) **Creation of a panel of VWF mutants for the mapping of the FXII binding site in the VWF protein**

   In order to map the FXII specific binding site in VWF, I will generate a panel of VWF A1 domain variants by mutating negatively charged residues in the α1 and α6 helices of VWF A1 to alanine. Mutations will be introduced in both the isolated VWF A1 domain as well as the full-length VWF protein in order to assess the role of the respective amino acids in the binding interaction between VWF and FXII.

3) **Identification of specific amino acid residues involved in the interaction between VWF and FXII**

   The effect of the introduced mutations on the VWF-FXII interaction subsequently will be assessed in static plate binding assays as well as surface plasmon resonance measurements. Binding of the different VWF mutants to FXIIa, FXII and FXII activated with its physiological activator kallikrein will be tested in order to identify amino acids responsible for the binding interaction between VWF and FXII(a).

4) **Analysis of FXII activation by different activators as well as VWF**

   A wide range of potential FXII activators has been reported in the literature (see chapter 1.4.2.1 and 1.4.2.2.). Activation of FXII zymogen in the presence of these activators will be tested in a substrate-based activation assay in order to confirm the activation potential

of the activators suggested. In addition, binding interaction between VWF and FXII(a) might result in the conversion of FXII zymogen to proteolytically active FXII which will be analysed in FXII activation assays alongside confirmed FXII activators.

5) **Analysis of the physiological relevance of the interaction between VWF and FXII(a)**

For the assessment of the physiological relevance as well as the potential therapeutic effect of the binding interaction between VWF and FXII(a), functional static plate-based and perfusion assays will be developed and carried out. Overall clot stability will be analysed in a thrombolysis assay specifically adapted to investigate the effect of inhibition of the FXIIa/VWF interaction on clot lysis times using either full-length VWF wildtype or full-length VWF mutants. In addition, collagen dependent perfusion assays mimicking thrombus formation and growth under pathological shear conditions will allow the characterisation of a potential therapeutic effect in relation to the disruption of the interaction between FXII-VWF.

2. Methodology

A schematic overview of the experimental methods employed during this project is shown in the following Figure 2.1.

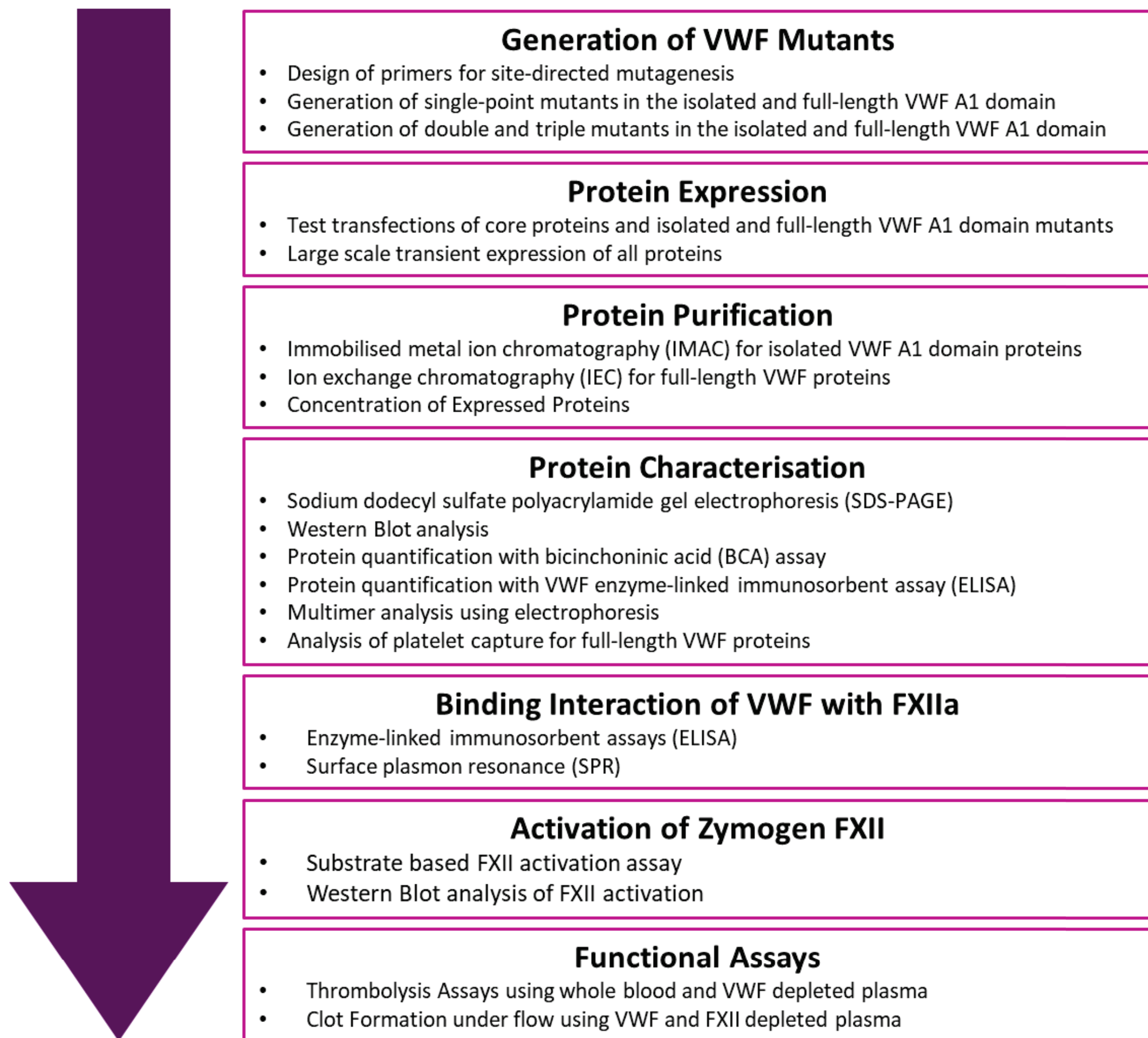

Figure 2.1: Overview of the experimental methodology used in this project.

2.1. Cell Culture

All tissue culture was performed in a class II flow cabinet (FASTER BHG 2004; JENCONS-PLS) in a separate laboratory specifically designated for tissue culture. While handling the cells aseptic technique was used at all times. Cells were grown and maintained in humidified incubators (BIOHIT; Biological Instrumentation Service; Lancashire) at 37°C and 5% CO_2 and were split when a confluency of 70-80% was reached. Culturing of cells was performed in minimum essential medium (MEM*; GIBCO*) supplemented with 10% fetal bovine serum (FBS; *Invitrogen*), 2mM L-glutamine (*Invitrogen*), 1U/ml penicillin and 0.1mg/ml streptomycin (*Invitrogen).* Cells were stored in 10% dimethyl sulfoxide (DMSO; *Thermo Fisher Scientific*) and 90% fetal bovine serum (FBS; *Invitrogen*) at -80°C (*New Brunswick Scientific*). Cultured cells were visualised under an inverted microscope CK2 (*Olypmus*).

2.1.1. Passage and Maintenance of Cells

For the expression of VWF proteins and VWF mutants, human embryonic kidney 293T (HEK293T) cells were used. Cells were maintained in MEM growth medium and expanded into T75 flasks (culture volume of 10 ml; *GelStar*) or T175 flasks (culture volume of 20ml; *GelStar*) once they had reached a confluency between 80 to 100%. For passaging, cells were washed twice with 10ml sterile phosphate buffered saline (PBS; 10mM phosphate buffer, 2.7mM potassium chloride, 137mM sodium chloride; *SIGMA*) and subsequently split 1:3 using 1x TripLE Express (0.5ml TriplE per T25 flask; 1ml TriplE per T75flask and 2ml TriplE per T175 flask; *GIBCO*). After detachment of the cells at 37°C for five minutes, trypsin activity was neutralised by the addition of 5-20ml MEM growth medium and cells were seeded appropriately.

2.1.2. Cell Revival and Cryopreservation

For their revival, cells were thawed at 37°C and subsequently added to a T25 flask (*GelStar*) containing 5ml of MEM growth media. Cells were grown at 37°C and 5% CO_2. Once cells had reached a confluency of 80 to 100%, cells were passaged as described above.

Cells were cryopreserved at -80°C. When confluent, cells were washed three times with sterile PBS and detached as described above. Cells were diluted in 10 ml MEM growth medium and

centrifuged at 400g for 5 minutes at room temperature. After discarding the supernatant, the cell pellet was resuspended in 1ml FBS supplemented with 10% dimethyl sulfoxide (DMSO; *Sigma*) per T175 flask. Resuspended cells were aliquoted in 1ml cryo-vials and stored in a cryo-freezing container (*Nalgene*) at -80°C for 24 hours before being transferred to a storage box at -80°C.

2.2. Generation of VWF Mutants

2.2.1. Cloning and Expression Vectors

Site-directed mutagenesis was carried out in the isolated VWF A1 domain present in the pcDNA3.1/Myc-His (A+) expression vector (Figure 2.2). In order to create VWF A1 domain mutants in full-length VWF, initial mutagenesis was carried out using pGEM-7Zf(+) cloning vector (Figure 2.2) containing the isolated VWF A1 domain. Mutated VWF A1 genes subsequently were sub-cloned into the pcDNA3.1/Myc-His (A+) expression vector.

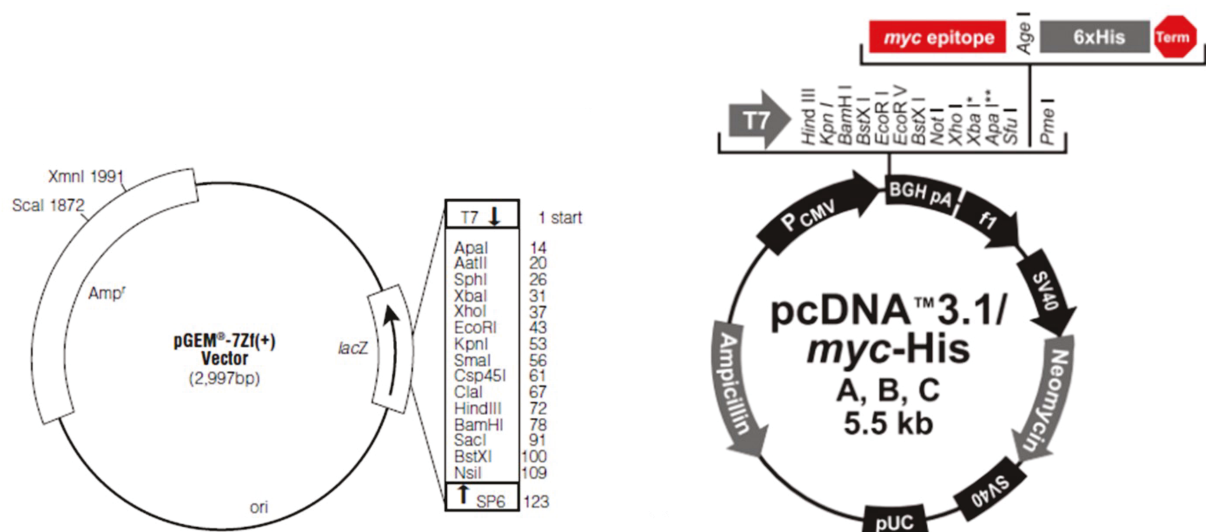

Figure 2.2: Vector maps of the pGEM-7Zf(+) cloning and the pcDNA3.1/Myc-His (A+) expression vector.

The pGEM-7Zf(+) (*Promega*) is a 3kb, high copy cloning vector. The vector contains a multiple cloning site (MCS), T7 and T3 RNA polymerase promoter sequences in the N-terminal portion of a lacZ fragment (Figure 2.2). A β-galactosidase coding sequence is interrupted by the large polylinker (MCS) which allows positive selection of bacterial colonies containing the desired

insert based on lacZ disruption and blue phenotype of the formed colonies. The vector additionally contains the pUC origin which enables high-copy number replication and growth in *E.coli* and an ampicillin resistance gene allowing antibiotic-based selection.

The pcDNA3.1 (*Invitrogen*) is a 5.5kb vector containing a human cytomegalovirus (CMV) and immediate-early promoter/enhancer that permits efficient and high-level gene expression in mammalian cells. A multiple cloning site allows cloning using different restriction enzymes and the pUC origin facilitates high-number replication and growth in *E.coli.* Furthermore, the vector contains a SV40 early promoter and origin for episomal replication in cells expressing SV40 large T antigen as well as ampicillin and neomycin resistance genes for selection purposes (Figure 2.2). The pcDNA3.1 vector used in this project also contains a myc epitope for immunodetection and a 6xHis C-terminal tag to allow efficient nickel-based protein purification (not needed for expression of full-length VWF protein and mutants).

2.2.2. Design of Primers for Site-directed Mutagenesis

Negatively charged amino acids present in the VWF A1 domain were mapped in silico (software PyMOL based on the crystal structure of the VWF A1 domain; PDB: 1auq) and surface exposed negatively charged amino acids were identified for mutation. In total, 12 amino acids were selected for single point site-directed mutagenesis in order to change aspartic and glutamic acid (negative charge) to alanine (neutral charge). In addition, three clones carrying double point mutations as well as one clone carrying a triple mutation were created in both recombinant full-length VWF wildtype and the isolated VWF A1 domain. The following table (Table 2.1) depicts a list of the primers specifically designed in order to carry out site-directed mutagenesis (*Eurofins*).

Clone	Name	Orientation	Sequence
E1290A	S1M1F	Forward	5`-AGGCTGTCCGCGGCTGAGTTT-3`
	S1M1R	Reverse	5`-GGAGGAGCCATCCAGCAG-3`
E1292A	A2P528F	Forward	5`-CCGCGGCTGCGTTTGAAGTGCTGAAGG-3`
	A2P528R	Reverse	5`-GCACTTCAAACGCAGCCTCGGACAGCC-3`
E1294A	S1M3F	Forward	5`-GCTGAGGCTGCGTTTGAAGTGC-3`
	S1M3R	Reverse	5`-CTCGGACAGCCTGGAGGA-3`
D1302A	A2P538F	Forward	5`-GTGGTGGCCATGATGGAGCGG-3`
	A2P538R	Reverse	5`-CTCCATCATGGCCACCACAAAGGCC-3`
E1305A	A2P541F	Forward	5`-GGACATGATGGCGCGGCTGCGC-3`
	A2P541R	Reverse	5`-CAGCCGCGCCATCATGTCCACC-3`
E1339A	A1P575F	Forward	5`-CGACCATCAGCGCTGCGGCGC-3`
	A1P575R	Reverse	5`-CCGCAGCGCTGATGGTCGCTTCCG-3`
D1444A	A6P680F	Forward	5`-GCAGTGTGGCTGAGCTGGAGCAGC-3`
	A6P680R	Reverse	5`-CCAGCTCAGCCACACTGCTCAGC-3`
E1445A	A6P681F	Forward	5`-GTGGATGCGCTGGAGCAGCAAAGGG-3`
	A6P681R	Reverse	5`-GCTCCAGCGCTACCACACTGCCT-3`
E1447A	S2M2F	Forward	5`-GATGAGCTGGCGCAGCAAAGG-3`
	S2M2R	Reverse	5`-CACACTGCTCAGCACGAA-3`
D1451A	A6P687F	Forward	5`-GCAAAGGGCCGAGATCGTTAGCTACC-3`
	A6P687R	Reverse	5`-CGATCTCGGCCCTTTGCTGCTCC-3`
E1452A	A6P688F	Forward	5`-GCAAAGGGACGCGATCGTTAGCTACC-3`
	A6P688R	Reverse	5`-GCTAACGATCGCGTCCCTTTGCTGC-3`
D1459A	A6P695F	Forward	5`-CCTCTGTGCCCTTGCCCCTGAAGC-3`
	A6P695R	Reverse	5`-GGGGCAAGGGCACAGAGGTAGC-3`
D1444/1445A	DouA6P681F	Forward	5`-GCAGTGTGGCTGCGCTGGAGCAGC-3`
	DouA6P681R	Reverse	5`-GCTCCAGCGCAGCCACACTGCTCAGC-3`
D1444/1447A	DouA6P683F	Forward	5`-GGCTGAGCTGGCGCAGCAAAGGG-3`
	DouA6P683R	Reverse	5`-CCCTTTGCTGCGCCAGCTCAGCC-3`
E1452/1459A	A6P688F + A6P695F	Forward	see above
	A6P688R + A6P695R	Reverse	see above
D1444/1445/1447A	DouA6P681F + S2M2F	Forward	see above
	DouA6P681R + S2M2F	Reverse	see above

Table 2.1: Overview of primer sequences. Specifically designed primers were used for site-directed mutagenesis in order to create VWF A1 domain mutants for the identification of the FXIIa binding site in VWF.

2.2.3. Site-directed Mutagenesis in the VWF A1 Domain

Mutations in the VWF A1 domain were introduced via polymerase chain reaction (PCR) using specifically designed primer pairs. PCR contained 1µg DNA (isolated VWF A1 domain in either

pcDNA3.1/Myc-His (A+) expression vector or pGEM-7Zf(+) cloning vector), a final concentration of 1µM of the customized forward and reverse primer (*Thermo Fisher Scientific*), 5µl PCR buffer (HiFi App Polymerase; *Appleton Woods*), 0.5µl HiFi Polymerase (2 U/µl; HiFi App Polymerase; *Appleton Woods*) and sterile water in 25µl reactions. PCR amplification consisted of an initial denaturation (95°C for 3 minute) and 18 cycles of i) denaturation (95°C for 45 seconds) ii) annealing (60°C for 45 seconds) iii) extension (68 to 76°C for 5 minutes) iv) final extension (68°C for 5 minutes) and v) storage (4°C). After PCR, samples were digested with 10U of DpnI (*New England BioLabs*) at 37°C for 1 hour in order to remove the methylated template DNA. In case of isolated VWF A1 domain mutants, mutagenesis reactions subsequently were transformed into bacterial cells described in section 2.2.9. In order to create full-length VWF A1 domain mutants, mutagenesis reactions were subjected to restriction digestion for sub-cloning into the pcDNA3.1/Myc-His (A+) expression vector.

Introduction of double or triple mutations was carried out using sequential site-directed mutagenesis with varying template DNA based on the protocol described above. Between each mutation step, vectors were isolated and sequenced as described in section 2.2.11 and 2.2.12. For the introduction of double mutations at position D1444/1445A and D1444/1447A, vector DNA containing the single point mutation at position 1444 was processed with the primers indicated in table 1 resulting in the incorporation of a second point mutation at position 1445 or 1447 respectively. Generation of triple mutant D1444/1445/1447A was achieved by PCR of the template DNA containing a single point mutation at positions 1444 with the relevant primers (Table 1). Double mutant E1452/1459A was generated by PCR of template DNA containing a single point mutation at position 1452 with the relevant primers (table 1) introducing a second mutation at position 1459.

2.2.4. Restriction Endonuclease Digestion of Plasmid DNA

In order to create full-length VWF mutants, pGEM-7Zf(+) cloning vectors containing isolated VWF A1 domain mutants as well as the target pcDNA3.1/Myc-His (A+) expression vector were subjected to digestion by endonuclease restriction enzymes. Restriction reactions consisted of 5µg DNA, 1µl BamHI and KpnI (20000U/µl for BamHI and 10000U/ml for KpnI; *NEB*), 3 µl smart cut buffer (*NEB*) and sterile water in 30 µl reactions. Restriction digestion was carried

out at 37°C for 1 hour and digestion products were analysed and separated with agarose gel electrophoresis.

2.2.5. Agarose Gel Electrophoresis

Horizontal gel electrophoresis was used in order to separate DNA molecules according to their molecular size (methodology according to Sambrook *et al*; 1989). In order to prepare agarose gels, 0.8g agarose powder was mixed with Tris-borate-ethylenediaminetetraacetic acid (EDTA) buffer (40mM Tris base; 40nM boric acid; 1 mM EDTA; pH8.0) in a final volume of 100ml. The solution was heated in a microwave until the powder was completely melted and SYBR™ (final concentration of 5µg/ml) was added in order to allow visualisation of the DNA after electrophoresis. Gels were poured and allowed to polymerise for 30 minutes at room temperature (RT). Samples were mixed with 6X gel loading buffer (30% (w/v) sucrose in deionised water containing 0.1% bromphenol blue; *Novex*) and subsequently loaded into the wells. Electrophoresis was carried out for approximately 1 hour at a constant voltage of 100V. DNA ladder molecular weight markers (CozyHI Prestained Protein Ladder; *highQu*) were run alongside the samples in order to determine the molecular size of the DNA fragments. Gels were examined on a Bio-Rad FluorS machine equipped with a UV filter to facilitate DNA visualisation.

2.2.6. Gel Extraction

After gel electrophoresis, DNA fragments of interest were excised from the agarose gels and purified using the QIAquick gel extraction kit (*Qiagen*) according to the manufacturer's instructions. Bands of interest were cut out of the gel using a clean razor blade, weighed and solubilised with 3 volumes of buffer QB at 50°C. Subsequently, 1 volume of isopropanol was added and the samples were transferred to silica-gel columns. Samples were centrifuged at 1400g for 1 minute and 0.5ml Buffer QG was added after discarding the supernatant. Samples were centrifuged at 1400g for 1 minute and 0.75ml Buffer PE was added to the samples after discarding the supernatant. Columns were centrifuged twice for 1 minute at 1400g before the DNA was eluted by addition of 30µl Buffer EB and centrifugation at 1400g for 1 minute.

2.2.7. Ligation of Plasmid Vector and Insert DNA

Ligation reactions were carried out after restriction digestion and gel purification of both pGEM-7Zf(+) cloning vector containing isolated VWF A1 domain mutants as well as the target pcDNA3.1/Myc-His (A+) expression vector. The ligation mixture contained vector and insert DNA at a ratio of 1:3, 5µl T4 DNA ligase buffer (*NEB*), 1µl T4 DNA ligase (*NEB*) and sterile water in a final volume of 20µl. Ligation was carried out for 1 hour at 37°C and samples subsequently were used for bacterial transformation.

2.2.8. Agar Plates and Luria-Bertani Media

Laura-Bertani (LB; *Sigma*) or LB agar (*Sigma*) were dissolved in distilled water according to the manufacturer`s instructions and autoclaved. Next, a final concentration of 100µg/ml ampicillin (*Sigma*) was added to the medium. For agar plates, approximately 10ml of molten LB agar was poured in $10cm^2$ petri dishes (*Fisher Scientific*) and allowed to set. LB medium and LB agar plates were stored for a maximum of 2 weeks at 4°C.

2.2.9. Transformation of Competent Bacterial Cells

Competent cells (NEB stable competent *E.coli*; NEB) were transformed with mammalian expression vector pcDNA3.1 containing i) full-length VWF wildtype (provided by Dr. McKinnon) ii) full-length VWFΔA1 (deletion of the VWF A1 domain from full-length VWF wildtype; provided by Dr. McKinnon) iii) the isolated VWF A1 domain (provided by Dr. McKinnon) iv) full-length VWF A1 domain mutants or v) isolated VWF A1 domain mutants according to the manufacturer's instructions. For transformation, 25µl cells were thawed on ice and mixed with 1µl of vector DNA. The mixture was incubated on ice for 30 minutes before cells were heat-pulsed at 42°C for 30 seconds. Cells then were incubated on ice for an additional 5 minutes. Next, 200µl preheated SOC medium (*NEB*) was added and the transformed bacteria were grown at 37°C and 220rpm for 1 hour. Subsequently, transformed bacteria were plated out on LB medium agar plates containing ampicillin and incubated overnight at 37°C.

2.2.10. Bacterial Culture of Plasmids

Transformed bacteria were cultured in order to allow isolation of large quantities of DNA. 5ml of ampicillin LB medium were inoculated with a single bacterial colony containing the pcDNA3.1 vector having incorporated the gene of i) full-length VWF wildtype ii) full-length VWFΔA1 iii) isolated VWF A1 domain iv) full-length VWF A1 domain mutants or v) isolated VWF A1 domain mutants. Cultures were incubated at 37°C and 220rpm for 6 hours. Subsequently, 1ml of the culture was used in order to inoculate 200ml LB medium containing ampicillin followed by an incubation at 37°C and 220rpm overnight.

2.2.11. Isolation of Plasmid DNA

Isolation of plasmid DNA from grown bacterial cultures was performed using different plasmid isolation kits. Miniprep kits (*NEB*) were used in order to isolate up to 20µg of plasmid for predominantly sequencing purposes. Maxiprep kits (*OMEGA Bio-Tek*) were used for the isolation of up to 500µg of plasmid DNA predominantly used for the transient transfection of mammalian cells in the context of protein expression. Both kits were used according to the manufacturer's instruction. DNA concentrations were determined measuring the absorbance at 260nm using a NanoDrop spectrophotometer (*ThermoScientific*).

2.2.12. Sequencing

All vectors and mutations generated were verified by sequencing in order to ensure absence of any unwanted PCR-introduced error as well as to confirm the presence of the mutations introduced by site-directed mutagenesis. DNA sequencing was performed by Genewiz.

2.3. Protein Expression

2.3.1. Transfection of HEK293T Cells

Transient transfection is used in order to produce recombinant protein without integrating the DNA into the host cell genome. HEK293T cells express high levels of SV40 large tumour (T)

antigen which allows episomal replication of SV-40 origin containing plasmids as for example pcDNA3.1. In order to express the various VWF proteins, HEK293T cells were transiently transfected using polyethylenimine (PEI; *Polysciences*). Cells were cultured in either flat bottom 6well cell culture plates (*Greiner BioOne*) or T175 flasks and MEM growth medium until a confluency of 70% was reached.

In order to determine ideal DNA concentration for mammalian cell transfections, HEK293T cells were transfected with 0.3µg/ml, 0.5µg/ml, 1.0µg/ml, 2.0µg/ml and 3.0µg/ml recombinant full-length VWF wildtype DNA and 20mM PEI that both were diluted in 0.15M sodium chloride. After dilution, DNA and PEI solutions were mixed dropwise and incubated for 20 minutes at room temperature. MEM was removed from cultured HEK293T cells and replaced with 2.7ml (6well plate) OptiMEM (*Thermo Fisher Scientific*). Next, 300µl of the DNA-PEI solution was added and cells were incubated at 37°C and 5% CO_2 for 3 days. Media containing expressed VWF proteins was harvested after 3 days, centrifuged at 4000g for 10 minutes and analysed using western blot.

Test transfection of all VWF based proteins used in this project were carried out as described above. Large-scale transfection of all VWF based proteins was carried out as described above using 1µg of DNA/ml and a final culture volume of 20ml. MEM culture medium was replaced by 18ml (T175 flask) OptiMEM (*Thermo Fisher Scientific*) and 2ml of the DNA-PEI solution was added. Cells were incubated at 37°C and 5% CO_2 for and media containing expressed VWF proteins was harvested after 3 days, centrifuged at 4000g for 10 minutes and further concentrated prior to protein purification.

2.4. Protein Purification

2.4.1. Tangential Flow Filtration of Isolated VWF A1 Domain Proteins

Tangential flow filtration (TFF) allows the concentration of high volumes of protein samples for further procession as for example fast protein liquid chromatography. Isolated VWF A1 domain mutants as well as the isolated VWF A1 domain itself were concentrated using TFF after HEK293T expression. 150 to 200ml of collected expression medium containing the VWF

proteins were subjected to TFF using a Labscale tangential flow filtration system (*Millipore*) with a molecular weight cut-off of 10 kDa. Samples were processed to a final volume of around 50ml and subsequently were further purified with nickel affinity chromatography.

2.4.2. Nickel Affinity Chromatography

Isolated VWF A1 domain as well as isolated VWF A1 domain mutants were purified with ion metal affinity chromatography (IMAC) using 1ml Ni^{2+}-His Trap chelating columns (Nickel Ion; *GE Healthcare*) which are capable of binding His-tagged proteins allowing removal of contaminants. Proteins were loaded onto the column and washed for 30 column volumes with washing buffer (20mM Tris and 100mM NaCl; pH7.4). Isocratic elution was carried out using elution buffer (20mM Tris; 100mM NaCl and 50mM imidazole; pH7.4) and eluted protein fractions were dialysed against 20mM Tris and 100mM NaCl (pH7.4) using a 10kDa molecular cut-off dialysis membrane (*Pierce*).

2.4.3. Concentration of VWF Proteins

VWF proteins were concentrated either directly after IMAC (isolated VWF A1 domain as well as isolated VWF A1 domain mutants), before and after ion exchange chromatography (IEC; full-length VWF wildtype) or after harvesting conditioned medium (full-length VWF A1 domain mutants). Concentration was carried out using Amicon Ultra 4 centrifugal filter units (*Millipore*) with molecular weight cut-offs of 10kDa for isolated VWF A1 domain and isolated VWF A1 domain mutants and 100kDa for full-length VWF wildtype, full-length VWFΔA1 and full-length VWF mutants. Samples were applied to the filter units and centrifuged at 4000g to a final volume of approximately 1 to 2ml. Concentrated full-length VWF wildtype was either directly aliquoted and stored at -80°C or purified using IEC alongside full-length VWFΔA1. full-length VWF A1 domain mutants, nickel purified isolated VWF A1 domain and nickel purified isolated VWF A1 domain mutants were directly aliquoted and stored at -80°C after concentration.

2.4.4. Ion Exchange Chromatography

Recombinant full-length VWF wildtype and full-length VWFΔA1 were purified using IC. Purification was carried out using a SK16 ion exchange chromatography column (self-packed with Tetramethylethylenediamine; *Merck*). Pre-concentrated VWF protein containing culture medium was loaded onto the column and samples were washed with 20mM Tris and 100mM NaCl (pH7.4) for 6 column volumes. Subsequently, proteins were isocratic eluted in 10mM Tris and 500mM NaCl (pH 7.4). Following elution, all proteins were dialysed against 20mM Tris and 100mM NaCl (pH7.4) and further concentrated as described in section (1.4.3.).

2.5. Protein characterisation

2.5.1. SDS Polyacrylamide Gel Electrophoresis

Purity of the different proteins after purification or concentration was monitored using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under both reducing and non-reducing conditions. Different sizes of NuPAGE Bis-Tris precast polyacrylamide gels (10-well; 12-well or 15-well gels; *Invitrogen*) were used in order to resolve proteins based on their different molecular weights. Samples to be analysed under non-reducing conditions were diluted with 4x SDS loading dye (200mM Tris; 8% SDS; 0.4.% bromophenolblue; 50% glycerol; *Novex*). Samples to be analysed under reducing conditions were diluted in the same buffer containing 4% w/v β-mercaptoethanol (*Sigma*). Reduced samples were heated at 90°C for 10 minutes and subsequently loaded onto the gel. Non-reduced samples were directly loaded onto the gel. A pre-stained molecular weight protein marker (CozyXL; highQu) was run alongside all samples. Electrophoresis was carried out at 150 to 200V for 60 to 90 minutes.

2.5.1.1. Coomassie Staining of SDS-PAGE Gels

Following SDS-PAGE, the gels were stained with Coomassie Brilliant Blue in order to visualise the protein bands. Gels were removed from the electrophoresis chambers and washed with ddH_2O for 5 minutes. Next, gels were placed in SimplyBlue™ SafeStain (*Invitrogen*) overnight at room temperature. Gels were destained with ddH_2O overnight at room temperature.

2.5.2. Western Blot Analysis

Western blot analysis was carried out in order to verify protein expression after test transfections of HEK293T cells, analyse multimer formation of full-length VWF proteins or test fragmentation of FXII upon activation. Therefore, proteins were transferred from polyacrylamide gels to nitrocellulose membranes (*Amersham Pharmacia*) using transfer buffer (*ThermoScientific*). Transfer was carried out for 15 minutes at 25V in a semi-dry blotting system (Pierce Power Blot; *ThermoScientific*) and membranes were washed with phosphate buffered saline (PBS) containing 0.1% (v/v) Tween (PBST). Next, membranes were blocked with 2.5% (w/v) non-fat milk powder in PBST for 2 hours on a shaker at room temperature or overnight at 4°C. After blocking, membranes were washed three times for 10 minutes with PBST and incubated in relevant horseradish peroxidase (HRP) conjugated antibody diluted in PBST on a shaker at room temperature for 3 hours or overnight at 4°C. For the detection of full-length VWF proteins and full-length VWFΔA1, a rabbit polyclonal anti-VWF HRP conjugated antibody (anti-VWF Ab HRP; *Dako*) was used in a 1:1000 dilution. Isolated VWF A1 domain proteins were visualised by incubation with a rabbit polyclonal anti-6xHis HRP conjugated antibody (anti-His Ab HRP; *abcam*) used in a 1:5000 dilution. Fragmentation of FXII upon activation was detected using a goat anti-human FXII HRP conjugated antibody (anti-FXII HRP Ab; *Affinity Biologics*) in a 1:5000 dilution. Excess antibody was removed by extensive washing with PBST and proteins were detected with an ECL chemiluminescence kit (Immobilion; *Millipore*). Briefly, equal amounts of solution A and B were applied to the membranes for 5 minutes which subsequently were exposed to Hyper-Films (*Amersham Pharmacia*) and developed.

2.5.3. Bicinchoninic Acid Assay for Concentration Estimation

The bicinchoninic acid (BCA) assay is a biochemical assay used for the determination of total protein levels in a solution. The method is based on the ability of proteins to reduce Cu^{2+} to Cu^{1+} (cuprous ion) in an alkaline medium resulting in the formation of a purple-coloured reaction product after the chelation of two BCA molecules with one cuprous ion. Colorimetric intensity correlates directly with the protein concentration of the sample. BCA assays for the determination of the protein concentration for the isolated VWF A1 domain as well as the isolated VWF A1 domain mutants were performed in flat bottomed uncoated 96well plates

(*Sigma*). BCA reagent (*Pierce*) was prepared immediately before use by adding 1ml cupric sulphate pentahydrate to 50ml of BCA. Bovine serum albumin (BSA) was used as standard with a concentration range from 8 to 1000µg/ml. VWF proteins were diluted 1:10 in PBS and subsequently analysed in a 1:10 dilution series. 25µl of each standard or sample were transferred to the wells of a 96well plate in duplicates and mixed with 200µl of the BCA reagent. Plates were incubated at 37°C for 30 minutes and the absorbance was measured at 562nm using a plate reader (*Biotek*). Protein concentration of the VWF containing samples was determined by reading from the BSA standard curve.

2.5.4. VWF Enzyme-linked Immunosorbent Assay

Protein concentrations for full-length VWF wildtype, recombinant full-length VWF A1 domain mutants and VWFΔA1 were determined using a Von Willebrand Factor enzyme-linked immunosorbent assay (VWF ELISA). Polyclonal rabbit anti-human VWF antibody (anti-VWF Ab; *Dako*) was immobilised on Maxisorp plates (final concentration of 3.1µg/ml in 100mM sodium carbonate and 100mM sodium bicarbonate; pH9.6; 100 µl/well; *Nunc*) overnight at 4°C. All subsequent incubations were carried out at room temperature and wells were washed three times with PBST (*Sigma*) between each incubation step. Wells were blocked with 1% BSA (200 µl/well; *Sigma*) in PBS for one hour. VWF containing samples were diluted in PBS (100 µl/well; *Sigma*) and added to the anti-VWF Ab coated wells for two hours. A dilution series of plasma derived VWF (initial concentration of 10µg/ml; subsequent 1:2 dilutions in PBS; 100 µl/well; *Technoclone*) was added to the anti-VWF Ab coated plate for two hours serving as reference standard. Binding of VWF to the anti-VWF Ab was detected by addition of a polyclonal rabbit anti-human VWF HRP conjugated antibody (anti-VWF Ab HRP; final concentration of 1.3µg/ml in PBS; 100 µl/well; *Dako*) for one hour. Wells were developed with chromogenic substrate o-phenylenediamine (SIGMAFAST™ OPD; 120 µl/well; *Sigma*) diluted in double-distilled water (ddH$_2$O). Colorimetric reaction was quenched with 3.2M sulphuric acid (H_2SO_4; 65 µl/well; *Sigma*) and absorbance was read at 492nm using an Epoch microplate spectrophotometer reader (*BioTek*).

Graphs were plotted in Microsoft Excel and the equation-of-the-line and R^2 values were calculated using the “add trendline” function. The equation-of-the-line was given in the format:

$$Y = mx + c$$

with y : absorbance value ; m: gradient of the slope; x: concentration of VWF y intercept. In order to calculate the VWF concentration of the different samples the equation was rearranged to:

$$x = (y - c) / m$$

Absorbance values (y) of the VWF samples were substituted into the equation and returned a VWF value. Unless stated otherwise, all VWF concentrations were expressed in µg/ml.

2.5.5. Gel Analysis of VWF Multimers

Formation of multimers in full-length VWF proteins was analysed using agarose gel electrophoresis under non-reducing conditions followed by western blot detection of the proteins. High resolution (1.4%) agarose gels (diameter of 1.5mm) were prepared by dissolving 1.4g high gelling temperature agarose (*Seakem*) in 100ml of gel buffer (200mM Tris; 100mM glycine; 0.1% SDS; pH9.0) and cast using a Bio-Rad mini-gel casting system. Samples were diluted with sample buffer (10mM Tris; 1mM EDTA; 2% SDS; 8M urea; 0.01% bromphenolblue; pH8.0) to a final concentration of 0.5µg/ml and incubated for 30 minutes at 60°C. Subsequently, gels were assembled in a Bio-Rad mini-gel tank and chilled running buffer (100mM Tris; 150mM glycine; 0.1% SDS; pH8.4) was applied to the tank which was placed in an ice filled box. 10µl of each sample were transferred onto the gel and electrophoresis was carried out at 65V for 20 minutes followed by 35V until the tracking dye had reached the end of the gel. Next, gels were incubated in *dichlorodiphenyltrichloroethane DDT; BioRad)* for 10 minutes, transferred onto nitrocellulose membranes and visualised using an anti-human VWF HRP conjugated antibody as described in section 2.5.2.

2.5.6. Platelet Capture of Full-Length VWF and VWF Mutants

Platelet capture ability of full-length VWF proteins under conditions of low shear was carried out in order to analyse binding capability of the proteins to both collagen type I as well as platelets ensuring normal protein conformation after introduction of mutations as well as expression and purification.

2.5.6.1. Preparation of Plasma Free Blood

Whole blood was collected from healthy donors into anticoagulant solution; 1:6 sodium citrate (ACD; 85mM sodium citrate; 111nM glucose; 71nM citric acid; pH4.5). In order to eliminate potential effects of endogenous VWF and other plasma proteins, plasma free blood was prepared. All centrifugation steps were performed without a brake at room temperature. Erythrocytes and platelet rich plasma (PRP) were separated by centrifugation at 120g for 15 minutes. The top phase containing PRP was transferred to a separate tube containing 10% v/v ACD, 100mU apyrase grade IV (*Sigma*) and 100nM prostaglandin E1 (PGE1; *Sigma*) preventing activation of platelets and was centrifuged at 1200g for 15 minutes in order to isolate the platelets. After centrifugation, the platelet poor plasma was discarded and the platelet rich pellet was resuspended in 10ml of platelet wash buffer (36mM citric acid; 5mM glucose; 5mM KCl; 1mM $MgCl_2$; 100mM NaCl; 2mM $CaCl_2$; pH6.5) containing 100mU apyrase, 100nM PGE1 and 3.5mg/ml BSA. Platelets were fluorescently labelled by the addition of 3,3'-Dihexyloxacarbocyanine Iodide ($DiOC_6$; *Thermo Fisher Scientific*) and subsequently centrifuged at 120g for 15 minutes. Next, the supernatant was discarded and the platelet pellet was resuspended in HEPES/Tyrodes buffer (10mM HEPES; 137mM NaCl; 2.68mM KCl; 0.42mM NaH_2PO_4; 1.7mM $MgCl_2$; 11.9mM $NaHCO_3$; 5mM glucose; pH7.4) supplemented with 3.5mg/ml BSA. Red blood cells were prepared alongside platelets. Following PRP removal, 50% v/v of 0.9% saline was added to the erythrocytes containing fraction. Following gentle mixing, the sample was centrifuged at 120g for 15 minutes. Next, the supernatant was removed, the red blood cells were resuspended in 50% v/v of 0.9% saline and centrifuged again at 120g for 15 minutes. This washing procedure was repeated two more times. After the final centrifugation, the supernatant was discarded and the erythrocytes were resuspended in HEPES/Tyrodes buffer supplemented with 3.5mg/ml BSA to the original volume of blood collected in order to maintain normal haematocrit function. After reconstitution of the original volume, isolated and labelled platelets were added back into the sample.

2.5.6.2. Flow Chamber and Digital Image Processing

Flow assays were performed using flow slides placed on an inverted epifluorescent microscope (*Olympus-CKX41*). All experiments were recorded in real time using a QImaging camera (*Rollera XR*) and Q Capture pro software. The digitalised images were collected, processed and analysed with the ImageJ software (*MacMaster Biophotonics*) and Virtual Dub

utility. Uncoated µ-slides 0.4 (*ibidi*) as well as fitting tubing and adapters manufactured by ibidi were used for the perfusion assays. Desired flow rates were achieved and maintained by adjusting syringe diameters and flow rates depending on a slide size according to conversion tables provided by the manufacturer. Slides were connected to a syringe pump Aladdin-1000 (*World Precision Instruments*) in order to generate continuous laminar flow at specific shear rates.

2.5.6.3. Preparation of Flow Slides

Slide channels were coated with collagen type I (CHRONO-PAR®; *Chronolog*). Collagen was diluted in acetic acid (25mM) to a final concentration of 5µg/ml. Subsequently, 20µl of collagen were transferred to a flow well and slides were incubated at room temperature for 15minutes. After incubation, the coating solution was carefully aspirated and the collagen coated wells were washed three times with PBS and blocked with 1.5% BSA in PBS for 1 hour. After blocking, the channels were washed with PBS again before perfusion of the individual samples.

2.5.6.4. Interaction of VWF Proteins with Collagen and Platelets

Binding ability of full-length VWF proteins to collagen type I and platelets was investigated by addition of full-length VWF wildtype or full-length VWF A1 domain mutants to plasma free blood containing labelled platelets (preparation described in section 2.5.6.1.). Samples contained a final concentration of 10µg/ml of the VWF proteins and were perfused over collagen type I coated slides for 4 minutes at a shear rate of $1500s^{-1}$. Short movies were recorded every minute of flow and the experiment was repeated three times for full-length VWF wildtype as well as full-length VWF mutants involved in FXII(a) binding. The number of accumulated platelets at each time point was assessed as surface coverage (%) using the ImageJ software.

2.6. Binding analysis of VWF to FXII

Binding interaction of VWF to activated and non-activated FXII was tested using different ELISA setups as well as surface plasmon resonance measurements.

2.6.1. Binding of VWF and VWF Mutants to FXII and FXIIa in ELISA`s

All ELISAs carried out in order to characterise the binding potential of VWF as well as VWF mutants to activated and nonactivated FXII were adapted from a general immunosorbent assay consisting of the following common steps. Antigens (FXIIa, *Enzyme Research Laboratories*; FXII, *Haematologic Technologies Incorporated*; rabbit polyclonal anti-VWF antibodies, *Dako* and goat anti-human FXII antibodies, *Enzyme Research Laboratories*) used in the binding assays were diluted in PBS and immobilised on 96well Maxisorp (*Nunc*) plates overnight at 4°C. FXII was activated with 290nM kallikrein (*Enzyme Research Laboratories*) for 1 hour at room temperature. Plates were washed with PBST between every blocking and incubation step. Blocking was performed with 2.5mg/ml BSA in PBS for 1 hour at room temperature. Samples were incubated on the target antigen for 2 hours and detection of binding interaction was monitored using HRP conjugated antibodies (rabbit polyclonal anti-VWF HRP conjugated antibodies for full-length VWF proteins, *Dako*; rabbit polyclonal anti-6xHis HRP conjugated antibodies for isolated VWF A1 domain proteins, *abcam* and goat anti-human FXII HRP conjugated antibodies for FXII, *Affinity Biologics*) for 1 hour. Wells were developed with chromogenic substrate o-phenylenediamine (SIGMAFAST™ OPD; 120 µl/well; *Sigma*) diluted in ddH_2O. Colorimetric reaction was quenched with 3.2M H_2SO_4 (65 µl/well; *Sigma*) and absorbance was read at 492nm using an Epoch microplate spectrophotometer reader (*BioTek*).

Binding of the VWF proteins to FXII and FXIIa was controlled by monitoring i) binding of isolated VWF A1 domain mutants to the immobilised isolated VWF A2 domain ii) binding of HRP conjugated antibodies to plastic iii) binding of HRP conjugated antibodies to the immobilised antigen iv) absence of the HRP conjugated antibodies v) colorimetric signal development in the absence of the immobilised antigen binding partner vi) disruption of protein complex formation by absence of one of the binding partners vii) cross reactivity of HRP conjugated antibodies to the respective binding partner after protein complex formation and viii) colorimetric signal development in the absence of HRP conjugated antibodies but presence of the formed protein complex.

The following table 2.2 summarises the different ELISA conditions.

Immobilised	Activated	Incubated with	Detected with
Human FXII	-	Plasma derived VWF wildtype	Rabbit anti-VWF Ab
		Recombinant VWF wildtype	
Human FXII	Kallikrein	Plasma derived full-length VWF wildtype	Rabbit anti-VWF Ab
		Recombinant full-length VWF wildtype	
Human FXIIa	-	Plasma derived full-length VWF wildtype	Rabbit anti-VWF Ab
		Recombinant full-length VWF wildtype	
		Recombinant full-length VWFΔA1	
		Isolated VWF A1 domain	Rabbit anti-His Ab HRP
Plasma derived full-length VWF wildtype	-	Human FXIIa	Goat anti-FXIIa Ab HRP
Rabbit anti-VWF Ab	-	Plasma derived full-length VWF wildtype + Human FXIIa complex	Goat anti-FXII Ab HRP
Goat anti-FXII Ab		Human FXIIa + Plasma derived full-length VWF wildtype complex	Rabbit anti-VWF Ab
Human FXIIa	-	Isolated VWF A1 domain	Rabbit anti-His Ab HRP
		Isolated VWF A1 domain mutants	
Human FXIIa	-	Recombinant full-length VWF	Rabbit anti-VWF Ab
		Recombinant full-length VWF mutants	
Human FXII	-	Recombinant full-length VWF	Rabbit anti-VWF Ab
		Recombinant full-length VWF mutants	
Human FXII	Kallikrein	Recombinant full-length VWF	Rabbit anti-VWF Ab
		Recombinant full-length VWF mutants	

Table 2.2: Overview about the different ELISA setups. ELISA was carried out in order to evaluate binding interaction between activated and non-activated FXII and VWF proteins.

2.6.1.1. Binding of Plasma derived VWF to FXIIa in a 4-way ELISA Format

Initial binding ability of plasma derived full-length VWF to FXIIa was tested in a 4-way ELISA set-up varying in the antigens immobilised and proteins or protein complexes detected. All ELISA`s were carried out as described in section 1.7.1 and conditions are summarised in the following table 2.3. Binding signals were read and binding potential of the two proteins was assessed in GraphPad Prism.

Immobilised	Incubated with	Detected with
100nM human FXIIa	200nM plasma derived full-length VWF wildtype	1.3µg/ml rabbit anti-VWF HRP Ab
200nM plasma derived full-length VWF wildtype	100nM human FXIIa	0.8µg/ml goat anti-FXII HRP Ab
Anti-VWF Ab	200nM plasma derived full-length VWF wildtype + 100nM human FXIIa	0.8µg/ml goat anti-FXII HRP Ab
Anti-FXII Ab	100nM human FXIIa + 200nM plasma derived full-length VWF wildtype	1.3µg/ml rabbit anti-VWF HRP Ab

Table 2.3: Layout of the 4-way ELISA. ELISA was carried out in order to evaluate binding interaction between human FXIIa and plasma derived full-length VWF wildtype in a single concentration static plate binding assay.

2.6.1.2. Binding of Plasma derived or Recombinant FL-VWF to FXII and FXIIa

Binding ability of plasma derived or recombinant full-length VWF to either activated or nonactivated FXII was tested by incubating a concentration range of VWF on immobilised FXIIa or kallikrein activated FXII. Binding interaction was detected as described in section 1.7.1. and binding signals as well as the estimated dissociation constant of the two proteins was assessed in GraphPad Prism. The following table 2.4 depicts the conditions of the ELISA used.

Immobilised	Activated with	Incubated with	Detected with
100nM human FXIIa	-	6.3 to 400nM plasma derived full-length VWF wildtype	1.3µg/ml rabbit anti-VWF HRP Ab
100nM human FXIIa	-	6.3 to 400nM recombinant full-length VWF wildtype	1.3µg/ml rabbit anti-VWF HRP Ab
100nM human FXIIa	-	6.3 to 400nM plasma derived full-length VWF wildtype	1.3µg/ml rabbit anti-VWF HRP Ab
100nM human FXIIa	-	6.3 to 400nM recombinant full-length VWF wildtype	1.3µg/ml rabbit anti-VWF HRP Ab
100nM human FXII	290nM Kallikrein	6.3 to 400nM plasma derived full-length VWF wildtype	1.3µg/ml rabbit anti-VWF HRP Ab
100nM human FXII	290nM Kallikrein	6.3 to 400nM recombinant full-length VWF wildtype	1.3µg/ml rabbit anti-VWF HRP Ab

Table 2.4: ELISA for the determination of the affinity between FXII and full-length VWF wildtype. ELISA was carried out in order to evaluate the binding interaction and affinity of plasma derived and full-length VWF wildtype and human FXIIa, human FXII and kallikrein activated FXIIa.

2.6.1.3. Binding of Recombinant FL-VWFΔA1 to FXIIa

Preliminary data obtained in the laboratory proposed the presence of a FXII binding site within the VWF A1 domain. In order to confirm this hypotheses, full-length VWF lacking the A1 domain (VWFΔA1) was incubated on immobilised FXIIa at a single concentration as well as a

concentration range. Binding signals were read and binding potential as well as the estimated dissociation constant (K_D) of the two proteins were assessed in GraphPad Prism. The following table 2.5 gives an overview about the ELISA conditions used.

<table>
<tr><th>Immobilised</th><th>Incubated with</th><th>Detected with</th></tr>
<tr><td rowspan="3">100nM human FXIIa</td><td>1.6 to 100nM plasma drived full-length VWF wildtype</td><td rowspan="3">1.3µg/ml rabbit anti-VWF HRP Ab</td></tr>
<tr><td>1.6 to 100nM recombinant full-length VWF wildtype</td></tr>
<tr><td>1.6 to 100nM full-length VWFΔA1</td></tr>
<tr><td rowspan="3">100nM human FXIIa</td><td>80nM plasma drived full-length VWF wildtype</td><td rowspan="3">1.3µg/ml rabbit anti-VWF HRP Ab</td></tr>
<tr><td>80nM recombinant full-length VWF wildtype</td></tr>
<tr><td>80nM full-length VWFΔA1</td></tr>
</table>

Table 2.5: Binding of full-length VWFΔA1 to human FXIIa. ELISA was carried out in order to evaluate the binding interaction and affinity between full-length VWFΔA1 and human FXIIa.

2.6.1.4. Binding of Isolated VWF A1 Domain to FXIIa

After having established that the VWF A1 domain contains the FXII binding site, binding potential of the isolated VWF A1 domain to FXIIa was tested by incubating a concentration range of the isolated VWF A1 domain on immobilised FXIIa. Binding signals as well as the estimated dissociation constant of the two proteins were assessed in GraphPad Prism. A general layout of the ELISA setup is summarised in table 2.6 below.

Immobilised	Incubated with	Detected with
100nM human FXIIa	14 to 900nM full-length VWFΔA1	0.2µg/ml rabbit anti-His Ab HRP

Table 2.6: Binding of the isolated VWF A1 domain to FXIIa. ELISA was carried out in order to evaluate the binding interaction and affinity between the isolated VWF A1 domain and human FXIIa.

2.6.1.5. Binding of Isolated VWF A1 Domain Mutants to FXIIa

Surface exposed negatively charged amino acids present in the VWF A1 domain were mutated in the isolated VWF A1 domain in order to identify residues involved in the binding interaction between FXII and VWF. Binding of 12 different isolated VWF A1 domain mutants to FXIIa was evaluated by incubation of the mutants on immobilised FXIIa at a single concentration. Binding

signals were read and binding potential of the two proteins was assessed in GraphPad Prism. The following table 2.7 contains the conditions used in the static plate binding assay.

Immobilised	Incubated with	Detected with
100nM human FXIIa	360nM isolated VWF A1 domain single-point mutant	0.2µg/ml rabbit anti-His Ab HRP
Mutants: E1290A; E1292A; E1294A; D1302A; E1305A; E1339A; D1444A; E1445A; E1447A; D1451A; E1452A; D1459A		
100nM human FXIIa	360nM isolated VWF A1 domain	0.2µg/ml rabbit anti-His Ab HRP
100nM human FXIIa	360nM isolated VWF A2 domain	0.2µg/ml rabbit anti-His Ab HRP

Table 2.7: Binding of the isolated VWF A1 domain single-point mutants to FXIIa. ELISA was carried out in order to evaluate the binding potential between single-point isolated VWF A1 domain mutants to FXIIa and identify amino acid residues responsible for the binding interaction.

2.6.1.6. Binding of Isolated VWF A1 Domain Double Mutants to FXIIa

Initial screening of the isolated VWF A1 domain mutants resulted in the identification of six amino acids potentially involved in the binding ability of VWF to FXIIa. A combination of identified surface residues was mutated in the isolated VWF A1 domain for the exact determination of the amino acids essential for protein-protein interaction. Isolated VWF A1 domain mutants containing either single-point mutations or a combination of mutations was screened against immobilised FXIIa at a single concentration. Binding signals were read and binding potential of the two proteins was assessed in GraphPad Prism. Table 2.8 below comprises the experimental setup used for this ELISA.

Immobilised	Incubated with	Detected with
100nM human FXIIa	360nM isolated VWF A1 domain single-point or double mutant	0.2µg/ml rabbit anti-His Ab HRP
Single-point mutants: D1444A; E1445A; E1447A; E1452A; D1459A Double mutants: D1444/1445A; D1444/1447A; E1452/1459A		
100nM human FXIIa	360nM isolated VWF A1 domain	0.2µg/ml rabbit anti-His Ab HRP
100nM human FXIIa	360nM isolated VWF A2 domain	0.2µg/ml rabbit anti-His Ab HRP

Table 2.8: Binding of isolated single-point and double VWF A1 domain mutants to FXIIa. ELISA was carried out in order to evaluate the binding potential between a selection of single-point and double isolated VWF A1 domain mutants to FXIIa and identify amino acid residues responsible for the binding interaction.

2.6.1.7. Binding of Isolated VWF A1 Domain Mutant E1452/1459A to FXIIa

Binding analysis of isolated VWF A1 domain double mutants on immobilised FXIIa led to the identification of two amino acids responsible for the binding ability of VWF to FXII. Binding of the isolated VWF A1 domain mutant E1452/1459A was characterised by incubation of the double mutant on immobilised FXIIa at a single concentration as well as a concentration range. Conditions of the ELISA used are summarised in table 2.9. Binding signals were read and binding potential as well as the estimated dissociation constant of the two proteins was assessed in GraphPad Prism.

Immobilised	Incubated with	Detected with
100nM human FXIIa	360nM isolated VWF A1 domain single-point mutant E1452A and D1459A	0.2µg/ml rabbit anti-His Ab HRP
	360nM isolated VWF A1 domain double mutant E1452/1459A	
	360nM isolated VWF A1 domain	
100nM human FXIIa	28 to 1800nM isolated VWF A1 single-point mutant E1452A and D1459A	0.2µg/ml rabbit anti-His Ab HRP
	28 to 1800nM isolated VWF A1 domain double mutant E1452/1459A	
	28 to 1800nM isolated VWF A1 domain	

Table 2.9: Characterisation of the interaction between isolated VWF A1 domain double mutant E1452/1459A and human FXIIa. ELISA was carried out in order to evaluate the binding potential between double isolated VWF A1 domain mutant E1452/1459D and FXIIa and confirm amino acid residues at position 1452 and 1459 as the FXII binding site in the VWF A1 domain.

2.6.1.8. Binding of FL-VWF A1 Domain Mutants to FXIIa

Surface exposed negatively charged amino acids present in the VWF A1 domain were mutated in full-length VWF in order to identify residues involved in the binding interaction between FXII and VWF. Binding of 12 different full-length VWF A1 domain mutants to FXIIa was evaluated by incubation the mutants on immobilised FXIIa at a single concentration. Binding signals were read and binding potential of the two proteins was assessed in GraphPad Prism. ELISA were carried out under the conditions displayed in table 2.10.

Immobilised	Incubated with	Detected with
100nM human FXIIa	360nM full-length VWF A1 domain single-point mutant	1.3µg/ml rabbit anti-VWF HRP Ab
Mutants: E1290A; E1292A; E1294A; D1302A; E1305A; E1339A; D1444A; E1445A; E1447A; D1451A; E1452A; D1459A		
100nM human FXIIa	360nM recombinant full-length VWF wildtype	0.2µg/ml rabbit anti-His Ab HRP

Table 2.10: Binding of the full-length VWF single-point mutants to FXIIa. ELISA was carried out in order to evaluate the binding potential between single-point full-length VWF A1 domain mutants to FXIIa and identify amino acid residues responsible for the binding interaction.

2.6.1.9. Binding of FL-VWF A1 Domain Double Mutants to FXIIa

Initial screening of full-length VWF A1 domain mutants resulted in the identification of eight amino acids potentially involved in the binding ability of VWF to FXIIa. Considering results obtained when screening the isolated VWF A1 domain mutants on FXIIa, a combination of identified surface residues was mutated in the full-length VWF A1 domain in order to identify specific amino acids essential for protein-protein interaction. Full-length VWF A1 domain mutants containing either single-point mutations or a combination of mutations was screened against immobilised FXIIa at a single concentration. Binding signals were read and binding potential of the two proteins was assessed in GraphPad Prism. The following table 2.11 depicts the conditions of the ELISA used.

Immobilised	Incubated with	Detected with
100nM human FXIIa	40nM full-length VWF A1 domain single-point or double mutant	1.3µg/ml rabbit anti-VWF HRP Ab
Single-point mutants: D1444A; E1445A; E1447A; E1452A; D1459A Double mutants: D1444/1445A; D1444/1447A; E1452/1459A		
100nM human FXIIa	360nM recombinant full-length VWF wildtype	1.3µg/ml rabbit anti-VWF HRP Ab

Table 2.11: Binding of single-point and double full-length VWF A1 domain mutants to FXIIa. ELISA was carried out in order to evaluate the binding potential between a selection of single-point and double full-length VWF A1 domain mutants to FXIIa and identify amino residues responsible for the binding interaction.

2.6.1.10. Binding of FL-VWF A1 Domain Mutant E1452/1459A to FXII and FXIIa

Binding analysis of full-length VWF A1 domain double mutants on immobilised FXIIa led to the identification of two amino acids responsible for the binding ability of VWF to FXII and results correlate with binding analysis carried out with isolated VWF A1 domain mutants. Binding of full-length VWF A1 domain mutant E1452/1459A was further characterised by incubation of the double mutant on immobilised activated and nonactivated FXII at a single concentration. Binding signals were read and binding potential as well as the estimated dissociation constant of the two proteins was assessed in GraphPad Prism. Conditions of the ELISA are summarised in the following table 2.12.

Immobilised	Activated with	Incubated with	Detected with
100nM human FXIIa	-	40nM full-length VWF A1 domain single-point mutant E1452A and D1459A	1.3µg/ml rabbit anti-VWF HRP Ab
	-	40nM full-length VWF A1 domain double mutant E1452/1459A	
100nM human FXIIa	-	40nM full-length VWF A1 domain single-point mutant E1452A and D1459A	1.3µg/ml rabbit anti-VWF HRP Ab
	-	40nM full-length VWF A1 domain double mutant E1452/1459A	
100nM human FXII	290nM Kallikrein	40nM full-length VWF A1 domain single-point mutant E1452A and D1459A	1.3µg/ml rabbit anti-VWF HRP Ab
	290nM Kallikrein	40nM full-length VWF A1 domain double mutant E1452/1459A	

Table 2.12: Binding of single-point and double full-length VWF A1 domain mutant to FXIIa, FXII and kallikrein activated FXIIa. ELISA was carried out in order to evaluate the binding potential of single-point and double full-length VWF A1 domain mutants E1452A, D1459A and E1452/1459A to FXIIa, FXII and kallikrein activated FXIIa.

2.6.2. Surface Plasmon Resonance

Surface plasmon resonance (SPR) is a well-established method for the determination of binding potential between two proteins in real time and allows monitoring of sequential binding events during protein-protein interaction. Surface plasmon resonance was carried out in order to determine the binding constant of different VWF proteins to immobilised FXIIa. All binding experiments were performed using a Biacore3000 (*GE Healthcare*). Flow cell 1, 2 and 3 of a COOH chip were activated at 22°C using amine coupling chemistry 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC)/ N-hydroxysuccinimide (NHS); *Thermo Fisher Scientific*). Subsequently, human FXIIa was diluted in SPR running buffer (10mM HEPES;

140mM NaCl; 1.5 mM $CaCl_2$; 40µM $ZnCl_2$, 0.001% Tween-20, pH7.4; filter sterilised and degassed) and immobilised at a flow rate of 20µl/min on flow cells 1 and 3 to final response units (RU) of 55 and 189 respectively. Flow cell 2 was used a reference cell. Remaining reactive succimide ester groups were deactivated by exposure to 1M ethanolamine at a flow rate of 20µl/min for 5 minutes. VWF proteins were diluted in SPR running buffer to a final concentration of 1µM and were perfused over immobilised FXIIa at a flow rate of 30µl/min using the OneStep® titration function with a 1-2-3 orientation and a 100% loop inject volume. Dissociation of VWF proteins was carried out for 600 seconds and the chip surface was regenerated by perfusion of 600mM imidazole, 50mM EDTA, 20mM ε-ACA (pH6) at a flow rate of 30µL/min for 10 seconds. Next, flow cells were equilibrated with running buffer at a flow rate of 30µL/min for 60 seconds before the next cycle was started. All data were sampled at 20 Hertz and binding of VWF proteins to FXIIa was repeated 3 times. A 3% sucrose bulk standard was included in the experiments in order to calibrate the apparent diffusion coefficient (m^2/s) of the analytes and the correction was applied to the binding data upon export from SPR to the QDAT software (Molecular Devices Ltd). For the appropriate calculation of the diffusion coefficients, the molecular weight of the different compounds was added to the data before export of the binding curves to the QDAT software. The blank was subtracted from all binding signals and the channels were aligned before analysis. Binding curves were analysed using a non-linear regression curve fitting with a pseudo-first-order 1:1 interaction. The simulated curve was fitted as closely as possible to the sensogram by altering the diffusion coefficient and analyte retention factor. The maximal retention values (R_{MAX}) were cross-checked between the theoretical and observed R_{MAX} and any discrepancies were corrected by inputting the theoretical R_{MAX} into the software and refitting of the curves.

2.7. Activation of FXII

Conversion of zymogen FXII into proteolytic ally active FXIIa was monitored using a substrate based static plate assay. The method for the determination of enzymatic activity hereby is based on the cleavage of a chromogenic substrate and subsequent evaluation of the optical density.

2.7.1. Determination of FXIIa Concentrations

In order to determine which concentration of FXII is most suitable for the screening of the activation potential of different activators, cleavage of a FXIIa specific chromogenic substrate was monitored using a range of FXIIa concentrations. A concentration range between 6.3 and 200nM of human FXIIa (*Haematologic Technologies*) was mixed with 0.8mM chromogenic substrate H-D-Prolyl-L-phenylalanyl-Larginine-p-nitroaniline dihydrochloride (Chromogenix S-2302™; *Diapharma*) in FXII activation assay buffer (50mM HEPES; 122mM sodium chloride; pH7.4) in a final volume of 100µl/well. Cleavage of the substrate by FXIIa was monitored at 405nm for 45 minutes using an Epoch microplate spectrophotometer reader (*BioTek*) and data was analysed with GraphPad Prism.

2.7.2. Activity of Plate Immobilised FXIIa

Retention of the activity of immobilised FXII, FXIIa as well as FXII after incubation with kallikrein used in the evaluation of binding interaction between VWF proteins and FXIIa was tested using FXII activation assays. A concentration range of human FXIIa (*Enzyme Research Laboratories*) and FXII (*Haematologic Technologies Incorporated*) between 16 and 125nM in PBS (100µl/well) was used to coat a Maxisorp plate (*Nunc)* overnight at 4°C. Wells were washed three times with PBST (*Sigma*) and FXII was activated by incubation with 290nM kallikrein (*Enzyme Research Laboratories)* for 1 hour at room temperature. After washing, 0.8mM chromogenic substrate H-D-Prolyl-L-phenylalanyl-Larginine-p-nitroaniline dihydrochloride (Chromogenix S-2302™; final volume of 100µl/well; *Diapharma*) was added. Cleavage of the substrate by FXIIa was monitored at 405nm for 40 minutes using an Epoch microplate spectrophotometer reader (*BioTek*) and data was analysed with GraphPad Prism.

2.7.3. Inhibition of FXIIa Activity

Inhibition of FXIIa activity was tested using an anti-FXII antibody and corn trypsin inhibitor (CTI). 100nM FXII (*Haematologic Technologies Incorporated)* were activated using 290nM kallikrein (*Enzyme Research Laboratories)* for 1 hour at room temperature. Subsequently, kallikrein activated FXIIa and 100nM FXIIa (*Enzyme Research Laboratories*) were mixed with 40µg/ml of either an goat anti-human FXII antibody (*Enzyme Research Laboratories*) or CTI

(*Enzyme Research Laboratories*) and 0.8mM chromogenic substrate H-D-Prolyl-L-phenylalanyl-Larginine-p-nitroaniline dihydrochloride (Chromogenix S-2302™; final volume of 100µl/well; *Diapharma*). Cleavage of the substrate by FXIIa was monitored at 405nm for 40 minutes using an Epoch microplate spectrophotometer reader (*BioTek*) and data was analysed with GraphPad Prism.

2.7.4. Activation of FXII by Different Activators

Activation potential of different prospective FXII activators was tested in FXII activation assays. 100nM of human FXII (*Haematologic Technologies Incorporated*) were mixed with potential activators listed in Table 2.13 and 0.8mM chromogenic substrate H-D-Prolyl-L-phenylalanyl-Larginine-p-nitroaniline dihydrochloride (Chromogenix S-2302™; *Diapharma*) in FXII activation assay buffer (50mM HEPES; 122mM NaCl; pH7.4) in a volume of 100µl/well. Cleavage of the substrate by converted FXIIa was monitored at 405nm for 45 to 300 minutes using an Epoch microplate spectrophotometer reader (*BioTek*) and data was analysed with GraphPad Prism.

Activator	Concentration tested	Supplier
Autoactivation	-	-
Calcium Chloride	400mM; 40mM and 4mM	*Sigma Aldrich*
Kallikrein	250µg/ml, 25µg/ml and 2.5µg/ml	*Enzyme Research Laboratories*
Kinninogen	25µg/ml	*Enzyme Research Laboratories*
Dextran	500µg/ml; 50µg/ml and 5µg/ml	*Sigma Aldrich*
Sulfatides	400µM; 40µM and 4µM	*Sigma Aldrich*
Phospholipids	500µM; 50µM and 5µM	*Avanti Polar Lipids*
Collagen Type I	100µg/ml; 10µg/ml and 1µg/ml	*Chronolog*
Heparin	10Units; 5Units and 1Units	*Fannin*
Shear Stress	-	-
Polyphosphates Long	100µM; 10µM and 1µM	*Kerafast*
Polyphosphates Short	100µM; 10µM and 1µM	*Kerafast*
Plasma Derived Full-length VWF	10µg/ml, 1µg/ml and 0.1µg/ml	*Technoclone*
Purified Plasma Derived Full-Length VWF	10µg/ml, 1µg/ml and 0.1µg/ml	*Technoclone*
Full-length Recombinant VWF wildtype	10µg/ml, 1µg/ml and 0.1µg/ml	*Self produced*
Isolated VWF A1 Domain	10µg/ml, 1µg/ml and 0.1µg/ml	*Self produced*
Full-length VWF A1 Domain Mutant E1452/1459A	10µg/ml and 1µg/ml	*Self produced*
Isolated VWF A1 Domain Mutant E1452/1459A	10µg/ml and 1µg/ml	*Self produced*

Table 2.13: Overview of activators used in FXII activation assays. FXII activation potential of a variety of activators was tested at different concentrations.

Autoactivation of FXII was determined by incubation of 100nM FXII diluted in FXII activation assay buffer with 0.8M Chromogenix S-2302™ and substrate cleavage was monitored at 405nM for 45 minutes at room temperature.

Activation of FXII by shear stress was determined by incubation of 100nM FXII diluted in FXII activation assay buffer on a vortex for 1 hour at room temperature. After vortexing, the sample was mixed with 0.8M Chromogenix S-2302™ and substrate cleavage was monitored at 405nM for 45 minutes at room temperature.

Activation of FXII and cleavage of the chromogenic substrate by FXIIa was controlled by readout of i) 100nM FXIIa with and without 0.8M Chromogenix S-2302™ ii) 100nM FXII without Chromogenix S-2302™ iii) the different activators with 0.8M Chromogenix S-2302™ iv) VWF proteins mixed with anti-VWF Ab and 0.8M Chromogenix S-2302™ v) 100nM FXII mixed with truncated VWF protein C1C6 and 0.8M Chromogenix S-2302™ vi) 0.8M Chromogenix S-2302™ alone and vii) activation assay buffer alone at 405nM for 45 to 300 minutes.

2.7.5. Western Blot Analysis of FXII Activation

Aside from FXII activation assays, activation and subsequent fragmentation of FXII was additionally monitored using western blot analysis. All samples and activators as well as the appropriate controls were diluted in FXII activation assay buffer (50mM HEPES; 122mM NaCl; pH7.4) in a final volume of 50µl per sample. 100nM FXII were incubated with:

- 25µg/ml Kallikrein
- Activation assay buffer (autoactivation)
- 40mM $CaCl_2$
- 25µg/ml Kininogen
- 50µM Phospholipids
- 50µg/ml Dextran
- 40µM Sulfatides
- 25µg/ml Collagen type I
- 5U Heparin
- 10µM Polyphosphate Mix
- 10µg/ml of plasma derived full-length VWF wildtype
- 10µg/ml of full-length VWFΔA1
- 10µg/ml of isolated VWF A1 domain
- 10µg/ml of full-length VWF A1 domain mutant E1452/1459A

- 10µg/ml of isolated VWF A1 domain E1452/1459A
- 10µg/ml of truncated VWF C1C6 for 1hour at 37°C.

Samples subsequently were processed for western blot analysis as described in section 2.5.1.2.. As controls, 100nM FXIIa as well as non-activated FXII were included into the western blot. Transferred proteins were detected by incubation of the nitrocellulose membranes with goat anti-FXII HRP conjugated antibodies (*Affinity Biologics*).

2.8. Functional Effect of VWF and FXII Interaction

Functional assays were carried out in order to analyse the effects of the interaction between VWF and FXII(a). In addition, functional assays served to investigate the benefits of the disruption of the binding relationship between the two proteins in relation to blood clot formation, stability and lysis times.

2.8.1. Thrombolysis Assay

Stability of formed blood clots was tested using an adapted thrombolysis assay and was carried out using whole blood in order to investigate overall clot lysis times while blocking VWF or FXII function or with VWF depleted plasma substituted with labelled platelets, purified erythrocytes and full-length VWF A1 domain mutants.

2.8.1.1. Thrombolysis Assay with Whole Blood

Whole blood was collected from healthy donors into ACD and platelets were labelled by addition of 1µl $DiOC_6$ (*Thermo Fisher Scientific*). VWF and FXII(a) function was blocked by incubating respective sample with either rabbit polyclonal anti-VWF antibodies (diluted 1:100; *Dako*), goat anti-human FXII antibodies (diluted 1:100; *Enzyme Research Laboratories*) or both antibodies for one hour at room temperature. Clot formation was induced by deposition of 5µl clotting solution (10pM Tissue Factor (TF); Dade Innovin; *Siemens* and 70mM $CaCl_2$) onto the bottom edge of the wells of a flat-bottomed 96well plate (Costar; *Fisher Scientific*). Subsequently, 25µl of the different blood samples was added in a circular motion around the edges of the well and plates were incubated at 37°C for 2 hours. Fibrinolysis was induced by

addition of 1nM or 5nM Tissue Plasminogen Activator (tPa; *Protein Specialists*) diluted in 70µl/well PBS to the formed clots and clot degradation was measured at 510nM every minute over a period of 3 hours using a BioTek plate reader. Thrombolysis assays were controlled by absence of the clotting solution to prevent clot formation and addition of PBS without tPa resulting in the absence of clot lysis.

2.8.1.2. Thrombolysis Assay with VWF depleted Plasma

Plasma free blood was prepared as described in section 2.5.6.1.. Volumes of VWF depleted plasma (*Enzyme Research Laboratories*) equalling the original volume of plasma volume were thawed at room temperature and mixed with isolated and labelled platelets as well as purified erythrocytes. Subsequently, blood samples were reconstituted with 10µg/ml of either full-length VWF wildtype or full-length VWF A1 domain mutants E1452A, D1459A or E1452/1459A. Clot formation as well as fibrinolysis was induced as described above and clot degradation was measured at 510nM every minute over a period of 3 hours using a BioTek plate reader. Thrombolysis assays were controlled by i) absence of the clotting solution to prevent clot formation (No TF) ii) addition of PBS without tPa resulting in the absence of clot lysis (No tPa) iii). VWF depleted plasma reconstituted with $DiOC_6$ labelled platelets and purified erythrocytes (No VWF/FXIIa) and iv) plasma free blood (No VWF/No FXIIa).

2.8.2. Clot Formation under Flow with VWF depleted Plasma

Formation of platelets rich blood clots with recalcified blood was monitored using an adapted flow-based platelet capture perfusion assay. Single lanes of Ibidi flow slides were coated with collagen type I and blocked as described in section 2.5.1.6.3.. Whole blood from healthy donors was collected and platelets as well as erythrocytes were purified according to the protocol in section 2.5.6.1.. VWF and FXII depleted plasma (*Enzyme Research Laboratories*) were reconstituted in 1ml of ddH_2O based on the manufacturer's instructions and was mixed with purified red blood cells in a ratio of 1:1. Samples were reconstituted with the $DiOC_6$ labelled platelets and recalcified by addition of 10mM $CaCl_2$. Next, 10µg/ml plasma derived full-length VWF wildtype, full-length VWF A1 domain mutant E1452, full-length VWF A1 domain mutant D1459A, full-length VWF A1 domain mutant E1452/1459A or 400nM human FXIIa were added and samples were perfused over collagen type I coated channels for 4

minutes at a shear rate of 1500^{-1}. Platelets capture was controlled by perfusion of i) recalcified whole blood ii) recalcified plasma free blood (No VWF/No FXIIa) iii) recalcified VWF depleted plasma reconstituted with $DiOC_6$ labelled platelets and purified erythrocytes (No VWF/FXIIa) and iv) recalcified FXII depleted plasma containing $DiOC_6$ labelled platelets and purified erythrocytes (VWF/No FXIIa). Short movies were recorded every minute of flow and the number of accumulated platelets at each time point was assessed as surface coverage (%) using the ImageJ software and GraphPad Prism.

3. Results

Based on recent data, an interaction between FXIIa with VWF is likely to be of significance for the formation of thrombosis and provides a novel link between primary and secondary haemostasis. Even though both VWF and FXII have been separately targeted for anti-thrombotic therapy in acute as well as chronic settings, manipulating the direct interaction of FXIIa with VWF may provide a new and effective anti-thrombotic strategy without significant bleeding complications.

3.1. Generation of VWF mutants

Preliminary data from our laboratory have shown that FXIIa is a previously unidentified binding partner of VWF. Direct interaction of FXIIa and VWF was demonstrated in static plate binding assays and further binding analysis using a VWF mutant lacking the VWF A1 domain indicated the presence of a FXII binding site within the A1 domain of the glycoprotein. Based on the data and the well characterised ability of FXII to bind negatively charged surfaces, negatively charged surface residues present in the VWF A1 domain were mapped in silico and subsequently mutated in order to identify the single amino acids involved in the binding interaction between FXII and VWF.

In total, 12 residues were selected for mutation from either aspartic or glutamic acid (negative charge) to alanine (neutral charge) using site-directed mutagenesis with specifically designed primers (Methods Table 1 for primer sequences; Figure 3.1.) in both the isolated as well as full-length VWF A1 domain. Mutations of the isolated VWF A1 domain were performed using the pcDNA3.1 expression vector carrying the gene for the VWF A1 domain (available in the lab) resulting in the generation of His-tagged mutants that subsequent to sequence confirmation can be purified using nickel affinity chromatography. Due to the size of VWF, direct introduction of mutations into full-length VWF protein has been proven to be of low efficiency leading to the construction of a range of specific single domain pGEM Z(+) cloning vectors (available in the lab) containing XhoI and KpnI restriction sites facilitating subsequent subcloning of generated mutants into the pcDNA3.1 expression vector. Full-length VWF mutants were therefore produced by site-directed mutagenesis of the VWF A1 domain present in a pGEM Z(+) cloning vector. Following sequence confirmation of the desired

mutations, the different XhoI/KpnI fragments were sub-cloned back into the pcDNA3.1 expression vector for efficient expression of the proteins.

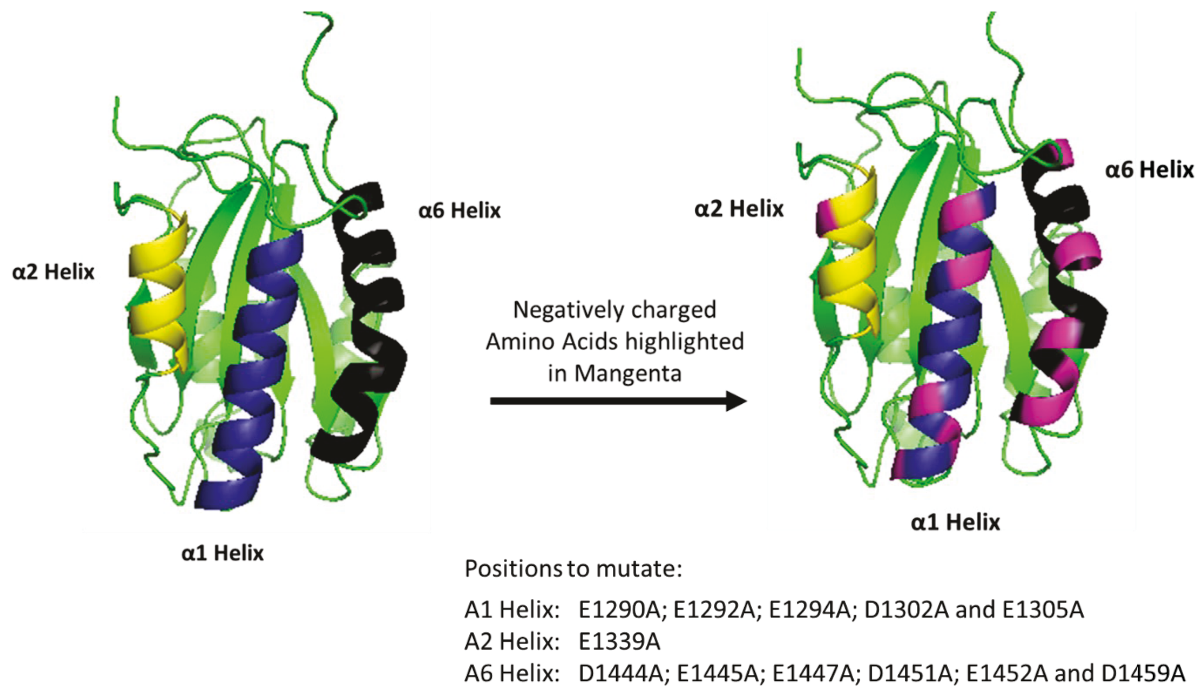

Figure 3.1: Mapping of negatively charged amino acids on the surface of the VWF A1 domain. Negatively charged amino acids were identified based on the protein crystal structure obtained from PDB (1auq) using the PyMOL software. In total, 12 different amino acids which are highlighted in mangenta were selected for site-directed mutagenensis. Subsequent to the introduction of the desired mutations, proteins were sequenced, expressed and screened for their binding ability towards FXII.

After initial screening of the different mutants for their binding ability towards FXII, double and triple mutants carrying two or three single point mutations which had exhibited reduced binding capabilities to FXIIa in static plate based assays were generated in both the isolated as well as the full-length VWF A1 domain as described above (Figure 3.2).

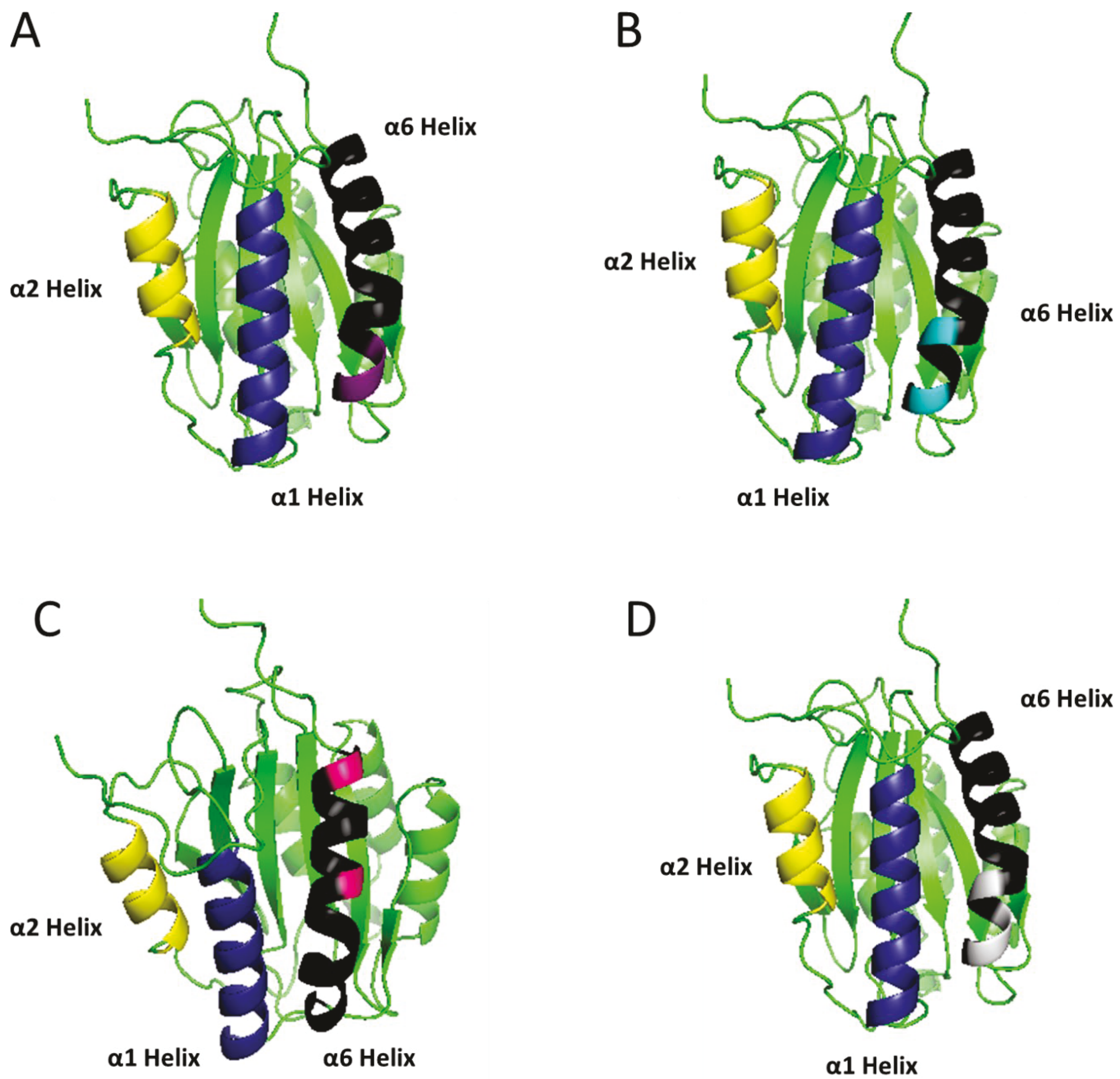

Figure 3.2: Crystal structure of the VWF A1 domain showing locations of double and triple mutants. Based on initial screening results evaluating the interaction capabilities of VWF A1 domain mutants to FXII, single-point mutations showing reduced binding ability towards FXIIa were combined. In total, three double and one triple mutant were generated. Proteins carrying the combined mutations were sequenced, expressed and subjected to further binding analysis in order to identify the exact amino acids involved in the binding interaction between VWF and FXII. A) Crystal structure of double mutant D1444/1445A. Single-point mutations at position 1444 and 1445 are highlighted in purple. B) Crystal structure of double mutant D1444/1447A. Single-point mutations at position 1444 and 1447 are highlighted in light blue. C) Crystal structure of double mutant E1452/1459A. Single-point mutations at position 1452 and 1459 are highlighted in pink. D) Crystal structure of triple mutant D1444/1445/1447A. Single-point mutations at position 1444, 1445 and 1447 are highlighted in white.

3.2. Protein Expression

All VWF proteins used for the characterisation of the binding interaction between FXII and VWF were transiently expressed in mammalian HEK293T cells aiming for high level protein expression without integration of the PEI transfected DNA into the host cell genome.

In a first step, HEK293T cells grown in a 6 well plate were transfected with different amounts of full-length VWF wildtype DNA (Figure 3.3) in order to evaluate whether varying DNA concentrations influenced overall protein expression levels. Transfection of the HEK293T cells with empty pcDNA3.1 vector was carried out as a negative control. western blot analysis of the screening experiment showed that transfection of HEK293T cells with different DNA concentrations resulted in high level protein expression with all concentration variations used. Based on the intensity the 250kDa band corresponding to full-length VWF wildtype, transfection of the cells with 1µg/ml full-length VWF wildtype DNA produced slightly elevated protein expression levels and was used for further test transfections and large-scale protein expression.

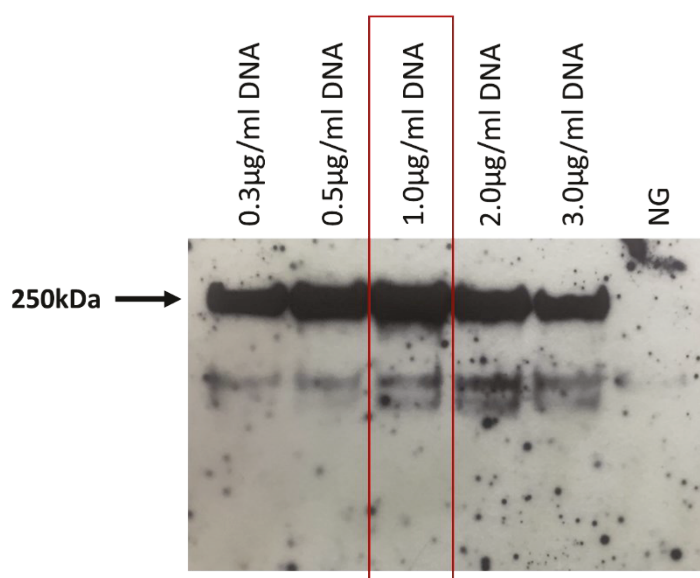

Figure 3.3: Western blot analysis of DNA concentration screening for HEK293T transfections. HEK293T cells were transfected with different amounts of full-length VWF wildtype DNA in order to evaluate whether DNA concentrations used influence overall protein expression levels. western blot results showed high level protein expression after transfection of the cells with all DNA concentrations tested. Intensity of the 250kDa band corresponding to full-length VWF wildtype after transfection with 1µg/ml DNA was slightly elevated compared to the remaining concentrations tested. Future protein expression was therefore carried out by transfection of HEK293T cells with 1µg/ml DNA. NG = negative control.

In order to evaluate whether mutation of the different surface residues within the VWF A1 domain has an effect on the expression and secretion profile of the proteins, initial transfection experiments with both isolated VWF A1 domain and full-length VWF A1 domain mutants were carried out in 6 well plates and expression levels of the mutants were evaluated. In addition to the VWF A1 domain mutants, cells also were transfected with the native isolated VWF A1 domain, recombinant full-length VWF wildtype as well as with empty pcDNA3.1 vector.

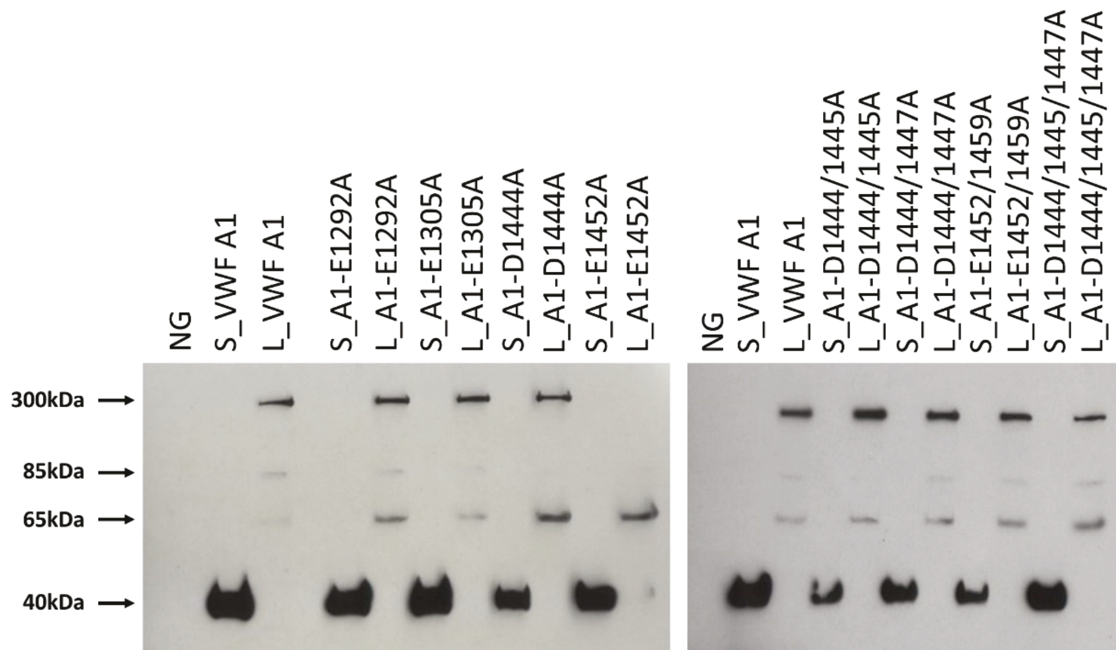

Figure 3.4: Protein expression and secretion profiles of the isolated VWF A1 domain and an example selection VWF A1 domain mutants. HEK293T cells were transiently transfected with the isolated VWF A1 domain as well as isolated VWF A1 domain single-point, double or triple mutants. Expression and secretion of the proteins was analysed by western blot of the conditioned medium and the cell lysate and, based on the formation of protein bands with a molecular weight of approximately 40kDa, showed similar protein expression and cell retention levels for nine out of 12 mutants compared to the isolated VWF A1 domain itself. Transfection of the cells with the empty pc3.1DNA vector led to absence of protein expression. Formation of additional protein bands at molecular sizes of approximately 300kDa, 85kDa and 65kDa indicate the formation of VWF A1 domain dimers or aggregates after high level protein expression. NG = negative control; S = supernatant; L = Lysate.

Western blot analysis of the conditioned medium as well as the cell lysate (Figure 3.4) showed uniform expression and secretion of the isolated VWF A1 domain and the majority of isolated VWF A1 domain mutants indicated by the formation of string protein bands of approximately 40kDa. Nine out of the 12 mutants were expressed at similar levels and a media to lysate ratio comparable to the isolated VWF A1 domain itself. Mutations at position 1302 and 1339

resulted in a significant reduction in protein expression indicating that introduction of mutations at these specific positions influences correct expression of the protein by HEK293T cells. Western blot of the cell medium and lysate showed the detection of additional protein bands corresponding to a molecular size of approximately 65kDa, 85kDa and 300kDa. Due to the fact that all proteins were detected using an anti-VWF specific antibody it is likely that these additional bands consist of VWF A1 domain mutant dimers or aggregates formed during high level expression of the proteins. As expected, transfection of the cells with the empty pc3.1DNA vector led to absence of protein expression.

As already observed with the isolated VWF A1 domain mutants, analysis of the expression and secretion profiles of full-length VWF A1 domain mutants showed adequate expression profiles for nine out of 12 mutants indicated by the formation of a protein band of approximately 250kDa. Comparison of the expression levels showed a low expression profile of mutants D1302A and E1339A. Full-length VWF wildtype as well as full-length VWFΔA1 exhibited reduced western blot signals compared to the full-length VWF A1 domain mutants (Figure 3.5). Interestingly, the full-length VWF A1 domain triple mutant carrying single point mutations at position 1444, 1445 and 1447 did not express at all in three separate transfection attempts indicating that simultaneous mutation of three amino acid residues prohibits expression of the protein by HEK293T cells. In contrast to the expression of isolated VWF A1 domain and isolated A1 domain mutants, cell retention levels varied between the different full-length VWF proteins with mutants E1290A, E1305A, D1444A, D1451A and D1444/1445A showing a higher media to lysate ratio compared to the remaining proteins expressed. Transfection of HEK293T cells with the empty pc3.1DNA vector again resulted in absent protein expression.

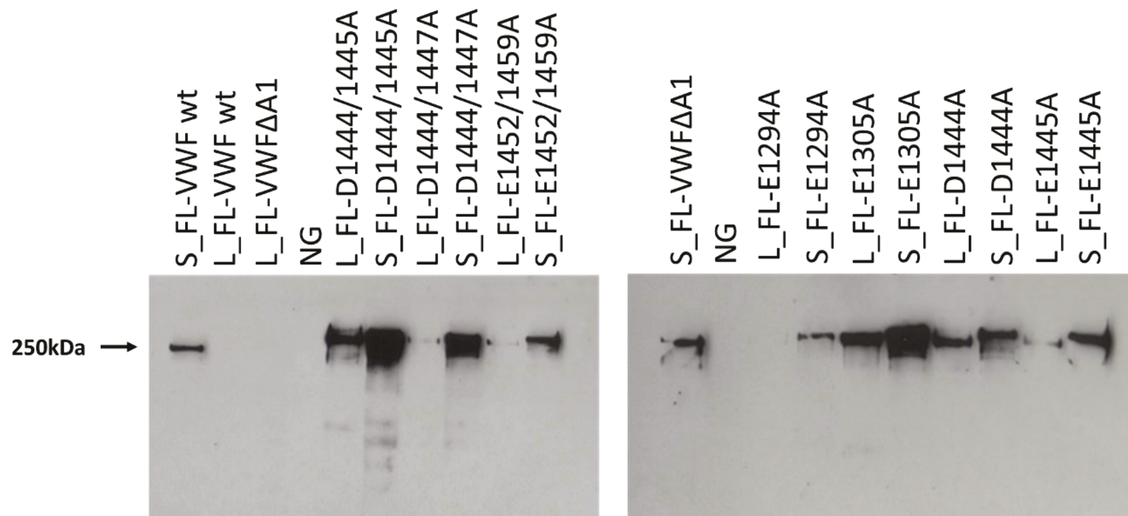

Figure 3.5: Analysis of protein expression and secretion profiles of full-length VWF wildtype, full-length VWFΔA1 and an exemplary selection full-length VWF A1 domain mutants. HEK293T cells were transiently transfected with full-length VWF wildtype, full-length VWFΔA1 as well as full-length VWF A1 domain single-point, double or triple mutants. Expression and secretion of the proteins was analysed by western blot of the conditioned medium and the cell lysate and showed adequate but varying protein expression and cell retention levels for nine out of 12 mutants indicated by the formation of protein bands with a molecular weight of approximately 250kDa. Expression levels of full-length VWF wildtype and full-length VWFΔA1 were reduced compared to full-length VWF A1 domain mutants. Transfection of the cells with the empty pc3.1DNA vector led to absence of protein expression. FL = full-length; wt = wildtype; NG = negative control; S = supernatant; L = Lysate.

Subsequent to small-scale transfections, expression of the isolated VWF A1 domain, the isolated VWF A1 domain mutants, full-length VWF wildtype, full-length VWFΔA1 as well as the full-length VWF A1 domain mutants were scaled up. Even though initial analysis of the expression levels of mutants D1302A and E1339A showed reduced expression levels in both the isolated VWF A1 domain as well as full-length VWF, both proteins were included in the up-scaling process. Based on absent expression of mutant in full-length VWF, the triple mutant E1444/1445/1447A was excluded from all further experiments.

3.3. Protein Purification

In order to characterise the interaction between VWF and FXII as well as identify the specific VWF amino acid residues involved, large quantities of the different proteins were needed. All VWF based proteins used in this project were expressed in HEK293T cells after large-scale PEI transfections. 150 to 200ml of expression medium was collected after three days and

centrifuged in order to separate cells and cellular debris from the proteins present in the supernatant. Subsequently, the volume of the different samples was reduced using either tangential flow filtration for the isolated VWF A1 domain and isolated VWF A1 domain mutants or Amicon Ultra 4 centrifugal filter units for full-length VWF wildtype, full-length VWFΔA1 and full-length VWF A1 domain mutants. After concentration, the isolated VWF A1 domain, all isolated VWF A1 domain mutants, full-length VWF wildtype as well as full-length VWFΔA1 were subjected to either immobilised metal affinity chromatography or ion exchange chromatography. Full-length VWF A1 domain mutants and half of the full-length VWF wildtype sample were not further processed after concentration but directly characterised in terms of their protein content and purity.

Isolated VWF A1 domain mutants as well as the isolated VWF A1 domain itself were IMAC purified using Ni^{2+}-His Trap chelating columns which allow efficient purification of His-tagged molecules. Proteins were loaded onto the column, washed and subsequently eluted with an imidazole concentration gradient (maximum 50mM imidazole). Protein elution occurred in a single and narrow peak at imidazole concentrations ranging between 37.5 and 50mM (Figure 3.6). All elution fractions were collected and analysed by SDS-PAGE followed by Coomassie staining. Fractions containing the protein of interest were dialysed against 20mM Tris and 100mM NaCl (pH7.4), concentrated using Amicon Ultra 4 centrifugal filters and protein concentration was determined by BCA assay.

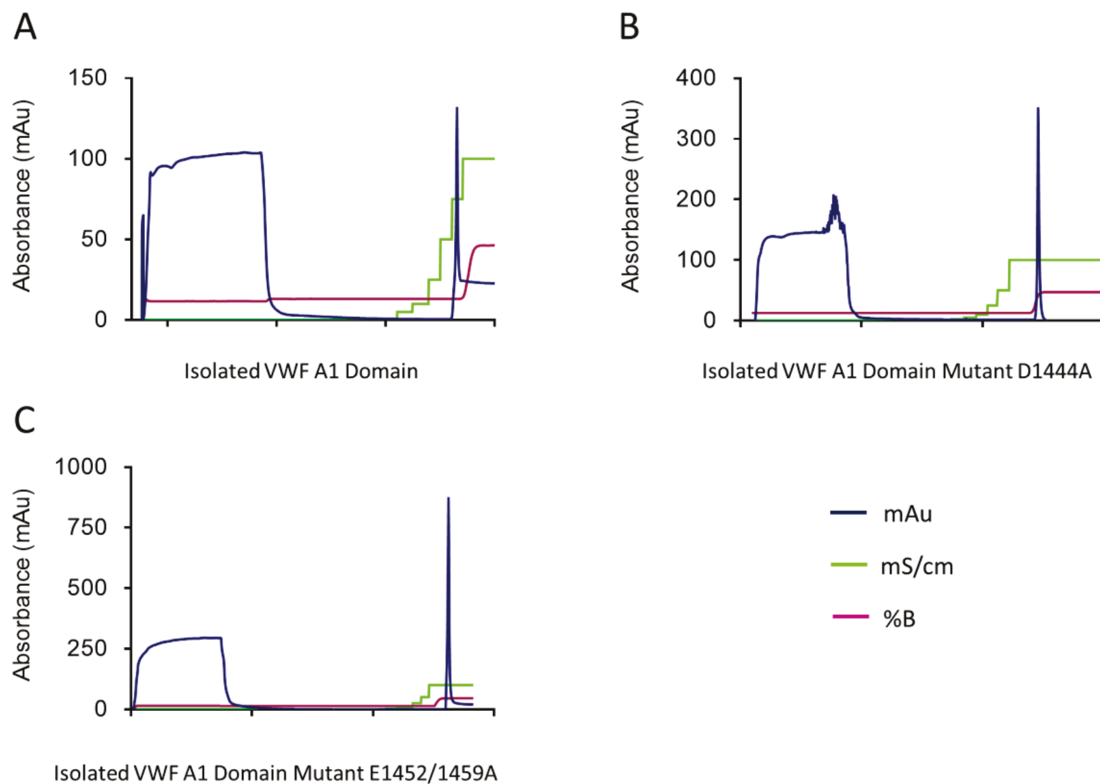

Figure 3.6: Immobilised metal affinity chromatography of the isolated VWF A1 domain and example isolated VWF A1 domain single-point and double mutants. Isolated VWF A1 domain and isolated VWF A1 domain mutants were transiently expressed using HEK293T cells and conditioned medium was collected, centrifuged and concentrated with tangential flow filtration. Subsequent to filtration, His-tagged proteins were purified with Ni^{2+}-His Trap chelating columns. Elution was carried out with an imidazole concentration gradient and elution fractions were collected for further analysis by SDS-PAGE. Elution fractions containing the proteins of interest additionally were dialysed, further concentrated and subjected to BCA in order to determine the protein concentration of the sample. IMAC chromatograms documented elution of the isolated VWF A1 domain (A), isolated VWF A1 domain mutant D1444A (B) and isolated VWF A1 domain mutant E1452/1459A (C) as single and narrow peaks. mAu = milli absorbance unit.

Full-length VWF wildtype and full-length VWFΔA1 were purified by ion exchange chromatography using a SK16 ion exchange chromatography column. Proteins were loaded onto the column, washed and subsequently eluted with 500mM NaCl (Figure 3.7). Proteins eluted as single peaks with low absorbance levels indicating a low protein concentration of the samples. The collected elution fractions containing the protein of interest were analysed by SDS-PAGE followed by Coomassie staining, dialysed against 20mM Tris and 100mM NaCl (pH7.4) and concentrated down using Amicon Ultra 4 centrifugal filters. Protein concentrations of the dialysed samples were determined using an in house VWF ELISA.

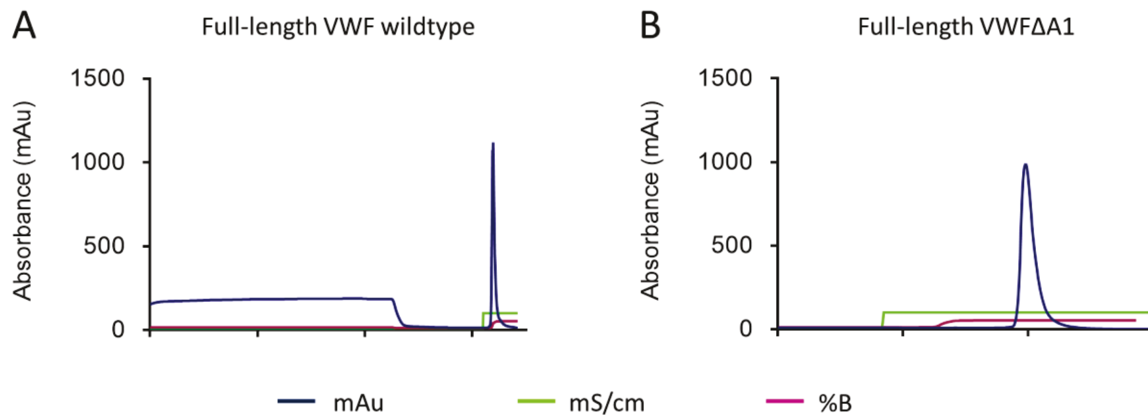

Figure 3.7: Ion exchange chromatography of recombinant full-length VWF wildtype and full-length VWFΔA1. Conditioned medium containing HEK293T expressed recombinant full-length VWF (A) and full-length VWFΔA1 (B) was collected, centrifuged and concentrated down using Amicon Ultra 4 centrifugal filters. Protein samples were loaded onto a SK16 ion exchange column, washed and eluted with 500mM NaCl and elution fractions were collected for analysis by SDS-PAGE. Elution fractions containing the proteins of interest additionally were dialysed, further concentrated and subjected to an in house VWF ELISA in order to determine the protein concentration of the sample.

3.4. Protein Characterisation

3.4.1. SDS-PAGE Analysis

The purity of all expressed proteins after purification or concentratio was analysed using SDS-PAGE followed by Coomassie staining. While samples collected during IEC or IMAC were run under non reducing conditions, final analysis of all purified VWF mutants in both the isolated VWF A1 domain as well as full-length VWF A1 domain were processed under reducing conditions.

Gel electrophoresis (non-reduced) of samples obtained during ion exchange chromatography of full-length VWF wildtype and full-length VWFΔA1 showed stepwise enrichment of both proteins which were retained on the column during the washing step and eluted in elution fraction 2. SDS-PAGE analysis of elution fraction 2 displayed formation of bands of approximately 250kDa corresponding to monomeric VWF and VWFΔA1 but also the presence of additional protein bands indicating contaminants and a low level of sample purity (Figure 3.8A). Subsequent to ion exchange, the proteins were further concentrated increasing the

concentration of all proteins present in the sample which was reflected by higher band intensities.

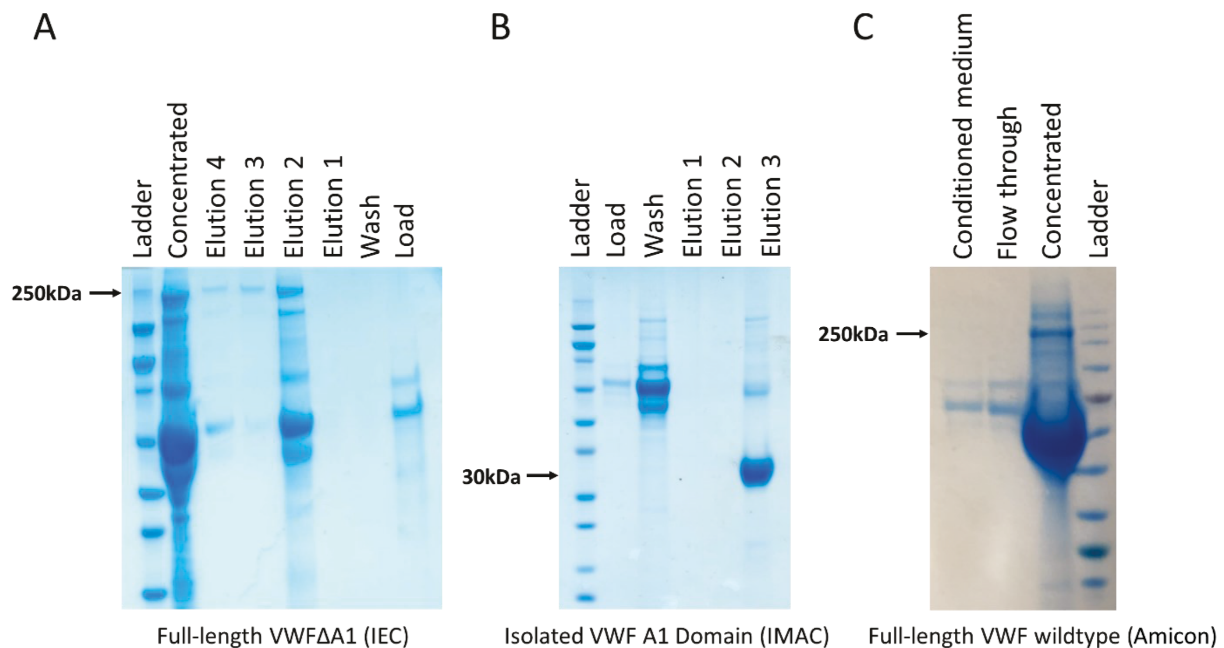

Figure 3.8: SDS-PAGE analysis of full-length VWF and isolated VWF A1 domain proteins. All proteins generated during this project were analysed by SDS-PAGE after purification or concentration. Samples were mixed with loading dye and run under non-reducing conditions. Gels subsequently were stained with Coomassie and protein purity as well as protein concentrations were assessed. A) SDS-PAGE analysis of ion exchange purified full-length VWFΔA1. Formation of a band of approximately 250kDa corresponding to monomeric VWF indicated elution of the protein of interest in predominantly elution fraction 2. Further concentration showed enrichment of full-length VWFΔA1 after purification. Presence of additional protein bands in both the elution fraction as well as the concentrated sample indicated low purity of the protein preparation. B) SDS-PAGE analysis of immobilised metal ion chromatography purified isolated VWF A1 domain. The isolated VWF A1 domain eluted in a single fraction (elution fraction 3) from the column. Formation of a strong band at approximately 30kDa corresponding to the monomeric VWF A1 domain indicated successful purification of the protein of interest and a high protein concentration of the sample. Presence of additional protein bands in elution fraction 3 might be due to the formation of VWF A1 dimers or aggregates or residual impurities contained in the protein preparations. C) SDS-PAGE analysis of concentrated full-length VWF wildtype. The majority of transiently expressed full-length VWF wildtype was directly concentrated using Amicon Ultra 4 centrifugal filters. Formation of a protein band of approximately 250kDa corresponding to monomeric VWF in the concentrated samples showed successful enrichment of the protein of interest. Presence of multiple additional protein bands indicated low purity of the samples. IEC = Ion exchange chromatography; IMAC = Immobilised metal affinity chromatography.

SDS-PAGE analysis (non-reduced) of the isolated VWF A1 domain and isolated VWF A1 domain mutants with immobilised metal ion chromatography showed successful purification of all proteins with high purity. Examination of both the loading and wash flow-through indicated that proteins were able to bind to the column via their His-tag (Figure 3.8B). All proteins were eluted in a single fraction which displayed formation of a protein band of approximately 30kDa

corresponding to the monomeric VWF A1 domain or VWA A1 domain mutants. Presence of additional faint bands indicated low-level dimerisation or aggregation of the isolated VWF A1 domain and VWF A1 domain mutants.

Different from full-length VWFΔA1 and a batch of full-length VWF wildtype, the majority of full-length VWF wildtype as well as all full-length VWF A1 domain mutants were not purified but directly concentrated after expression of the proteins by HEK293T cells. Concentration was carried out using Amicon Ultra 4 centrifugal filters and SDS-PAGE analysis of the sample showed formation of a protein band of approximately 250kDa corresponding to monomeric VWF (Figure 3.8C). All samples were of low purity and were characterised by the presence of multiple bands on the gels in addition to the desired protein.

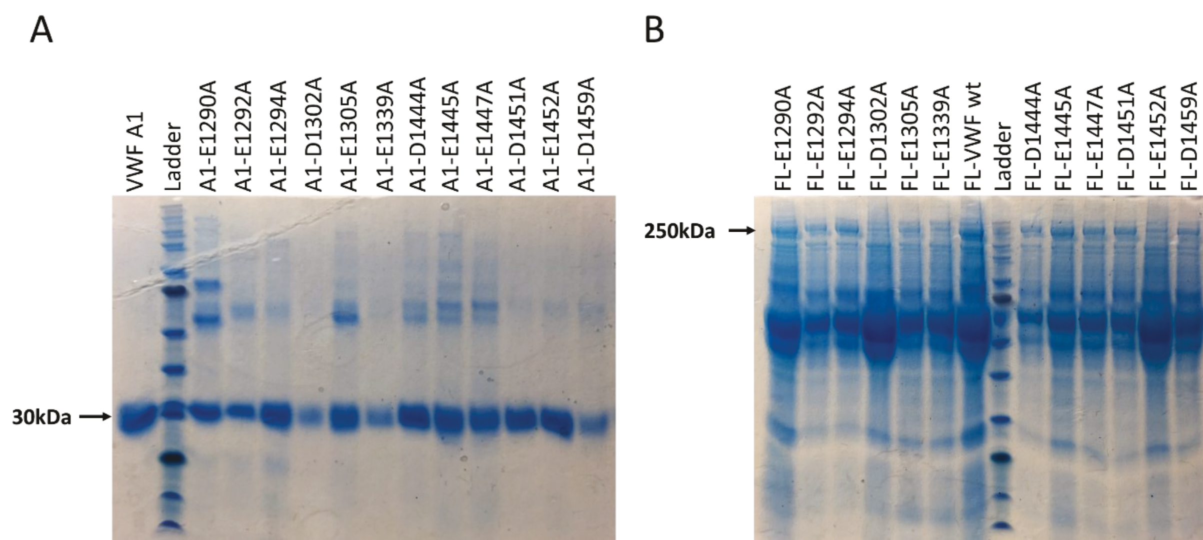

Figure 3.9: SDS-PAGE analysis of isolated VWF A1 domain and full-length VWF proteins. All proteins generated during this project were analysed by SDS-PAGE after purification or concentration. Samples were mixed with loading dye ± β-Mercaptoethanol and run under reducing conditions. Gels subsequently were stained with Coomassie and protein purity as well as protein concentrations were assessed. A) SDS-PAGE analysis of immobilised metal ion chromatography purified isolated VWF A1 domain and isolated A1 domain mutants. Formation of a strong band at approximately 30kDa corresponding to the monomeric VWF A1 domain indicated successful purification of all proteins. Protein concentrations were comparable between most samples with mutants D1302A and E1339A showing slightly reduced expression levels. Purity of all samples was moderate to high. Formation of additional protein bands might be due to residual impurities in the protein preparation. B) SDS-PAGE analysis of down-concentrated full-length VWF wildtype and full-length VWF A1 domain mutants. Analysis of all concentrated samples was characterised by the formation of multiple bands indicating low purity of the protein preparations. Formation of a protein band of approximately 250kDa corresponding to monomeric VWF verified presence of the proteins of interest. Band intensities indicated low concentration for all full-length VWF A1 domain mutants. A1 = isolated VWF A1 domain; FL = full-length; wt = wildtype.

After expression and purification or concentration, all proteins necessary for the characterisation of the binding interaction between VWF and FXII were analysed by SDS-PAGE under reducing conditions in order to allow a direct comparison of the sample purity as well as an initial estimation of the protein concentration. Samples containing either the isolated VWF A1 domain or isolated VWF A1 domain mutants generally showed moderate to high levels of purity and similar estimated protein concentrations for 11 out of the 13 proteins analysed. SDS-PAGE of isolated VWF A1 domain mutants D1302A and E1339A indicated lower protein concentrations compared to the remaining samples (Figure 3.9A). Full-length VWF wildtype as well as full-length VWF A1 domain mutants were not purified but concentrated directly after expression resulting in protein preparations of low purity reflected by the formation of multiple bands during SDS-PAGE analysis (Figure 3.9B). Based on the electrophoresis results, overall concentration of both full-length VWF wildtype as well as full-length VWF A1 domain mutants was estimated to be low.

Aside from single-point VWF mutants, full-length VWF and isolated VWF A1 domain double mutants were subjected to SDS-PAGE under reducing conditions for the full-length proteins and non-reducing conditions for the isolated VWF A1 domain mutants (Figure 3.10). As already observed before, the overall purity of the samples containing full-length VWF A1 domain mutants was low. Comparison of the band intensities indicated a higher protein concentration for mutant D1444/1445 compared to mutants D1444/1447A and E1452/1459A. Even though formation of multiple bands was observed, samples containing the isolated VWF A1 domain double mutants showed a higher purity compared to full-length VWF A1 domain mutants. Additional protein bands might be caused by the formation of A1 domain dimers or aggregates during expression and purification of the proteins or might be due to the presence of residual impurities in the protein preparations. Concentrations of the three isolated VWF A1 domain mutants were comparable indicating similar expression levels for all proteins.

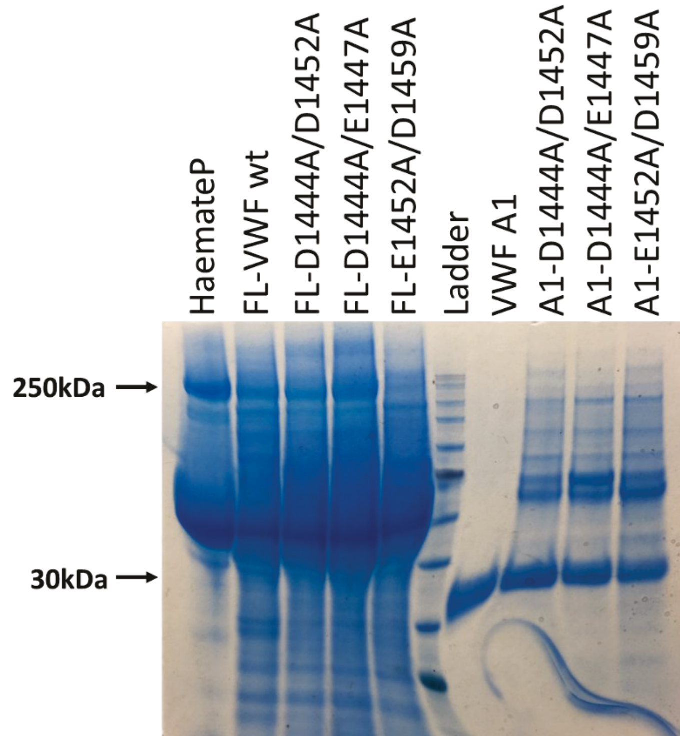

Figure 3.10: SDS-PAGE analysis of isolated VWF A1 domain and full-length VWF double mutants. In addition to single-point mutants, isolated VWF A1 domain and full-length VWF A1 domain double mutants were analysed by SDS-PAGE after purification or concentration. Samples were mixed with loading dye ± β-Mercaptoethanol and run under reducing (full-length VWF proteins) or non-reducing (isolated VWF A1 domain proteins) conditions. Gels subsequently were stained with Coomassie and protein purity as well as protein concentrations were assessed. A) SDS-PAGE analysis of down-concentrated full-length VWF double mutants. Samples containing full-length VWF double mutants were of low purity and showed formation of multiple protein bands. Bands at approximately 250kDa verified presence of the proteins of interests and band intensity indicated a higher protein concentration for mutant D1444/1447A compared to the remaining two double mutants. B) SDS-PAGE analysis of immobilised metal ion chromatography purified isolated VWF A1 domain double mutants. Gel analysis of the isolated VWF A1 double mutants showed successful purification of all three double mutants. Aside from bands of approximately 30kDa corresponding to the monomeric A1 domain, presence of multiple additional bands indicated formation of protein dimers and aggregates or the presence of residual impurities in the respective samples. Band intensities of the different protein preparations were comparable insinuating similar protein concentrations of the different samples.

3.4.2. Determination of Protein Concentrations

Subsequent to SDS-PAGE analysis which allowed an initial estimation of the protein concentration, the exact concentrations of all protein preparations were determined by either BCA for the isolated VWF A1 domain and all isolated VWF A1 domain mutants or a VWF specific ELISA for recombinant full-length VWF wildtype, full-length VWFΔA1 as well as all full-length VWF A1 domain mutants. All samples were measured in triplicates and mean

concentrations values were used for all future calculations. Table 3.1 depicts an overview of the concentrations of all VWF based proteins relevant for this project.

While preparations of full-length VWF wildtype (510µg/ml), full-length VWF A1 domain mutant E1290A (230µg/ml) and full-length VWF A1 domain double mutant D1452/1447A (554µg/ml) showed a high protein content, the majority of the full-length VWF A1 domain mutants displayed low protein concentrations ranging between 10 to 96µg/ml. Concentration of samples containing the isolated VWF A1 domain and isolated VWF A1 domain mutants were significantly higher compared to full-length VWF variants and ranged from 80 µg/ml for clone D1459A to 449 µg/ml for the isolated VWF A1 domain.

No.	Protein	Name	Concentration (ug/ml)
1	FL-VWF	VWF-wt	510
2	FL-VWF	VWFΔA1	96
3	FL-VWF	E1290A	230
4	FL-VWF	E1292A	60
5	FL-VWF	E1294A	70
6	FL-VWF	D1302A	24
7	FL-VWF	E1305A	40
8	FL-VWF	E1339A	10
9	FL-VWF	D1444A	64
10	FL-VWF	E1445A	77
11	FL-VWF	E1447A	56
12	FL-VWF	D1451A	54
13	FL-VWF	E1452A	23
14	FL-VWF	D1459A	44
15	FL-VWF	D1444/1445A	45
16	FL-VWF	D1444/1447A	554
17	FL-VWF	E1452/1459A	48

No.	Protein	Name	Concentration (ug/ml)
18	A1 Domain	VWF A1	449
19	A1 Domain	E1290A	299
20	A1 Domain	E1292A	109
21	A1 Domain	E1294A	353
22	A1 Domain	D1302A	20
23	A1 Domain	E1305A	208
24	A1 Domain	E1339A	10
25	A1 Domain	D1444A	221
26	A1 Domain	E1445A	220
27	A1 Domain	E1447A	100
28	A1 Domain	D1451A	165
29	A1 Domain	E1452A	175
30	A1 Domain	D1459A	80
31	A1 Domain	D1444/1445A	90
32	A1 Domain	D1444/1447A	100
33	A1 Domain	E1452/1459A	630
-	-	-	-

Table 3.1: Concentrations of all VWF based proteins relevant for the characterisation of the interaction between VWF and FXII. Proteins were transiently expressed in HEK293T cells, purified using either IMAC or IEC or directly-down concentrated. Concentrations of the protein preparations were determined by BCA for the isolated VWF A1 domain and the isolated VWF A1 domain mutants and an VWF specific ELISA for all full-length VWF proteins. Concentrations of the majority of full-length proteins was significantly lower compared to the isolated VWF A1 domain variants. Contradictory to results obtained during SDS-PAGE analysis, concentration determination of both full-length and isolated VWF A1 domain mutants D1302A and E1339A showed very low protein concentrations indicating that introduction of the mutations prohibits effective expression of the protein. While change of the amino acid residue at position 1452 results in low protein concentration of the isolated VWF A1 domain mutant, introduction of the same mutation in the full-length protein does not seem to affect expression of the mutant. No. = number; FL = full-length.

Interestingly, concentrations of clones D1302A and E1339A in both the isolated A1 (20 and 10 µg/ml respectively) as well as full-length VWF A1 domain (24 and 10 µg/ml respectively) were very low indicating that introduction of these mutations prohibits effective expression of the proteins. In addition, introduction of a mutation at position 1452 resulted in low protein concentrations (175µg/ml) in the isolated VWF A1 domain which did not correlate with expression of the mutant in the full-length protein (23µg/ml).

3.4.3. Gel Analysis of VWF Multimers

Physiological activity of full-length VWF is heavily dependent on the formation of large multimeric protein complexes and multimeric size has been shown to directly correlate to the activity levels of VWF. Expressed and purified full-length VWF proteins were analysed in terms of their multimer formation in order to ensure correct expression and post-translational processing as well as maintained integrity of the proteins after purification. Plasma derived, recombinant full-length VWF as well as the 12 full-length VWF A1 domain single-point mutants and the three double mutants were subjected to gel electrophoresis followed by detection of the proteins by western blot (Figure 3.11 for a selection of full-length VWF A1 domain mutants). Results of the multimer analysis demonstrated formation of a range of multimer patterns for all proteins tested. Direct comparison of plasma derived full-length VWF wildtype to the different full-length VWF A1 domain mutants and recombinant full-length VWF wildtype displayed absence of a band correlating to ultra-high multimers in all expressed proteins indicating a limitation in multimer assembly during protein expression or loss of this protein fraction during purification. All proteins will be tested in flow-based platelet capture assays in order to ensure full functionality in terms of collagen and platelet binding.

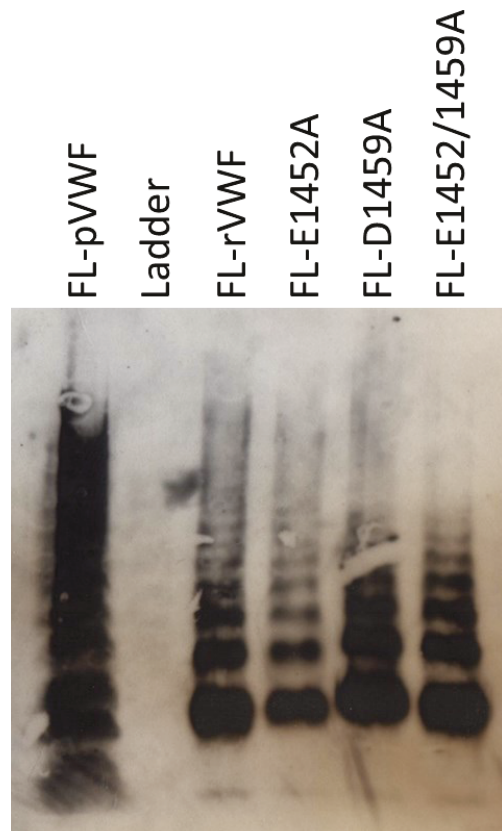

Figure 3.11: Multimer gel analysis of recombinant full-length VWF wildtype and full-length VWF A1 domain mutants. Formation of VWF multimers after protein expression and purification was analysed using gel electrophoresis and subsequent detection of the multimer patterns by western blot. 0.5µg/ml of the individual proteins were loaded onto self-made multimer gels and run at 65V for 20 minutes and 35V until the tracking dye had run out of the gel. Proteins were transferred onto western blot membranes and detected with an anti-VWF Ab HRP conjugate. Results of the multimer analysis showed formation of a range of multimers for all proteins tested. Compared to multimeric patterns formed by plasma derived full-length VWF wildtype both recombinant full-length VWF wildtype as well as the different full-length VWF A1 domain single-point and double mutants displayed an absence of a band correlating to ultra-large multimers. Impact of this slightly extenuated formation of multimers on the overall functionality of the proteins will be tested in platelet capture experiments verifying collagen and platelet binding ability of all full-length VWF proteins. FL = full-length; pVWF = plasma derived full-length VWF wildtype; rVWF = recombinant full-length VWF wildtype.

3.4.4. Platelet Capture of Full-Length VWF and VWF Mutants

VWF has a crucial role during primary haemostasis and facilitates the initial capture of platelets at sites of vascular injury. VWF circulates the blood in a globular conformation in which its multiple binding sites are shielded preventing unwanted interaction of the glycoprotein with its various binding partners. Upon endothelial injury, VWF is able to bind to exposed subendothelial collagen via specific collagen binding sites present in the A1 and A3 domain and eventually adopts a linear conformation. Once linearised, VWF can interact with

platelets via its GP1bα binding site in the A1 domain resulting in the effective arrest of platelets from the circulation.

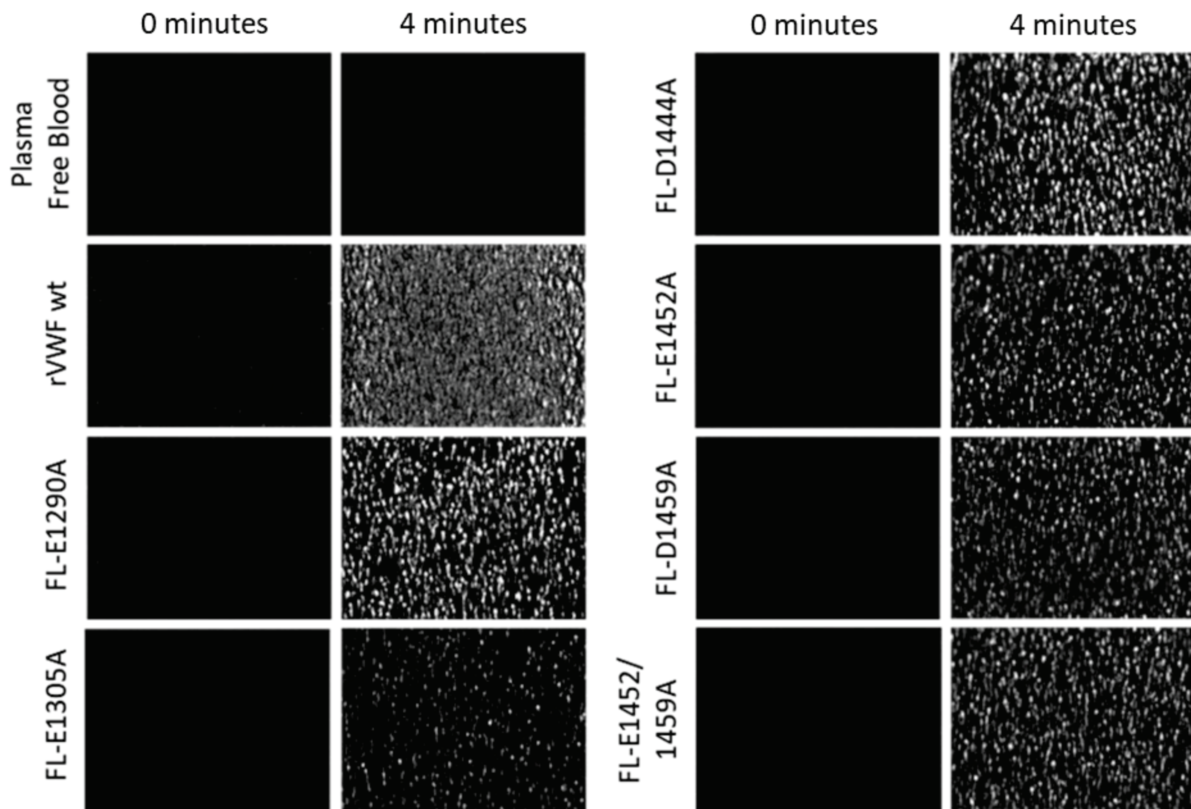

Figure 3.12: Platelet capture profiles of full-length VWF wildtype and selected full-length VWF A1 domain single and double mutants. In order to ensure integrity of the collagen and platelet binding sites present in the A1 domain of full-length VWF A1 domain mutants, plasma free blood containing labelled platelets was reconstituted with 10µg/ml of the different proteins and subsequently perfused over collagen type I coated ibidi slides at a shear rate of 1500^{-1}s. Platelet capture was recorded and assessed after four minutes. Perfusion of plasma free blood alone showed no attachment of platelets to the collagen type I coated slides. Platelet surface coverage after perfusion of samples containing the different full-length VWF A1 domain mutants was comparable to samples with full-length VWF wildtype. FL = full-length

Due to the fact that all VWF mutants generated in this project contained amino acid changes in the A1 domain which together with the A3 domain facilitates binding of VWF to collagen and platelets, integrity of platelet capture mediated by the different VWF variants had to be ensured. Platelet capture profiles of full-length VWF proteins in plasma free blood containing fluorescently labelled platelets were analysed under conditions of flow on collagen type I coated ibidi slides. The experimental setup allowed demonstration of protein binding ability to both collagen type I and platelets under physiological conditions. Perfusion of the samples was carried out for four minutes and platelet capture was assessed at the beginning and the

end of the experiment. Results showed that recombinant full-length VWF wildtype exhibited platelet capture profiles comparable to nine out of 12 full-length VWF A1 domain mutants tested (Figure 3.12).

As well as optical examination of the platelet capture profiles, the number of accumulated platelets after perfusion of the full-length VWF A1 domain mutants was assessed as surface coverage and compared to platelet capture profiles of full-length VWF wildtype using the ImageJ software (Figure 3.13). Platelet capture of all full-length VWF A1 domain mutants as well as recombinant full-length VWF was carried out as a single experiment and was subsequently repeated for full-length VWF wildtype, full-length VWF A1 domain mutant E1452A, D1459A and double mutant E1452/1459A, which had been shown to be involved in the binding interaction between VWF to FXII (binding analysis is described in section 3.5). Comparison of the platelet capture profiles showed that the majority of full-length VWF mutants had similar platelet capture ability as full-length VWF wildtype (Figure 3.13) with surface coverage percentages ranging from 67% (mutant E1305A) to 95% (mutant E1445A). Platelet numbers counted after perfusion of clones D1302A, E1305A and E1339A showed a reduction of attached platelets correlating with low expression profiles and protein concentration for clones D1302A and E1339A. Overall, results obtained in this experiment show that mutation of the different amino acid residues did not compromise either the collagen or the platelet binding ability of the proteins tested. Full-length VWF mutants can therefore be used for a more in-depth analysis of the interaction between FXII and VWF in order to identify and verify specific amino acids involved in binding of the two proteins.

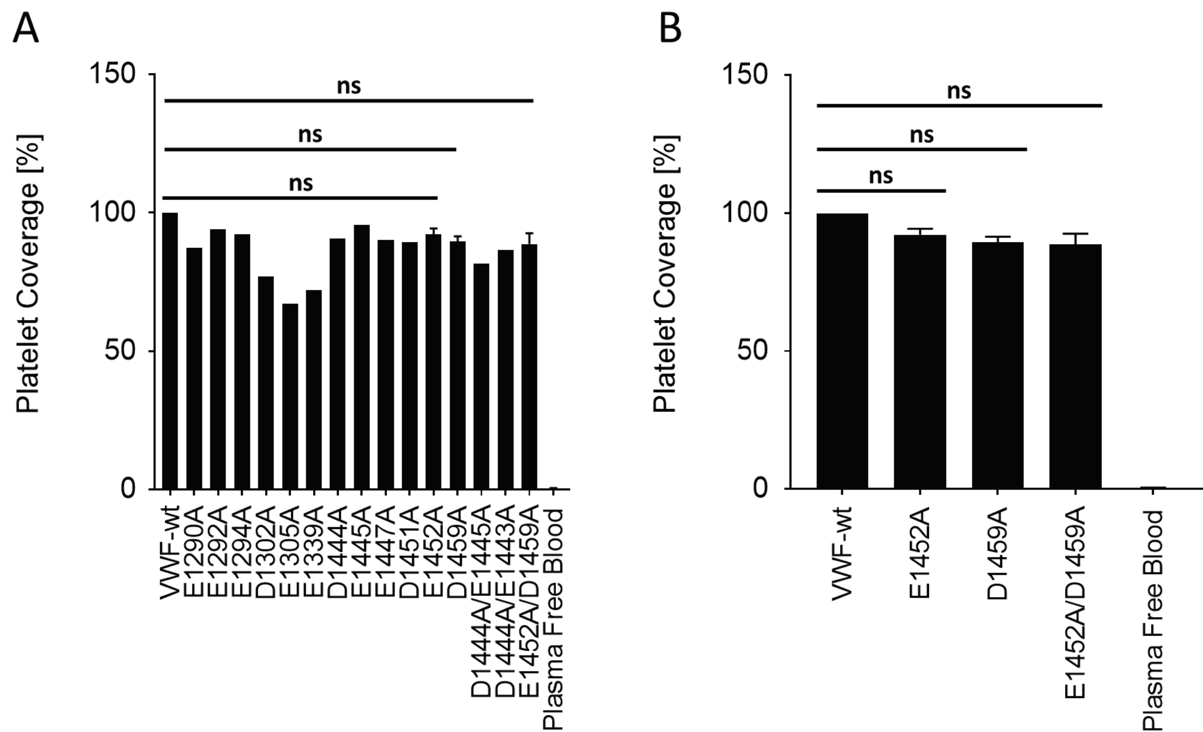

Figure 3.13: Platelet surface coverage of recombinant full-length VWF wildtype and full-length VWF A1 domain single and double mutants. In order to ensure integrity of the collagen and platelet binding ability of full-length VWF A1 domain mutants, plasma free blood containing labelled platelets was reconstituted with 10µg/ml of the different proteins and subsequently perfused over collagen type I coated ibidi slides at a shear rate of 1500^{-1}s. Platelet capture was recorded and platelet surface coverage was assessed after four minutes. Perfusion of plasma free blood alone showed no attachment of platelets to the collagen type I coated slides. A) Platelet surface coverage of all full-length VWF mutants compared to full-length VWF wildtype. Platelet surface coverage after perfusion of samples containing the different full-length VWF A1 domain mutants was comparable to samples with full-length VWF wildtype for the majority of the variants tested. Platelet coverage varied between 67% (mutant E1305A) and 95% (mutant E1445A). Three separate clones (D1302A, E1305A and E1339A) showed reduced platelet capture ability which correlated with low protein expression levels for clones D1302A and E1339A. B) Platelet surface coverage of single-point and double full-length VWF A1 domain mutant involved in the binding interaction between VWF and FXII (binding analysis described in section 3.5) compared to full-length VWF wildtype. All three proteins tested showed similar numbers of platelets attached after four minutes of flow. Conclusively, results show that mutation of the negatively charged amino acid residues in the VWF A1 domain has no significant influence on the ability of the different mutants to bind to collagen and capture platelets. The proteins therefore can be used for the further characterisation of the binding interaction between VWF and FXII. Data presented as mean ± SEM; P-value < 0.0001. n = 1 to 3.

3.5. Binding Analysis of VWF to FXII

Preliminary data from our laboratory postulated a novel binding interaction between VWF and FXII that was tested in a variety of plate based binding assays and subsequently confirmed with surface plasmon resonance measurements.

Initial binding interaction between the two proteins was tested in a 4-way ELISA (Figure 3.14) in which either FXIIa, plasma derived full-length VWF wildtype or antibodies capable of capturing formed FXIIa/VWF complexes were immobilised and subsequently incubated with the respective binding partner.

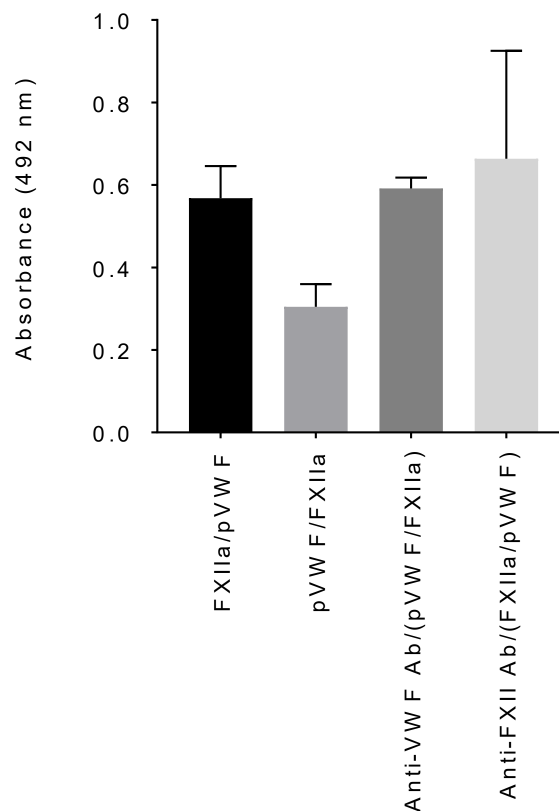

Figure 3.14: 4-way ELISA testing the general binding ability of plasma derived full-length VWF wildtype to FXIIa. Binding capability of plasma derived VWF to FXIIa was analysed using different ELISA in which either VWF, FXIIa or antibodies which are able to capture formed VWF/FXIIa complexes were immobilised and incubated with their respective binding partners. Binding of the different proteins was detected using anti-VWF or anti-FXII HRP conjugated antibodies. Binding signals showed that plasma VWF is able to directly bind towards FXIIa and that the two proteins are capable of forming stable complexes in solution. Binding signal intensities were slightly decreased after immobilisation of plasma derived full-length VWF wildtype resulting in the decision to further characterise the binding interaction between the two proteins using FXIIa immobilisation followed by incubation with VWF proteins. pVWF = plasma derived full-length VWF; Ab = antibody. Data presented as mean ± SEM; n = 3.

Binding signals obtained confirmed direct binding ability of plasma derived full-length VWF wildtype towards FXIIa as well as formation of stable FXIIa/VWF complexes in solution. Signal intensity was slightly reduced when plasma VWF was immobilised and incubated with FXIIa compared to immobilisation of FXIIa and subsequent addition of plasma derived VWF. Based

on these results it was decided to immobilise FXII(a) for future analysis of the binding interaction between the two proteins.

After having established general binding capability of VWF to FXIIa, the binding interaction of the two proteins to each other was further characterised by incubation of a concentration range of plasma derived or recombinant full-length VWF wild-type on immobilised FXIIa, unactivated FXII and FXII activated with kallikrein subsequent to protein immobilisation (Figure 3.15).

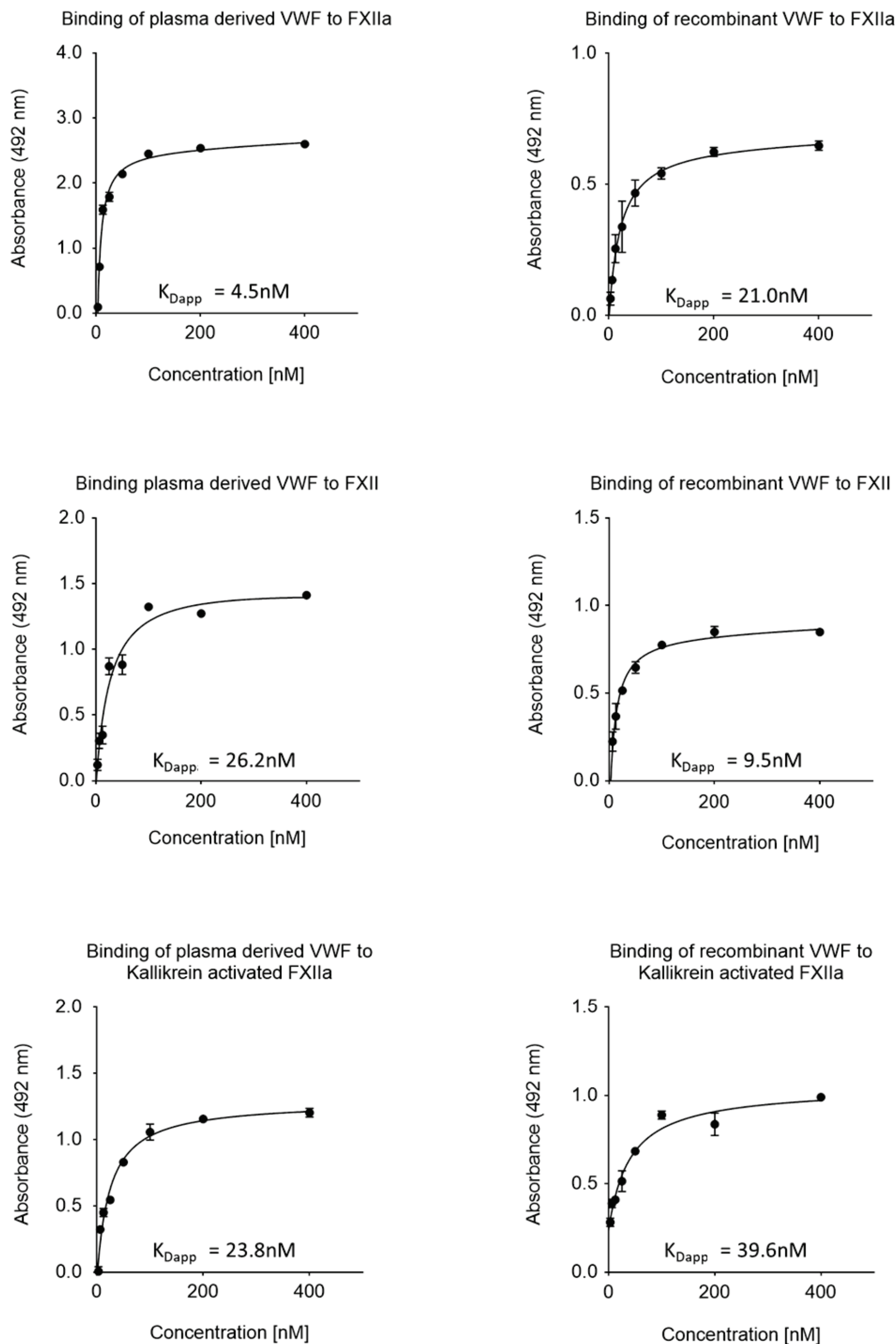

Figure 3.15: Binding of plasma derived and recombinant full-length VWF wildtype towards FXIIa, FXII and kallikrein activated FXII. 100nM activated, non-activated FXII as well as kallikrein activated FXII were immobilised and incubated with a concentration range of plasma derived and recombinant full-length VWF wildtype. Binding interaction of the two proteins was detected using an anti-VWF HRP conjugated antibody. Binding signals confirmed the ability of both plasma derived and recombinant VWF to stably bind towards immobilised FXIIa, FXIIa and kallikrein activated FXII in a concentration dependent manner. Analysis of the binding interaction allowed the determination of the affinity of plasma derived and recombinant full-length VWF wildtype to immobilised FXII(a) which was calculated as the following: K_{Dapp} = 4.5nM for plasma derived VWF to FXIIa; K_{Dapp} = 26.2nM for plasma derived VWF to FXII; K_{Dapp} = 23.8nM for plasma derived VWF to kallikrein activated FXIIa; K_{Dapp} = 21.0nM for recombinant VWF to FXIIa; K_{Dapa} = 9.5nM for recombinant VWF to FXII; K_{Dapp} = 39.6nM for recombinant VWF to kallikrein activated FXIIa. Analysis of affinity using one site total nonlinear fit. Data presented as mean ± SEM. n = 3.

Results of the second set of ELISA confirmed initial observation of the binding interaction between VWF and FXII. In addition, binding signals also displayed a concentration dependent ability of both plasma derived as well as recombinant full-length VWF wildtype to bind towards activated, non-activated FXII and FXII that has been activated using kallikrein prior to incubation with VWF. Analysis of full protein concentration ranges allowed for the determination of the affinity of the different proteins tested and are listed below.

- Plasma derived VWF to FXIIa K_{Dapp}= 4.5nM
- Plasma derived VWF to FXII K_{Dapp}= 36.2nM
- Plasma derived VWF to kallikrein activated FXIIa K_{Dapp}= 23.8nM
- Recombinant VWF to FXIIa K_{Dapp}= 21.0nM
- Recombinant VWF to FXII K_{Dapp}= 9.5nM
- Recombinant VWF to kallikrein activated FXIIa K_{Dapp}= 39.6nM

Initial data suggested the presence of a FXII binding site within the A1 domain of VWF. In order to confirm this hypothesis, plate bound FXIIa was incubated with a single concentration of 80nM (Figure 3.16A) as well as a concentration range (1.6 to 100nM; Figure 3.16B) of recombinant full-length VWF lacking the A1 domain (VWFΔA1).

Results of the binding analysis confirmed the presence of a FXII binding site within the VWF A1 domain since binding signals were significantly reduced after incubation of recombinant full-length VWFΔA1 on immobilised FXIIa compared to incubation of both plasma derived as well as recombinant full-length VWF wildtype in which the VWF A1 domain is present.

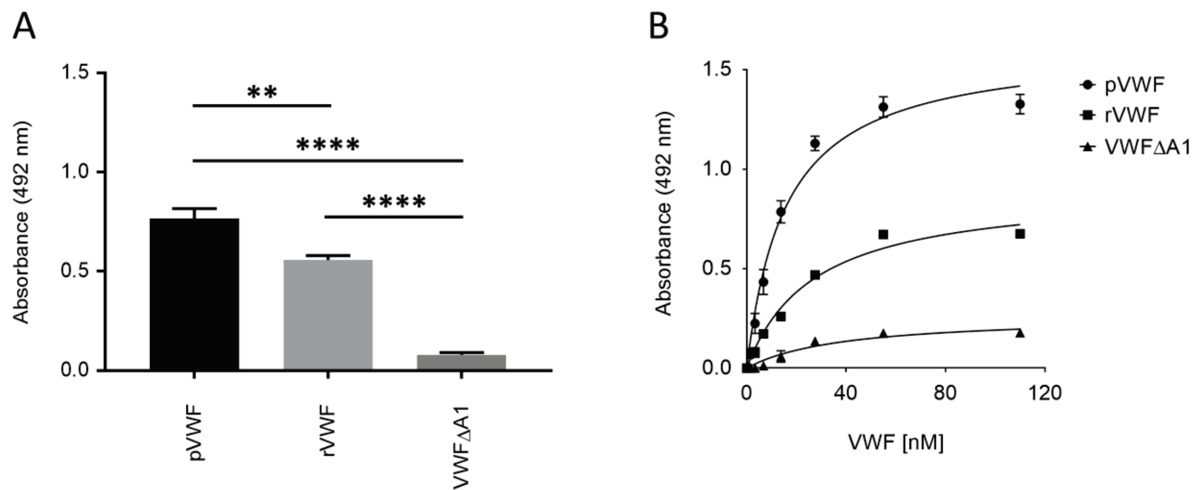

Figure 3.16: Confirmation of the presence of a FXII binding site within the VWF A1 domain by ELISA. Preliminary binding data suggested that the VWF binding site for FXII is located within the VWF A1 domain. In order to confirm this hypothesis, plasma derived and recombinant VWF as well as recombinant full-length VWF lacking the A1 domain (VWFΔA1) were incubated on immobilised FXIIa. Binding interaction was detected using an anti-VWF HRP conjugated antibody. A) 80nM of either plasma derived, recombinant full-length VWF wildtype or recombinant full-length VWFΔA1 were incubated on immobilised FXIIa. Analysis of the binding signals showed significantly reduced signal intensities after incubation of VWFΔA1 on immobilised FXIIa confirming presence of the FXII binding site within the VWF A1 domain. B) Incubation of a concentration range (1.6 to 100nM) of plasma derived, recombinant full-length VWF wildtype or recombinant full-length VWFΔA1 on immobilised FXIIa again confirmed that the FXII binding site is located in the A1 domain of VWF since binding signals were significantly reduced after incubation of VWFΔA1 on FXIIa compared to plasma derived and recombinant full-length VWF wildtype. pVWF = plasma derived full-length VWF wildtype; rVWF = recombinant full-length VWF wildtype. Data presented as mean ± SEM. Analysis of affinity using one site total nonlinear fit. Statistical analysis with t-test; **p = 0.0027; ****p = 0.0001. n = 3.

Confirmation of a FXII binding site within the A1 domain of VWF warranted a closer analysis of the binding interaction between the VWF A1 domain and FXII. Therefore, a concentration range of isolated VWF A1 (14 to 900nM) was incubated on immobilised FXIIa (Figure 3.17).

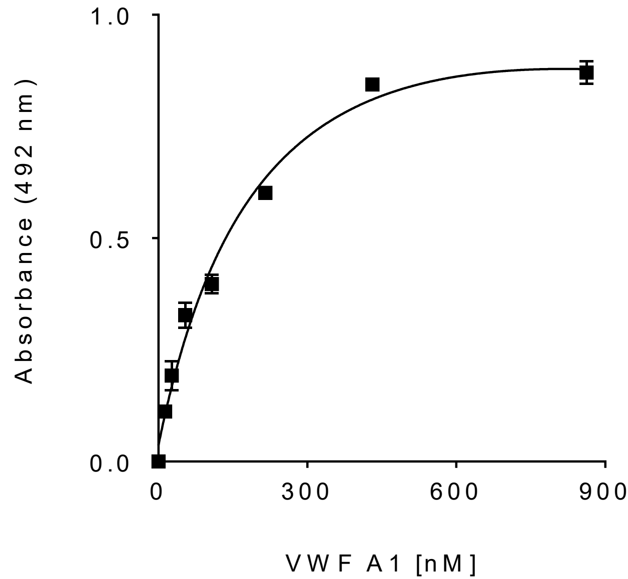

Figure 3.17: Binding of the isolated VWF A1 domain to FXIIa. After confirmation of a FXII binding site within the A1 domain of VWF, binding interaction of an isolated VWF A1 domain concentration range (14 to 900nM) to immobilised FXIIa was tested. ELISA signals confirmed binding ability of the isolated VWF A1 domain towards FXIIa in a concentration dependent manner. Analysis of the interaction allowed for the determination of an affinity (K_{Dapp}) of 139nM. Data presented as mean ± SEM. Analysis of affinity using one site total nonlinear fit. n = 3

Results of the binding assay showed a concentration dependent binding interaction between the isolated VWF A1 domain and immobilised FXIIa. Analysis of a full concentration range allowed for the determination of the affinity (K_{Dapp}) of the isolated VWF A1 domain towards FXIIa which was calculated to be 139nM.

In order to identify specific amino acids involved in the binding interaction between VWF and FXII, surface exposed negatively charged amino acids present in the A1 domain were mutated in both the isolated VWF A1 domain as well as in the A1 domain present in the full-length VWF protein. In total, 24 different mutants (12 in the isolated VWF A1 domain and 12 in the full-length VWF A1 domain protein) were created using site-directed mutagenesis. Generated clones subsequently were screened for their binding ability towards immobilised FXIIa assuming that a reduction or complete abolishment of binding signals positively correlates with the role of the respective amino acid in VWF/FXII binding (Figure 3.18).

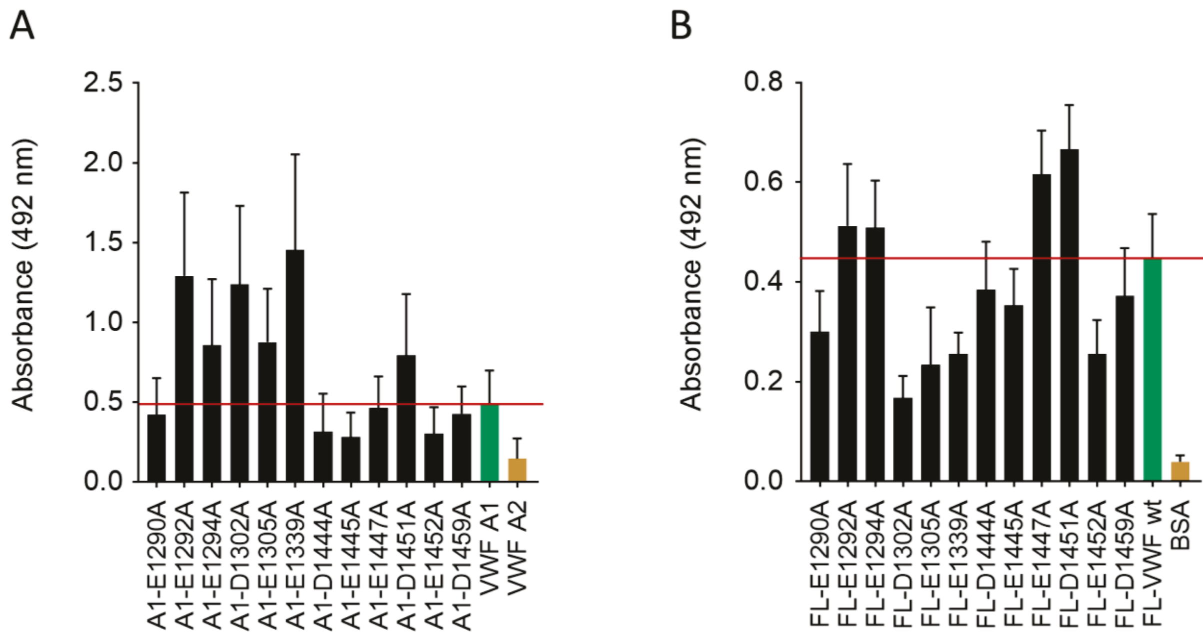

Figure 3.18: Binding analysis of isolated and full-length VWF A1 domain mutants to immobilised FXIIa. Surface exposed negatively charged amino acids were mutated in both the isolated VWF A1 domain as well as the A1 domain of full-length VWF using site-directed mutagenesis in order to identify specific amino acids involved in the binding interaction between VWF and FXII. Generated mutants subsequently were screened in terms of their binding ability towards immobilised FXIIa by ELISA. A) Binding of isolated VWF A1 domain mutants to immobilised FXIIa. 360nM of the different mutants were incubated on FXIIa and protein interaction was detected using an anti-VWF HRP conjugated antibody. Six of the mutants tested (E1209A; D1444A; E1445A; E1447A; E1452A and D1459A) displayed reduced FXIIa binding abilities while the remaining six mutants (E1292A; E1294A; D1302A; E1305A; E1339A and D1451A) exhibited increased FXIIa binding signals compared to the isolated VWF A1 domain itself. B) Binding of full-length VWF A1 domain mutants to immobilised FXIIa. 40nM of the different full-length VWF A1 domain mutants were incubated on FXIIa and binding interaction of the two proteins was detected using an anti-VWF HRP conjugated antibody. As already observed during the screening of the isolated VWF A1 domain mutants, full-length VWF A1 domain mutants showed either reduced (E1290A, D1302A; E1305A; E1339A; D1444A; E1445A; D1452A and E1459A; eight out of 12 mutants) or enhanced (E1292A; E1294A; E1305A; E1447A; four out of 12 mutants) binding ability towards FXIIa compared to full-length VWF wildtype. A1 = isolated VWF A1 domain; FL = full-length; wt = wildtype; BSA = bovine serum albumin. Data presented as mean ± SEM. n = 3.

Binding analysis of isolated VWF A1 domain mutants (360nM) to immobilised FXIIa showed that mutation of the negatively charged amino acids present on the surface of the individual proteins influenced the overall binding ability of the proteins to FXIIa. Interestingly, mutation of the different residues resulted in the generation of mutants exhibiting either increased or decreased FXIIa binding ability compared to the isolated VWF A1 domain itself. While half of the mutants (E1292A; E1294A; D1302A; E1305A; E1339A and D1451A) showed enhanced FXIIa binding capabilities, the remaining six isolated VWF A1 domain mutants (E1209A; D1444A; E1445A; E1447A; E1452A and D1459A) displayed a reduced binding ability towards FXIIa.

Incubation of the isolated VWF A2 domain on immobilised FXIIa served as negative control and showed minimal binding activity towards FXIIa.

As already observed during the screening of the isolated VWF A1 domain mutants, mutation of the different surface exposed amino acids also influenced the FXIIa binding abilities of full-length VWF A1 domain mutants. Introduction of the different mutations resulted in reduced FXIIa binding signals for eight (E1290A, D1302A; E1305A; E1339A; D1444A; E1445A; D1452A and E1459A) out of the 12 clones generated while the remaining four clones (E1292A; E1294A; E1305A; E1447A) exhibited enhanced binding towards FXIIa. BSA was incubated on immobilised FXIIa as a negative control and did not exhibit significant binding signals.

Based on the results obtained after screening of both isolated VWF A1 domain and full-length VWF A1 domain mutants, mutations resulting in an either reduced or enhanced binding ability of the respective clones were traced back to their original location within the VWF A1 domain (Figure 3.19A for the isolated VWF A1 domain mutants and Figure 3.19B for the full-length VWF A1 domain mutants). Mapping of the different amino acid changes showed that the majority of mutations leading to enhanced FXIIa binding signals were located on the α1 helix present in the A1 domain while mutations correlating with reduced FXIIa binding capabilities predominantly were located on the α6 helix.

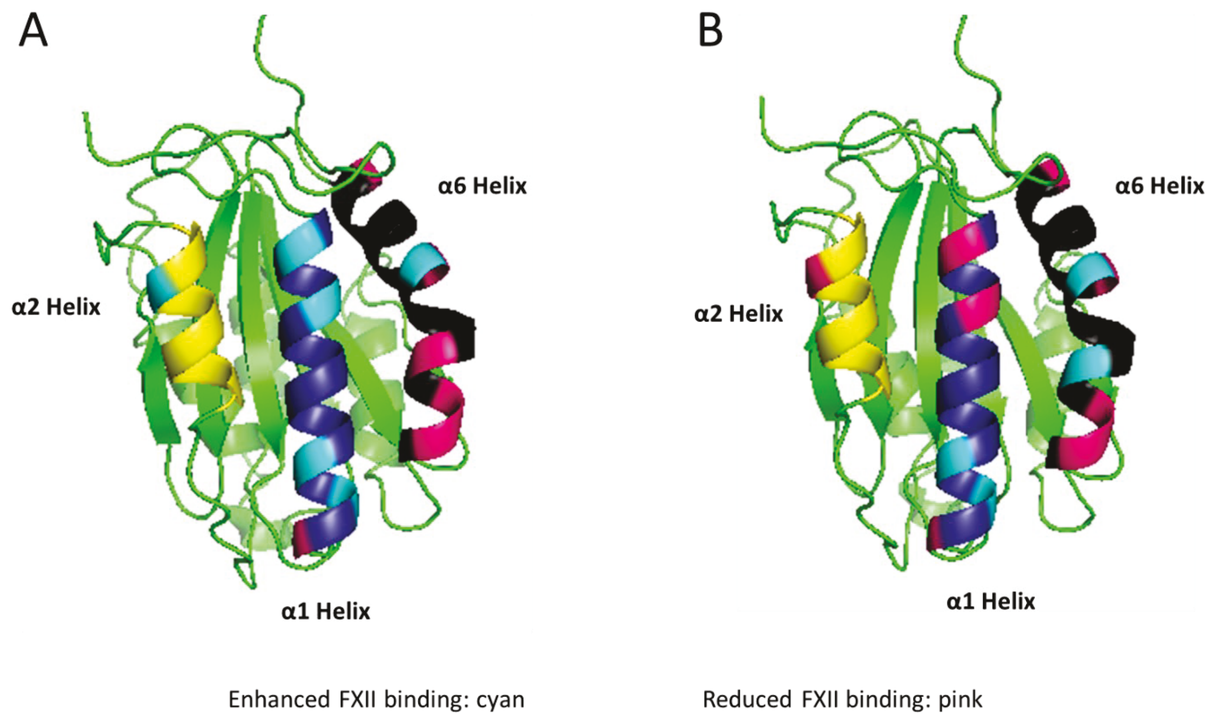

Figure 3.19: Mapping of VWF A1 domain mutations resulting in either enhanced or reduced FXIIa binding ability. Isolated and full-length VWF A1 domain mutants were screened in terms of their binding capability towards immobilised FXIIa. Results of the binding assays showed that mutation of negatively charged amino acids present on the surface of the A1 domain directly influenced the binding interaction between VWF and FXII. Mutations displaying enhanced (cyan) or reduced (pink) binding to FXIIa were traced back to their original location in the A1 domain. A) Mutations resulting in either enhanced or reduced FXIIa binding based on the screening of isolated VWF A1 domain mutants on immobilised FXIIa. Binding analysis showed that six (E1292A; E1294A; D1302A; E1305A; E1339A and D1451A) out of the 12 mutants exhibited increased in FXIIa binding signals compared to the isolated VWF A1 domain itself. The remaining six mutants (E1209A; D1444A; E1445A; E1447A; E1452A and D1459A) generated displayed a reduction in FXIIa binding ability. B) Summary of mutants showing either an increase or a reduction in FXIIa binding ability based on screening results using full-length VWF A1 domain mutants on immobilised FXIIa. Results of the binding assays led to the identification of four mutants (E1292A; E1294A; E1305A; E1447A) with increased FXIIa binding ability compared to full-length VWF wildtype. The remaining eight clones (E1290A, D1302A; E1305A; E1339A; D1444A; E1445A; D1452A and E1459A) demonstrated reduced FXIIa binding signals.

Even though the increase in FXIIa binding ability displayed by some of the mutants generated was considered as an interesting observation, it was decided to primarily focus on the mutants exhibiting reduced binding capabilities to FXIIa in order to further identify the exact amino acids responsible for the binding interaction between VWF and FXII. Combined results of the screen with both isolated VWF A1 domain mutants as well as full-length VWF A1 domain mutants led to the identification of five mutants (E1290A; D1444A; E1445A; E1452A and D1459A; Figure 3.20) with reduced FXIIa binding abilities indicating that the amino acids at

position 1290, 1444, 1445, 1452 and 1459 might be involved in the interaction of VWF with FXII.

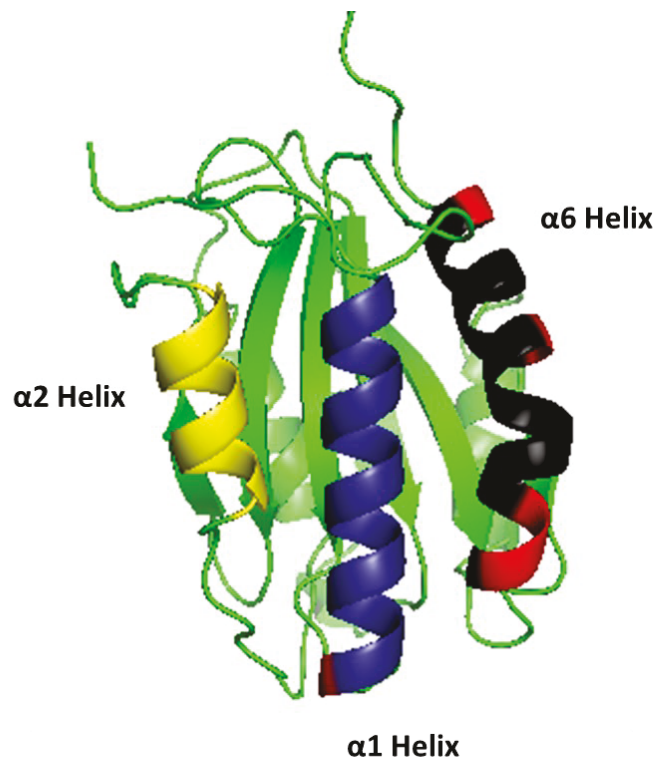

Figure 3.20: Combination of different mutations resulting in reduced FXIIa binding ability of the VWF A1 domain. Screening of both isolated VWF A1 domain as well as full-length VWF A1 domain mutants allowed for the identification of five mutants displaying reduced FXIIa binding abilities in both formats. Amino acids at position 1290, 1444, 1445, 1452 and 1459 or combinations thereof are assumed to be necessary for the interaction between FXII and VWF. In order to identify the exact amino acids involved in the binding interaction between the two proteins additional mutants containing multiple mutations were generated and screened in terms of their binding capability towards FXIIa. Relevant mutations resulting in reduced FXIIa binding are displayed in red.

Since mutation of neither of these residues resulted in a completed abolishment of VWF mutant/FXIIa binding, it was assumed that more than one amino acid is responsible for the interaction between the two proteins leading to the decision to create mutants containing two or more mutations for the further identification of the exact FXII binding site in the VWF A1 domain. In total, three double mutants with combined mutations at position 1444/1445, 1444/1447 and 1452/1459 as well as a triple mutant comprising mutations at position 1444/1445/1447 (Figure 3.25 in chapter 3.5.1) were created and subsequently tested in terms of their binding ability towards FXIIa.

Mutants containing single-point mutations that have been shown to exhibit reduced FXIIa binding ability were screened against immobilised FXIIa alongside the newly generated double mutants (Figure 3.21). Analysis of the binding behaviour of the triple mutants towards FXIIa was not possible since the protein could not be expressed properly. While ELISA with the single-point mutants showed the same binding behaviour already observed in the initial screening of the proteins, introduction of multiple mutations partly resulted in an increase in FXIIa binding. Isolated VWF A1 domain double mutant D1444/1445A and D1444/1447A for example showed significantly increased FXIIa binding signals compared to both the isolated VWF A1 domain as well as single-point mutants D1444A, E1445A and E1447A (Figure 3.21A). Interestingly, introduction of multiple mutation into the full-length VWF A1 domain showed a consistent reduction in FXIIa binding ability in static plate based binding assays (Figure 3.21B). Despite the differing results obtained after incubation of isolated VWF A1 domain and full-length VWF A1 domain double mutants on immobilised FXIIa, combination of single-point mutations at positions 1452 and 1459 resulted in abolished FXIIa binding ability in both protein formats indicating that these two amino acids (aspartic acid at position 1452 and glutamic acid at position 1459) comprise the FXII binding site in the VWF A1 domain.

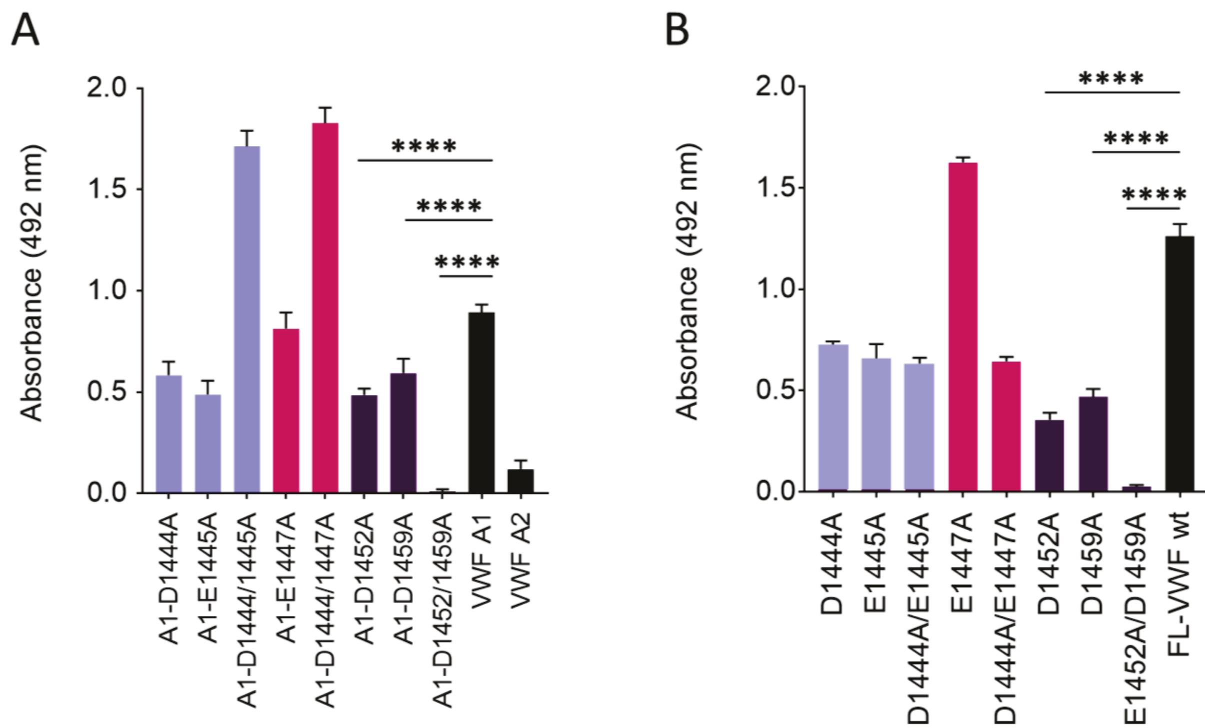

Figure 3.21: Static plate binding assay of isolated VWF A1 domain and full-length VWF A1 domain single-point and double mutants on immobilised FXIIa. Binding of the VWF based proteins to FXIIa was detected using an anti-VWF HRP conjugated antibody. A) Binding analysis of isolated VWF A1 domain mutants (360nM) carrying singe-point or combined mutations on immobilised FXIIa. Binding signals obtained after incubation of single-point isolated VWF A1 domain mutants on FXIIa reflected binding behaviour previously observed during initial screening of the mutants. Combination of the different mutations resulted in an increase in FXIIa binding for mutants D1444/1445A and D1444/1447A. No FXIIa binding of mutant D1452/1459A was detected. B) ELISA with single-point and double full-length VWF A1 domain mutants. 40nM of the different mutants were incubated on plate bound FXIIa. As already observed in the initial screening experiments, single-point mutants showed a reduced binding ability towards FXIIa. All three double mutants displayed a reduction in FXIIa binding compared to full-length VWF wildtype with mutant D1452/1459A exhibiting no binding ability to FXIIa. Results of FXIIa binding assays carried out with isolated VWF A1 domain and full-length VWF A1 domain double mutants led to the identification of aspartic acid at position 1452 and glutamic acid at position 1459 as the FXII binding site within the VWF A1 domain. A1 = isolated VWF A1 domain; FL = full-length. Data presented as mean ± SEM. Statistical analysis with t-test; ****p = 0.0001. n = 3.

In order to confirm identification of the FXII binding site in the VWF A1 domain, the isolated VWF A1 domain mutant D1452/1459A was tested in terms of its binding ability towards FXIIa again. Binding of the double mutant to plate bound FXIIa hereby was analysed at a single concentration of 360nM (Figure 3.22A) and a concentration range varying between 28 and 1800nM (Figure 3.22B). Due to limited availability of full-length VWF A1 domain mutants the experiment was only carried out using the isolated VWF A1 domain mutants. Results of the binding assay showed significantly reduced binding of mutant D1452/1459A to FXIIa in both assay setups and confirmed aspartic acid at position 1452 and glutamic acid at position 1459

as the FXII binding site in the VWF A1 domain. Analysis of a full concentration range of mutant E1452/1459A allowed for the determination of the affinity (K_{Dapp}) between the two proteins which was calculated to be 419.4nM.

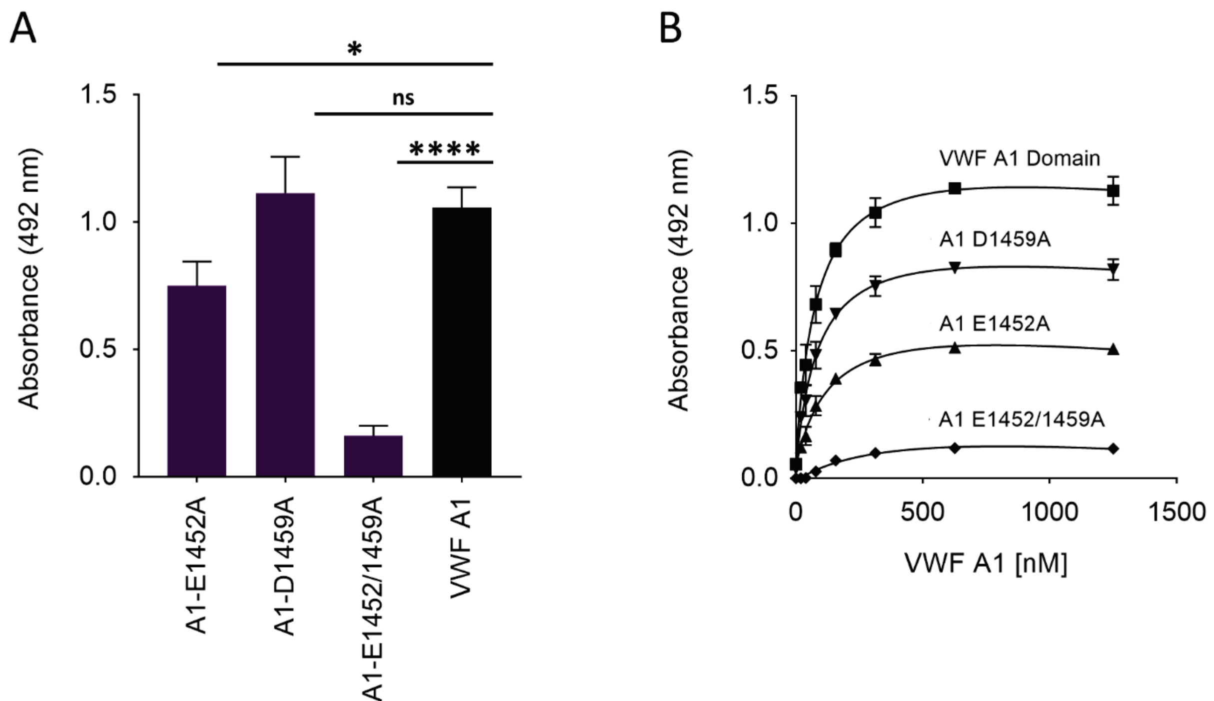

Figure 3.22: Confirmation of the FXII binding site within the VWF A1 domain using plate based FXII binding assays. In order to confirm the identification of the FXII binding site in the VWF A1 domain binding of the relevant double mutant to FXIIa was tested again. Experiments were carried out using the isolated VWF A1 domain mutant due to the limited availability of the full-length VWF A1 domain proteins. Binding of the VWF based proteins to FXIIa was detected with an anti-VWF HRP conjugated antibody. A) Binding analysis of double mutant E1452/1459A to immobilised FXIIa. 360nM of the different mutants were incubated on plate bound FXIIa. Binding results showed a reduction in FXIIa binding for single-point mutant E1452A, similar FXIIa binding signals for single-point mutant D1459A and a significant decrease in FXIIa binding for double mutant E1452/1459A compared to the isolated VWF A1 domain alone. B) Binding analysis of a concentration range of double mutant E1452/1459A. Relevant single point as well as the double mutant E1452/1459A were incubated on immobilised FXIIa in a concentration range varying between 28 and 1800nM. Both single-point mutants showed a reduced FXIIa binding ability compared to the native isolated VWF A1 domain. Incubation of the double mutant on FXIIa confirmed the significantly reduced binding capabilities of this mutant and therefore established aspartic acid at position 1452 and glutamic acid at position 1459 as the FXII binding site within the VWF A1 domain. Analysis of a full concentration range allowed for the determination of the binding affinity (K_{Dapp}) between the two proteins which was calculated to be 419.4nM. A1 = isolated VWF A1 domain. Data presented as mean ± SEM. Analysis of affinity using one site total nonlinear fit. Statistical analysis with t-test; *p = 0.05; ****p = 0.0001. n = 3.

Apart from binding to activated FXII, the binding ability of the double mutant E1452/1459A was additionally tested towards the FXII zymogen as well as kallikrein activated FXII (Figure 3.23). The experiment was carried out using full-length VWF A1 domain mutants in order to

reflect binding behaviour of physiologically relevant VWF proteins as closely as possible. Aside from the relevant double mutant, single-point full-length VWF A1 domain mutants E1452A and D1459A were included. As already observed in previous assays, results of the binding analysis showed that plasma derived full-length VWF wildtype is able to bind both activated and nonactivated FXII as well as FXIIa that has been activated by the addition of kallikrein. Similar to full-length wildtype VWF, full-length VWF A1 domain single-point mutants E1452A and D1459A displayed binding ability towards all three forms of FXII and therefore are capable of binding both activated as well as nonactivated FXII. Consistent with the binding data obtained during the initial screening as well as the subsequent characterisation assays, incubation of the double mutant E1452/1459A on FXIIa, FXII and kallikrein activated FXIIa did not result in the exhibition of any binding signal; again confirming the necessity of amino acids aspartic acid at position 1452 and glutamic acid at position 1459 for the binding interaction between FXII and VWF.

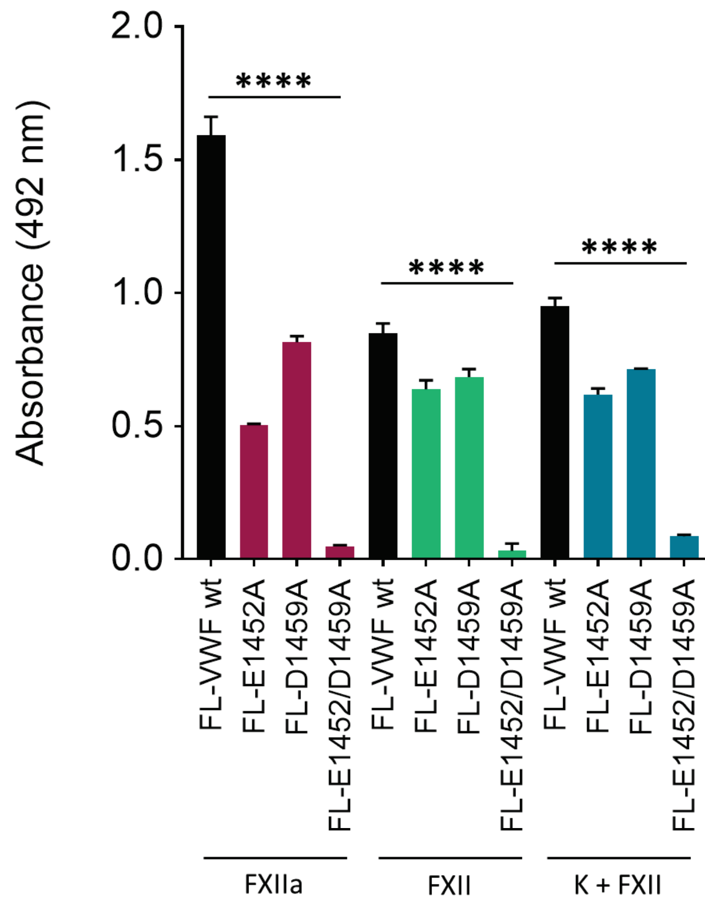

Figure 3.23: Binding analysis of full-length VWF A1 domain single-point mutants E1452A and D1459A as well as double mutant E1452/1459A to FXIIa, FXII and kallikrein activated FXII. 40nM of the different full-length VWF A1 domain mutants were incubated on immobilised FXII, zymogen FXII or FXII which has been activated by kallikrein prior to incubation with the VWF mutants. Binding analysis showed that full-length VWF wildtype as well as both single-point full-length VWF A1 domain mutants were capable to bind to FXIIa, FXII as well as kallikrein activated FXII. Introduction of a double mutation at position 1452 and 1459 lead to abolished binding ability of the respective proteins to any form of FXII. Results confirmed that the FXII binding site of VWF is comprised of the amino acids aspartic acid at position 1452 and glutamic acid at position 1459. FL = full-length; wt = wildtype; K = kallikrein. Data presented as mean ± SEM. Statistical analysis with t-test; **** = 0.0001. n = 3.

3.5.1. Surface Plasmon Resonance

In order to confirm binding data obtained by carrying out static plate binding assays, the interaction between FXIIa and selected VWF proteins was further analysed using surface plasmon resonance. 1µM of recombinant full-length VWF wildtype, the isolated VWF A1 domain or the isolated VWF A1 domain mutant E1452/1459A was perfused over FXIIa immobilised on a COOH chip (Figure 3.24). Association and dissociation of the VWF proteins to FXIIa was monitored for 600 seconds at 20 Hertz. SPR binding curves confirmed binding ability of recombinant full-length VWF wildtype as well as the isolated VWF A1 domain.

Evaluation of the binding curves demonstrated a slower association but similar FXIIa dissociation constants for the isolated VWF A1 domain compared to full-length VWF wildtype. Binding analysis of the isolated VWF A1 domain double mutant E1452/1459A displayed a significant reduction in both the association and dissociation rate of the protein to FXIIa indicating a loss of affinity and therefore a general inability of the mutant to bind to FXIIa.

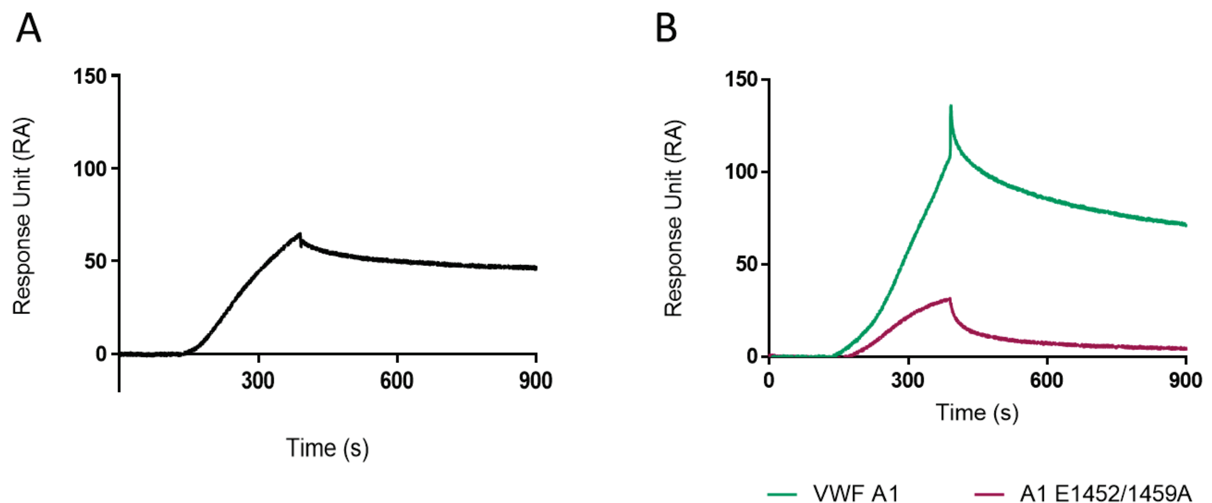

Figure 3.24: Surface plasmon resonance analysis of the binding interaction between FXIIa and different VWF proteins. 1µM of recombinant full-length VWF wildtype, the isolated VWF A1 domain and the isolated VWF A1 domain mutant E1452/1459A was perfused over immobilised FXIIa. Protein-protein interaction was monitored at 200 Hertz for 10 minutes. Evaluation of the different binding curves confirmed that recombinant full-length VWF wildtype as well as the isolated VWF A1 domain are capable of binding towards FXIIa with similar association and dissociation of the proteins to immobilised FXIIa. Significantly reduced association and dissociation constants of the isolated VWF A1 domain mutant E1452/1459A to FXIIa verified the inability of this mutant to bind to FXIIa and therefore confirmed amino acids aspartic acid at position 1452 and glutamic acid at position 1459 as the FXII binding site residing in the VWF A1 domain. n = 3.

All binding data obtained were corrected in terms of the apparent diffusion coefficient and the affinities of the different proteins to FXIIa were calculated based on the molecular weight (Table 3.2). Comparison of the calculated K_{Dapp} values obtained using static plate binding assays and SPR measurements showed similar values for recombinant full-length VWF wildtype, the isolated VWF A1 domain and the isolated VWF A1 domain double mutant E1452/1459A.

	K_{Dapp} Static Assay (nM)	K_{Dapp} SPR (nM)
Recombinant full-length VWF wildtype	21	31
Isolated VWF A1 Domain	139	80
Isolated VWF A1 Domain Mutant E1452/1459A	419	564

Table 3.2: Comparison of the calculated affinities of VWF proteins to immobilised FXIIa. Direct comparison of the K_{Dapp} values demonstrated similar binding abilities of recombinant full-length VWF wildtype, the isolated VWF A1 domain and the isolated VWF A1 domain double mutant E1452/1459A to FXIIa. Mean values of n = 3.

Conclusively, data from both static plate based binding assays as well as SPR measurements showed a loss of FXIIa binding ability for the isolated VWF A1 domain and full-length VWF A1 domain mutant E1452/1459A which identifies the amino acids aspartic acid at position 1452 and glutamic acid at position 1459 as the FXII binding sited located in the VWF A1 domain (Figure 3.25).

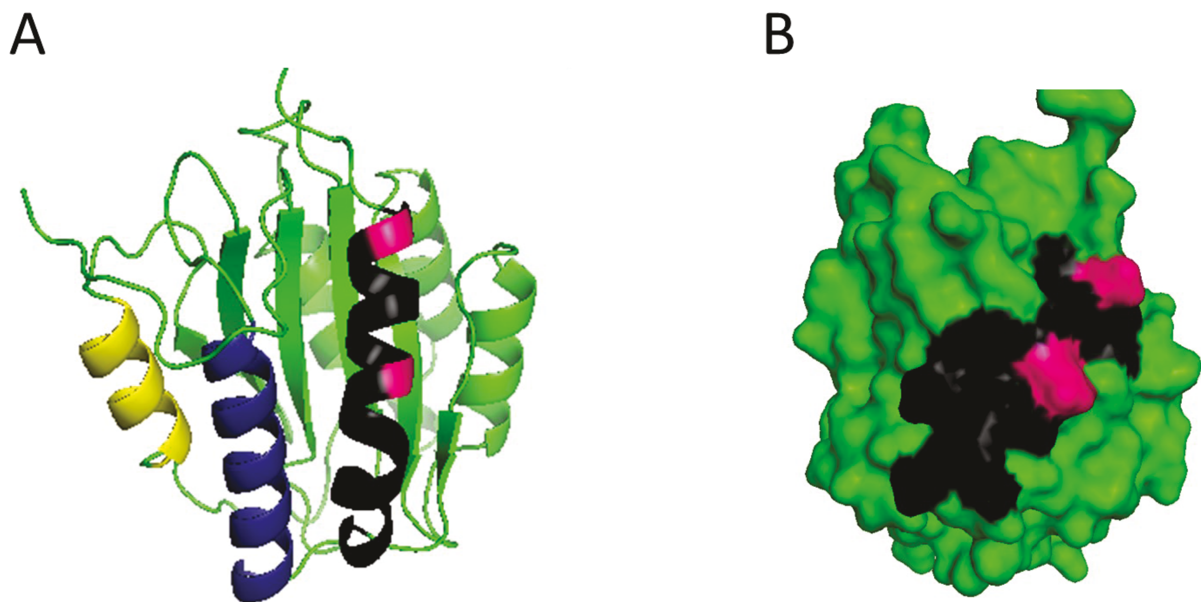

Figure 3.25: Visualisation of the protein structure of double mutant E1452/1459A. Crystal (A) and surface (B) structure of the VWF A1 domain with amino acids 1452 (aspartic acid) and 1459 (glutamic acid) which have been demonstrated to compose the FXIIa binding site highlighted in pink.

3.6. Activation of FXII

FXII is expressed and secreted into the circulatory system as a zymogen and therefore does not exhibit any proteolytic activity under normal physiological conditions. In order to interact and cleave its various substrates inactive FXII has to be converted into its active form FXIIa which can be achieved by either binding of FXII to negatively charged surfaces and subsequent autoactivation or direct activating cleavage of FXII by predominantly kallikrein.

In this project, FXIIa activity was evaluated using a substrate based static plate assay which is depended on the cleavage of a chromogenic substrate by activated FXII. In order to confirm the activation capabilities of the different activators described in the literature, FXII activity was determined based on substrate cleavage signals at 405nM after 45minutes as well as the overall activation potential (%) of the activators.

In a first step, a concentration range of FXIIa (6.3 to 200nM) was screened (Figure 3.26A) in terms of the substrate conversion rate in order to determine a suitable FXIIa concentration for the detailed analysis of FXIIa activity and potential FXII activators. Results of the FXIIa concentration screening showed that the substrate (0.8M) used is cleaved by FXIIa in a concentration and time dependent manner with concentrations of 200nM and 100nM displaying the strongest signal intensity after 10 and 40 minutes respectively. Based on these results, all future FXII activation assays were carried out using a FXII(a) concentration of 100nM and an activation assay readout length of at least 45 minutes.

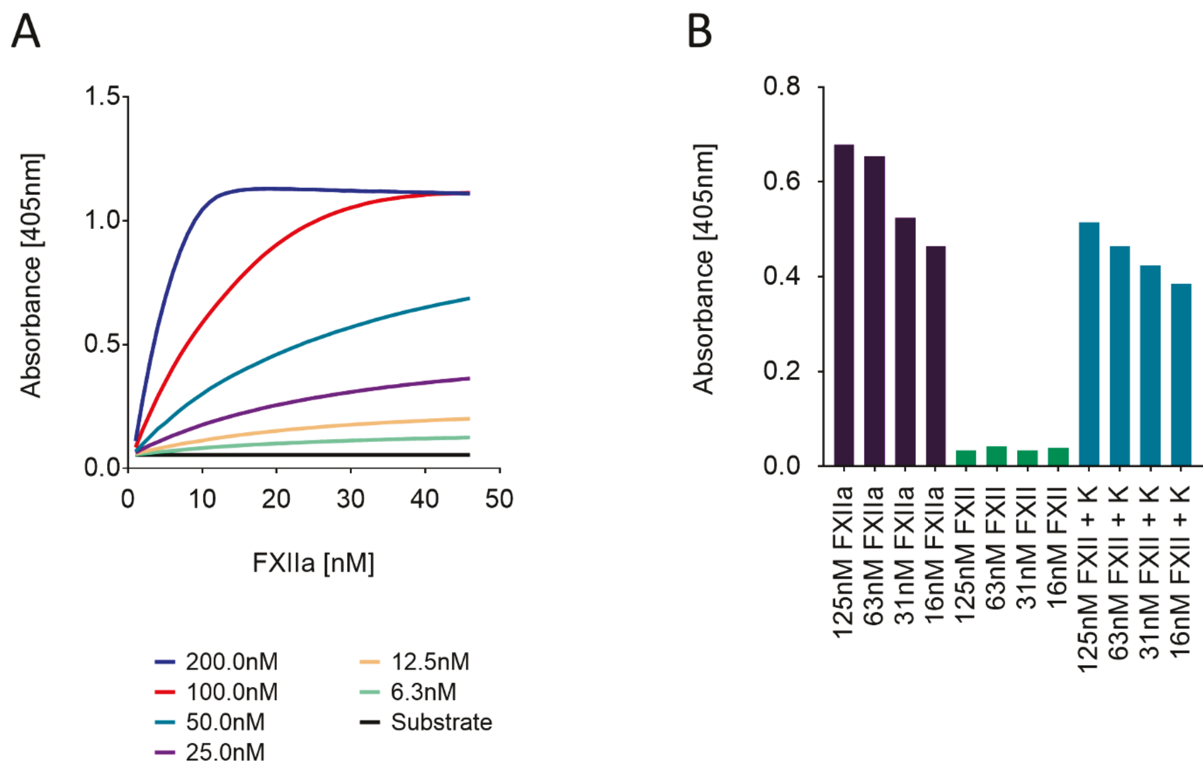

Figure 3.26: FXIIa concentration finding and evaluation of activity retention. Cleavage of 0.8M substrate by FXIIa was monitored at 405nm over 45 minutes. A) Suitable FXII(a) concentration for future assays testing both FXIIa activity as well as the activation potential of substances described in the literature were determined by incubation of a FXIIa concentration range varying between 6.3nM and 200nM with the activation assay substrate. Absorbance curves showed that FXIIa is capable of cleaving the substrate in a concentration and time dependent manner. Based on the results, future FXII activation assays will be carried out using 100nM FXII(a) and a minimum readout length of 45 minutes. B) Binding of VWF to FXII(a) predominantly will be analysed in static plate binding assays using immobilised FXII or FXIIa. Retention of FXIIa activity and activity level of kallikrein activated FXII was tested by incubation of the proteins with the activation assay substrate. Assay readouts showed that both FXIIa as well as kallikrein activated FXIIa were capable of cleaving the substrate in a concentration dependent manner while immobilised FXII itself did not display any activity. Results of the assay validated planned binding assays evaluating the binding interaction between VWF to FXII and FXIIa since activity of neither of the proteins was influenced by activation subsequent to immobilisation or immobilisation alone. K = kallikrein.

Since the majority of the planned FXII/VWF binding assays would be carried out using plate bound FXIIa (see chapter 3.5), retention of the protein`s activity after immobilisation was tested by incubation of a FXIIa concentration range varying between 16 and 125nM with 0.8M of the activation assay substrate. In addition, immobilised FXII was activated with kallikrein and subsequently incubated with the substrate as well in order to determine whether activation of plate bound FXII is possible and can be efficiently monitored (Figure 3.26B). Optical density observed showed that both immobilised FXIIa as well as kallikrein activated FXIIa showed proteolytic activity in a concentration dependent manner while nonactivated

immobilised FXII did not display any cleavage of the activation assay substrate. Results therefore confirmed that immobilisation of FXIIa and activation of FXII after immobilisation have no influence on FXIIa activity and therefore can be used in order to evaluate the binding behaviour of VWF towards both FXII and FXIIa in static plate biding assays.

Inhibition of FXIIa activity was tested by incubation of 100nM FXIIa as well as kallikrein activated FXIIa with 40µg/ml of either corn trypsin inhibitor (CTI) or an anti-FXII antibody (anti-FXII Ab; Figure 3.27) and 0.8M of the activation assays substrate. Absorbance signals lower than 0.1 showed that incubation of FXII and kallikrein activated FXIIa with CTI as well as the anti-FXII Ab effectively blocked the conversion of the substrate and therefore the proteolytic function of FXIIa.

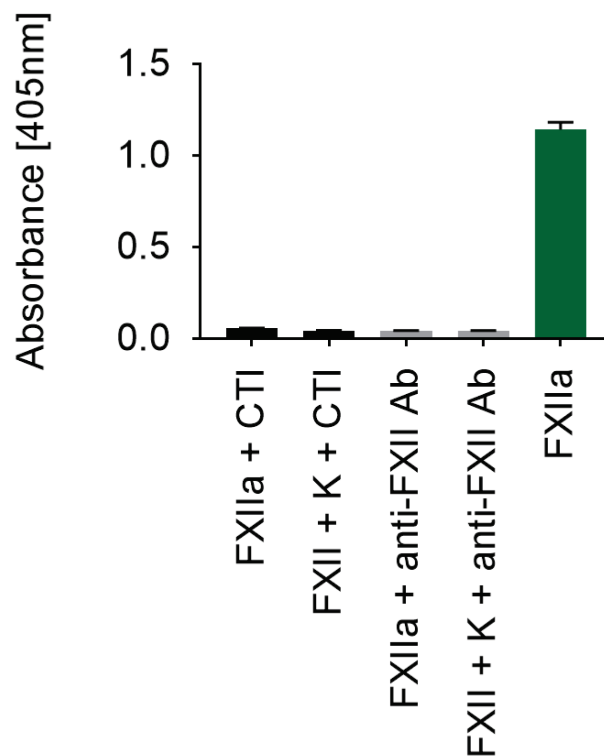

Figure 3.27: Blockage of FXIIa activity with CTI and an anti-FXII Ab. Activity of 100nM FXIIa and kallikrein activated FXIIa was blocked with 40µg/ml of either CTI or an anti-FXII Ab. Cleavage of the substrate (0.8M) by FXIIa was monitored for 45 minutes at 405nm. Absorbance values ≤ 0.1 indicated complete inhibition of FXIIa activity by both CTI and the anti-FXII antibody. CTI = corn trypsin inhibitor; Ab = antibody; K = kallikrein. Data presented as mean ± SEM. n = 3 to 12.

Over the last few decades, a multitude of potential activators promoting the conversion of FXII to FXIIa have been described and tested in different assay setups. Before determining the

FXII activation potential of VWF itself, FXII activation assays with the most commonly described FXII activators were carried out in order to confirm the capability of these substances to convert inactive FXII to FXIIa. All potential activators were tested at three different concentrations reflecting their relevant physiological concentration as well as a (decimal) concentration in- or decrease respectively. Optical density of FXII samples incubated with 0.8M substrate and the different activators was compared to OD values of commercially available FXIIa after 45 minutes (Figure 3.28). In addition, the calculated FXII activation potential of the different substances was compared to the activation potential exhibited by kallikrein which is considered as the most relevant physiological activator of FXII (Figure 3.29).

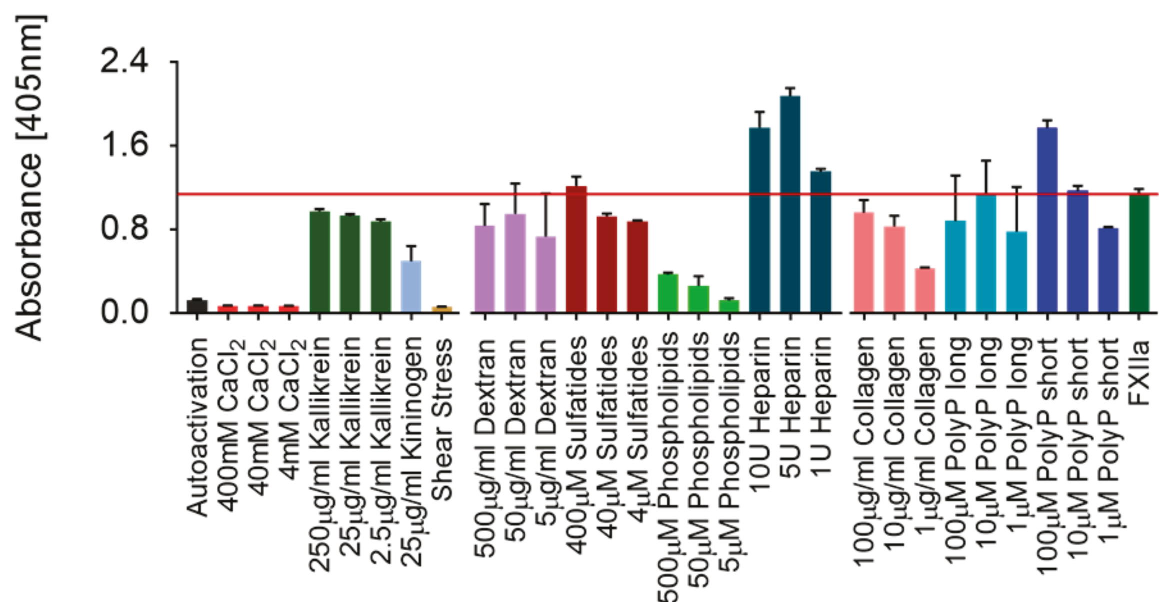

Figure 3.28: FXII activation assays with previously described FXII activators. 100nM FXII were incubated with different concentrations of a variety of substances previously described as FXII activators. Cleavage of 0.8M activation assay substrate by the FXII/activator mix or FXIIa was monitored for 45 to 300 minutes at 405nm. Optical density values of the different samples after 45 minutes were compared to commercial FXIIa. Figure shows optical density of FXIIa and FXII samples incubated with different activators after 45 minutes. Comparison of the OD values confirmed sulfatides, heparin and short polyphosphates as potent activators of FXII. Eight out of the 12 substances or conditions tested displayed the ability to promote conversion of the zymogen into the active protease. Subjection of FXII to shear stress, incubation with $CaCl_2$ as well as measurement of FXII autoactivation resulted in low OD valued indicating that these processes or substances do not elicit relevant FXII activation. Data presented as mean ± SEM. n = 3 to 12

Results of the activation assay confirmed a set of substances previously described as FXII activators. Evaluation of the OD values measured indicated sulfatides (OD of 1.2 for 400µM sulfatides), heparin (OD of 2.1 for 5U heparin) as well as long polyphosphates (100µM with an OD of 1.8 and 10µM with an OD of 1.2) as the most potent activators of FXII with FXIIa itself

displaying a mean OD value of 1.1. In general, the majority (8 out of 12) of the substances tested displayed FXII activating capabilities to some degree which could be monitored by the cleavage of the substrate added. Interestingly, some of the substances or conditions that were reported to have FXII activating abilities such as $CaCl_2$ and shear stress did not result in significant activation of the protease (OD of 0.1 for both). Completion of these assays furthermore showed minimal autoactivation of FXII (OD of 0.1) under the conditions used which might be a reflection of FXII autoactivation rates within the circulatory system highlighting the importance of the kallikrein-FXII(a) feedback loop.

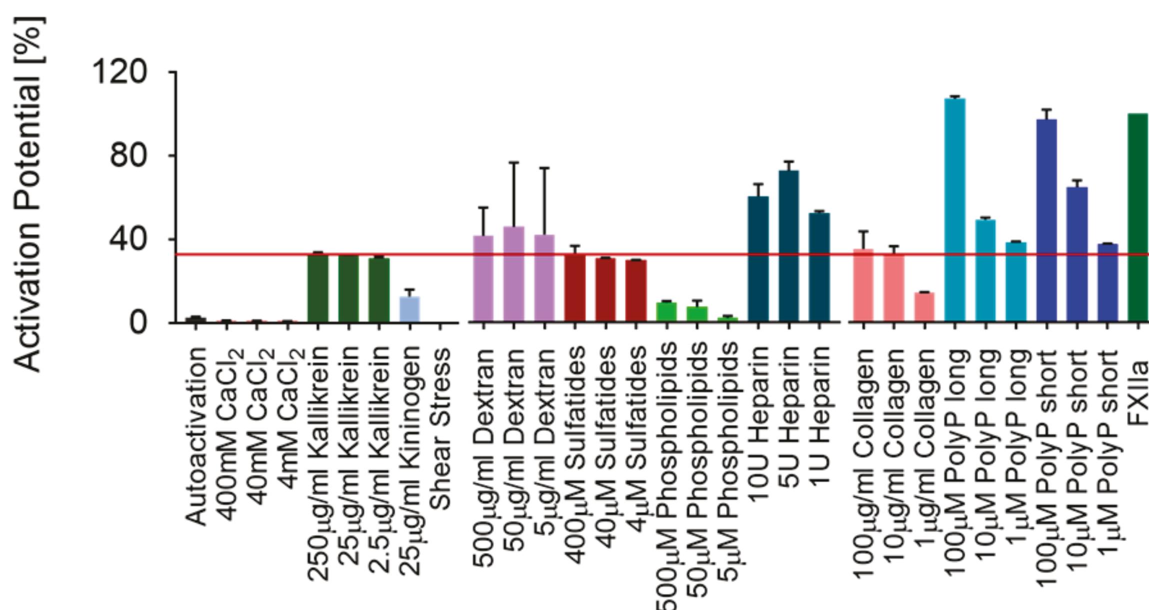

Figure 3.29: FXII activation potential previously described FXII activators. 100nM FXII were incubated with different concentrations of a variety of substances previously described as FXII activators. Cleavage of 0.8M activation assay substrate by the FXII/activator mix or FXIIa was monitored for 45 to 300 minutes at 405nm. Conversion of FXII to FXIIa (activation potential) of the different activators was determined based on the slopes of the individual substrate cleavage curves using a linear regression model with R square values ≥0.9. Calculated slopes subsequently were normalised against FXIIa activity rates (100%) and compared to the activation potential displayed by kallikrein (32.7%) which is the most relevant physiological activator of FXII. Eight out of the 12 substances and conditions tested showed varying degrees of FXII activation with shear stress, $CaCl_2$ and autoactivation exhibiting a low activation potential. Results confirmed sulfatides, heparin as well as short polyphosphates as potent FXII activators and additionally showed that dextran and long polyphosphates displayed high FXII activation potentials as well. U = units; PolyP = polyphosphates. Data presented as mean ± SEM. n = 3 to 12

Even though measurement of the optical density after 45 minutes allows a direct comparison of substrate cleavage rate between commercially available FXIIa and FXII that has been

activated with different activators, this readout methods does not consider the maximum substrate conversion levels over an extended period of time and additionally does not factor in how fast FXII is converted to FXIIa after incubation of the zymogen with the different activators. In order to include these two aspects, the activation potential of the different activators was determined by evaluating the slopes of the individual substrate cleavage curves at 405nm using a linear regression model with R square values ≥0.9. Calculated slopes were normalised against FXIIa activity rates (100%) and compared to kallikrein which exhibited a mean activation potential of 32.7%. Optical illustration of the activation potential of the substances tested confirmed sulfatides, heparin as well as short polyphosphates as potent FXIIa activators. While most of the substances and conditions tested resulted in varying degrees of FXII activity, dextran as well as long polyphosphates exhibited a high FXII activation potential (45.9% for 50µg/ml dextran and 107.1% for 100µM long polyphosphates) and therefore can be considered as FXII activators alongside sulfatides, heparin and short polyphosphates. As already observed when evaluating the OD values at 405nM, shear stress and $CaCl_2$ are not capable of triggering conversion of FXII to FXIIa (activation potentials of 0.2% and 1.2% respectively).

Mean values of the optical densities measured and the calculated activation potential of all substances and conditions tested are summarised in the following table 3.3.

Activator	Concentration	Absorbance (405nm)	Activation Potential (%)
FXIIa	100nM	**1,1**	-
Autoactivation	-	0,1	2,9
Calcium Chloride	400mM	0,1	1,2
	40mM	0,1	1,2
	4mM	0,1	1,1
Kallikrein	250µg/ml	1,0	33,5
	25µg/ml	0,9	**32,7**
	2.5µg/ml	0,9	31,3
Kinninogen	25µg/ml	0,5	13,0
Shear Stress	-	0,1	0,2
Dextran	500µg/ml	0,8	**41,5**
	50µg/ml	0,9	**45,9**
	5µg/ml	0,7	**42,2**
Sulfatides	400µM	**1,2**	**32,8**
	40µM	0,9	30,7
	4µM	0,9	29,9

Activator	Concentration	Absorbance (405nm)	Activation Potential (%)
Phospholipids	500µM	0,4	9,5
	50µM	0,3	7,5
	5µM	0,1	2,3
Heparin	10U	**1,8**	**60,4**
	5U	**2,1**	**72,7**
	1U	**1,4**	**52,5**
Collagen	100µg/ml	1,0	**35,3**
	10µg/ml	0,8	32,7
	1µg/ml	0,4	14,3
Polyphosphates Long	100µM	0,9	**107,1**
	10µM	1,1	**49,3**
	1µM	0,8	**38,3**
Polyphosphates Short	100µM	**1,8**	**97,1**
	10µM	**1,2**	**64,8**
	1µM	0,8	**37,6**
-	-	-	-

Table 3.3: Mean optical densities and calculated activation potentials of substances tested in FXII activation assays. Optical densities at 405nm after 45 minutes were measured for all FXII/substance and condition combinations and compared to commercially available active FXIIa (highlighted in red). OD values higher than 1.1 are marked in bold and indicate higher substrate conversion signals of the FXII/activator mix than exhibited by FXIIa itself. The activation potential of all activators tested was calculated by evaluation the slopes of the individual substrate cleavage curves. Slopes were normalised against FXIIa activity and compared to the activation potential of the physiological FXII activator kallikrein (highlighted in red). Values marked in bold refer to substances with an increased FXII activation potential compared to kallikrein. Data presented as mean ± SEM. n = 3 to 12.

Even though VWF has not been reported as a potential FXII activator in the literature, different binding assays have demonstrated a binding interaction between the two proteins. In order to investigate whether this interaction is capable of promoting the conversion of non-active FXII into the enzymatically active protease FXIIa, different preparation of VWF were tested at different concentrations (summarized below; physiological concentration of VWF is 10-12 mcg/ml which equals 35nM) in FXII activation assays.

- (Purified) plasma derived full-length VWF at
  0.1µg/ml = 0.4nM
  1.0µg/ml = 4.0nM
  10.0µg/ml = 40.0nM
  50.0µg/ml = 200.0nM
  100.0µg/ml = 400.0nM

- Recombinant full-length VWF at
  0.1µg/ml = 0.4nM
  1.0µg/ml = 4.0nM
  10.0µg/ml = 40.0nM

- Full-length VWF A1 domain mutant E1452/1459A — 1.0 µg /ml = 4.0nM
10.0µg/ml = 40.0nM

- Recombinant isolated VWF A1 domain at — 0.1µg/ml = 3.3nM
1.0µg/ml = 33.3nM
10µg/ml = 333.3nM
50 µg/ml = 1666.5nM
100µg/ml = 3333.0nM

- Isolated VWF A1 domain mutant E1452/1459A — 50.0µg/ml = 1666.5nM

In a first step, 100nM FXII were incubated with either 1µg/ml, 10µg/ml or 100µg/ml of plasma derived full-length VWF wildtype (Figure 3.30A) or the isolated VWF A1 domain (Figure 3.30B) and 0.8M of the activation assay substrate. As previously described, substrate cleavage was monitored at 405nm for 120 minutes. In order to verify that FXII activation is driven by the binding interaction between VWF and FXII, the isolated VWF A2 domain which is assumed to be void of any FXII binding site was included into this experiment as a negative control (Figure 3.30C). Results of the activation assay demonstrated that both plasma derived full-length VWF wildtype as well as the isolated VWF A1 domain were capable of activating FXII in a concentration and time dependent fashion. Substrate cleavage curves furthermore indicated that plasma derived full-length VWF wildtype is a more potent activator than the isolated VWF A1 domain. Negative controls which were run alongside the VWF proteins verified that FXII itself is not capable of cleaving the activation assay substrate and that the protease cannot be activated by a non-specific VWF based protein (isolated VWF A2 domain). Further controls additionally showed that neither plasma derived VWF nor the isolated VWF A1 domain interact with the substrate without FXII being present.

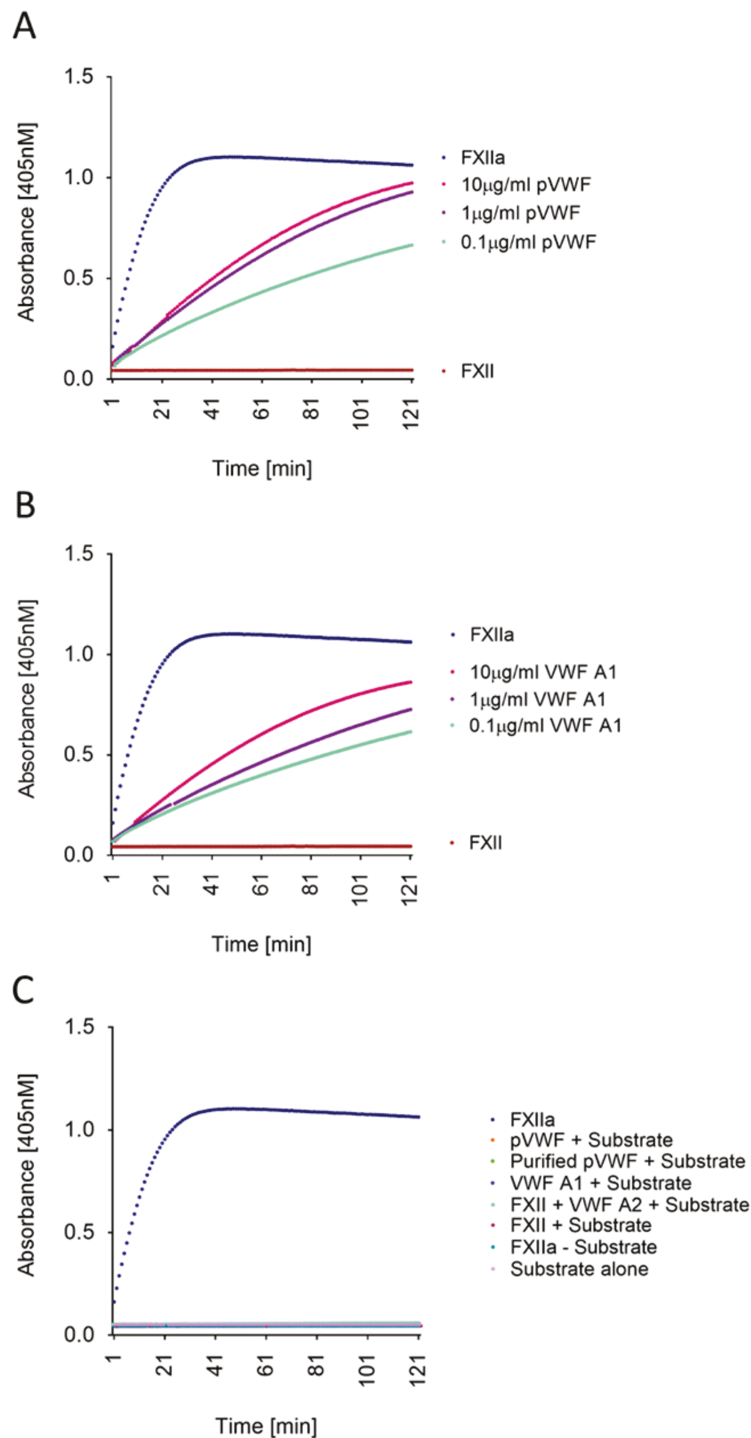

Figure 3.30: FXII activation assay with plasma derived full-length VWF wildtype and the isolated VWF A1 domain. 100nM FXII were incubated with different concentrations (1µg/ml,10µg/ml and 100µg/ml) of the isolated VWF A1 domain and plasma derived full-length VWF wildtype in the presence of 0.8M activation assay substrate. Cleavage of the substrate by activated FXIII was measured at 405nm for 120 minutes. Optical density signals showed concentration and time dependent activation of FXII by plasma derived VWF (A) and the isolated VWF A1 domain (B). Absorbance signals of the controls (C) demonstrated that activation of FXII is specific to plasma derived full-length VWF wildtype and the isolated VWF domain since incubation of FXII with the isolated VWF A2 domain which does not harbour a FXII binding site did not promote the conversion of FXII to FXIIa. In addition, neither VWF proteins nor FXII itself showed any cross-reactivity with the activation assay substrate. pVWF = plasma derived VWF; VWF A1 = isolated VWF A1 domain; VWF A2 = isolated VWF A2 domain. Data presented as mean ± SEM. n = 3.

After having established that VWF indeed is capable of converting inactive FXII to FXIIa, different VWF proteins (plasma derived full-length VWF wildtype, purified plasma derived full-length VWF wildtype, recombinant full-length VWF wildtype and the isolated VWF A1 domain; all tested at 0.1µg/ml,1µg/ml and 10µg/ml) were analysed in terms of their FXII activation potential. Activation of FXII was measured at 405nm for up to 300 minutes. Optical density values and the calculated activation potentials were compared to signals displayed by commercially available FXIIa and kallikrein respectively (Table 3.4). Evaluation of the individual optical density measurements after 45 minutes (Figure 3.31A) showed that all forms of VWF tested triggered activation of FXII and subsequent cleavage of the activation assay substrate in a concentration dependent manner. Incubation of FXII with both forms of plasma derived full-length VWF wildtype as well as the isolated VWF A1 domain showed increased substrate cleavage signals compared to FXII that was incubated with recombinant full-length VWF wildtype. Assessment of the activation potential (Figure 3.31B) of the different VWF proteins confirmed the observations made when evaluating the absorbance at 405nm and again showed a concentration dependent activation potential of VWF for FXII to a similar degree as kallikrein (Table 3.4).

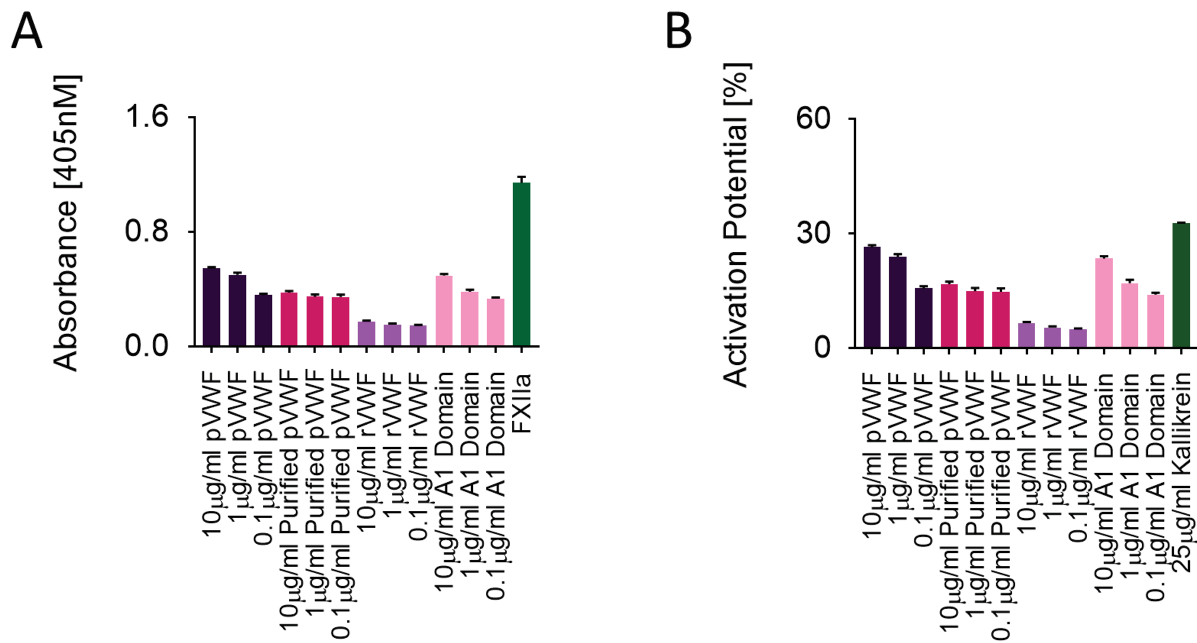

Figure 3.31: FXII activation assay with plasma derived, purified plasma derived, and recombinant full-length VWF wildtype as well as the isolated VWF A1 domain. 100nM FXII were activated with 0.1µg/ml, 1µg/ml and 10µg/ml of the different VWF proteins and cleavage of 0.8M substrate by VWF activated FXIIa was monitored for up to 300 minutes at 405nm. Optical density values were compared to OD values generated by substrate cleavage with commercially available FXIIa. Calculated activation potentials (linear regression model with R square values ≥0.9; normalised against FXIIa) were compared to the activation potential of the physiologically relevant FXII activator kallikrein. A) Optical density of substrate cleavage by VWF activated FXII after 45minutes. Results showed that all forms of VWF were capable of activating FXII in a concentration dependent manner. In direct comparison, both forms of plasma derived full-length VWF wildtype as well as the isolated VWF A1 domain promoted conversion of the zymogen into FXIIa to a higher degree than recombinant full-length VWF wildtype. B) Calculated activation potentials of VWF proteins in comparison to kallikrein. Evaluation of the activation potentials confirmed the results of the absorbance measurements and additionally showed that the activation potential of both forms of plasma derived full-length VWF wildtype as well as the isolated VWF A1 domain were in the range of kallikrein which is the main physiologically relevant activator of FXII. pVWF = plasma derived VWF; rVWF= recombinant VWF; A1 domain = isolated VWF A1 domain. Data presented as mean ± SEM. n = 3 to 12

Mean optical density values as well as the mean calculated FXII activation potential of plasma derived, purified plasma derived, recombinant full-length VWF wildtype and the isolated VWF A1 domain are displayed in the following table 3.4.

Activator	Concentration	Absorbance (405nm)	Activation Potential (%)
FXIIa	100nM	1,1	-
Kallikrein	25µg/ml	-	32.7
Plasma derived VWF	10µg/ml	0,5	26,5
	1µg/ml	0,5	24,0
	0.1µg/ml	0,4	15,7
Purified plasma derived VWF	10µg/ml	0,4	16,8
	1µg/ml	0,3	15,0
	0.1µg/ml	0,3	14,8
Recombinant VWF	10µg/ml	0,2	6,6
	1µg/ml	0,2	5,5
	0.1µg/ml	0,1	5,0
Isolated VWF A1 Domain	10µg/ml	0,5	23,5
	1µg/ml	0,4	17,1
	0.1µg/ml	0,3	14,1

Table 3.4: Mean optical densities and calculated activation potential for plasma derived, purified plasma derived, recombinant full-length VWF wildtype and the isolated VWF A1 domain. Evaluation of the different measurements confirmed VWF as an activator of FXII with an activation potential in a similar range to kallikrein (highlighted in red) for plasma derived full-length VWF wildtype and the isolated VWF A1 domain. OD of positive control FXIIa is highlighted in red. Mean of n = 3 to 12.

Static plate binding assays which were carried out in order to characterise the binding interaction between VWF and FXII(a) allowed the identification of a mutant carrying a combination of single-point mutations at position 1452 and 1459 which showed significantly reduced binding abilities towards both FXII and FXIIa. After having demonstrated that both full-length VWF wildtype as well as the isolated VWF A1 domain are capable of activating FXII, activation assays using 10µg/ml and 1µg/ml of the full-length and isolated VWF A1 domain mutant E1452/1459A were performed. Results of the assay confirmed that plasma derived VWF is capable of activating FXII (Figure 3.32 and Table 3.4) to a similar degree as observed when incubating FXII with kallikrein (activation potential of 32.7% for kallikrein and 26.5% for plasma derived full-length VWF wildtype). Evaluation of the optical density as well as comparison of the activation potential for the full-length and isolated VWF A1 domain double mutant displayed minimal FXII activating abilities for both sets of proteins with an activation potential of less than 10% and OD values of 0.1 and 0.2 compared to an OD of 1.1 measured after incubation of commercially active FXIIa with the activation assay substrate.

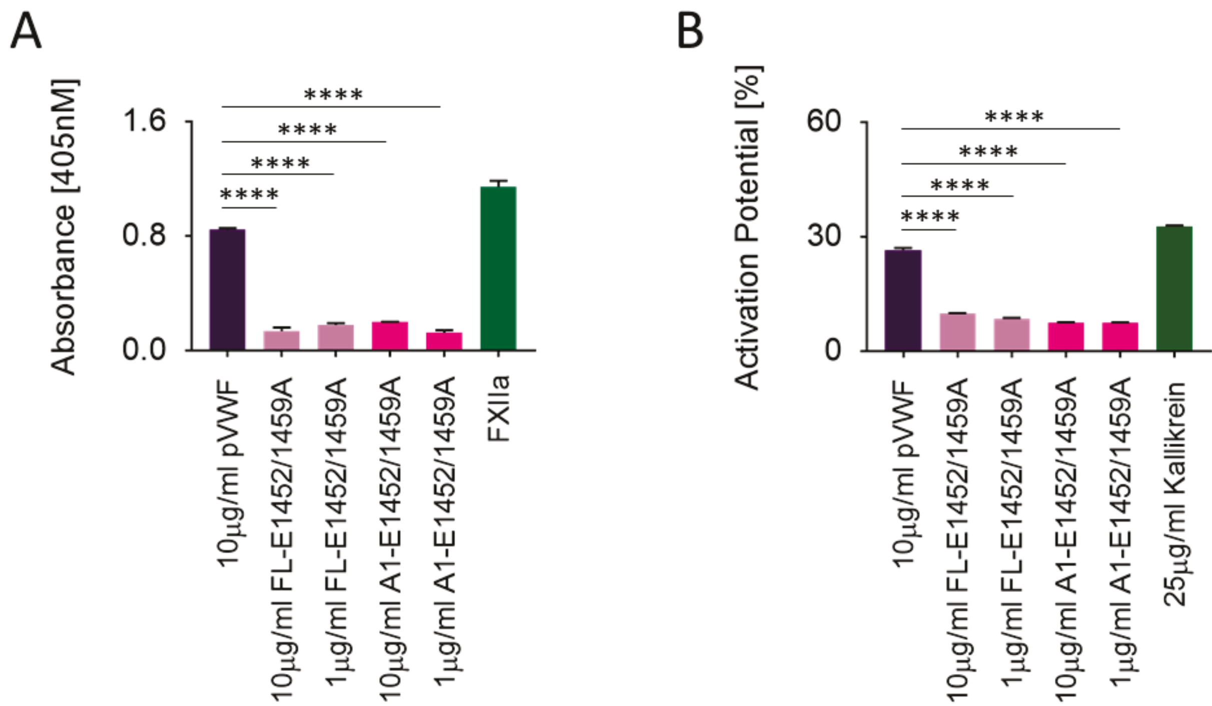

Figure 3.32 FXII activation assays with full-length and isolated VWF A1 domain double mutant E1452/1459. Binding data obtained after carrying out static plate binding assays demonstrated significantly reduced FXII(a) binding capabilities of a VWF mutant carrying single-point mutations at position 1452 and 1459. 100nM FXII were incubated with 10µg/ml and 1µg/ml of the full-length and isolated VWF A1 domain mutant E1452/1459A and 0.8M of the activation assays substrate in order to evaluate whether the double mutant is able to trigger conversion of FXII to FXIIa. Substrate cleavage by mutant activated FXIIa was monitored at 405nm for 300 minutes. Optical density values were compared to absorbance values of commercially available FXIIa. Calculated activation potentials (linear regression model with R square values ≥0.9; normalised against FXIIa) were compared to the activation potential of kallikrein. A) Optical density of mutant activated FXIIa substrate cleavage after 45 minutes. Results confirm activation of FXII after incubation with plasma derived full-length VWF wildtype. Incubation of FXII with both full-length and isolated VWF A1 domain mutant E1452/1459A showed minimal activation of the protease. B) FXII activation potential of full-length and isolated VWF A1 domain mutant E1452/1459A. Calculation of the activation potential demonstrated a similar activation potential of plasma derived full-length VWF wildtype as observed for kallikrein. Activation potentials of full-length and isolated VWF A1 domain mutants were significantly reduced. pVWF = plasma derived VWF; FL = full-length; A1 = isolated VWF A1 domain. Data presented as mean ± SEM. Statistical analysis with t-test; **** = 0.0001. n = 3 to 12

Mean optical density values as well as the mean calculated FXII activation potential of plasma derived full-length VWF wildtype as well as the full-length and isolated VWF A1 domain double mutant E1452/1459A are summarised in the table 3.5 below.

Activator	Concentration	Absorbance (405nm)	Activation Potential (%)
FXIIa	100nM	1,1	-
Kallikrein	25µg/ml	-	32.7
Plasma derived VWF	10µg/ml	0,8	26,5
FL-E1452/1459A	10µg/ml	0,1	9,8
	1µg/ml	0,2	8,5
A1-E1452/1459A	10µg/ml	0,2	7,5
	1µg/ml	0,1	7,4

Table 3.5: Mean optical densities and calculated activation potential for plasma derived full-length VWF wildtype and full-length as well as isolated VWF A1 double mutant E1452/1459A. Evaluation of the results of the activation assays confirmed plasma derived full-length VWF wildtype as an activator of FXII with a similar activation potential as kallikrein (highlighted in red). Both full-length and isolated VWF A1 domain mutant E1452/1459A exhibited minimal FXII activation potential. Absorbance at 405nm of positive control FXIIa is highlighted in red. Data presented as mean ± SEM. n = 3 to 12.

In order to verify whether conversion of zymogenic FXII into proteolytically active FXIIa is specifically triggered by VWF, FXII activation assays in which binding of VWF to FXII was inhibited by the addition of an anti-VWF antibody were performed. 100nM FXII were activated with 50µg/ml plasma derived full-length VWF wildtype, the isolated VWF A1 domain and isolated VWF A1 domain double mutant E1452/1459A in the presence and absence of 100µg/ml of an anti-VWF antibody. In addition, FXII activation also was tested using 50µg/ml of a truncated VWF protein (VWF C1C6) lacking the FXII binding site containing VWF A1 domain. Cleavage of 0.8M of the activation assay substrate was monitored at 405nm for up to 300 minutes and absorbance values as well as the calculated activation potentials were compared to signals displayed by commercially available FXIIa and kallikrein respectively (Figure 3.33 and Table 3.6). Measurement of the optical density demonstrated that both plasma derived full-length VWF wildtype and the isolated VWF A1 domain are capable of eliciting substrate cleavage by VWF activated FXIIa while incubation of FXII with the double mutant as well as VWF C1C6 only displayed minimal FXIIa activity. Blockage of the binding interaction between FXII and the different VWF proteins resulted in a complete inhibition of substrate cleavage and therefore FXII activation under all conditions tested (Figure 3.33A). Comparison of the activation potentials (Figure 3.33B) calculated for the proteins used in this assay displayed increased activation potentials of plasma full-length VWF wildtype and the isolated VWF A1 domain compared to kallikrein and additionally confirmed the inability of the remaining proteins to convert FXII into FXIIa. Again, blockage of VWF/FXII binding by an anti-

VWF antibody resulted in the complete inhibition of FXII activation demonstrating that binding interaction of VWF to FXII is mandatory for the conversion of FXII to FXIIa.

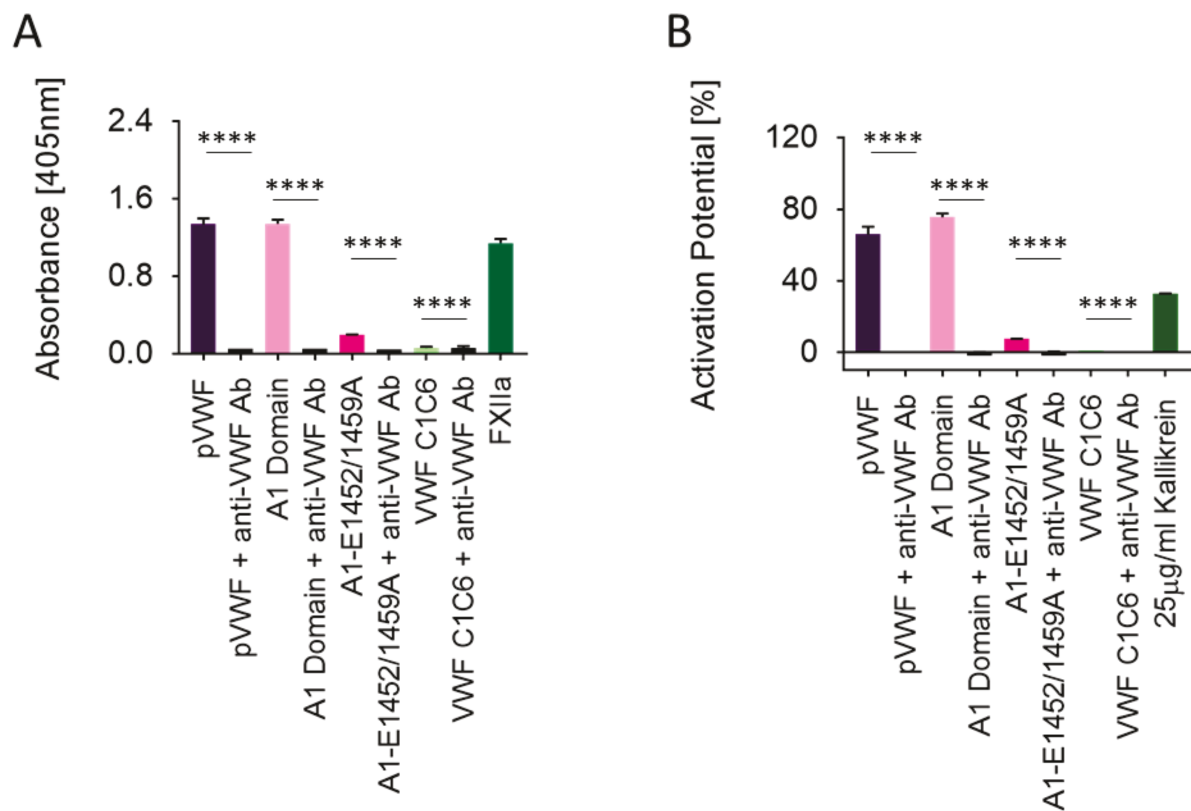

Figure 3.33: FXII activation assay testing the specificity of VWF as an activator of FXII. 100nM FXII were incubated with 50µg/ml plasma derived full-length VWF wildtype, the isolated VWF A1 domain and the isolated VWF A1 domain double mutant E1452/1459A in the presence and absence of 100µg/ml of an anti-VWF Ab in order to test whether activation of FXII can be prohibited by inhibition of the binding interaction between VWF and FXII. In addition, FXII activation by 50µg/ml VWF C1C6 which is lacking the FXII binding site containing A1 domain was evaluated. Cleavage of 0.8M activation assay substrate by VWF activated FXIIa was monitored at 405nm for 300 minutes. Measured optical densities as well as the calculated activation potential (linear regression model with R square values ≥0.9; normalised against FXIIa) were compared to substrate cleavage signals displayed by commercially available FXIIa and kallikrein (25µg/ml) respectively. A) Optical densities of substrate cleavage by VWF activated FXIIa after 45 minutes. Absorbance signals showed that plasma derived full-length VWF wildtype and isolated VWF both promote conversion of zymogenic FXII to FXIIa and subsequent cleavage of the activation assay substrate. Incubation of FXII with the isolated VWF A1 domain mutant E1452/1459A and VWF C1C6 showed minimal FXIIa activity indicating that neither protein is capable of activating FXII. Blockage of the binding interaction between VWF and FXII with an anti-VWF Ab resulted in complete absence of substrate cleavage and therefore FXII activation. B) Calculated activation potentials of VWF proteins in the presence and absence of an anti-VWF Ab prohibiting interaction between VWF and FXII. Comparison of the activation potentials between plasma derived full-length VWF wildtype, the isolated VWF A1 domain and kallikrein showed an enhanced potential of the VWF proteins to activate FXII. Results furthermore confirmed that blockage of the binding interaction between VWF and FXII effectively prevents conversion of FXII into FXIIa. Activation potentials of the double mutant and VWF C1C6 confirmed their inability to promote FXII activation. pVWF = plasma derived VWF; Ab = antibody; A1 domain = isolated VWF A1 domain; A1- = isolated VWF A1 domain. Data presented as mean ± SEM. Statistical analysis with t-test; **** = 0.0001; n = 3.

The following table 3.6 displays the mean optical density values and the mean calculated FXII activation potential of plasma derived full-length VWF wildtype, the isolated VWF A1 domain, the isolated VWF A1 domain mutant E1452/1459A and VWF C1C6 in the absence and presence of an anti-VWF Ab.

Activator	Blockage	Absorbance (405nm)	Activation Potential (%)
FXIIa	-	1,1	-
Kallikrein	-	-	32.7
Plasma derived VWF	-	1,3	66,1
	Anti-VWF Ab	0,1	0,0
Isolated VWF A1 Domain	-	1,3	75,5
	Anti-VWF Ab	0,1	0,1
Isolated VWF A1 Domain mutant E1452/1459A	-	0,2	7,5
	Anti-VWF Ab	0,0	0,2
VWF C1C6	-	0,1	1,2
	Anti-VWF Ab	0,1	0,0

Table 3.6: Specificity of FXII activation by VWF proteins. Comparison of the optical densities measured after 45 minutes showed that both plasma derived full-length VWF wildtype as well as the isolated VWF A1 domain are able to activate FXII. Incubation of FXII with the isolated VWF A1 domain mutant E1452/1459, VWF C1C6 and blockage of the binding interaction between VWF and FXII with an anti-VWF antibody prevented cleavage of the activation assay substrate and therefore activation of the protease. Calculated activation potentials demonstrated an increased activation potential of plasma derived full-length VWF wildtype and the isolated VWF A1 domain compared to kallikrein (highlighted in red). Calculation of the different activation potentials furthermore confirmed the inability of VWF C1C6 and the isolated VWF A1 domain mutant E1452/1459A to activate FXII and additionally verified that binding interaction between VWF and FXII is necessary for the conversion of FXII into FXIIa. Data presented as a mean of n = 3.

3.6.1. Western Blot Analysis of FXII Activation

Aside from the conventional FXII activation assays which are based on the conversion of a substrate by activated FXII, activation of FXII also can be monitored using western blot analysis since incubation of zymogen FXII with different activators results in the cleavage and subsequent fragmentation of the protease (Figure 3.34).

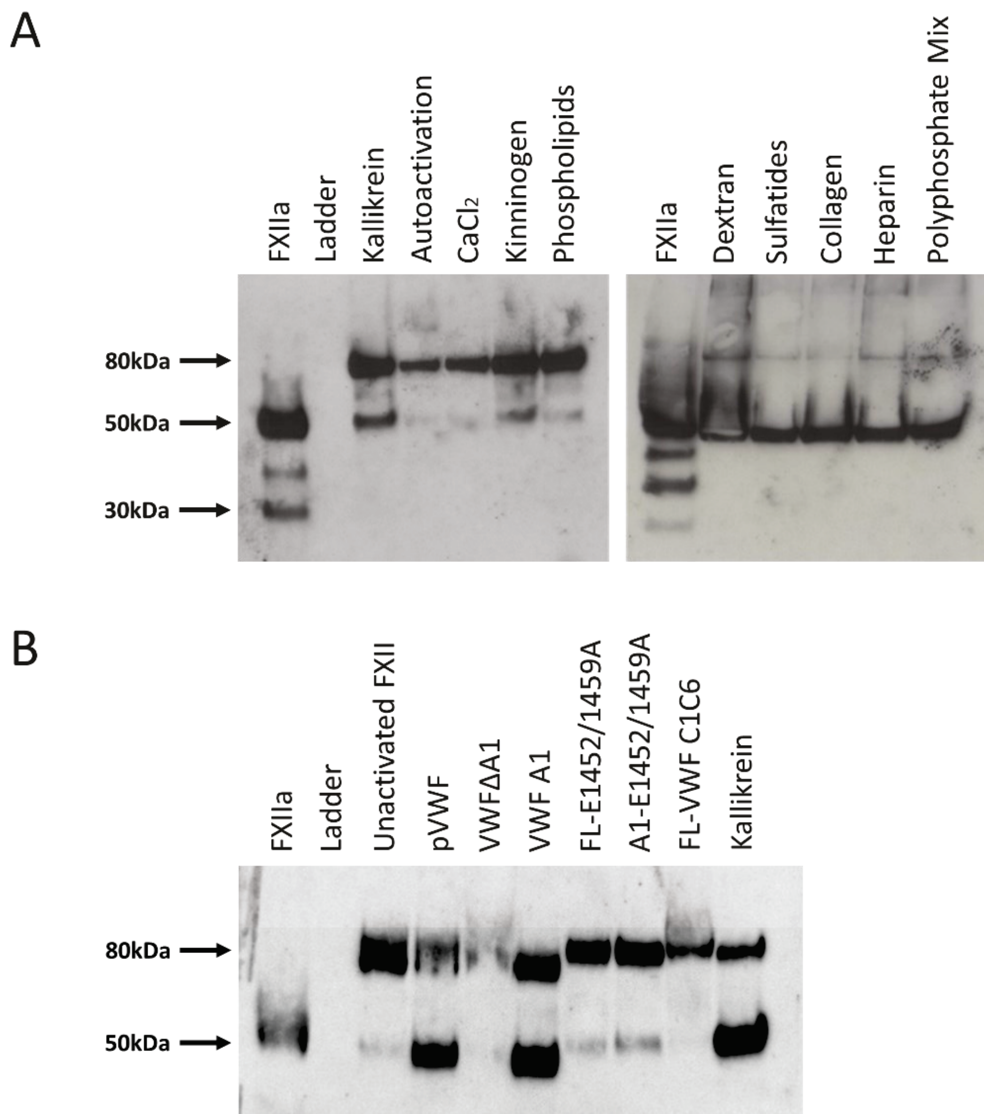

Figure 3.34: Western blot analysis of FXII activation using various activators. Activation of FXII is characterised by fragmentation of the 80kDa zymogen in a heavy and a light chain of 50kDa and 30kDa respectively. FXII was incubated with different activators for one hour at 37°C and protein fragmentation subsequently was analysed by western blot. Blotting results correlated with the activation potential determined using substrate-based activation assays and confirmed the activation potential of the different substances used. Based on the combined results, the activators were classified as strong to medium (s/m), weak (w) as well as non-activators (n) A) Western blot analysis of FXII activation using kallikrein (s/m), autoactivation (n), $CaCl_2$ (n), kininogen (w), phospholipids (w), dextran (s), sulfatides (s), collagen type I (s) heparin (s) and a mix of short and long polyphosphates (s). B) Western blot analysis of FXII activation using various VWF based proteins. While incubation of FXII with plasma derived full-length VWF wildtype (s) and the isolated VWF A1 domain (s) resulted in activation and fragmentation of FXII, full-length VWFΔA1 (n), full-length VWF C1C6 (n) as well as both the full-length VWF and the isolated VWF A1 domain mutant E1452/1459A (n/n) displayed no activation potential.

Western blot results showed different degrees of fragmentation of full-length FXII (80kDa) into a heavy (50kDa) and a light (30kDa) chain after incubation of the zymogen with various activators. The fragmentation of FXII therefore correlated with activation signals observed

when carrying out substrate-based activation assays and confirmed the activation potential or lack thereof of the activators used.

Based on the results, the different activators were divided into different groups summarised in table 3.7 below.

Strong/Medium Activators	Weak Activators	Non-activators
Kallikrein	Kininogen	$CaCl_2$
Dextran	Phospholipids	Autoactivation
Sulfatides	-	VWFΔA1
Collagen	-	FL-E1452/1459A
Heparin	-	A1-E1452/1459A
Polyphosphates	-	VWF C1C6
pVWF	-	-
VWF A1 domain	-	-

Table 3.7: Summary of the different FXII activators and their FXII activation potential. Kallikrein, dextran, sulfatides, collagen type I, heparin, polyphosphates, pVWF and the isolated VWF A1 domain showed medium to strong FXII activation potential. Weak FXII activation was exhibited by Kininogen and Phospholipids. $CaCl_2$, autoactivation, VWFΔA1, FL-E1452/1459A, A1-1452/1459A as well as VWF C1C6 are not capable to activate FXII.

3.7. Thrombolysis Assay

After having demonstrated that VWF is as a previously unrecognised binding partner of FXII(a) and that binding interaction between the two proteins promotes activation of the zymogen, the functional effects of these observations were further investigated.

In a first step, stability of blood clots formed using whole blood was tested in an adapted thrombolysis assay monitoring overall blood lysis times of blood clots generated under static conditions. In addition, clot lysis times of whole blood samples in which the activity of either VWF, FXIIa or both proteins was blocked with antibodies (40µg/ml of an anti-VWF or anti-FXII antibody) were evaluated in order to investigate whether the two proteins as well as their interaction with each other influence the stability and integrity of the formed blood clots. Clot formation was initiated with a mixture of 10pM tissue factor (TF) and 70mM $CaCl_2$ followed by incubation at 37°C for two hours. Generated clots were lysed by addition of 1nM tissue plasminogen activator (tPa). Lysis was monitored by measuring the absorbance at 510nm for

three to six hours. A rise in OD values hereby correlated with progressive lysis of the formed clots characterised by coverage of the wells by red blood cells released from the degrading blood clot. All thrombolysis assays were controlled by absence of the clotting solution (equivalent to completely disrupted clot formation) and addition of PBS without tPa to the formed clots (absence of clot lysis).

Initial thrombolysis assays served to verify the general setup of the assay and demonstrated the suitability of the relevant controls comprising conditions preventing clot formation or lysis in whole blood (Figure 3.35A) as well as effectiveness of the anti-VWF (Figure 3.35B) and anti-FXII antibodies (Figure 3.35C) or a combination thereof (Figure 3.35D). Results furthermore showed effective clot lysis over time after addition of 1nM tPa and indicated a contribution of VWF, FXIIa and potentially the interaction between the two proteins to the overall stability of the generated blood clots.

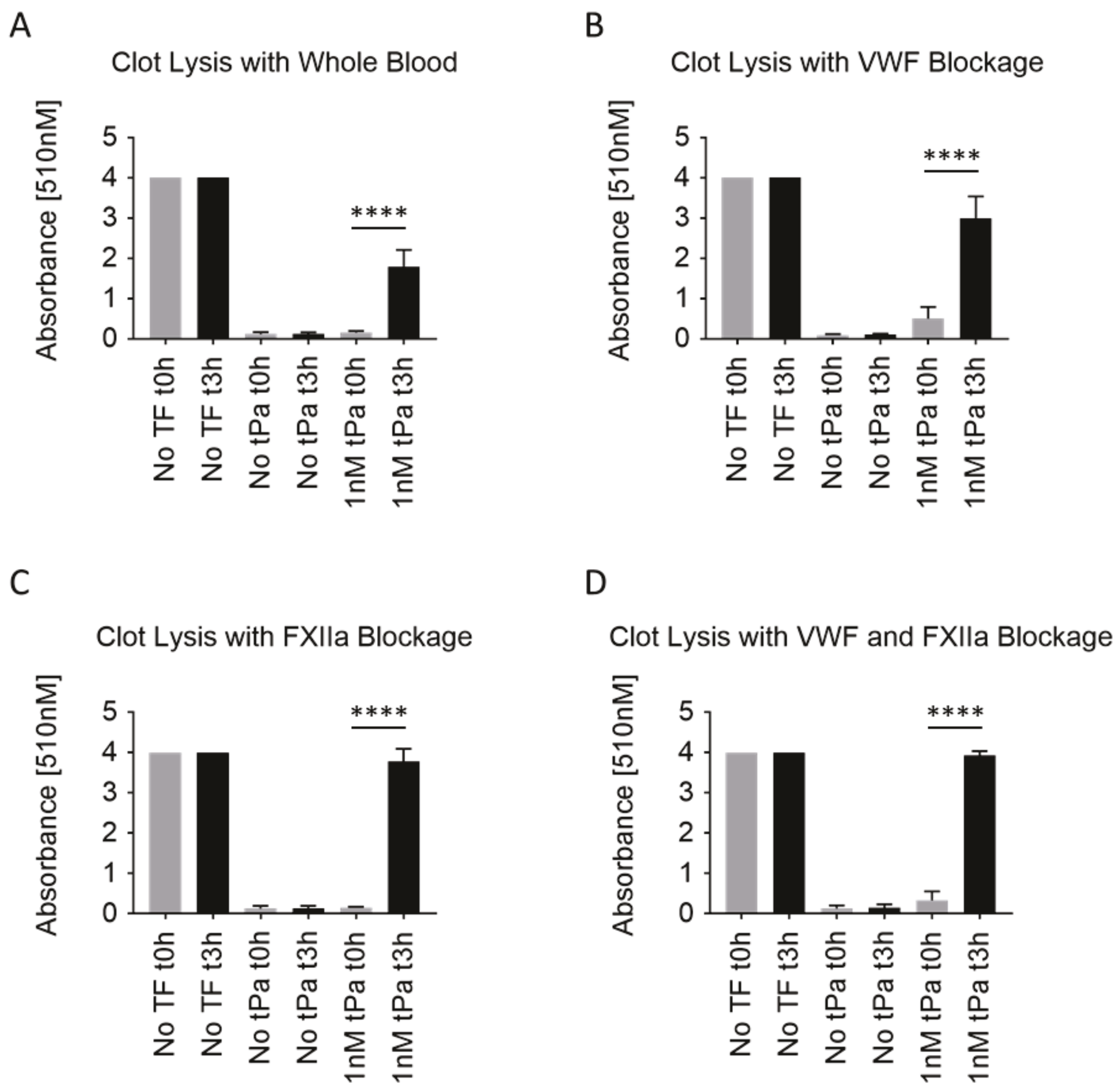

Figure 3.35: Adapted thrombolysis assay with whole blood. Thrombolysis assays were carried out in order to investigate the physiological relevance of the previously established interaction between VWF and FXIIa in whole blood samples (A). Activity of VWF and FXIIa was blocked by incubation of whole blood samples with 40µg/ml of an anti-VWF Ab (B), an anti-FXII Ab antibody (C) or a combination thereof (D) for one hour prior to clot formation. Clotting was initiated with 10pM TF and 70mM $CaCl_2$ at 37°C for two hours. Clot lysis was performed using 1nM tPa and lysis was monitored at 510nm for three hours. Lysis conditions were controlled by clot formation in the absence of the clotting mixture as well as monitoring of clot lysis in the absence of tPa. Results of the control samples verified the suitability of the conditions tested. Mean optical density values after addition of tPa (t0) and three hours (t3) were plotted and indicated a contribution of VWF, FXIIa and potentially of the interaction between the two proteins to the overall blood clot stability. Data presented as mean ± SEM. Statistical analysis with t-test; **** = 0.0001. n = 3 to 12

Subsequent to the verification of the general assay setup, the role of VWF and FXIIa in clot stability and lysis was further analysed by monitoring clot lysis times of whole blood clots as well as clots generated from whole blood samples containing either an anti-VW Ab, and anti-

FXIIa antibody or both antibodies simultaneously. Optical density values after two (Figure 3.36A and 3.36C) and three hours (Figure 3.36B and 3.36D) showed significantly decreased clot lysis times with both 1nM (Figure 3.36A and B) and 5nM tPa (Figure 3.36 C and D) after blockage of VWF activity that was further accentuated by inhibition of FXIIa alone and combined VWF and FXIIa activity compared to lysis times observed for clots formed from whole blood. Results therefore indicated that activity of VWF and FXIIa and potentially the interaction between these two proteins have a significant impact on the maintenance and integrity of stable blood clots.

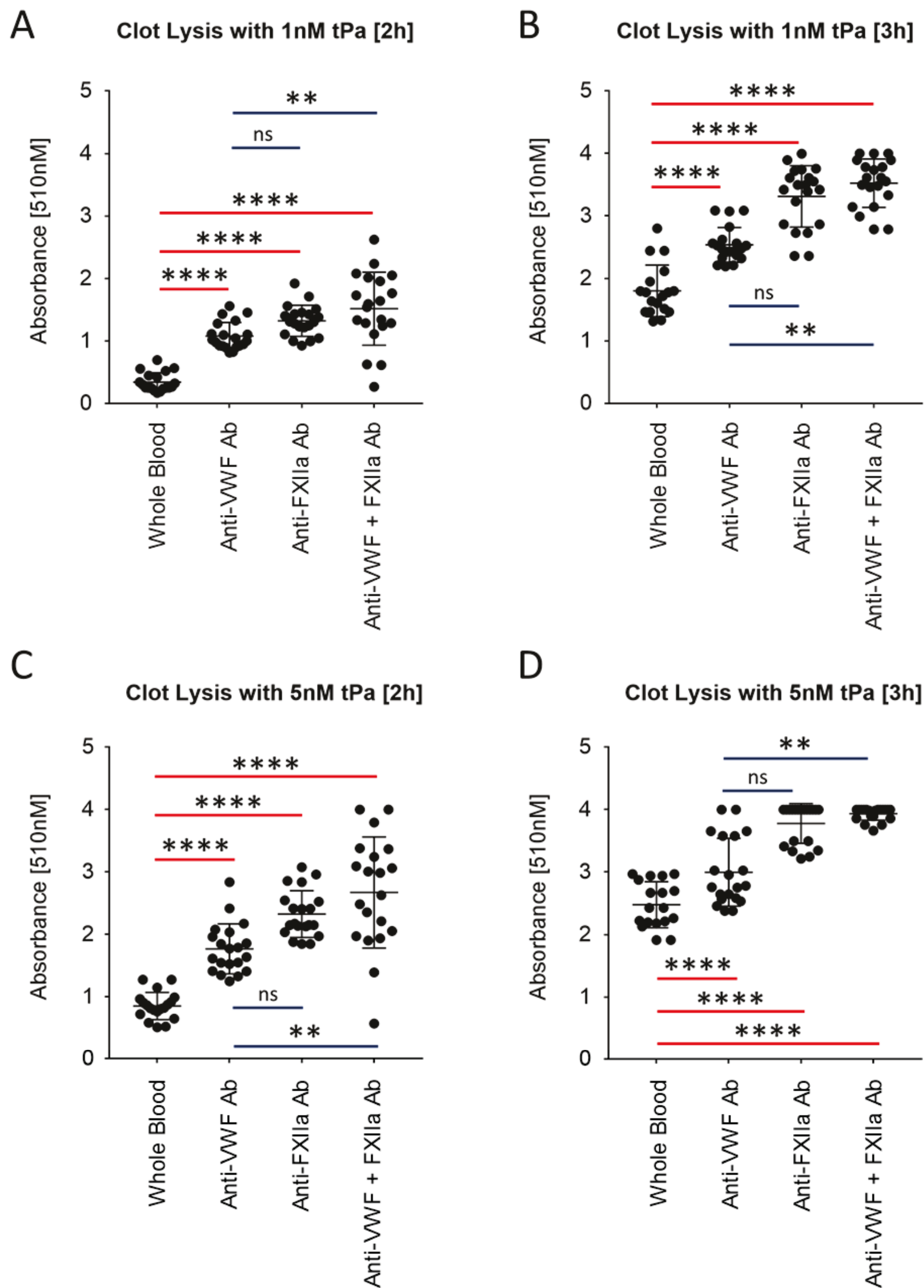

Figure 3.36: Adapted thrombolysis assay investigating the role of VWF and FXIIa in overall stability of blood clots. Formation of blood clots from whole blood or blood samples containing 40µg/ml of either an anti-VWF antibody, an anti-FXIIa antibody or a mix of the two antibodies was initiated with 10pM TF and 70mM $CaCl_2$ for two hours at 37°C. Clot lysis was performed by addition of 1nM (A and B) and 5nM tPa (C and D). Lysis was monitored at 510nm for two (A and C) and three (B and D) hours. Assessment of the optical densities showed that blockage of VWF activity resulted in a significant decrease of clot lysis times compared to whole blood clot lysis. Lysis times were further accelerated by inhibition of FXIIa activity and simultaneous blockage of the activity of both proteins. Results therefore indicated a significant contribution of both VWF and FXIIa as well as potentially the interaction between the two proteins to the overall stability of blood clots under all conditions tested. Ab = antibody; tPa = tissue plasminogen activator. Data presented as mean ± SEM. Statistical analysis with t-test; ** < 0.02; ****< 0.0001. n = 18 to 20

In order to further evaluate the influence of the binding interaction between VWF and FXIIa on the stability and integrity of blood clots, thrombolysis assays with VWF depleted plasma reconstituted with either recombinant full-length VWF wildtype or full-length VWF A1 domain mutants that have been shown to exhibit reduced FXII binding ability were carried out. Red blood cells and platelets were isolated and purified from whole blood samples and subsequently were mixed with VWF depleted plasma. Next, 10µg/ml of either recombinant full-length VWF wildtype or the full-length VWF A1 domain mutants E1452A, D1459A or E1452/1459A were added to the individual samples which then were used to form blood clots. Lysis of the generated clots was performed with 2.5nM tPa and was monitored at 510nM for five hours. Based on the optical density values measured, time points at which 50% of the clots had dissolved were determined (Figure 3.37A). The results confirmed that blood clots formed from blood samples void of VWF as well as VWF and FXII have accelerated clot lysis times and therefore decreased clot stability compared to clots containing recombinant full-length VWF wildtype. Interestingly, interference of the binding interaction between VWF and FXIIa displayed an even more pronounced reduction in the 50% lysis time with clots containing the double mutant E1452/1459D which is not capable of interacting with FXIIa not being able to form stable clots at all (Table 3.8). Absorbance curves monitoring clot lysis over time (Figure 3.37B) again demonstrated reduced stability of clots containing the different full-length VWF A1 domain mutants compared to clots formed from a mixture of VWF depleted plasma, red blood cells, platelets and the recombinant full-length VWF wildtype. Combined results of the thrombolysis assay with VWF depleted plasma revealed a significant role of both VWF and FXIIa as well as their binding relationship in the formation and stability of blood clots since reductions in clot lysis times directly correlated with a reduced ability of VWF to bind to FXIIa.

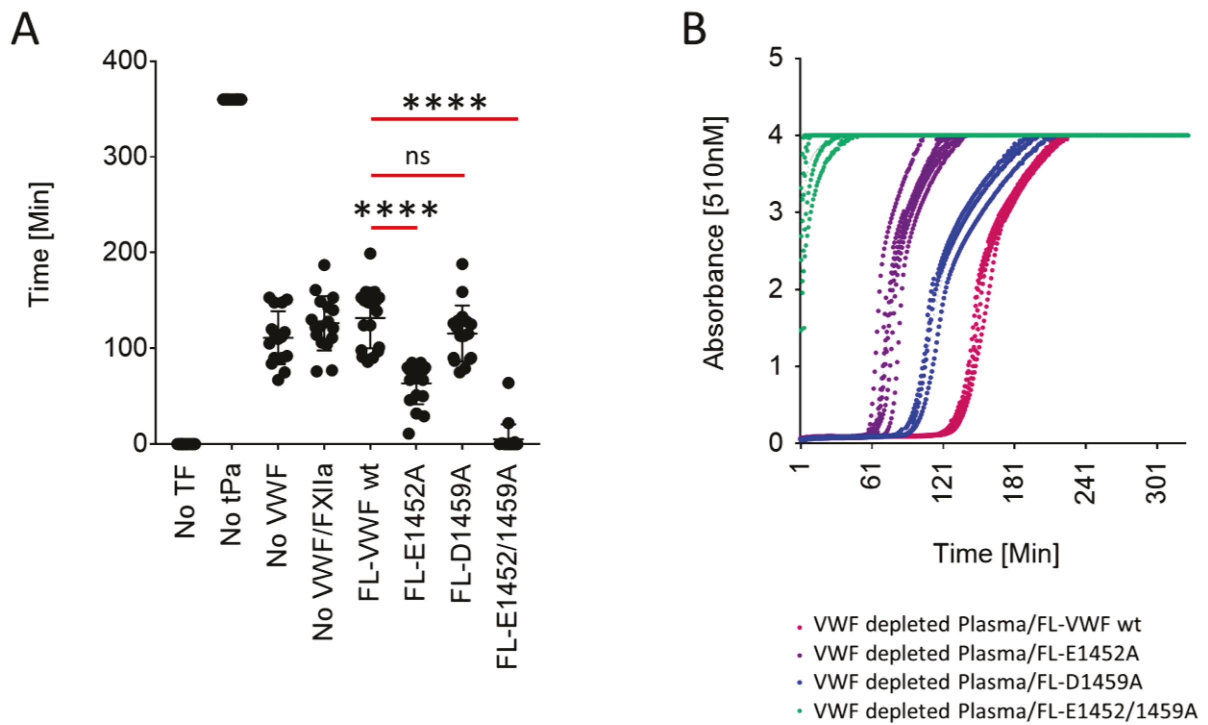

Figure 3.37: Thrombolysis assay with VWF depleted plasma testing the effect of the VWF/FXII binding interaction on clot formation and stability. VWF depleted plasma was mixed with isolated and purified red blood cells and platelets. Samples were reconstituted with 10µg/ml of either recombinant full-length VWF wildtype or the full-length VWF A1 domain mutants E1452A, D1459A or E1452/1459A. Clots were formed at 37°C for two hours using 10pM TF and 70mM $CaCl_2$. Clot lysis was monitored at 510nm for five hours using 2.5nM tPa. A) 50% lysis times of clots formed from VWF depleted plasma samples in the presence and absence of recombinant full-length VWF wildtype and full-length VWF A1 domain mutants. Time points after which 50% of the clots had been lysed were selected for the direct comparison of the clot stability. Results showed that absence of VWF and a combination of VWF and FXII led to decreased lysis times compared to sample containing recombinant full-length VWF wildtype. Lysis times were further reduced when clots contained full-length VWF A1 domain mutants E1452A or D1459A. Formation of clots was completely abolished in blood samples reconstituted with the double mutant E1452/1459A. B) Clot lysis curves of VWF depleted blood clots reconstituted with recombinant full-length VWF wildtype, full-length VWF A1 domain mutant E1452A, D1459A or the double mutant E1452/1459A. Comparison of the lysis times again showed decreased lysis times of clots containing the different full-length VWF A1 domain mutants and almost complete absence of clot formation with samples containing the VWF double mutant E1452/1459A. Combined results indicated a significant role of the binding interaction between VWF and FXII in the formation and stability of blood clots since interference in binding of VWF to FXIIa by the use of VWF mutants with reduced FXIIa binding abilities directly correlated with decreased clot lysis times or absence of clot formation. TF = tissue factor; tPa = tissue plasminogen activator; FL = full-length; wt = wildtype. Data presented as mean ± SEM. Statistical analysis with t-test; **** < 0.0001. n = 16 to 18

Mean values of the 50% clot lysis times for clots formed from VWF depleted plasma samples reconstituted with either recombinant full-length VWF wildtype or full-length VWF A1 domain mutants E1452A, D1459A and E1452/1459A are depicted in table 3.8.

	50% Lysis (min)
No VWF	111,1
No VWF/FXII	126,2
Full-length VWF wildtype	131,6
Full-length VWF A1 domain mutant E1452A	61,4
Full-length VWF A1 domain mutant D1459A	115,4
Full-length VWF A1 domain mutant E1452/1459A	5,0

Table 3.8: Mean 50% clot lysis times. Blood clots formed with VWF depleted samples containing 10µg/ml recombinant full-length VWF wildtype, full-length VWF A1 domain mutants E1452, D1459A or the double mutant E1452/1459A were lysed and the time point at which 50% of the clot had been disseminated were determined. Comparison of the 50% lysis times showed a decrease in lysis time in the absence of VWF and a combination of VWF and FXII. Lysis times were further decreased in the presence of full-length VWF A1 domain mutants which displayed reduced FXII binding abilities. Samples containing the double mutant E1452/1459A were characterised by either complete absence of clot formation or generation of highly unstable clots. Thrombolysis results with VWF depleted plasma therefore showed a significant role of the binding interaction between VWF and FXII in the formation and stability of blood clots. min = minutes. Mean of n = 16 to 18.

3.8. Clot Formation under Flow with VWF depleted Plasma

Based on the results of the adapted thrombolysis assays, the physiological relevance of the interaction between VWF and FXII was further investigated by analysing the formation of platelet rich blood clots under conditions of flow. VWF and FXIIa depleted plasma was mixed with isolated and purified red blood cells as well as fluorescently labelled platelets. Subsequently, samples were reconstituted with 10µg/ml recombinant full-length VWF wildtype or the full-length VWF A1 domain mutants E1452, D1459A, double mutant E1452/1459A or 400nM human FXIIa. Next, samples were recalcified using 10mM $CaCl_2$, perfused over collagen type I coated ibidi slides for four minutes under conditions of shear ($1500s^{-1}$) and platelet capture was assessed as percentage of surface coverage (Figure 3.38). Optical evaluation of the platelet capture obtained by perfusion of the different samples demonstrated regular platelet capture profiles of VWF depleted plasma samples that were reconstituted with recombinant full-length VWF wildtype compared to whole blood sample which contained both VWF and FXIIa. Control samples which were void of either VWF or both VWF and FXIIa did not exhibit any significant platelet attachment. Platelet surface coverage of FXII depleted plasma samples which naturally contain full-length VWF wildtype displayed reduced platelet capture compared to whole blood or samples that had been reconstituted

with full-length VWF wildtype and FXIIa indicating a role of FXIIa in the formation of platelet rich blood clots during haemostasis. Arrest of platelets in samples reconstituted with the full-length VWF A1 domain mutants E1452A, D1459A and the double mutant E1452/1459A showed significantly reduced platelet surface coverage profiles compared to whole blood and full-length VWF wildtype runs.

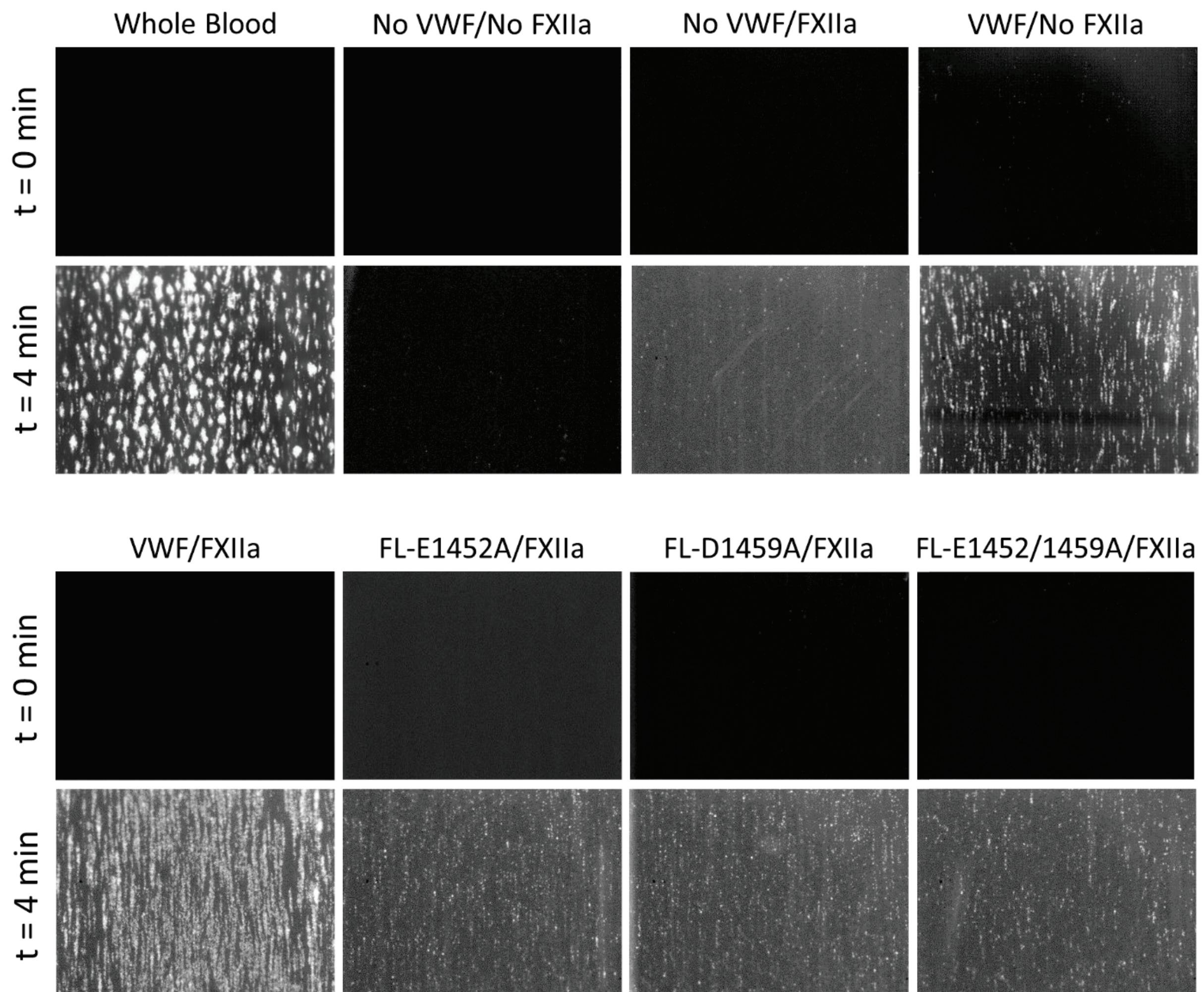

Figure 3.38: Exemplary platelet capture profiles of full-length VWF proteins under shear in recalcified VWF depleted plasma. VWF and FXII depleted plasma was mixed with isolated and purified red blood cells and $DiOC_6$ labelled platelets. Samples subsequently were reconstituted with 10µg/ml of recombinant full-length VWF wildtype or the full-length VWF A1 domain mutants E1452A, D1459A and the double mutant E1452/1459A. After recalcification with 10mM $CaCl_2$, samples were perfused over collagen type I coated ibidi slides at a shear rate of 1500^{-s} and platelet attachment was monitored for four minutes. Results of the flow assay demonstrated normal platelet capture profiles for whole blood and VWF depleted plasma to which recombinant full-length VWF wildtype had been added. FXII depleted plasma samples showed reduced platelet attachment compared to whole blood and VWF depleted plasma samples reconstituted with recombinant full-length VWF wildtype. Perfusion of samples void of either VWF or VWF and FXIIa resulted in absent platelet capture. Platelet surface coverage profiles of samples containing the different full-length VWF A1 domain mutants exhibited significantly reduced platelet capture profiles (D1459A>E1452A>E1452/1459A) compared to whole blood and recombinant full-length VWF wildtype samples. t = time; min = minutes; FL = full-length; n = 3 to 4.

Platelet surface coverage of the individual samples additionally was normalised against the platelet capture observed with samples of VWF depleted plasma containing red blood cells, labelled platelets, FXIIa and 10µg/ml of the recombinant full-length VWF wildtype (Figure 3.39). Comparison of the platelet coverage confirmed similar platelet capture profiles for whole blood and VWF depleted plasma samples that had been reconstituted with recombinant full-length VWF wildtype, a reduced platelet capture profile for samples void of FXIIa and absence of platelet attachment for samples missing either VWF or VWF and FXIIa simultaneously. Platelet surface coverage of samples containing the isolated VWF A1 domain mutants was significantly reduced compared to whole blood and recombinant full-length VWF wildtype samples. Reduction of platelet coverage after perfusion of VWF depleted plasma reconstituted with the double mutant E1452/1459A which has been demonstrated to be incapable of binding to FXIIa were comparable to platelet coverage levels of samples void of VWF. Conjunctively, the results demonstrated that the binding interaction between VWF and FXIIa is mandatory for the attachment of platelets to VWF under conditions of high shear stress as well as that interference in this interaction has a definite detrimental effect on the overall formation of platelet rich blood clots.

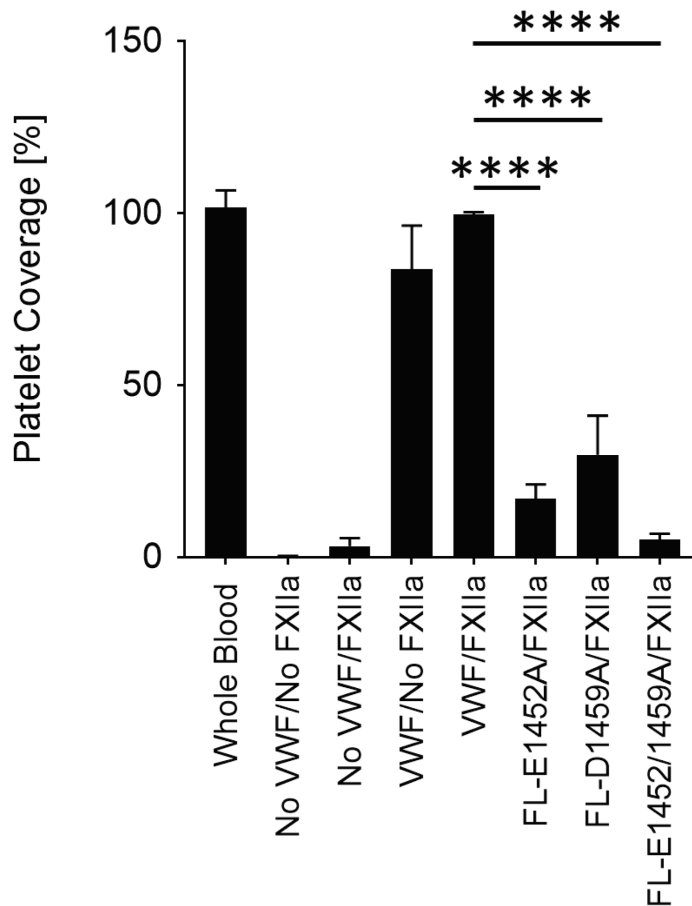

Figure 3.39: Percentage of platelet coverage of full-length VWF proteins under shear in recalcified VWF depleted plasma. VWF and FXII depleted plasma was mixed with isolated and purified red blood cells and $DiOC_6$ labelled platelets. Samples subsequently were reconstituted with 10µg/ml of recombinant full-length VWF wildtype or the full-length VWF A1 domain mutants E1452A, D1459A and the double mutant E1452/1459A. After recalcification with 10mM $CaCl_2$, samples were perfused over collagen type I coated ibidi slides at a shear rate of 1500^{-s} and platelet attachment was monitored for four minutes. Percentages of platelet coverage were normalised against VWF depleted plasma samples containing the recombinant full-length VWF wildtype. Results demonstrated similar platelet capture abilities for whole blood and VWF depleted plasma samples reconstituted with recombinant full-length VWF wildtype. While absence of FXIIa resulted in a decrease in platelet capture, no platelet attachment in samples void of VWF or VWF and FXIIa was observed. Substitution of VWF with either full-length VWF E1452A and D1459A resulted in a significant reduction in bound platelets. Platelet capture levels of samples void of full-length VWF were comparable to samples containing the full-length VWF A1 domain double mutant E1452/1459A which is not capable of binding FXIIa indicating that not only VWF but also the interaction between VWF and FXIIa plays a crucial role in the formation of platelet rich clots and that interference in this binding interaction circumvents clot formation. Data presented as mean ± SEM. Statistical analysis with t-test; **** < 0.0001. n = 3 to 5.

4. Discussion

4.1. Conclusions

The purpose of this thesis was the characterisation of a novel binding interaction between VWF and FXII(a) and its functional effects on the development of pathological thrombi. The results summarised below suggest that inhibition of the VWF-FXII(a) complex formation would be a suitable target for the prevention of pathological thrombus formation and therefore might present an alternative avenue for the development of new and safe anticoagulant therapies.

1) Verification of a binding interaction between VWF and FXII

The first part of the research project was dedicated to establishing a general binding interaction between VWF and FXII which was confirmed using two different experimental methods (ELISA and SPR). Static plate binding assays demonstrated a binding interaction between both plasma derived as well as recombinant full-length VWF and FXIIa after immobilisation of one of the binding partners on plastic and subsequent incubation with the respective second protein. In order to eliminate the possibility of inaccurate binding signals due to conformational changes after immobilisation on a static surface, formation of a VWF/FXIIa complex in solution which subsequently was detected in ELISA experiments was carried out alongside and confirmed the general binding of VWF to FXIIa. Affinity of recombinant as well as plasma derived VWF towards immobilised FXIIa, FXII zymogen and FXII activated with kallikrein after immobilisation of the protease was determined with the following $K_{D,app}$ values:

Plasma derived VWF to FXIIa	4.5nM	ELISA
Plasma derived VWF to FXII	36.2nM	ELISA
Plasma derived VWF to kallikrein activated FXII	23.8nM	ELISA
Recombinant VWF to FXIIa	21.0nM	ELISA
	31nM	SPR
Recombinant VWF to FXII	9.5nM	ELISA
Recombinant VWF to kallikrein activated FXII	39.6nM	ELISA

Binding assays using recombinant full-length VWF lacking the A1 domain (VWFΔA1) showed significantly reduced binding towards immobilised FXIIa suggesting the presence of the FXII(a) binding site withing the A1 domain which subsequently was confirmed by ELISA as well as SPR measurements characterising an affinity of the isolated VWF A1 domain towards FXIIa with $K_{D,app}$ values of 139nM (ELISA) and 80nM (SPR) respectively.

2) Creation of a panel of VWF mutants for the mapping of the FXII binding site in the VWF protein

After having confirmed the general binding interaction between VWF and FXII(a) as well as verifying the presence of the FXII(a) binding site within the A1 domain of the glycoprotein, a set of VWF A1 mutants was created in order to identify specific amino acid residues involved in the binding of VWF towards FXII(a). Due to FXII having been described to bind to negatively charged surfaces, negatively charged amino acids in the VWF A1 domain were identified and mutated in both the isolated VWF A1 domain as well as the full-length VWF protein. The proteins subsequently were transiently expressed in HEK293T cells, purified or concentrated and subjected to protein concentration determination assays.

Apart from mutants D1302A and E1339A, expression levels of the isolated VWF A1 domain mutants were comparable to expression levels observed for the isolated wildtype A1 domain. In contrast, expression of the full-length VWF mutants revealed generally lower expression levels of the majority of mutants compared to both full-length VWF wildtype as well as the isolated VWF A1 domain mutants. After initial screening of the VWF mutants in both the isolated VWF A1 domain and the full-length protein in terms of their binding ability towards immobilised FXII(a), a set of double and one triple mutant were generated in order to identify specific amino acids responsible for the binding interaction between the two proteins. Transient HEK293T expression of the generated double and triple mutants showed moderate expression levels for FL-VWF D1444/1445A, FL-VWF E14352/1459A, A1 D1444/1445A as well as A1 D1444/1447A and high expression of mutants FL-VWF D1444/1447A and A1 E14352/1459A. Analysis of the expression level of the triple mutant with mutations at position 1444, 1445 and 1447 showed virtually no expression of the protein.

Further characterisation of the different single-point and double full-length VWF mutants included multimer gel analysis as well as platelet capture assays. Determination of the

multimer patterns of the respective full-length VWF mutants showed formation of a range of VWF multimers comparable to recombinant full-length VWF. Perfusion platelet capture assays confirmed the binding ability of all full-length VWF mutants towards both collagen as well as platelets indicating that the relevant binding sites were not compromised by the introduction of the different mutations and therefore did not impair the physiological function of VWF.

3) Identification of specific amino acid residues involved in the interaction between VWF and FXII

After characterisation, all full-length and isolated A1 domain mutants were tested in terms of their binding ability towards immobilised FXIIa in static plate binding assays. Results of the ELISA´s showed that introduction of the different single-point mutation led to either an increase or a decrease in FXIIa binding. In total, five proteins with mutations at position 1290, 1444, 1445, 1452 and 1459 showed a reduction in FXIIa binding in both the isolated A1 domain as well as full-length VWF. Since none of the mutations introduced led to a complete abolishment of FXIIa binding, three double (D1444/1445A, D1444/1445A, E1452/1459A) and one triple mutant (D1444/1445/1447A; did not express) were created based on their location on the surface of the A1 domain in order to further map the specific amino acids responsible for the interaction between the glycoprotein and the protease. After expression and further characterisation, binding of all mutants to immobilised FXIIa was tested in static plate binding assays. Binding signals demonstrated that the combination of mutations at position 1452 and 1459 led to an inability of the protein to bind FXIIa, FXII and FXII that previously had been activated with kallikrein. ELISA results subsequently were confirmed by SPR measurements identifying amino acids aspartic acid at position 1452 and glutamic acid at position 1459 as the FXII binding site within the A1 domain of VWF.

4) Analysis of FXII activation by different activators as well as VWF

Activation of zymogen FXII by different molecules was tested using plate-based activation assays with a colorimetric readout after cleavage of substrate S-2302 by the protease. Results of the activations assays allowed separation of the substances analysed into three different groups (see below) based on their potential to trigger conversion of inactive FXII to FXIIa.

Based on the results of VWF-FXII(a) binding assays which established a binding interaction between the two proteins, activation of FXII zymogen by different VWF proteins was analysed. Determination of the activation potential demonstrated specific activation of FXII after incubation of the zymogen with plasma-derived full-length VWF, purified plasma derived full-length VWF as well as the isolated VWF A1 domain. In contrast, exposure of inactive FXII to the full-length and isolated VWF A1 domain mutant E1452/1459A did not result in the activation of the protease confirming that binding of VWF to FXII is mediated by aspartic acid at position 1452 and glutamic acid at position 1459.

Results of the substrate plate-based activation assays subsequently were confirmed with western blot analysis showing fragmentation of zymogen FXII after incubation with the different substances mentioned above.

- Strong/medium activators: Kallikrein
Dextran
Sulfatides
Collagen
Heparin
Polyphosphates
Plasma-derived VWF
Isolated VWF A1 domain
- Weak activators: Kininogen
Phospholipids
- Non-activators: $CaCl_2$
Autoactivation
FL-E1452/1459A
A1-E1452/1459A

5) Analysis of the physiological relevance of the interaction between VWF and FXII(a)

The physiological relevance of the binding interaction between VWF and FXII(a) was assessed using adapted versions of a thrombolysis as well as a perfusion assay.

The influence of VWF, FXIIa as well as the interaction of the two proteins on clot stability was analysed in thrombolysis assays and results showed that samples in which the activity of VWF was blocked using a VWF specific antibody formed less stable thrombi compared to samples containing whole blood. This effect was further enhanced after inhibition of FXIIa activity by a

FXII specific antibody. Simultaneous inhibition of the activity of both VWF and FXIIa led to highly instable clot formation indicating that interaction between the two proteins is essential for the generation of stable thrombi. Substitution of parental full-length VWF with full-length VWF A1 mutants E1452A, D1459A and E1452/1459A in the samples used for clot formation confirmed that the interaction between VWF and FXII(a) directly influences clot stability since a reduction in the binding ability of VWF to FXIIa correlated with an increased clot instability.

Perfusion assays investigating the generation of platelet rich blood clots under conditions of pathological shear demonstrated enhanced VWF mediated platelet capture in the presence of FXIIa suggesting that the interaction of the two proteins influences the overall ability of VWF to interact with platelets. Results of the perfusion assays furthermore confirmed the previously made observation of a direct correlation between the binding ability of VWF to FXIIa and the generation of stable thrombi since substitution of the blood samples with full-length VWF mutants E1452A, D1459A and E1452/1459A instead of full-length VWF wildtype resulted in reduced or even absent formation of platelet rich blood clots.

Combined analysis of the physiological relevance of the interaction between VWF and FXII(a) demonstrated that the binding interaction between the glycoprotein and protease is essential for VWF mediated platelet capture under conditions of high shear stress and that inhibition of VWF-FXIIa binding has a prohibitive effect on the overall formation and stability of thrombi.

4.2. Critical Analysis

4.2.1. Transient Expression of VWF A1 Domain Mutants

In order to identify amino acids involved in the proposed binding interaction between VWF and FXII(a), a set of 12 different VWF1 A1 domain mutants in both the isolated VWF A1 domain as well as full-length VWF were generated. All proteins subsequently were transiently expressed by HEK293T cells. Western blot analysis showed minimal retention of the isolated VWF A1 domain as well as the 12 isolated VWF A1 domain mutants indicating that introduction of the various single-point mutations did not compromise neither the general structure nor the expression of the proteins. In contrast, intracellular retention of the full-length VWF mutants was slightly higher compared to full-length VWF wildtype.

Differences in the secretion profiles between the full-length VWF wildtype and the full-length VWF A1 domain mutants could be due to the introduction of the various mutations and their potential effect on the structure of the respective protein. While the isolated VWF A1 domain is a relatively small protein (30kDa) and is not associated with any further VWF domains, full-length VWF is a large glycoprotein and its final conformation is dependent on the interaction of the different VWF domains within the protein. Therefore, introduction of mutations in any of the VWF domains could potentially alter its primary, secondary and tertiary structure causing a less efficient secretion by for example impaired presentation of the secretory signal peptide due to alternative folding patterns of the VWF proteins.

4.2.2. Protein Purification

After expression, the different VWF proteins were either directly concentrated or purified using ion-based chromatography. Subsequently, the concentration and purity of the proteins in the different sample preparations was assessed by SDS-PAGE analysis.

Presence of a His-taq allowed the effective purification of the isolated VWF A1 domain as well as isolated VWF A1 domain mutants using IMAC which resulted in protein preparations of high purity. While analysis of the purified proteins by SDS-PAGE showed a single and strong protein band for the isolated VWF A1 domain, formation of dimers or aggregates was observed for seven out of the 12 clones generated. Generally, VWF belongs to a class of proteins that is highly dependent on the formation of disulfide bonds between individual VWF molecules and therefore contains a high number of cysteine residues in its sequence. Presence of this amino acid could potentially allow the association of several isolated VWF A domain which each other resulting in the formation of protein aggregates. Alternatively, aggregation of proteins also can be caused by high protein concentrations or altered surface properties leading to an unspecific binding of the proteins to each other. Considering that the overall band intensity of the isolated VWF A1 domain is stronger compared to bands present in the lanes containing the different isolated VWF A1 domain mutants, the aggregation of the proteins therefore also could be due to a change in the surface properties after introduction of the different amino acids in the individual mutants.

Due to the lack of a tag in the full-length VWF sequence, full-length VWFΔA1 and a part of the full-length VWF wildtype were purified using IC which resulted in protein samples of adequate

purity. The remaining full-length VWF wildtype sample as well as all full-length VWF A1 domain mutants were not purified but concentrated which led to protein preparations of very low purity. Concentration was carried out directly after expression of the proteins using the HEK293T expression medium containing the secreted VWF proteins as well as other proteins produced by the cells during expression. Usage of the centrifugation tubes therefore did not lead to a separation of the desired VWF from the remaining proteins present in the medium but an increase in concentration of all molecules apparent by the formation of multiple strong bands on the corresponding SDS-PAGE gels. Presence of such high concentrations of VWF unspecific proteins in the different samples potentially can cause problems in all assays involving the respective full-length VWF samples used for the characterisation of the binding interaction between VWF and FXII(a).

4.2.3. Protein Characterisation

After purification or concentration, the different samples were further characterised in order to analyse the integrity of the different proteins in terms of their concentration, multimer formation and ability to simultaneously bind to collagen and platelets under conditions of flow.

4.2.3.1. Concentration Determination

Concentration of the protein samples containing the isolated VWF A1 domain as well as the isolated VWF A1 domain mutants was determined using a BCA assay which aims at the detection of all proteins present in the test sample. Due to the presence of the His-taq that allowed the purification of all isolated VWF A1 based proteins using affinity chromatography leading to protein preparations of high purity, it was assumed that the results of the BCA assay reflect the actual concentration of VWF proteins present in the samples. As already observed during SDS-PAGE analysis, results of the BCA assays furthermore confirmed good expression levels for ten out of the 12 of the isolated VWF A1 domain mutants as well as the isolated VWF A1 domain itself.

Different to the isolated VWF A1 domain proteins, BCA assays were not a suitable method to determine the protein concentration of the full-length VWF proteins due to the high amount of impurities present in the protein preparations. In order to allow specific detection of the

full-length VWF proteins in the different samples, an in-house VWF ELISA was used. Results of the VWF ELISA showed generally lower protein concentrations for all full-length VWF proteins apart from the full-length VWF wildtype compared to the isolated VWF A1 domain samples. Even though the higher specificity of the ELISA allows a more accurate evaluation of the actual VWF concentrations compared to the BCA assas, the overall low concentrations of the full-length VWF proteins potentially could have a negative impact on the assays planned for the characterisation of the binding interaction between VWF and FXII(a) since higher volumes of the respective samples are necessary in order to use comparable amounts of full-length and isolated VWF. At the same time, an increase in full-length VWF sample volume correlates with higher amounts of impurities present in the protein preparations which again could negatively influence the binding assays planned.

Interestingly and in contrast to results obtained after initial test transfections, determination of the concentration of mutants D1302A and E1339A showed low protein levels for both the isolated VWF A1 as well as the full-length VWF A1 domain samples indicating that introduction of the mutations at those positions negatively affects their expression. One possible solution could be the generation of stably transfected HEK293T cells with the respective mutants since subsequent selection of transfected cells having integrated the desired gene in their genome has been shown to lead to a more reliable and successful expression of unstable proteins in some cases[331].

4.2.3.2. Formation of Multimers

After expression, purification and concentration determinations, all full-length VWF proteins were further characterised in terms of their ability to form a full range of VWF multimers using a combinational approach of electrophoresis and western blotting. Results of the experiments showed formation of multimers for full-length VWF wildtype and all 12 mutants generated. However, comparison between the recombinant proteins and plasma derived VWF wildtype showed an additional high molecular weight band corresponding to ultra-large VWF for the plasma derived glycoprotein. Multimerisation of full-length VWF occurs by association of several VWF dimers and is dependent on the presence of cysteines in the sequence of the proteins as well as the conformation of the individual VWF monomers. In addition, generation of large VWF multimers is supported by the VWF domains D1 and D2 which act as chaperones. In general, it is possible that introduction of the different mutations might have led to a slightly

altered conformation of the proteins which subsequently prevented the formation dimeric bouquets characteristic for VWF multimerisation. Alternatively, changes in the conformation also might have prohibited the VWF chaperon domains from exhibiting their intended function during the multimerisation process stopping the formation of ultra-large VWF multimers. However, since direct comparison of the multimer patterns did not show any significant differences between full-length VWF wildtype and the various full-length VWF A1 domain mutants, absence of ultra large VWF multimers in the different samples most likely is not caused by the mutations introduced into the VWF A1 domain. A potential explanation for these discrepancies could be a difference in expression and protein procession by the cells used to source the individual proteins. While plasma derived VWF predominantly is expressed in human endothelial cells and megakaryocytes, recombinant full-length VWF wildtype as well as the all full-length VWF mutants were expressed using human embryonic kidney cells. Even though both systems employ human cell lines, multimerisation of the VWF proteins might not be as efficiently regulated in the kidney cells which would result in a decrease in overall multimer size. Since the haemostatic capacity of VWF directly correlates with the size of the multimers and larger multimers exhibit more pronounced physiological functions, absence of the largest multimers in the recombinant protein samples might have an impact on subsequent assays used for the analysis of the binding between VWF and FXII(a) as well as the characterisation of the physiological relevance of this interaction. In addition, reduced multimer sizes influences the molar concentrations of the different VWF domain in the protein samples since comparatively ultra-large multimers contain more for example VWF A1 domains than smaller sized VWF multimers. These difference again might cause problems in the planned binding and activation assays and potentially could impede a direct comparison of results generated with the isolated VWF A1 domain and the full-length VWF protein preparations.

4.2.3.3. Collagen Binding and Platelet Capture Profiles

In order to exclude detrimental conformational changes in the full-length VWF proteins after introduction of the different mutations, binding of all full-length proteins to immobilised collagen and platelets was tested in perfusion assays. Apart from mutants E1305A and E1339A, results of the flow experiments demonstrated similar platelet capture profiles for all full-length VWF mutants and the full-length VWF wildtype indicating that introduction of the

different mutations did not compromise neither the platelet nor collagen binding site of the proteins tested. As previously mentioned, concentration of the protein preparations for full-length VWF A1 domain mutant E1305A and E1339A was very low resulting in difficulties to reach the target concentration of 10µg/ml set for the perfusion assays. The necessity of using high volumes of the respective protein samples therefore might have compromised the overall composition of the flow samples resulting in slightly reduced platelet capture profiles of the respective mutants compared to full-length VWF wildtype.

While binding analysis showed efficient attachment of the different recombinant VWF proteins to collagen under flow, the general setup of this experiment is not suitable for the verification of the collagen binding site present in the A1 domain. The collagen used in these assays belongs to the family of collagen type I which is known to associate with the VWF A3 domain. Functionality of the VWF A1 domain binding site therefore would require binding analysis of the recombinant full-length VWF proteins to collagen type IV or VI in order to fully exclude an influence of the A1 domain mutations on the overall structure of the proteins.

4.2.4. Binding Analysis of VWF to FXII(a)

Subsequent to the expression, purification and characterisation of all VWF based proteins, the binding ability of VWF to FXII(a) was tested in different variations of static plate bindings assays as well as SPR.

4.2.4.1. Enzyme-linked Immunosorbent Assays

While ELISA`s are a convenient and reliable method for the characterisation of binding interactions between proteins, the technique also has some disadvantages. One major drawback consists of the immobilisation of one of the binding partners in order to allow the formation of a binding complex during subsequent assay steps. In general, coating of the plastic surfaces does not allow a directed orientation of the respective molecules which carries the risk of protein immobilisation in a way that potentially could shield the binding site of the respective binding partner leading to reduced assays signals and inaccurate assay readouts. In order to verify that immobilisation of the different binding partners does not interfere with the general ability of the proteins to bind to each other due to conformational changes upon coating, an approach in which VWF and FXIIa were allowed to form a complex in solution was

tested. Formed protein complexes subsequently were captured by surface immobilised VWF or FXIIa specific antibodies and the respective secondary binding partner was recognised using a target specific detection antibody. Readouts of the assays exhibited ELISA signals comparable to the conventional strategies (immobilisation of one of the binding partners) suggesting that coating of neither FXIIa nor VWF compromises the ability of the proteins to interact with their respective binding partner.

Another problem related to the coating characteristics in the binding assays used is the unspecific attachment of proteins to the plastic leading to the immobilisation of all proteins present in the individual samples. Therefore, coating of the full-length VWF protein preparations that were highly impure could result in the immobilisation of an overall lower concentration of the actual VWF proteins compared to the isolated VWF A1 domain samples which were purified to a higher standard. This effect also might be the reason for the reduced ELISA signals observed in the 4-way ELISA (Figure 3.14). One variation in this initial binding assays consisted of the direct immobilisation of recombinant VWF which, as already mentioned above, was a very impure protein preparation and therefore most probably resulted in the coating of multiple other proteins alongside VWF on the plastic surface. Based on this assumption, all subsequent binding assays were carried out by coating commercially purified FXII which negated immobilisation of assay irrelevant proteins present in the protein preparations. In this assay setup, use of the impure full-length VWF samples as well as a larger sample volume in order to reach the defined VWF target concentrations should not have a negative effect on the binding characterisation since it is assumed that the interaction between FXIIa and VWF is specific which would allow the removal of all irrelevant proteins present in the samples during the different washing steps incorporated into the ELISA protocol.

After having established a general binding ability of VWF to FXIIa, binding of a concentration range of both plasma derived as well as recombinant full-length VWF wildtype towards plastic immobilised FXIIa, FXII and kallikrein activated FXII was tested in order to determine the binding affinities ($K_{D,app}$) of the various proteins to each other. Results of the assays showed differences between plasma derived and recombinant full-length VWF regarding their affinity (summarised below) to the FXII samples used.

FXIIa	Plasma derived VWF wildtype = 4.5nM	
	Recombinant VWF wildtype = 21nM	~ 5 fold increase
FXII	Plasma derived VWF wildtype = 36.2nM	
	Recombinant VWF wildtype = 9.5nM	~ 4 fold decrease
FXII + K	Plasma derived VWF wildtype = 23.8nM	
	Recombinant VWF wildtype = 39.6nM	~ 2 fold increase

While the differences in affinity between plasma derived and recombinant VWF wildtype to kallikrein activated FXII can be considered to be in an acceptable range, a variation of 4 to 5 fold as observed for the binding of the two VWF proteins to FXIIa and FXII is relatively high and indicates a discrepancy in overall protein structure . As already discussed above, multimer analysis of plasma derived full-length VWF wildtype showed the formation of additional ultra-large high molecular weight multimers compared to recombinant full-length VWF wildtype. Considering that absence of these multimers leads to a lower molar concentration of the VWF A1 domains, a lack of ultra-large VWF multimers could be the reason of a reduced binding ability of the recombinant protein towards FXIIa. Another major difference between plasma derived and recombinant full-length VWF wildtype consists of the glycosylation of the two proteins. As described in chapter 1.3.2., VWF contains multiple glycosylation sites and structural analysis has shown that changes in these glycosylation pattern directly influence the binding ability of the glycoprotein to for example ADAMTS13[332]. Even tough recombinant full-length VWF was generated using an expression system based on human embryonic kidney cells, plasma derived VWF wildtype originally is expressed by endothelial cells and megakaryocytes. While glycosylation patterns in different human cells types are similar, it is possible that expression of the recombinant VWF proteins in kidney cells slightly altered the overall glycosylation of the VWF proteins. Interestingly, none of the reported VWF glycosylation sites reside in the VWF A1 domain itself, multiple O-linked glycosylation sites have been identified in the adjacent linker regions. Alterations in these regions potentially could influence the accessibility of the VWF A1 domain and therefore could lead to differences in the FXII(a) binding capacities of plasma derived and recombinant full-length VWF wildtype.

Initial data generated in our laboratory suggested the presence of a FXIIa binding site in the A1 domain of VWF. This hypothesis was verified by carrying out different static plate binding assays using full-length VWF proteins lacking the A1 domain (VWFΔA1) as well as the isolated VWF A1 domain itself. Results of the ELISA`s testing the binding of full-length VWFΔA1 to immobilised FXIIa confirmed that the protease binding site indeed is located in the A1 domain

of VWF. Further binding assays characterising the interaction of the isolated VWF A1 domain to plastic immobilised FXIIa led to the determination of an $K_{D,app}$ of 139nM which is lower compared to the affinity measured after incubation of full-length VWF on FXIIa (4.5nM for plasma derived VWF wildtype and 36.2nM for recombinant VWF wildtype). This discrepancy in affinity is surprising considering that the same concentrations of plasma derived, recombinant full-length VWF and the isolated VWF A1 domain was used. Despite of full-length VWF consisting of several VWF monomers and therefore containing multiple VWF A1 domains, samples of the isolated VWF A1 domain are solely comprised of the proteins carrying the FXIIa binding site. It therefore was expected that the affinity of the isolated VWF A1 domain to FXIIa would be higher compared to the full-length proteins. One potential explanation of these results could be the binding behavior of VWF since the glycoprotein is known to interact with a multitude of different proteins and generally is considered as "sticky". This characteristic might have caused the full-length VWF proteins to unspecifically bind to the plastic surface at high concentrations. In addition, both plasma derived as well as the recombinant full-length VWF samples contained impurities which might have adhered to the plastic surface during the first incubation step of the ELISA protocol. If full-length VWF subsequently is capable of interacting with these additional proteins, bound VWF would be detected during the following steps of the ELISA protocol leading to increased binding signals. Another explanation for the increased affinity might be an aggregation of the full-length VWF proteins at high concentrations which would result in the presence of more VWF molecules in the respective samples, higher signals in the ELISA binding curves and therefore calculation of an higher affinity of the full-length VWF proteins compared to the isolated VWF A1 domain.

Subsequent to the verification of the presence of a FXII(a) binding site within the VWF A1 domain, a set of 12 different VWF mutants in both the isolated VWF A1 domain as well as the full-length VWF protein were generated. As already discussed earlier, negatively charged amino acids were selected for site-directed mutagenesis since FXII is known to interact with negatively charged surfaces. After expression, purification and characterisation, both sets of mutants were tested in terms of their binding ability to immobilised FXIIa in static plate binding assays with the expectation that mutation of the different residues will have an direct impact on the protein-protein interaction. Interestingly incubation of the isolated VWF A1 domain and the full-length VWF A1 domain mutants on FXIIa showed that 6 out of the 12 isolated VWF A1 domain and 8 out of the 12 full-length VWF A1 domain mutants

demonstrated reduced FXIIa binding abilities while the remaining clones exhibited elevated binding signals compared to their respective parental proteins. Based on the hypothesis that at least some of the negatively charged amino acids are necessary in order to mediate VWF A1 domain binding, a reduction in ELISA signals after site-directed mutagenesis was assumed to indicate a direct involvement of the respective surface residue or a combination of multiple residues in the binding interaction between VWF and FXIIa. Observation of increased ELISA signals after incubation of some of the VWF mutants on FXIIa were unexpected but potentially could be explained once again by a change in the tertiary structure of the proteins after introduction of the different mutations. While analysis of the expression profiles and for some clones formation of multimer patterns as well as platelet capture and collagen binding abilities did not show significant differences between the isolated VWF A1 domain or full-length VWF A1 domain mutants and their respective parental proteins, introduction of the various mutations might have had an effect on a restricted surface area of the proteins after all. Alternate folding after association of the VWF A1 domain with predominantly the VWF A2 and D1 to D3 domains for example could lead to the creation of an extended negatively charged patch on the surface of the respective proteins which subsequently could result in an enhanced binding of the mutants to FXIIa. Aside from differential FXIIa binding signals, direct comparison of the ELISA results using either isolated VWF A1 domain or full-length-VWF A1 domain mutants additionally showed fundamental discrepancies in FXIIa binding behaviour of four clones. While mutants D1302A, E1305A and E1339A exhibited enhanced FXIIa binding signals when tested in the isolated VWF A1 domain form, the same set of mutants demonstrated reduced binding to the protease as full-length VWF proteins. In contrast, reduced FXIIa binding signals of isolated VWF A1 domain mutant E1447A but increase in FXIIa binding for the full-length VWF mutant towards FXIIa were observed. Taking into account that the only difference between these proteins consists of the presence of the remaining VWF domains, it is very likely that the differences in FXIIa binding are due to the overall structure the proteins adopted after introduction of the various mutations. As already discussed earlier, mutation of the different amino acid residues might affect the overall structure of the full-length protein and therefore influence the physiologically observed association of the VWF A1 domain with the A2 as well as D1 to D3 domains. A change in these domain interactions might lead to the formation of areas with an increased negative charge correlating with enhanced FXIIa abilities or alternatively might influence the conformation of the protein in a way that at

least partly shields the FXIIa binding site resulting in an decrease in FXIIa binding. Consequently, both variations in the structure of the full-length VWF mutants would explain the differences in FXIIa binding abilities observed between the isolated VWF A1 and full-length VWF A1 domain mutants.

Since ELISA results showed that mutation of individual negatively charged amino acids present in the VWF A1 domain led to a reduction but not a complete abolishment in FXIIa binding, it was hypothesised that more than one residue is responsible for the mediation of the binding interaction between the protease and the glycoprotein. Based on the reduced FXIIa binding properties measured for a subset of mutants in both the isolated VWF A1 as well as full-length VWF A1 domain and their distribution on the protein surface, a total of five amino acids (D1444A, E1445A, E1447A, E1452A and D1459A) were selected for the generation of double and triple mutants. Subsequent to expression, purification or concentration and further characterisation, double mutants D1444/1445A, D1444/1447A and E1452/1459A were tested in terms of their binding ability towards immobilised FXIIa. Results of the binding assays confirmed the previously observed reduced FXIIa binding properties of single-point mutants D1444A, E1445A, E1452A and D1459A which were used as controls in these experiments. Interestingly, incubation of the double mutants D1444/1445A and D1444/1447A on plastic coated FXIIa resulted in increased ELISA signals for the isolated VWF A1 domain but not the full-length VWF proteins compared to their respective single-point mutants. As already previously discussed, introduction of amino acid changes can have a profound effect on the overall structure of a protein. Considering that the proteins tested contained a combination of two individual mutations, the potential effect on the overall structure of the proteins could be even more pronounced. Especially in case of the full-length VWF mutants, simultaneous substitution of two amino acids therefore might have led to a conformation in which the FXIIa binding site was masked resulting in differential binding results for the isolated VWF and full-length VWF A1 domain mutants. Considering these aspects, only ELISA results that showed reduced FXIIa binding with both the isolated VWF and the full-length VWF mutants were considered as reliable leading to the identification of mutant E1452/1459A which showed virtually no binding towards FXII in neither the isolated VWF A1 domain nor the full-length VWF protein.

After initial identification of VWF mutant E1452/1459, the binding behaviour of the protein towards different forms of FXII was further characterised in order to verify whether amino

acids aspartic acid at position 1452 and glutamic acid at position 1459 indeed comprise the FXII(a) binding site in the VWF A1 domain. Incubation of a concentration range of isolated VWF A1 domain mutant E1452/1459A on FXIIa confirmed a reduction in FXIIa binding for single-point mutants D1459A and E1452A and virtually no binding of mutant E1452/1459A to the immobilised protease. Further experiments additionally showed an inability of full-length VWF A1 domain mutant E1452/1459A to interact with plastic immobilised FXIIa, FXII and FXII that previously had been activated with kallikrein. Interestingly, results of the ELISA analysing the binding behaviour of full-length VWF A1 domain double mutant E1452/1459A towards FXII and FXIIa demonstrated reduced binding signals of the control full-length VWF wildtype to both FXII as well as kallikrein activated FXIIa. Differences in binding signals might be based on the general protein structure of FXII(a) during immobilisation. In general, full-length FXII zymogen is activated upon cleavage by either kallikrein or plasmin which is accompanied by an irreversible conformational change of the protease. Therefore, immobilisation of FXII zymogen and activated FXII might result in the display of the proteins in different orientations which could at least partly shield the surface areas VWF is capable to recognise and bind. This hypothesis is supported by structural analysis of FXII carried out by Clark et al.[CITATION] which postulates that the fibronectin type II domain present in the heavy chain of FXII is masking the activation site of the protease in order to allow directed activation of the protein. Based on these descriptions, it is likely to assume that the VWF binding site is partly overlapping with one of the concealed areas and therefore is not fully accessible after immobilisation of FXII which would be an explanation for a decrease in binding signals after incubation of full-length VWF wildtype on FXII and FXII that has been activated with kallikrein subsequent to its immobilisation.

4.2.4.2. Surface Plasmon Resonance Measurements

In order to confirm the results obtained by carrying out different variations of a static plate binding assay, the binding interaction between full-length VWF wildtype as well as the isolated VWF A1 domain and isolated VWF A1 domain mutant E1452/1459A additionally was characterised using SPR measurements. In general, SPR analysis carried out was restricted by the limited availability of full-length VWF protein samples and therefore only allowed the confirmation of a binding interaction between full-length VWF wildtype and FXIIa. In addition, perfusion of the isolated VWF A1 domain as well as isolated VWF A1 domain mutant

E1452/1359A over immobilised FXIIa showed a significantly reduced binding ability of the mutant to the protease which verified amino acids aspartic acid at position 1452 and glutamic acid at position 1459 as the FXIIa binding site within the A1 domain of VWF.

4.2.5. Activation of FXII

As already described in chapter 1.4.2., a multitude of different substances which trigger the conversion of inactive FXII to FXIIa have been described and were analysed in FXII activation assays. In these experiments, activation of the FXII zymogen resulted in the enzymatic cleavage of a substrate (S-2302) allowing a colorimetric readout and evaluation of the FXIIa activity as well as the activation potential of the substances tested. One limitation encountered while using this assay consisted of the cross-reactivity of the substrate chosen. While S-2302 is well suited in order to test the activity of FXIIa, it also can be enzymatically processed by kallikrein, FXIa, trypsin, thrombin, plasmin and tPa. Considering that kallikrein was used as a control in this experiment, readout signals of the control reactions could be slightly elevated due to the fact that the substrate was processed by both activated FXII as well as kallikrein.

Before testing different substances in terms of their FXII activation potential, a general assay setup was carried out in order to determine FXII concentrations for future activation assays. Screening of different concentrations of FXII showed optimal substrate conversion curves for 100nM which subsequently was used as the FXII(a) target concentration for all activation assays. Further experiments were carried out in order to verify that cleavage of the substrate used was specific to activated FXII but not FXII zymogen. These set of experiments furthermore showed that addition of kallikrein to FXII results in the efficient conversion of the substrate by activated FXII which validates the general assay setup for both the FXII activation assays as well as all binding assays using kallikrein activated FXII.

In order to evaluate the activation potential of a list of potential activators, FXII zymogen was incubated with three different concentrations of the substances to be tested. The concentrations used reflected the physiologically reported concentration of the individual activators as well as a 10 fold de- or increase in concentration in order to evaluate the overall strength and sensitivity of FXII activation. Results of the activation assays showed minimal autocactivation of FXII after 45 minutes leading to the categorisation of the process as a non-

activator. However, autoactivation plays a significant role in the physiological activation of the protease and has been described in the literature extensively. Since autoactivation of FXII almost exclusively is associated with the binding of the protease to a negatively charged surface, it might be possible that the plastic used in this experiment does not present a suitable surface for the activation of FXII in an autoactive manner. In addition, autoactivation has been characterised as a relative slow process with activation times between 90 and 120 minutes. Since autoactivation of FXII was already assessed after 45 minutes, the overall assay length might not have been sufficient to truly evaluate autoactivation of the protease.

After having established a general binding interaction between FXII(a) and VWF, activation of the zymogen after incubation with different VWF proteins was tested in order to identify VWF as a potential activator of FXII. In a first step, the activation potential of plasma derived full-length VWF wildtype and the isolated VWF A1 domain was determined. Results of the activation assays showed that incubation of both proteins resulted in the conversion of inactive FXII to FXIIa. Considering that equal concentrations of both the plasma derived full-length VWF wildtype and the isolated VWF A1 domain were used in these assays, the activation potential of the isolated VWF A1 domain samples is estimated to be higher compared to full-length VWF wildtype preparations due to the fact that the relative number of VWF A1 domains comprising the FXII(a) binding site that facilitates interaction of the two proteins is lower in the full-length VWF wildtype compared to the isolated VWF A1 domain samples.

Direct comparison of the activation potential of purified plasma derived full-length VWF wildtype, plasma derived full-length VWF wildtype, recombinant full-length VWF wildtype and the isolated VWF A1 domain showed a slightly increased activation potential of plasma derived full-length VWF wildtype compared to purified plasma derived full-length VWF wildtype. This observation potentially is based on the fact that plasma derived full-length VWF wildtype samples still can contain traces of other molecules present in the blood plasma such as for example kallikrein which is a known FXII activator. In contrast, purified plasma derived full-length VWF wildtype completed a second chromatographic purification process efficiently removing any remaining plasma contaminants. Elevated FXII activation after incubation of the zymogen with plasma derived full-length VWF wildtype therefore could be due to residual activity of kallikrein impurities present in the protein preparations used. Incubation of FXII with recombinant full-length VWF wildtype led to a further decrease in FXII activation

indicating a lower activation potential of the recombinant protein compared to the plasma derived VWF samples. As already previously discussed, protein preparations containing the full-length VWF wildtype were highly impure which could be a reason for a diminished FXII activation. In addition, recombinant full-length VWF showed absence of ultra-large VWF multimers which directly correlates with a decrease in the relative concentration of the VWF A1 domain in the respective samples. Since the FXII(a) binding site which mediates the interaction between the glycoprotein and the protease is present in the VWF A1 domain, lower concentrations of the domain therefore would result in a decrease in FXII activation after incubation of FXII zymogen with recombinant full-length VWF wildtype. Based on the same rationale, activation assay results carried out with the isolated VWF A1 domain demonstrated a higher activation potential compared to plasma derived full-length VWF which likely again is due to the elevated relative concentration of the VWF A1 domain present in the isolated VWF A1 domain samples. Activation assays using both isolated VWF and full-length VWF A1 domain mutants E1452A, D1459A as well as double mutant E1452/1459A demonstrated that incubation of these proteins with FXII resulted in a very limited conversion of the zymogen to FXIIa. While residual activation of FXII might be mediated by amino acids adjacent to the positions mutated, it is more likely that FXIIa activity observed in these assays is based on FXII autoactivation since these assays were carried out for 300 minutes which would be enough time for the autoactivation of the protease which has been reported to occur after 90 to 120 minutes. Furthermore, results of the FXII activation assays with the different VWF mutants are in line with the observations made during the characterisation of the binding interaction between VWF and FXII(a) in which aspartic acid at position 1452 and glutamic acid at position 1459 were identified as the FXIIa binding site within the VWF A1 domain. Taking into consideration that direct interaction between VWF and FXII is necessary in order to trigger conversion of the zymogen into the active protease, incubation of FXII with the different VWF mutants would not result in the activation of FXII due to their inability to bind to the protease. These conclusions were further substantiated by FXII activation assays in which the interaction between different VWF proteins and FXII were blocked using an anti-VWF antibody. Substrate conversion mediated by activated FXII was virtually absent whenever the binding interaction between the protease and the glycoprotein was prohibited. Simultaneously, presence of the anti-VWF antibody in the samples that contained either full-length VWF or isolated VWF A1 domain mutants E1452/1459A did not result in a decrease in

FXII activation confirming that the VWF proteins are not able to bind to and therefore convert FXII zymogen to FXIIa.

4.2.6. Functional Analysis of the VWF-FXII Interaction

Subsequent to the characterisation of the binding interaction between VWF and FXII(a) as well as identification of VWF as a new physiological activator of FXII zymogen, the functional effects of this interaction were analysed using adapted thrombolysis and modified perfusion assays.

4.2.6.1. Thrombolysis Assay

Thrombolysis assays originally were described for the high throughput screening of whole blood lysis characteristics in order to identify new thrombolytic agents. Based on the general protocol, the assay subsequently was adapted for the analysis of the role of FXIIa, VWF and the interaction between the proteins on clot stability and lysis times. Results of the assays demonstrated a decrease in whole blood clot stability whenever the activity of VWF was inhibited by an anti-VWF antibody. This effect was further enhanced whenever FXII(a) instead of VWF activity was blocked using an anti-FXII antibody. In general, formation of blood clots heavily depends on the aggregation and activation of platelets which predominantly is mediated by VWF exposed to the flowing blood. Consequently, a decrease in clot stability after inhibition of VWF activity most likely based in the inability of the glycoprotein to bind to and activate platelets present in the samples. Based on the current literature, the relevance of FXII(a) for the formation of blood clots has been questioned. However, it is well known that FXIIa is capable of initiating the intrinsic pathway of coagulation which eventually leads to the formation of fibrin necessary to cross-link loosely packaged platelet rich blood clots therefore increasing the overall stability of the thrombus. Inhibition of FXII(a) activity in this assay therefore most probably had a direct influence on the generation of fibrin fibres leading to the formation of less stable blood clots. Interestingly, simultaneous inhibition of VWF and FXII(a) activity further pronounced clot instability indicating that the formation of stable thrombi is dependent on both VWF and FXIIa activity due to the following reasons: while inhibition of the activity of VWF might have an effect on the overall size and integrity of the blood clots generated during this assay, the blood samples used still contained normal plasma concentrations of FXIIa which is able to initiate the intrinsic pathway of coagulation eventually leading to the generation of fibrin and therefore stabilisation of the clot. Inhibition of VWF

therefore will only slightly impact the overall stability of the thrombi formed. Based on this principle, blockage of the FXIIa activity is bound to have a greater effect on the stability of the respective thrombi due to a lack of fibrin fibres genertion which will lead to the formation of loosely packed thrombi. Combined inhibition of VWF and FXIIa however prevents both FXIIa driven fibrin formation as well as VWD mediated platelet aggregation resulting in the formation of highly unstable blood clots.

In order to elucidate the role of the binding interaction between VWF and FXII(a) in clot stability, thrombolysis assays using VWF depleted plasma reconstituted with full-length VWF A1 domain mutants E1452A, D1459A and E1452/1459A were carried out. In theory, VWF mediated platelet aggregation and activation should not be compromised in these samples since the mutants used showed platelet capture capacities comparable to recombinant full-length VWF wildtype. Simultaneously, VWF depleted plasma still contains both FXII as well as FXIIa which should allow the generation of fibrin fibres that subsequently serve to increase the overall stability of the blood clots. Since neither the activity of VWF nor FXIIa should be compromised in the samples used, stability of the thrombi generated is solely dependent on the interaction between the glycoprotein and the protease. Interestingly, clot lysis times measured demonstrated a direct correlation between the ability of the VWF proteins to bind towards FXII(a) and the stability of the clots formed. Furthermore, samples reconstituted with full-length VWF A1 domain double mutant E1452/1459A which is not able to interact with FXII(a) formed highly unstable clot with significantly reduced 50% lysis times compared to samples containing mutants E1452A, D1459A or the recombinant full-length VWF wildtype. Results therefore strongly indicate that the formation of stable blood clots is not only dependent on the activity of VWF and FXIIa alone but requires direct interaction between the glycoprotein and the protease. One potential explanation for this effect might be the absence of any FXII activators and therefore interrupted formation of fibrin since the VWF depleted plasma in theory should not contain kallikrein, plasmin or polyphosphates. Furthermore, all full-length VWF A1 domain mutants used showed either reduced or absent binding capabilities towards FXII(a) and therefore were not capable of triggering conversion of FXII zymogen into the active protease which would suggest that VWF actually plays a crucial role in the physiological activation of FXII and subsequent generation of fibrin fibres.

4.2.6.2. Clot Formation under Flow

Apart from thrombolysis assays, the functional effect of the binding interaction between VWF and FXII(a) additionally was analysed in perfusion assays with flow conditions mimicking high shear rates in order to simulate perfusion encountered in pathological settings. For the purpose of characterising the formation of pathological thrombi in the context of the newly identified binding ability of VWF towards FXII(a), both VWF or FXII depleted plasma reconstituted with isolated and purified red blood cells, platelets and recombinant full-length VWF wildtype, full-length VWF A1 domain mutants E1452A, D1459A, E1452/1459A or FXIIa respectively was used. Results of the flow assays showed enhanced platelet capture in samples containing both recombinant full-length VWF wildtype as well as FXIIa compared to samples lacking FXIIa confirming that the presence of the protease supports VWF mediated platelet capture. One possible explanation for this effect might be an increase in VWF availability. After initial binding of VWF to the collagen immobilised on the flow slides, the glycoprotein is capable of binding and therefore arresting platelets from flow. This binding subsequently induces the activation of the platelets leading to the secretion of polyphosphates which then can act as an activator for FXII. Colocalisation studies have shown that FXII tends to stay associated to the platelet surface subsequent to its activation. Taking into consideration that FXIIa is able to bind to VWF, it is possible that the presence of FXIIa on the surface of the platelets leads to a kind of coating of the platelets with VWF which in turn is able to capture new platelets resulting in increased platelet capture profiles compared to samples lacking FXII(a). As already expected based on the results of the thrombolysis assays, samples containing the different full-length VWF A1 domain mutants showed significantly reduced platelet capture profiles which correlated directly with the ability of the individual proteins to bind to FXII(a). Following this observations, perfusion of samples containing full-length VWF A1 double mutant E1452/1459A led to platelet capture profiles comparable to samples which did not contain any VWF again suggesting that the binding interaction between VWF and FXIIa plays a crucial part for the formation of thrombi under pathological shear.

4.3. Interaction of FXIIa to VWF

Results generated during this project demonstrated and characterised a previously unknown interaction between blood coagulation factor XII and the plasma glycoprotein VWF. More

specifically, my data show that VWF is capable of binding to both the activated as well as the inactive form of FXII and that this protein-protein interaction is driven by amino acid residues present in the A1 domain of VWF. Based on the previously established knowledge that FXII predominantly binds to negatively charged surfaces, I hypothesised that the interaction between VWF and FXII was likely to be driven or at least supported by this phenomenon. Therefore, I carried out detailed binding analysis with VWF mutants in which all 12 negatively charged surface residues present in the A1 domain had been mutated from either glutamic or aspartic acid to neutrally charged alanine, which ultimately allowed the identification of aspartic acid at position 1452 and glutamic acid at position 1459 as the residues involved in the binding between VWF and FXII. After having established the ability of the two proteins to interact, I proceeded to demonstrate that VWF is not only capable of binding to FXII but also that the interaction of the two proteins with each other triggers conversion of FXII zymogen into the enzymatically active protease FXIIa and therefore identified VWF as a new physiological activator of blood coagulation factor FXII.

This newly identified binding interaction, as well as the potential of VWF to activate FXII, has a range of potential implications on different physiological but also medical processes in which either VWF, FXII or both proteins are involved.

As discussed in the introduction, FXII is the initiator of the intrinsic pathway of coagulation and therefore can participate in the formation of thrombin and ultimately fibrin which are essential for sealing vascular breaches and the prevention of excessive blood loss[312]. Interestingly, FXII deficiency has not been associated with any form of bleeding complications which led to the widespread belief that the role of FXII in coagulation is negligible, simultaneously raising the question of the actual physiological function of this protein for haemostasis[229]. Research conducted over the past 50 years has led to a better understanding of FXII and its involvement in coagulation and showed that FXII indeed does not contribute to the initial formation of thrombin and fibrin which predominantly is driven by TF and FVIIa. Extensive studies of the formation of blood clots however have demonstrated that the TF/FVIIa complex eventually is inhibited by tissue factor pathway inhibitor released by activated platelets and endothelial cells which effectively limits continuous production of fibrin and stabilisation as well as growth of the forming blood clot[27,52]. Interestingly, inhibition of the complex does not lead to a complete arrest of blood clot growth indicating that an alternative pathway for the generation of thrombin and fibrin has to compensate for the loss

of TF/FVIIa activity[1,35]. Analysis of the mechanisms leading to the formation of a stable blood clot as well as the different components necessary for these events showed that thrombi predominantly are composed of aggregated platelets that have been linked by a mesh of fibrin fibres serving to increase the overall stability of the clot structure[23]. Simultaneously, initial binding of platelets at sites of a vascular breach as well as their subsequent aggregation triggers their activation which is accompanied by degranulation, releasing a range of molecules such as coagulation factors FV and FXIII, ADP, adhesion molecules (P-selectin) and the fibrin precursor fibrinogen which are all essential for the generation of a stable blood clot[2,35]. Another type of molecule secreted by activated platelets is structural units of linked phosphates molecules which are commonly referred to as polyphosphates[217,233]. Polyphosphates have been shown to function, alongside kallikrein and now VWF, as a physiological activator of FXII which has been confirmed by polyphosphate based FXII activation assays carried out in this thesis. This discovery provides a plausible explanation for the continuous generation of thrombin and fibrin after inhibition of the TF/FVIIa complex by TFPI since activation of FXII and therefore the contact pathway of coagulation by platelet secreted polyphosphates presents an alternative route for the formation of fibrin fibres.

The possibility of FXII being activated by polyphosphates has the potential to redefine the role of FXII in the formation of blood clots as it would indicate that FXII is involved in the continued growth and stabilisation of thrombi preventing premature dissolution of the platelet-fibrin fibre network which might happen without the contribution of the contact pathway. Alternatively, it is also possible that FXII, even though dispensable for the initial formation of the blood clot, plays a part in the subtle reinforcement of the overall thrombus structure and stability during clot formation due to the activity of the classic components of haemostasis.

Taking these aspects into account, the binding and activating interaction between VWF and FXII provides a new insight into the formation of a thrombus by expanding the accepted theory of FXII being activated solely by kallikrein and polyphosphates and additionally proposes co-localisation of VWF and FXII within the blood clot structure (Figure 4.1).

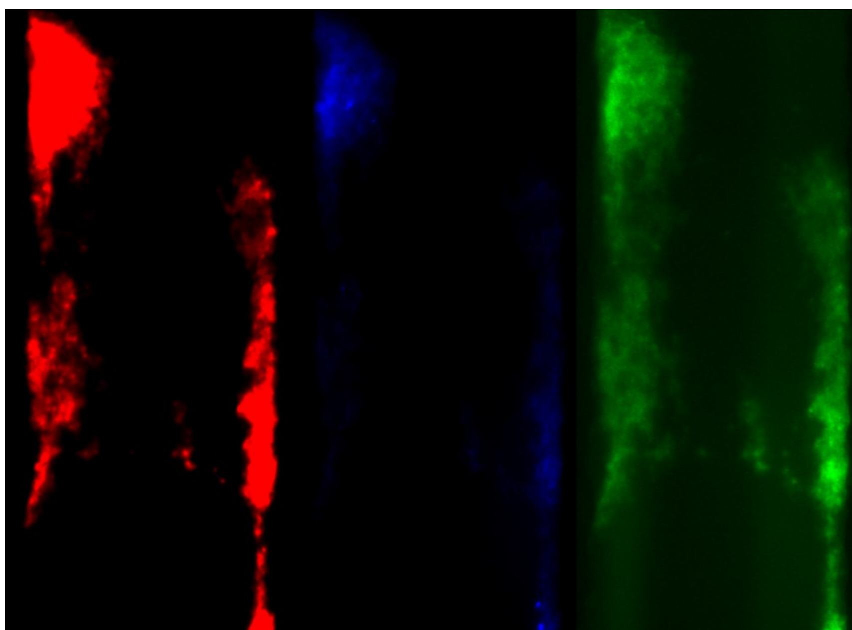

Figure 4.1: Co-localisation of platelets, VWF and FXIIa on collagen type I under flow. Perfusion assay using whole blood with red fluorescent protein labelled platelets (red), pacific blue stained VWF (blue) and green fluorescent protein labelled FXIIa (green) were carried out in order to analyse simultaneous binding of platelets and FXIIa to collagen type I bound VWF. Fluorescent signals show significant attachment of both platelets and FXIIa to VWF rich areas. Interestingly, FXIIa signals extend beyond localised VWF areas and overlap with platelet binding signals confirming direct binding of FXIIa to thrombocytes. Results of the flow assay furthermore indicate localised formation of complexes consisting of VWF, platelets and FXIIa which I assume is leading to the activation of FXII and therefore baseline production of thrombin as well as fibrin fibres supporting thrombus growth and stabilisation (preliminary data).

VWF is a well characterised plasma glycoprotein which plays an essential role in the initial capture of platelets at sites of vessel injury[144]. In cases of a vascular breach, VWF is capable of binding to exposed subendothelial collagen via its A3 domain and subsequently, due to the interaction with collagen as well as shear forces exhibited by the flowing blood, undergoes a conformational change exposing its platelet binding site present in VWF A1 domain[137]. Binding of the platelets to VWF initiates their activation which as described above results in their degranulation and therefore release of polyphosphates which in turn are capable of activating FXII[4,27]. In this thesis, I have shown that the platelet binding site present in the VWF A1 domain is positioned opposite the FXII(a) binding site and that VWF mediated platelet capture is not impaired in the presence of FXII indicating that simultaneous binding of both platelets as well as FXII(a) to the VWF A1 domain is possible.

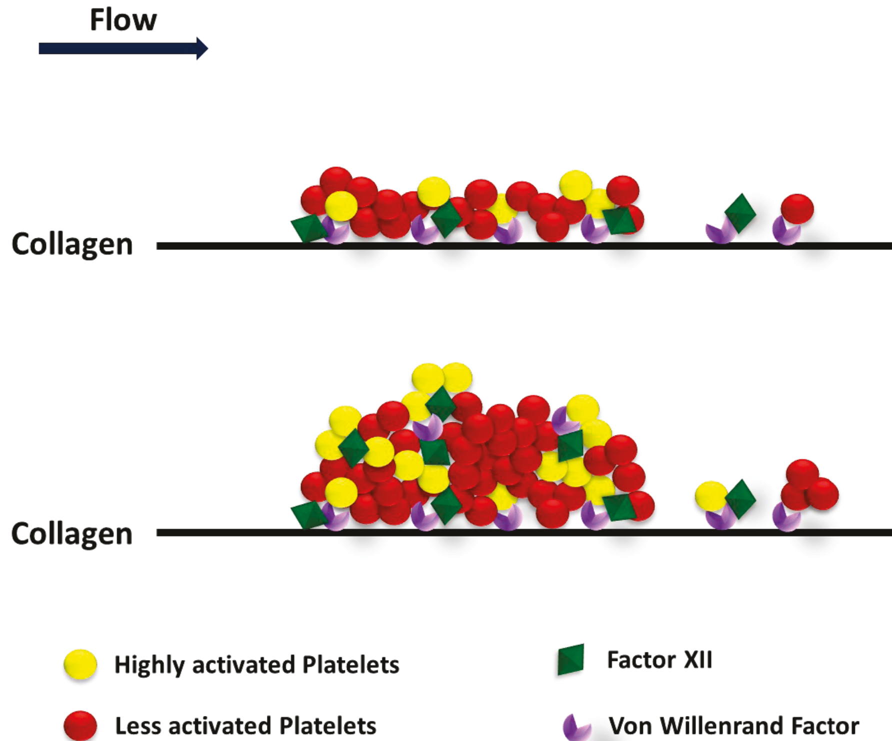

Figure 4.2: Hypothetical model of the role of FXII in the formation of blood clots. Even though FXII is classified as a coagulation factor, its role in haemostasis appears to be negligible since the majority of clotting reactions seem to be driven by the more prominent TF/FVIIa complex pathway. Analysis of the formation of thrombi however have demonstrated that clot formation seems to slowly progress even after the eventual inhibition of the TF/VIIa complex by TFPI. This continued formation of thrombin as well as fibrin alongside ongoing capture of platelets potentially can be attributed to the activity of FXII as well as its interaction with VWF. Upon vascular injury, sub-endothelial collagen is exposed presenting a surface for the attachment of VWF which subsequently captures platelets from the circulation. After binding to VWF, platelets become activated, attract further platelets and finally aggregate resulting in the formation of platelet rich clots. Furthermore, activated platelets degranulate and release polyphosphates which have been shown to act as an activator for FXII. Co-localisation studies furthermore have demonstrated that FXII subsequently can stay associated to the membrane of the activated platelets. At the same time, FXII also is capable of directly binding to VWF without compromising the platelet capture function of the glycoprotein and additionally has been shown to be activated by VWF itself. Consequently, complexes of VWF/FXII(a) and activated platelets will form throughout the growing thrombus adding to its overall size as well as stability due to prolonged formation of thrombin and fibrin fibres.

In the case of blood clot formation this phenomenon suggests the following sequence of events: subendothelial collagen is exposed upon vascular injury allowing VWF to bind at the site of the injury accompanied by a conformational change of the glycoprotein. Exposure of VWF’s multiple binding sites subsequently provides a focal point for the localisation of FXII(a) as well as platelets which are both capable of binding to the VWF A1 domain. Interaction of VWF with FXII results in the conversion of the zymogen into active FXIIa which subsequently

is able to initiate the contact pathway, culminating in the generation of thrombin and ultimately fibrin fibres. In addition, binding of the platelets to VWF results in their activation, degranulation and release of polyphosphates which in turn can act on the VWF bound FXII further adding to its activation. Co-localisation studies carried out by Mitchell et al have demonstrated that polyphosphate-activated FXIIa is capable of associating with the membrane of activated platelets which theoretically could create another focal point for the attachment of VWF and subsequent recruitment of platelets[333]. It is very likely that generation of thrombin as well as the formation of fibrin via the contact pathway should be viewed as an underlying ongoing process within the framework of normal haemostasis comparable to the parallel events directed by the TF/FVIIa complex: the results of my study together with the current understanding of FXII activation by polyphosphates therefore imply that FXII(a) not only exhibits its coagulative function after TFPI inhibition of the TF/FVIIa complex but throughout thrombus growth (Figure 4.2).

The newly established interaction between VWF and FXII(a) also has implications for currently used medical procedures. The majority of cardiovascular diseases are treated by mechanical intervention using medical equipment such as stents, heart valves as well as pacemakers and extravascular circuits which all come into direct contact with flowing blood[93]. Unlike the endothelium, which exhibits antithrombotic functions, artificial surfaces have been shown to initiate coagulative events by protein adsorption, platelet adhesion and activation of the contact pathway[93,334]. Based on several in vitro and in vivo studies predominantly carried out in rabbit and baboon models, it is assumed that medically induced coagulation is triggered by adsorption of FXII to the foreign artificial surface which ultimately results in the autoactivation of the protease[93,334]. Activated FXII subsequently is capable of cleaving and therefore activating pre-kallikrein which in turn mediates additional conversion of FXII to FXIIa initiating the contact pathway of coagulation eventually leading to the generation of thrombin and fibrin[269]. While the predominant function of thrombin lies in the generation of fibrin fibres by cleavage of fibrinogen, the protease also acts as a potent platelet agonist initiating the aggregation and activation of local platelets which have been observed to get entangled in the formed fibrin mesh resulting in the formation of small thrombi on the surface of the medical device[93,286]. These events have been shown to carry a high risk of device failure resulting in life threatening complications for the patient[93]. In addition, thrombi have been shown to eventually detach from the artificial surface leading to further complications in the form of

strokes and embolism as well as occluded vessels at other sites within the circulatory system[93]. Even though this model largely conforms to the clotting reactions observed after introduction of an artificial surface into a patient, the size of the formed thrombi as well as the short time periods between adsorption of FXII and formation of the platelet rich blood clot have been regarded as unusual because it was assumed that the relatively small amounts of thrombin generated by the contact pathway would be quickly inhibited or too marginal to facilitate proper thrombus formation[93,335]. The ability of FXII to bind to VWF however could provide a possible explanation for this phenomenon. Once FXII has absorbed to the artificial surface, small amounts of the zymogen will undergo autoactivation leading to the events described above. At the same time VWF binds to the adsorbed protease and due to the shear forces exhibited by the flowing blood, undergoes a conformational change resulting in the exposure of its platelet binding sites. Captured platelets subsequently become activated, aggregate and eventually degranulate releasing polyphosphates which in turn mediate further activation of FXII. As already mentioned, polyphosphate activated FXIIa has been shown to stay associated to the membrane of activated platelets functioning as a platform for further VWF and subsequently platelet recruitment while simultaneously generating small amounts of thrombin via the contact pathway[333]. In this scenario, the VWF-FXII interaction therefore would support thrombus growth using two different pathways potentially increasing the overall size while decreasing the generation time of the platelet rich thrombus.

Apart from coagulation, VWF- and FXII-driven accelerated formation or increased size of platelet rich thrombi could also be of importance in other diseases in which FXII has been shown to exhibit an increased proteolytic activity. As already discussed in chapter 1.4.3., FXII plays a significant role in inflammatory processes and has been shown to play a role in the development of sepsis[262], rheumatoid arthritis[280] as well as anaphylaxis[289]. The contribution of the protease in these disease settings heavily relies on its function within the kallikrein-kinin pathway which ultimately leads to the generation of a potent inflammatory initiator referred to as bradykinin[286]. Interestingly and despite the fact that the effect of FXII on dysregulated coagulation events has been deemed negligible, the majority of diseases in which FXIIa activity is a crucial factor for the development of the condition have been associated with an increased thrombogenic risk[324,336,337].

In sepsis for example, inflammatory processes are thought to be mediated by predominantly pro-inflammatory cells which secrete both chemo- as well as cytokines in response to

pathogens such as bacteria, viruses or fungi entering the blood stream[338–340]. Simultaneously, sepsis is characterised by dysregulated activation of the coagulation system which is assumed to be caused by the expression of tissue factor on activated monocytes and endothelial cells. Subsequent generation of thrombin and eventually fibrin via the TF/FVIIa complex leads to the formation of a closely interwoven fibrin meshwork in which pathogens are captured[339,340]. Even though formation of fibrin has been shown to be dependent on the expression of tissue factor on inflamed cells, it has been suggested that there might be alternative mechanisms supporting the generation of fibrin in this context[338,339]. Interestingly, inflammatory stimulation in sepsis is heavily influenced by bradykinin which, as already mentioned, is generated via the FXIIa induced kallikrein-kinin pathway[272]. At the same time, activation studies have shown, that FXII is capable of being activated by polysaccharides present in the cell membrane of bacteria which would support the assumption that FXIIa is likely to be present in or on the surface of the bacteria which have been trapped by the formed fibrin fibres[341,342]. Closer analysis of the pathogen-fibrin structures also showed the presence of platelets in this network which was assumed to be due to the platelets becoming entangled in the fibrin meshwork due to their size[262,275]. The results of my study however provide an alternative explanation for this observation. The presence of FXII in the pathogen-fibrin meshwork might function as a focal point for localised VWF mediated platelet capture and therefore thrombus growth. The ability of VWF to bind to FXII together with FXII activation by both VWF itself as well as platelet secreted polyphosphates would contribute to the creation of a highly pro-coagulant environment resulting in the formation of pathological thrombi which subsequently can contribute to organ dysfunction and eventually failure characteristic of sepsis.

Aside from sepsis, enhanced FXIIa activity also has been observed in rheumatoid arthritis and cohort studies have shown that patients suffering from RA have a 3.36 times elevated risk to develop deep vein thrombosis and a 3.07 times increased risk of experiencing a pulmonary embolism compared to healthy participants[280,343,344]. The link between rheumatoid arthritis and the coagulation system is poorly understood but is thought to be based on an interplay of endothelial dysfunction and an increase in tissue factor activity of the synovial membranes present in the affected joints[282,342]. Interestingly, rheumatoid arthritis also has been associated with elevated VWF levels at the site of the inflamed joints and simultaneously both the severity of joint inflammation and damage directly correlates with levels of FXII mediated

bradykinin production[343,344]. It therefore is possible that dysregulation of the endothelium at the site of the inflammation leads to VWF binding to the inflamed joint areas resulting in the capture of both platelets as well as FXII. Subsequent activation of FXII by VWF alone or platelet-released polyphosphates could initiate the localised activation of FXII resulting in the generation of bradykinin, further driving the inflammatory reaction and ultimately damage of the joints. At the same time, interaction between VWF and FXII can also lead to the formation of thrombin and fibrin which together with platelets arrested by VWF would be capable of forming platelet rich thrombi. Consequently, an increasing size of the blood clot correlates with an augmented risk of a thrombus breaking loose and embolising resulting in arterial occlusion or pulmonary embolism.

Anaphylaxis also has been associated with abnormal FXII activation and subsequent bradykinin production[345,346]. The underlying cause of anaphylaxis is based on the local recruitment of immunological cells in response to a form of foreign antigen[262,275]. Activation of these immunological cells leads to their degranulation and release of heparin which has been shown to trigger conversion of FXII to FXIIa[276,277]. The activated protease subsequently is capable of mediating large-scale generation of bradykinin via the kallikrein-kininogen pathway[262]. Severity of the anaphylactic response has been directly linked with the amount of heparin released from predominantly mast cells and the increase in FXII activation as well as the generation of bradykinin[288]. Simultaneously, anaphylaxis often seems to be accompanied by changes in coagulation and platelet response as well as an elevated thrombotic risk[346]. Based on the results of our study, the interaction of VWF and FXIIa may have an effect on the formation of pathological thrombi during anaphylaxis. As already mentioned, the primary anaphylactic inflammatory mediator is bradykinin which is generated by FXIIa via the kallikrein-kinin pathway[272]. It therefore can be assumed that both FXII zymogen as well as FXIIa are present at the site of the inflammation and activation of FXII is driven by heparin released by recruited mast cells. Even though it has not been demonstrated yet, FXII might be able to undergo a heparin driven cell surface associated activation similar to the activation process observed during degranulation of platelets which would result in FXII being bound to the surface of the mast cells. Bound FXII would subsequently be able to bind to VWF which in turn is responsible for the capture of platelets from the circulation. Together with the coagulant function of FXII, this VWF-platelet construct then would be capable of initiating the formation of platelet rich thrombi causing vascular occlusion, embolism and infarction.

Another set of conditions in which the interaction of VWF and FXII might play a role are general vascular occlusions and arteriosclerosis. Zhu *et al.* have demonstrated that VWF accumulates and undergoes its characteristic conformational change in areas of turbulent flow which primarily can be found at vessel junctions and vessels with reduced diameter as for example observed at sites of arteriosclerosis[347]. The use of fluorescently labelled VWF in these experiments demonstrated enhanced capture of platelets at these sites. Interestingly, further analysis of the areas exhibiting turbulent flow additionally indicated the formation of small blood clots even under conditions of low TF levels or complete absence of the cell surface receptor[348–350]. A likely explanation for the generation of these thrombi again is the interaction between VWF, FXII and VWF captured platelets. Based on the data obtained, arrest of VWF from the circulation would not only result in the localised capture of platelets but also binding of FXII to the VWF fibres, subsequently leading to the activation of the protease by either VWF itself or the polyphosphates secreted by the VWF bound platelets. Initiation of the contact pathway consequently would result in the generation of small amounts of thrombin and fibrin and therefore formation of small thrombi. Interestingly, formation of arteriosclerotic plaques is accompanied by inflammatory reactions that at least partly are driven by FXII driven bradykinin production[232,351,352]. This observation confirms the assumption that FXIIa is present at the site of the plaque formation, further supporting my hypothesis about the role of the VWF/FXII interaction in vascular occlusions as well as arteriosclerosis.

Even though the newly characterised protein-protein interaction has the potential to explain several features of different diseases involving either VWF, FXII or both proteins simultaneously, the actual impact of the binding and activation interaction between the glycoprotein and the protease described in this thesis remains hypothetical. In contrast to these theoretical models, the role of both VWF as well as FXII in thrombosis is well studied.

High levels of VWF predominantly have been associated with the occurrence of arterial thrombosis and clinical studies have shown a direct link between increased VWF released by stimulated endothelium and ischemic heart diseases as well as myocardial infarction frequency[353,354]. Especially the increased shear rates associated with thrombotic conditions are thought to majorly influence VWF's haemostatic function enforcing the characteristic unravelling of the glycoprotein accompanied by first transient and later stable aggregation as well as activation of captured platelets resulting in the formation of platelet rich plugs[355–357]. In addition, failure in the regulation of VWF activity as for example observed in TTP directly

results in the augmented generation of microthrombi supporting the claim that the glycoprotein is capable of at least supporting thrombotic events[358]. However due to its essential role in haemostasis, inhibition of VWF for the sake of further elucidating the involvement of VWF in thrombosis or achieving a thromboprotective effect is not feasible without simultaneously risking life-threatening bleeding complications[359,121].

As already previously discussed, FXII deficiency is not associated with either spontaneous or injury-related increased bleeding tendencies but is characterised by a normal haemostatic capacity[2,269]. These observations were confirmed by the generation of FXII deficient mouse models in 2005 which allowed a detailed analysis of FXII deficiency in the wider context of thrombosis[214]. Several studies involving FXII deficient mice elegantly demonstrated defective thrombus formation in both arterial as well as venous settings that could be entirely reversed after reconstitution with wildtype FXII clearly indicating an active role of FXII in pathological thrombus formation[114,316,319,320]. In addition to FXII deficient mice, treatment of wildtype mice, rats, rabbits as well as baboons with FXII inhibitors resulted in a thromboprotective effect and effectively prevented or at least diminished the occurrence and severity of thromboembolic diseases such as stroke, atherothrombosis and pulmonary embolism[322,326,327,360].

Considering that both proteins involved in this project have been associated with thrombotic events independently, it is likely that the binding as well as activation interaction between VWF and FXII has a significant influence on the development of pathological thrombi. While the initial haemostatic response heavily relies on the rapid sealing of a vascular breach and therefore generation of large amounts of thrombin and subsequently fibrin fibres serving to stabilise the initial platelet plug, formation of a pathological blood clot is characterised by a predominantly slow accumulation and fibrin based cross-linkage of aggregated platelets[66,67,361]. The relatively slow formation of pathological thrombi corresponds to the previously formulated hypothesis that binding and subsequent activation of FXII by VWF results in a continuous but low-level generation of thrombin accompanied by conversion of fibrinogen to fibrin fibres. Consequently, it is possible that the formation of pathological thrombi can be solely ascribed to a constant interaction between VWF and FXII resulting in increased FXII dependent VWF mediated platelet capture, the activation of the protease which will be further enhanced by polyphosphates secreted by the captured and activated platelets and the subsequent initiation of the intrinsic pathway of coagulation by FXIIa.

As already previously discussed, binding and subsequent activation of FXII by VWF offers an explanation for thrombotic events observed in a multitude of different diseases such as rheumatoid arthritis, sepsis and thrombosis. At the same time, activity of FXIIa has been associated with the generation of bradykinin which is a potent inflammatory agent and therefore drives a wide range of inflammatory reactions. Interestingly, results of my studies showed that VWF is capable of binding towards both FXII zymogen as well as the activated form of the protease indicating that the glycoprotein is involved in both the conversion of FXII to FXIIa as well as subsequent FXIIa mediated processes. Consequently, this observation raises the question whether binding of VWF to FXII or FXIIa has a differential effect on the events following the interaction between the two proteins. In order to discuss this, it is necessary to distinguish whether FXII, FXIIa or VWF is considered as the initial binding partner driving the protein-protein interaction. VWF primarily would be available after endothelial injury or under pathological conditions leading to an exposure or accumulation of the glycoprotein in certain areas throughout the vasculature. Under these conditions, VWF predominantly would encounter inactive FXII present in the blood circulation. After binding of the protease to VWF, the glycoproteins subsequently would be able to trigger conversion of the zymogen into proteolytic active FXIIa leading to the localized generation of fibrin fibers and potentially bradykinin. The majority of pathological conditions such as sepsis, anaphylaxis or rheumatoid arthritis in which the interaction between VWF and FXII would offer a suitable explanation for some of the phenomena observed (as for example increased thrombogenesis) however relies on the presence of FXIIa defining the active protease as the primary binding partner. In these cases, binding of VWF to FXIIa would present a focal point for the generation of small platelet rich thrombi accompanied by generation of fibrin fibers and again potentially bradykinin. Based on the literature review and considering the data generated in this project, interaction between VWF and FXII in which the zymogen would be considered as primary binding partner would only occur to a limited extend as for example after insertion of artificial medical devices into the circulatory system. In these cases, FXII has been shown to absorb to the plastic surface coating the device with a layer of FXII. Subsequent binding of VWF could potentially initiate the formation of small thrombi which have been associated with device failure and in case of a detachment of the thrombus additionally can cause strokes and heart attacks. Independent on whether VWF binds to FXII or FXIIa, interaction between these two proteins therefore would result in VWF mediated platelet capture, FXII activation, FXIIa driven fibrin fibers as well

as bradykinin generation in all scenarios which would indicate that binding of VWF to FXII zymogen or the active protease has no differential effect on the subsequent VWF and FXIIa dependent processes but determines the location of the initial thrombus or bradykinin formation.

4.4. VWF and FXII dependent Clot Formation

The functional implications of the interaction between VWF and FXIIa were further analysed by adapting a commonly used thrombolysis assay as well as setting up a flow-based assay allowing for the assessment of thrombus formation and stability dependent on the degree of interaction between VWF and FXII.

The general impact of FXII on the formation and stability of thrombi is poorly characterised which partly is due to the low number of people being affected or aware of a FXII deficiency and therefore a reduced availability of patient samples[362]. However, a recently published study by Govers-Riemslag *et al.* examined the impact of a deficiency in intrinsic coagulation factors on thrombin generation, thrombus formation and clot stability using plasma derived from severely FXII deficient patients[363]. The results obtained during this study demonstrated that absence of FXII directly translated into significantly delayed thrombin formation as well as decreased overall thrombin generation accompanied by a 10-fold increase in clotting times. In addition, clots formed using FXII deficient patient samples were significantly smaller and less stable compared to thrombi formed with blood obtained from healthy donors.

The results of this newly published study are in line with the observations made in this project in which thrombolysis assays were used to determine the overall stability of a blood clot formed in the absence and presence of VWF and FXII activity as well as under conditions in which the binding of VWF to FXII was either reduced or inhibited. Results of the thrombolysis assays showed that addition of an anti-VWF antibody blocking the activity of the glycoprotein resulted in a decreased stability of the blood clot. Blockage of VWF function in this scenario was assumed to result in a reduction in platelet activation, subsequent aggregation as well as a decreased release of polyphosphates from the platelets present in the samples. Asidw fom interrupting the interaction between platelets and VWF, blockage of VWF activity also lead to an impaired FXII activation, supressed initiation of the contact pathway and therefore

subdued generation of thrombin and fibrin necessary for the stabilisation of the formed thrombus. Apart from VWF, the proteolytic activity of FXIIa was also inhibited by addition of an anti-FXIIa antibody resulting in the further decrease of clot stability compared to blockage of VWF activity alone. Since clot formation is initiated via the TF pathway in this assay, the increased instability of the formed thrombi is directly related to blockage of FXIIa and therefore failure in FXIIa mediated fibrin formation, which supports the hypothesis that despite the current belief of FXIIa being of negligible importance in the context of coagulation, the protease plays a role in overall blood clot growth and stability after all. Consequently, inhibition of the activity of both FXIIa and VWF resulted in a further decrease in thrombus stability since fibrin formation via the FXIIa pathway, VWF induced platelet aggregation as well as VWF and polyphosphate mediated activation of FXII were impaired in the respective samples. Based on these observations, we investigated whether disruption of the interaction between VWF and FXII has an influence on thrombus stability by reconstituting VWF depleted plasma with VWF mutants that showed reduced FXIIa binding capabilities. Results of these assays clearly showed that clot stability directly correlated with the ability of VWF to bind to FXIIa with virtually no thrombus formation in assays using VWF mutant 1458/1459 which is not capable of binding FXII. An almost complete absence of clot formation is very interesting since the double mutant demonstrated VWF wildtype-like platelet capture capabilities under conditions of flow. These captured platelets subsequently are likely to be activated, aggregate and secrete polyphosphates. The fact that the released polyphosphates which are known to trigger conversion of FXII to FXIIa are not sufficient to compensate for the loss of VWF/FXIIa interaction even in the presence of TF driven fibrin formation in terms of thrombus growth and stabilisation furthermore indicates a crucial role of the binding and activation interaction between VWF and FXII in the formation, structure and stability of blood clots.

After having demonstrated decreased clot stability whenever activity of VWF, FXIIa or both proteins are blocked or binding of the glycoprotein towards the protease is impaired in a static setting, I analysed blockage of the effects of the protein-protein interaction under more relevant physiological conditions using an adapted flow based platelet capture assay. In this experimental setup, I aimed to reproduce the formation of platelet rich blood clots by immobilising collagen type I on ibidi slides and subsequently perfusing either whole blood or VWF depleted plasma that was reconstituted with isolated and purified platelets, red blood cells and VWF mutants that have demonstrated reduced FXII binding capabilities. The

presence of TF and re-calcification of the samples before perfusion theoretically should allow for the generation of fibrin fibres which subsequently would cross-link VWF captured platelets resulting in the formation of platelet rich plugs as observed during predominantly primary haemostasis. As already previously mentioned, I demonstrated that introduction of the different mutations in the A1 domain resulted in an impaired binding ability of VWF to FXII but did not interfere with VWF A1 domain mediated platelet capture. A reduction in platelet capture observed in the described flow assay therefore can be directly attributed to the interruption of the binding and activation interaction between VWF and FXII. Results of the flow assay demonstrated two interesting observations. Firstly, perfusion of samples that contained VWF but lacked FXII resulted in slightly decreased platelet capture profiles indicating that the interaction of VWF and FXII supports VWF mediated platelet capture under physiological conditions. Secondly, platelet capture profiles in experiments using VWF mutants with reduced FXII binding ability were significantly decreased compared to both whole blood as well as VWF depleted plasma controls reconstituted with full-length wildtype VWF. As already observed in the thrombolysis assays, reduction in the numbers of platelets captured by VWF directly correlated with the ability of the A1 domain to interact with FXII. The double mutant E1452/1459E, which is not capable of binding to FXII at all showed a platelet capture profile comparable to samples devoid of VWF. Based on these results, I therefore hypothesise that the interaction between VWF and FXII is mandatory for the formation of stable thrombi and that inhibition of this interaction potentially can be used to prohibit the formation of pathological clots characteristic for different diseases.

4.5. Therapeutic Potential

As already discussed in previous chapters, both VWF and FXII belong to the group of haemostatic proteins[4]. While VWF plays an important role in the coagulative response by mediating the initial platelet capture at sites of vascular injury, FXII is the initiator of the intrinsic pathway of coagulation as well as being responsible for generation of the inflammatory peptide bradykinin via the kinin-kallikrein pathway[32,341]. Apart from the formation of the haemostatic clot necessary for the prevention of excessive blood loss, both proteins individually also have been associated with different forms of venous and arterial thrombosis such as DVT, pulmonary embolism and stroke[144,166,207,217,337,341].

Current strategies for the prevention and treatment of thrombotic events predominantly focus on the usage of anticoagulants such as warfarin or heparin which aim to reduce procoagulant activity[105]. Alternatively, thrombosis also can be treated with antiplatelet therapies in which for example aspirin ADP inhibitors are applied in order to decrease platelet aggregation[96]. In addition, formed blood clots can be lysed with thrombolytic agents such as tissue plasminogen activator, ultrasound or can be surgically removed in order to reinstate normal blood flow[94,95]. One common drawback of these treatment options is that all currently used anti-coagulant agents or antiplatelet therapies are characterised by bleeding side effects and combination of the two therapies has been shown to proportionally increase the risk of bleeding which severely limits their therapeutic application[121,106]. Therefore, identification of a novel anti-platelet therapy that does not induce a bleeding risk would offer both an alternative treatment strategy for patients that currently are taking anti-platelet medication and the possibility of a safer combined therapy with an anticoagulant.

In my studies, I have shown that FXII is a previously unrecognised binding partner of VWF and that the binding interaction between the two proteins is capable of converting zymogen FXII to the enzymatically active protease FXIIa. The results of my work furthermore demonstrate that inhibition of the interaction between VWF and FXIIa effectively prohibits thrombus formation under both static conditions as well as under flow. Based on the results obtained during my work, together with the current understanding of the role of FXII in haemostasis described in the literature, I hypothesise that the binding interaction between the glycoprotein and the coagulation factor is involved in the continuous growth and formation of pathological thrombi in different settings which are described in more detail in chapter 4.1 and 4.2. Consequently, inhibition of the binding interaction would be beneficial for different thrombotic situations and therefore would be a potentially suitable alternative to currently used anti-thrombotic treatment strategies.

In order to mimic the anti-thrombotic effects observed in this study in a therapeutic context, either VWF, FXIIa or the direct interaction between the two proteins was blocked or prohibited respectively. Due to its critical role in the formation of an initial platelet plug during primary haemostasis, neutralisation of VWF itself would result in certain bleeding complications as for example observed in VWD patients and therefore cannot be considered as a suitable treatment option[208].

However, targeting the enzymatic activity of FXIIa has garnered considerable interest over the past few years. As already previously discussed, low levels or a general FXII deficiency are not associated with any bleeding phenotype leading to a widespread unawareness of the individuals affected. For a long time, it was generally believed that only a small portion of the overall population (1 out of 1 million) exhibits a FXII deficiency. Opposing studies analysing clotting factor levels of 300 participants however showed that 2.3% of the individuals tested showed low or no FXII expression indicating that the percentage of FXII deficient people might be significantly higher than initially anticipated. Interestingly, a study carried out in 2006 in which almost 9000 individuals with varying FXII levels were enrolled, proposed a bell-shaped association between the activity of FXIIa and an increased thrombogenic potential. The study suggested that while FXIIa activity above 80% or below 20% did not influence the occurrence of thrombotic events, a general decrease in FXII activity levels was linked to an enhanced risk of experiencing arterial and venous thrombosis as well as increased mortality rates[364–366]. Data gathered from this study is in line with a variety of experiments carried out using FXII deficient mice which showed that absence of the protease interferes with pathological thrombus formation indicating that blockage of FXIIa might have a thromboprotective effect[329].

The currently available FXII inhibitors include small peptides, natural and recombinant proteins, antibodies as well as antisense RNA. The oldest known FXIIa inhibitor is the naturally occurring protein corn trypsin inhibitor (CTI)[217]. Even though the protein is capable of blocking FXIIa with a high potency, a clinical application of CTI is difficult due to the fact that, alongside FXIIa, the protein also has been shown to inhibit the activity of FXa as well as plasmin which both are mandatory to guarantee normal clotting reactions[367]. Aside from CTI, the activity of FXIIa also can be inhibited using the synthetic peptide H-D-Pro-Phe-Arg-chloromethylketone (PCK)[217,341]. Utilisation of the peptide in a clinical setting might be problematic due to the fact that inhibition of FXIIa with PCK is irreversible which will not only affect the generation of unwanted thrombi but also restrict any inflammatory responses FXIIa is involved in[368]. Two more recently developed therapeutics targeting FXII are the recombinant protein rHA-infestin-4 and the human monoclonal antibody 3F7. rHA-infestin-4 consists of domain 4 of the serine protease infestin which originally was isolated from a kissing bug[369,370]. The small protein has been tested as both a prophylactic as well as acute treatment for ischemic stroke in rat models and additionally showed promising anti-thrombotic effects in mice and rabbits suffering from surface induced venous and arterial thrombosis[369]. Despite the promising

results of the studies, rHA-infestin-4 has exhibited immunogenic properties, making a standardised therapy challenging[369–371]. Different to rHA-infestin-4, the human monoclonal antibody 3F7 is characterised by a low immunogenic profile and has been shown to efficiently prevent pathological thrombus formation both in vitro under flow as well as in thrombotic mice and rabbits[328,329,372]. While presenting many advantages compared to the other FXII inhibitors described so far, production of a human monoclonal antibody is cost-intensive and administration of an antibody based therapeutic requires regular injections or infusions. Both factors potentially could, at least in the case of a prophylactic treatment course, outweigh the benefits mentioned. The latest development in the field of FXII targeting molecules is the use of antisense RNA which has been demonstrated to reduce both arterial as well as venous thrombosis in mouse and rabbit models[327]. Unfortunately, blockage of FXII activity with antisense RNA is characterised by a slow onset and would require a ambulant treatment scheme consisting of repeated dosing in order to guarantee a continuous inhibition of the protease[373].

Based on the currently available anti-FXII therapeutics which have been demonstrated to have a beneficial effect in thrombotic settings, development of new agents targeting the previously unknown interaction between FXIIa and, in the case of this study, VWF might address an as yet unmet medical need. Considering the results obtained during my studies, generation of a molecule capable of directly inhibiting the binding and activation interaction between FXII and VWF potentially could attenuate formation of pathological thrombi without compromising normal haemostatic functions. Therefore, this molecule might have a beneficial effect on the treatment of all diseases involving the interaction between the glycoprotein and the protease. Blockage of the binding and activation interaction between the two proteins for example could potentially reduce the formation of pathological thrombi after insertion of certain types of medical devices and decrease VWF mediated platelet capture at locations with a high burden of bacteria as observed in sepsis. Additionally, prevention of the interaction also might reduce the extend as well as intensity of inflammatory reactions during anaphylaxis and rheumatoid arthritis as well as other processes in which FXII activity is either dependent or enhanced by the presence of VWF. Due to the fact that the exact amino acids involved in the binding interaction between FXII and VWF have been identified, generation of for example a small peptide targeting the surface residues aspartic acid at position E1452A and glutamic acid at position D1459A within the VWF A1 domain would prevent any form of interaction between

the two proteins. Compared to the currently available FXII targeting alternatives, a small peptide additionally would be easy to manufacture and process in large scale production schemes and together with in silico modification could be modified in order to pose a minimal immunogenic risk. Due to the general nature of small peptides, the therapeutic furthermore potentially could be formulated in order to allow both oral as well as intravenous administration which would create the possibility of the drug to be used in both prophylactic as well as acute settings.

4.6. Experimental Alterations and Future Research

Experiments carried out in the context of this project aimed at the elucidation of the binding interaction between VWF and FXII(a) and the effect this interaction has on the formation of pathological thrombi. Even though, the results generated confirmed a general binding ability of the glycoprotein to the protease and additionally identified VWF as a new physiological activator of FXII, some alterations within the general experimental layout as well as additional experiments would have allowed a mor in depth analysis of the protein-protein interaction.

4.6.1. Protein Expression and Characterisation

While expression of all isolated VWF A1 domain proteins necessary for the characterisation of the interaction between VWF and FXII(a) yielded protein samples of good purity and concentration, expression of especially the full-length VWF A1 domain mutants resulted in protein samples containing high amounts of impurities and low protein concentrations. Both circumstances subsequently limited the analysis of the VWF-FXII(a) binding interaction as well as the extent of the assays planned in order to assess the physiological relevance of the protein-protein interaction. Considering that the initial phase of the project was dedicated to the generation of different VWF A1 domain mutants as well as the expression, purification and characterisation of all necessary proteins, larger-scale protein expression strategies should have been exploited in order to ensure sufficient amounts of protein for all assays planned. Especially in case of the full-length VWF proteins, higher expression volumes would have allowed further concentration of the respective samples which would have made a more in-depth analysis by for example SPR screenings possible.

Even though expression of the different proteins in increased volumes would have provided larger amounts of protein for the various assays planned, this strategy would not have circumvented the presence of a high proportion of impurities in the full-length VWF samples. A potential solution for this problem is the introduction of a protein tag into the sequence of the respective proteins which would allow a directed purification with for example IMAC. In the past, this strategy was not applied due to the overall size of full-length VWF which frequently has caused problems during cloning steps and often exceeds insertion capacities of the cloning vectors that are currently in use. Therefore, protein tags have only been added to the isolated VWF A1 domain sequences which subsequently were sub-cloned into expression vectors containing the remaining VWF domains. Adaptation of this strategy might allow the generation of His-tagged full-length VWF proteins and enable the generation of highly pure protein preparations provided that introduction of the taq would not have any influence on the overall structure of the full-length VWF proteins.

After expression and purification, all VWF proteins were characterised in terms of their concentration. Full-length VWF proteins additionally were tested for their ability to form multimeric patterns as well as their capacity to bind to collagen and capture platelets under conditions of flow. Even though the perfusion assays allowed a direct comparison of the platelet capture profiles between recombinant full-length VWF wildtype and the 12 different full-length VWF A1 domain mutants, the assay did not provide any data for the capacity of plasma derived full-length VWF wildtype to arrest platelets from flow. Consequently, results of the assays did not determine whether plasma derived and recombinant full-length VWF proteins have similar platelet capture profiles and therefore did not evaluate the integrity of the overall protein structure of the recombinant VWF variants compared to the physiologically available VWF. This issue could be circumvented by including whole blood sample controls into the perfusion runs allowing the analysis of the platelet capture capacities of plasma derived full-length VWF alongside recombinant full-length VWF wildtype. As already previously mentioned, simultaneous binding of the full-length VWF proteins to platelets and collagen type I was tested in platelet capture perfusion assays in order to verify that introduction of different mutations in the VWF A1 domain did not compromise the overall structure of the proteins. Even though the assay provided a suitable method to establish the intactness of the platelet GPIbα binding site present in the VWF A1 domain, it did not allow verification of the VWF A1 collagen binding site since the collagen used in the flow assays is

known to associate with the VWF A3 domain. In order to confirm the structural integrity of the collagen binding site present in the A1 domain, it would be necessary to immobilise collagen type IV or VI instead of type I on the flow slides used. Alternatively, binding of the full-length VWF proteins to collagen type IV or VI also could be tested in a separate binding assay.

Structural as well as functional comparison between recombinant full-length VWF and plasma derived full-length VWF wildtype is essential in order to ensure validity of the data generated using the recombinant protein. SDS-PAGE analysis and formation of multimer patterns allowed a limited assessment of the equality in protein expression and formation of adequate secondary as well as tertiary structures. As already described above, including whole blood samples in the platelet capture perfusion assays would have provided another mean in order to compare the functional as well as structural integrity of both plasma derived and recombinant full-length VWF wildtype in terms of their collagen and platelet binding abilities. In addition to the perfusion assays, it would have been beneficial to implement a set of static plate binding assays analysing the integrity of for example the GP1Bα or heparin binding site present in the VWF A1 domain. Aside from additional assays focusing solely on the integrity of the binding sites present in the VWF A1 domain, further assays analysing the interaction of the full-length VWF mutants with for example FVII, β2 integrins, ADAMTS13 as well s GPIIbIIIa would ensure that the mutations introduced in the VWF A1 domain do not negatively affect the overall protein structure of the proteins. These assays therefore would present an additional method for the evaluation of reported binding capacities of plasma derived, recombinant full-length VWF wildtype as well as the different full-length VWF A1 domain wildtypes generating another data set confirming that neither mutations nor expression of the proteins by HEKT293T cells did influence the overall structure and therefore functionality of the respective proteins.

4.6.2. Binding Analysis

The binding interaction between VWF and FXII(a) predominantly was characterised using static plate binding assays in which FXII(a) was immobilised on a plastic surface at a concentration of 100nM. While the coating concentration of the protease was kept constant

throughout all experiments, incubation of the different VWF proteins on immobilised FXII(a) was carried out at various concentrations (Table 4.1).

Assay Name	Style	Concentration	Molecule
4-way ELISA	Single concentration	200nM	Plasma derived full-length VWF wildtype
Affinity Determination	Concentration range	6.3 to 400nM	Plasma derived and recombinant full-length VWF wildtype
VWFΔA1	Single concentration	80nM	Full-length VWFΔA1, plasma derived and recombinant full-length VWF wiltype
	Concentration range	1.6 to 100nM	Full-length VWFΔA1, plasma derived and recombinant full-length VWF wiltype
Isolated VWF A1 Domain	Concentration range	14 to 900nM	Isolated VWF A1 domain
Isolated VWF A1 Domain Mutant	Single concentration	360nM	Single-point isolated VWF A1 domain mutants, isolated VWF A1 domain and isolated VWF A2 domain
Isolated VWF A1 Domain Double Mutant			Single-point and double isolated VWF A1 domain mutants, isolated VWF A1 domain and isolated VWF A2 domain
Isolated VWF A1 Domain Double Mutant E1452/1459A	Single concentration	360nM	Single-point isolated VWF A1 domain mutants E1452A and D1459A, isolated VWF A1 domain double mutant E1452/1459A and isolated VWF A1 domain
	Concentration range	28 to 1800nM	
Full-length VWF A1 Domain Mutant	Single concentration	40nM	Single-point full-length VWF A1 domain mutants and recombinant full-length VWF wildtype
Full-length VWF A1 Domain Double Mutant			Single-point and double full-length VWF A1 domain mutants and recombinant full-length VWF wildtype
Full-length VWF A1 Domain Double Mutant E1452/1459A			Single-point full-length VWF A1 domain mutants E1452A and D1459A, full-length VWF A1 domain double mutant E1452/1459A and recombinant full-length VWF wildtype

Table 4.1: Summary of VWF concentrations used for the binding analysis between VWF and FXII(a). The binding interaction between the glycoprotein and the protease was characterised using static plate binding assays. VWF proteins were incubated on immobilised FXII(a) in various single concentrations or concentration ranges.

In retrospect, direct comparison of the binding behavior between the different VWF proteins and FXII(a) could have been simplified by using the same protein concentrations throughout all assays. Significant differences in the target concentrations especially between assays carried out with the full-length VWF and isolated VWF A1 domain samples were due to the overall lower concentrations of the full-length VWF preparations. Disregarding this aspect, even assays carried out with full-length VWF or isolated VWF A1 domain samples alone were not consistent in their protein target concentrations complicating both the evaluation as well as the comparison of the binding interaction displayed by the different full-length VWF A1 domain mutants or even the full-length VWF wildtype proteins between assays.

Initial results of the binding assays carried out with full-length VWF proteins lacking the VWF A1 domain suggested the presence of a FXII(a) binding site in the A1 domain of the glycoprotein. Even though further experiments confirmed specific binding of the isolated VWF A1 domain to FXIIa, a thorough characterisation of this interaction would have included additional assays in which binding of the isolated VWF A1 domain towards both FXII zymogen as well as FXII which previously had been activated with kallikrein was tested.

Incubation of both isolated as well as full-length VWF A1 domain mutants led to the identification of amino acids aspartic acid at position 1452 and glutamic acid at position 1459 as the FXIIa binding site within the VWF A1 domain. The selection of the positions to be mutated was based on the hypothesis that FXII which has been reported to predominantly bind to negatively charged surfaces is likely to interact with negatively charged amino acids on the surface of the VWF A1 domain. Even though simultaneous change of amino acids at position 1452 and 1459 led to an abolishment in FXIIa binding, it is possible that the interaction between the glycoprotein and the protease is facilitated by additional amino acids in structurally close proximity of the mutated surface residues. This possibility could be further explored by the creation of another set of VWF mutants containing different combination of amino acid changes near aspartic (1452) and glutamic acid (1459).

Aside from static plate binding assays, the binding interaction between VWF and FXIIa additionally was tested using SPR measurements. Due to the limited availability of the full-length VWF proteins, this analysis was very restricted and only allowed the determination of binding affinities between recombinant full-length VWF wildtype but not the full-length VWF A1 domain mutants and FXIIa. Considering the divergent results obtained after ELISA screening of all full-length VWF A1 domain and isolated VWF A1 domain mutants on FXIIa, SPR experiments determining the binding behaviour between FXIIa and all VWF mutants might have supported the decision to concentrate on residues 1444, 1445, 1447, 1452 and 1459 for the generation of VWF double mutants. Another limitation encountered during the SPR measurements was the restricted capacity and availability of suitable BIAcore facilities. Ideally, SPR measurements would have included the examination of the binding interaction between FXIIa, FXII, kallikrein activated FXIIa and plasma derived and recombinant full-length VWF wildtype, recombinant full-length VWFΔA1, the isolated VWF A1 domain and all VWF A1 domain mutants in both the full-length VWF as well as the isolated VWF A1 domain format.

In general, all binding assays aiming at the characterisation of the binding interaction between VWF and FXIIa were carried out under non-physiological conditions. Considering that binding of VWF to FXII has not been previously described, experiments proving a direct interaction between the glycoprotein and the protease in their natural environment would be of great interest. One technique allowing this kind of analysis is called immunoprecipitation which is based on the protein of interest being captured by an immobilised antibody exhibiting a high binding specificity towards the target protein. Considering that antibodies specific for both FXII as well as VWF are commonly available and that the glycoprotein and the protease seem to be able to form a complex in solution, it would theoretically be possible to demonstrate the presence of FXII(a), VWF and the protein complex in physiologically relevant samples as for example whole blood or blood plasma.

4.6.3. Activation of FXII

Similar to a vast variety of different proteases, FXII is expressed and circulates the blood in a zymogen state in which the protein does not exhibit any enzymatic activity. In order to mediate proteolytic cleavage, FXII therefore needs to undergo a conformational change resulting in the conversion of FXII zymogen into the active protease FXIIa. Directed activation predominantly is facilitated by kallikrein and plasmin while unspecific activation of the zymogen has been observed after interaction of FXII with negatively charged surfaces in a process called autoactivation. Examination of the autoactive potential of FXII was tested in FXII activation assays using plastic as the respective activating surface. Interestingly, only limited conversion of FXII to FXIIa was observed in these experiments after 45 minutes. Extended literature research showed that autoactivation as a relatively slow process with average conversion times between 90 and 120 minutes. Based on this information, the activation assays testing the autoactivation of FXII should be repeated with extended time frames allowing the conversion of the FXII substrate for a minimum of 90 minutes.

Subsequent to the characterisation of the FXII activation by a multitude of different substances, conversion of FXII to FXIIa after incubation of the zymogen with different VWF proteins was tested. Activation of the protease was determined using both full-length VWF wildtype as well as isolated VWF A1 domain samples. Even though the results of these assays showed a consistent activation of FXII, use of equal full-length VWF wildtype and isolated VWF

A1 domain concentrations did not account for the physiological distribution of these two molecules. As already previously described, VWF is a large multimeric protein which is composed of individual VWF monomers. Consequently, full-length VWF molecules used in these experiments contained more than one VWF A1 domain raising the overall molar concentration of the A1 domain in the respective samples. At the same time, preparations of the isolated VWF A1 domain were solely comprised of the A1 domain itself. Considering that the VWF A1 domain has been identified as essential for the interaction of the glycoprotein with FXII(a), activation assays utilising the same molar concentration of the A1 domain in both the full-length VWF as well as the isolated VWF A1 domain samples therefore would allow a more accurate comparison of the activation potential of the two VWF preparations.

In order to verify that activation of FXII after incubation with different VWF proteins is specific to the glycoprotein, FXII activation assays in which the activity of VWF was blocked using an anti-VWF antibody were carried out. Even though the general setup of this assay was sufficient for the demonstration of VWF specificity, additional control reactions might have been beneficial. For example, control sampled comprising FXIIa as well as the anti-VWF antibody used in these experiments would have allowed to demonstrate that the general activity of the protease is not compromised by the addition of the antibody. Furthermore, samples verifying that FXII zymogen can be activated by for example kallikrein or polyphosphates even in the presence of the anti-VWF antibody would have circumvented residual questions about the specificity of the FXII activation mediated by VWF.

Apart from FXII activation assays, conversion of FXII zymogen into proteolytically active FXIIa after incubation of the protease with different substances additionally was verified by western blot analysis. Despite of the blots showing clear fragmentation of FXII after its activation, inclusion of FXII zymogen into the experiments would have allowed a direct comparison between the inactive protease, commercially available FXIIa and FXII samples that previously had been activated by a variety of potential activators.

4.6.4. Functional Effect of the Interaction between VWF and FXII(a)

After having established a general binding ability of VWF towards FXII(a) which additionally led to the identification of VWF as a previously unrecognised activator of the protease, the

interaction between the two proteins was further characterised in terms of their physiological relevance.

In a first step, the stability of blood clots that had been generated under different conditions was measured in order to analyse the role of VWF, FXIIa as well as the interaction between the glycoprotein and the protease on clot lysis times. After having carried out initial experiments using whole blood samples, assays were repeated with VWF depleted plasma that was reconstituted with isolated and purified red blood cells, platelets and full-length VWF A1 domain mutants exhibiting reduced FXIIa biding abilities. Results of these assays showed a direct correlation between the capability of the VWF proteins to interact with FXIIa and the stability of the respective blood clots. Especially clots generated using samples containing the full-length VWF A1 domain double mutant E1452/1459A which is not able to bind towards FXIIa showed a significant increase in clot instability leading to virtually no thrombus formation. While control samples in this experiments included VWF depleted plasma reconstituted with red blood cells, platelets and the recombinant full-length VWF wildtype, inclusion of a range of additional controls would have allowed a more thorough analysis of clot stability and the role of the different proteins on clot lysis times. First of all, an additional control consisting of whole blood samples would have allowed to evaluate whether the use of recombinant VWF instead of VWF present in unprocessed blood plasma had any detrimental effects on the formation of the respective blood clots. A second control that would have been useful in order to confirm previous results showing that blockage of FXIIa activity decreases thrombus stability should comprise VWF depleted plasma reconstituted with red blood cells, platelets, recombinant full-length VWF and additionally an anti-FXII antibody. These samples theoretically would equal preparations of whole blood in which solely the activity of FXIIa was inhibited. Another valuable assay readout would have been the evaluation of cot lysis times for thrombi formed with either whole blood or VWF depleted plasma reconstituted with red blood cells, platelets and recombinant full-length VWF wildtype that contained both anti-VWF as well as anti-FXII antibodies. Activity of both the protease as well as the glycoprotein would have been blocked in these samples and therefore would have allowed a comparison between prohibition of protein activity versus the effect of the interaction between VWF and FXII(a) which for example was achieved by substitution of the VWF wildtype with the full-length VWF A1 domain double mutant E1452/1459A. In addition to the controls described above, blood clots formed from samples containing an additional

FXII activator would have been of interest in order to determine whether the extend of FXII activation has a direct influence on the overall stability and lysis time of the respective thrombi. Considering that for example polyphosphates have demonstrated a superior FXII activation potential compared to VWF, VWF depleted plasma samples reconstituted with red blood cells, platelets and for example 10µM polyphosphates could have been used for this purpose. In addition, the described samples might have given an initial insight into a potential differential effect of FXII zymogen and FXIIa on the overall stability of thrombi. Since plasma samples naturally contain both the active as well as the inactive form of FXII it is very likely that, even in the presence of VWF, conversion of FXII to FXIIa would not be exhaustive. Addition of another activator such as polyphosphates therefore might increase this conversion rate resulting in a higher concentration of FXIIa in the respective samples which in turn potentially could provide another mean to prove a direct correlation between FXII activity and clot stability.

Aside from thrombolysis assays, the effect of the interaction between VWF and FXII(a) also was tested in platelet capture perfusion assays. The results obtained in these experiments confirmed the previously made observation that the ability of VWF to interact with FXIIa directly influences the formation of blood clots. Furthermore, the assays demonstrated that inhibition of the interaction between the protease and the glycoprotein prevents the generation of platelet rich clots under conditions of high shear observed in for example thrombotic arteries or veins. In general, assays described in this thesis focused on the formation of pathological thrombi using shear rates above 1500s-1. It would be interesting to evaluate whether there is a significant difference between physiological and pathological shear rates in regard to VWF and FXII(a) mediated platelet capture and therefore thrombotic clot formation. Consequently, perfusion assays with a whole range of shear rates mimicking both physiological as well as pathological shear could be used in order to demonstrate that the interaction of VWF and FXIIa plays an important role in the formation of unwanted thrombi but is negligible under normal conditions.

4.6.5. Future Research

The role of FXII in haemostasis has been controversially discussed for the past decade due to the fact that individuals with a FXII deficiency do not present a bleeding phenotype.

Generation of FXII deficient mice even led to the hypothesis that absence of the protease in fact might have a thromboprotective effect. While the necessity of FXII in the context of haemostasis has been questioned, the protease also is responsible for the release of the inflammatory mediator bradykinin which is generated via the kinin-kallikrein. Structural as well as activation analysis have shown that interaction of FXII zymogen with its activators or negatively charged surfaces results in either cleavage of FXI in the context of coagulation or production of bradykinin depending on which protease domains are involved[223]. Results of these biochemistry studies additionally showed that the majority of the events triggering the conversion of FXII zymogen into proteolytically active FXIIa result in the formation of bradykinin but less frequently in the activation of FXI. Based on these findings it would have been interesting to carry out additional activation assays further characterising the VWF mediated activation of FXII. Especially analysis whether the interaction between the protease and the glycoprotein predominantly drives the initiation of coagulation or the generation of bradykinin would have given a new insight into whether binding of VWF to FXII(a) under physiological as well as pathological conditions is only important in haemostasis or also influences critical inflammatory processes.

All of the experiments described in this thesis focused on the in vitro characterisation of the interaction between VWF and FXII(a). While some assays as for example initial evaluation of clot lysis times as well as the parts of the perfusion assays were carried out using entirely human components in the form of plasma or plasma derived VWF, a big proportion of the assays described in this thesis were based on the analysis of recombinant proteins. In addition, even though both thrombolysis as well as the perfusion assays were set up with the intend to mimic physiologically relevant situations, more in-depth studies of the interaction between the glycoprotein and the protease in their natural environment would require in vivo experiments. In order to conduct in vivo studies, suitable models preferably exhibiting a thrombogenic phenotype would be necessary. In these models, application of for example mechanical or chemical injury to the aorta or carotid artery results in a range of thrombotic events such as arterial thrombosis, pulmonary thromboembolism, vessel occlusion or cerebral ischemia. Simultaneous use of anti-VWF and anti-FXII antibodies subsequently would allow the evaluation of the duration and severity of the pathologic thrombus formation in regards to the activity of VWF and FXII. Furthermore, it would be possible to analyse whether inhibition of FXIIa, VWF or activity of both proteins would have a beneficial effect on recovery

times and subsequent functional test as for example assessment of the global neurological function or mechanical grip exercises. Unfortunately, the current state of the project would not allow to directly test the effect of the interaction between VWF and FXII(a) on the occurrence of thrombotic events due to the fact that VWF deficient mice either die in utero or present a bleeding phenotype which would interfere with the surgical procedures mentioned above. An alternative would be the use of FXII deficient mice. In order for this model to present a viable alternative, it would be necessary to identify the exact VWF binding site within the FXII protein. After identification of the amino acids mediating binding of the protease to VWF, the relevant amino acids could be mutated creating FXII proteins incapable of binding the glycoprotein. These newly created mutants then could be injected in FXII deficient mice generating a model in which not the activity of VWF or FXIIa but the interaction between the two proteins van be analysed. The design of the proposed in vivo studies is entirely based on the fact that the generation of FXII deficient mice allowed extensive research focusing on the role of FXII in normal haemostasis and pathological thrombus formation and eventually led to the conclusion that absence of the protease has a thromboprotective effect. Despite the conclusive data obtained from experiments with FXII deficient mice, there has been no evidence that these findings can be directly translated to humans so far. Therefore, all assumptions drawn from in vivo studies analysing the effect of prohibiting the interaction between VWF and FXIIa additionally would need to be verified in clinical trials on human individuals.

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Publications arising from this Work

- P. Henne et al; “Mapping the interaction of VWF with FXIIa and its potential as a new anti-coagulant target”; manuscript in preparation

- Oral presentation “Mapping the interaction of VWF with FXIIa and its potential as a new anti-coagulant target” at the International Society on Thrombosis and Haemostasis congress 2019 in Melbourne; Australia

- Oral and poster presentation “Mapping the interaction of VWF with FXIIa and its potential as a new anti-coagulant target” at the Imperial College London rising scientist day 2018 in London; United Kingdom

- Poster presentation “Mapping the interaction of VWF with FXIIa and its potential as a new anti-coagulant target” at the British Society for Haemostasis and Thrombosis conference 2017 in Warwick; United Kingdom