



universität
wien

DIPLOMARBEIT

Titel der Diplomarbeit

„Influence of coagulation inhibitors on the
procoagulant properties of an activated prothrombin
complex concentrate“

verfasst von

Margarita Löschberger

angestrebter akademischer Grad

Magister ODER Magistra der Pharmazie (Mag.pharm.)

Wien, 2014

Studienkennzahl lt. Studienblatt:

A 449

Studienrichtung lt. Studienblatt:

Diplomstudium Pharmazie

Betreut von:

Univ.-Doz. Dr. Peter Turecek

DANKSAGUNG

Ich möchte an dieser Stelle Herrn Univ.-Doz. Dr. Peter Turecek für die freundliche Betreuung und Unterstützung meiner Diplomarbeit danken.

Mein Dank gilt ebenfalls Frau Dr. Katalin Váradi und Dr. Gerald Schrenk, die mich ebenfalls zu jedem Zeitpunkt vorbildlich betreut und unterstützt haben.

Danke an alle lieben Kollegen und Kolleginnen – allen voran Sylvia Peyrer-Heimstät – ,die im Labor immer besonders hilfsbereit waren.

Vielen Dank an meinen Freund und meine Familie, die mich alle ohne Vorbehalt und zu jedem Zeitpunkt während meiner Ausbildung tatkräftig unterstützt haben.

Table of Contents

1	Introduction	7
1.1	Hemostasis	7
1.1.1	Vascular Reaction.....	7
1.1.2	Cellular Reaction (Primary Hemostasis)	7
1.1.3	Plasmatic Hemostasis (Secondary Hemostasis)	9
1.1.4	Regulation of Hemostasis.....	17
1.1.5	New concept of coagulation.....	23
1.2	Hemophilia	24
1.2.1	Development of inhibitors.....	27
1.2.2	Acquired inhibitors.....	28
1.3	Activated Prothrombin Complex Concentrate (APCC).....	30
1.4	Recombinant factor VIIa	34
2	Materials and Methods.....	35
2.1	Thrombin Generation Assay	35
2.1.1	Reagents and Equipment	39
2.1.2	Test Procedure	42
2.2	Determination of total TFPI.....	45
2.2.1	Reagents and Equipment	45
2.2.2	Test Procedure	46
2.2.3	Validation	48
2.3	Determination of TFPI Activity	49
2.3.1	Reagents and Equipment	49
2.3.2	Test Procedure	50
2.3.3	Validation	51
2.4	Immunoprecipitation.....	52
2.4.1	Reagents and Equipment	53
2.4.2	Test Procedure	54
2.5	Electrophoresis.....	56
2.5.1	SDS PAGE - Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis.....	56
2.5.2	Western Blot.....	58
3	Results	66
3.1	Detailed examination of different APCC samples	66
3.1.1	Characterization of APCC Lots.....	67

Table of Contents

3.1.2	<i>Influence of TFPI in plasma and TFPI in APCC on the APCC activity.....</i>	<i>79</i>
3.1.3	<i>TFPI spiking experiments.....</i>	<i>87</i>
3.1.4	<i>Comparison of APCC and synthetic APCC-Mix.....</i>	<i>91</i>
3.2	Investigation of plasma samples of healthy donors and of patients with hemophilia A with inhibitors.....	97
3.2.1	<i>Comparison of normal plasma (from healthy donors) and patient plasma (patients with hemophilia A with FVIII inhibitors).....</i>	<i>97</i>
3.2.2	<i>Characterization of patient plasma samples with hemophilia A with inhibitors before and after APCC application</i>	<i>104</i>
4	Conclusion and Discussion	110
5	Summary (English).....	119
6	Summary (German)	121
7	Appendix.....	123
7.1	List of Tables	123
7.2	List of Figures	125
7.3	List of Abbreviations.....	128
7.4	References.....	129
8	Curriculum Vitae	135

1 Introduction

1.1 Hemostasis

Hemostasis refers to all physiological processes that result in the stop of bleeding without loss of rheological properties.

An injured venous or arterial blood vessel can thus be rapidly closed without interrupting the blood flow. The whole process serves the organism as a protection against greater blood loss.

Blood clotting is reached by a complex interaction of vascular, cellular and plasmatic reactions (Witt I and Dörner K. 2006).

After hemostasis follows fibrinolysis, in which the formed clot is slowly dissolved. Both processes are in a dynamic equilibrium, which assures a controlled coagulation process. If equilibrium is located more on the side of fibrinolysis, it can lead to bleeding, whereas there is an increased risk of thrombosis, when blood clotting dominates.

1.1.1 Vascular Reaction

As a result of injury there is a stimulation of smooth muscle cells. This leads to a reflex contraction of the blood vessels by the sympathetic nervous system. This reflex contraction happens due to the release of serotonin and catecholamines. In addition, thromboxane A₂ is released by thrombocytes. By contraction of the vessels the blood flow is slowed down, which is an essential condition for blood clotting.

1.1.2 Cellular Reaction (Primary Hemostasis)

If damage to a vessel occurs, subendothelial collagen fibers are exposed. To these fibers thrombocytes can adhere (adhesion). The adhesion is performed using the von-Willebrand factor (VWF), which represents the link between the collagen fiber and the

Introduction

glycoprotein Ib-receptor, which is located on the surface of the platelets. Due to the contact with the extracellular matrix thrombocytes get activated (Estler CJ. 2000). So once adherent to subendothelium, platelets spread out on the surface, and additional platelets, delivered by the flowing blood, adhere first to the basal layer of adherent platelets and eventually to one another, forming a mass of aggregated platelets (Colman RW, et al. 2005). Thus, on the one hand there is a change of shape and aggregation of platelets and on the other hand a secretion of substances essential for coagulation.

From the granules of platelets substances are released, which support firstly the aggregation of thrombocytes and secondly the endogenous coagulation system. Among the most important substances there are ADP, thromboxane A₂, PAF (platelet activated factor), serotonin and thrombospondin.

Serotonin and thromboxane A₂ show vasoconstrictive functions. ADP, PAF, thrombospondin and thromboxane A₂ encourage aggregation. Thrombospondin leads to irreversible aggregation because this glycoprotein stabilizes the fibrinogen bridges. Additionally, negatively charged phospholipids, platelet factors (PF) 3 and 4, are available on the surface of the platelets. These bind plasmatic coagulation factors (secondary hemostasis). PF₃, a coagulation-active phospholipid, is required as a complexing agent during the plasmatic coagulation (activation of factor X and prothrombin). PF₄ binds heparin and neutralizes its effect.

Due to the activation of thrombocytes, the glycoprotein IIb/IIIa–receptor is exposed on their surface. This receptor also supports the formation of aggregates together with von-Willebrand factor. At the same time thrombocytes perform a shape change, get spherical and develop pseudopods, whereby aggregation can take place. The glycoprotein IIb/IIIa – receptor shows high affinity to fibrinogen, fibronectin and calcium (Estler CJ. 2000). Binding of these substances results in a strengthening of the clot.

Fibrinogen promotes the interconnection of thrombocytes. Fibronectin is essential for the linking of platelets on the subendothelial matrix.

With this process, the primary hemostasis is completed. The thrombus is still not very stable and could be washed away by the blood stream. Only by the conversion of fibrinogen to fibrin and the storage of erythrocytes, the thrombus is stabilized (secondary hemostasis) (Mutschler E, et al. 2005). The time that passes from the beginning of the vascular injury to the primary, not yet stable, closure of the injury is called bleeding time. It normally takes 2 to 3 minutes.

1.1.3 Plasmatic Hemostasis (Secondary Hemostasis)

Object of the secondary hemostasis is to close the - for now - unstable sealed defect and make it mechanically stable until the final repair by scarring is done. In addition to the platelets, numerous plasmatic factors and tissue factors are involved. They are collectively referred to as blood clotting factors (Table 1) (Mutschler E, et al. 2008).

Traditionally, two pathways of blood coagulation have been distinguished: the intrinsic and the extrinsic pathway. The names allude to the fact that the intrinsic pathway leads to thrombin formation, using only protein factors that are present in plasma (Halkier T. 2009). Contact with negative charges on the surface of injured endothelium activates the intrinsic pathway (Mutschler E, et al. 2005). Activation via the intrinsic system succeeds within a few minutes. The extrinsic pathway requires the presence of tissue factor (FIII), which is released from the tissue when tissue injury happens. The activation of the extrinsic system proceeds within seconds – far more quickly than the intrinsic pathway (Mutschler E, et al. 2008).

However, the division of coagulation process into two branches proves to be somewhat artificial, since several components of the two options appear to interact with one another.

Introduction

The process of coagulation is classically regarded as a cascade reaction, as originally suggested. But recent developments suggest that the cascade concept has outlived its usefulness, because a number of the enzymatic reactions leading to the formation of thrombin require the presence of thrombin-activated co-factors if they are to proceed at a physically relevant speed. It therefore seems closer to the truth to describe the coagulation system as a cyclic reaction network (Halkier T. 2009).

Between the two pathways there exist a number of inter-relationships and dependencies, whereby a separation in vivo is not possible (Mutschler E, et al. 2005). Because activated FVII (FVIIa) intervenes not only in the extrinsic pathway but also in the intrinsic coagulation system (Mutschler E, et al. 2008).

For both systems of the coagulation activation negatively charged phospholipids must be available on the surface of platelets. In the end both pathways lead to the activation of factor X, which activates prothrombin (FII) to thrombin (FIIa). FIIa supports the conversion of fibrinogen to fibrin. Fibrin penetrates the loose platelet plug and solidifies it with the formation of a three-dimensional network.

The membranes of mammalian cells are asymmetrical. On the outside positive charges are located, whereas the inside is covered with negatively charged phospholipids. When an injury to a vessel happens the negatively charged site turns to the surface and supports the activation of coagulation factors. During the process of coagulation a number of central reactions take place, such as the complexation of coagulation factors, calcium-ions and phospholipids. The negatively charged surface of the phospholipids is used as a reactive surface in the complex where coagulation factors can be bound together with calcium. Phospholipids, especially phosphatidyl-serin and phosphatidyl-cholin, can thereby be described as coagulant phospholipids. Moreover, also calcium is a very important mediator of the binding and activation of coagulation factors.

1.1.3.1 Coagulation Factors

The coagulation factors of the blood plasma are all glycoproteins, with the exception of calcium ions. Many of these glycoproteins are produced in the liver. The von-Willebrand factor (VWF) instead is synthesized in the endothelium of the vessels and also in megakaryocytes. Vitamin K is essential for the synthesis of factor II, VII, IX and X as well as for the synthesis of inhibitor protein C and its co-factor protein S. In the synthesis of these factors Vitamin K operates as a co-enzyme, more specifically in the γ -carboxylation of glutamic acid – containing side chains of the factors. The resulting carboxylglutamyl-compounds are now able to form calcium-complexes with phospholipids (Mutschler E, et al. 2005).

Blood coagulation factors either operate as enzymes (mostly serine proteases) or as co-factors. They are available in the plasma in their inactive form (= zymogen). This is the way they can be regulated not to trigger uncontrolled processes. A second protection is the half-life of the factors. Even if there is only a simple, small wound, thrombin for example would be able to coagulate the blood of the whole body if it had a longer half-life.

The activation of serine proteases respectively occurs by the previously activated factor (indicated by a small suffix "a") of the coagulation cascade. The inactive factor is proteolytically cleaved, in order to expose the active center of the enzyme. This activated site is responsible for the catalytic activity.

Only factors II, IX, X, XI, and XII are active pro-enzymes of serine proteases. Factor V and VIII do not have any enzymatic activity. They, together with fibrinogen act as co-factors, because of thrombin, a peptide cleavage occurs in FV and FVIII, which leads to a change of conformation. Only then they can act as co-factors.

Introduction

Factor		Function		Half-life	Location of synthesis
I	Fibrinogen	Substrate (glycoprotein)	Adhesive protein that forms the fibrin clot	96-120h	Liver
II	Prothrombin	Zymogen,	Activated form is the main enzyme of coagulation	41-72h	Liver, vitamin K-dependent
IIa	Thrombin	Enzyme (serine protease)			
III	Tissue factor Thromboplastin= thrombokinas	Zymogen	Lipoprotein initiator of extrinsic pathway	very short	Subendothelium (smooth musculature), monocytes, leukocytes, thrombocytes, circulating micro particles, fibroblasts
(IV)	Calcium	Catalyst	Metal cation necessary for coagulation reactions		
V	Proaccelerin	Accelerator	Cofactor for activation of prothrombin to thrombin	12-15h	Liver
Va	Accelerin	Accelerator			
VII	Proconvertin	Zymogen	With tissue factor, initiates extrinsic pathway	2-5h	Liver, vitamin K-dependent
VIIa	Convertin	Enzyme (serine protease)		<2h	
VIII	Antihemophilic factor	Accelerator	Cofactor for intrinsic activation of factor X	8-12h	Liver, megakaryocytes
VWF	Von-Willebrand-Factor	- Mediation of platelet adhesion (binding on glycoprotein Ib) - Mediation of platelet aggregation (binding on glycoprotein IIb/IIIa)		10-12h	Endothelium, megakaryocytes

Introduction

		- Binding of FVIII (protection against proteolysis)			
IX	Christmas factor	Zymogen (serine protease)	Activated form is enzyme for intrinsic activation of factor X	18-30h (FIXa: <12h)	Liver, vitamin K-dependent
X	Stuart-Prower factor	Zymogen (serine protease)	Activated form is enzyme for final common pathway activation of prothrombin	20-42h (FXa: <4h)	Liver, vitamin K-dependent
XI	Plasma thromboplastin antecedent	Zymogen (serine protease)	Activated form is intrinsic activator of factor IX	10-20h	Liver
XII	Hageman factor	Zymogen (serine protease)		10-20h	Liver
XIII	Fibrin stabilizing factor	Zymogen (serine protease)	Transamidase that cross-links fibrin clot	100-200h	Liver, thrombocytes
(XIV)	Fitzgerald factor	Zymogen (high-molecular-weight kininogen)	Cofactor, circulates in a complex with F XI		
(XV)	Prekallikrein Fletcher factor	Zymogen (serine protease)			

Table 1: List of coagulation factors ((Barthels M and Depka M. 2002)/ (Golenhofen K. 2006)/ (Pötzsch B and Madlener K. 2010)/ (Hagemann O. 2013)/ (Lefkowitz JB. 2008))

1.1.3.2 Activation Phase (Initiation Phase)

1.1.3.2.1 Intrinsic System

If blood is added to a bin with negatively charged surface, for example a glass tube, it clots. Therefore all factors necessary for blood clotting must be available in the blood. Thus it is known as the intrinsic coagulation.

Prekallikrein (FXV), high-molecular-weight kininogen and FXII start the intrinsic pathway. They bind to the negatively charged surface, for example collagen, which gets exposed because of an injury. Consequently, the intrinsic system is also known as the “contact activation”.

First, FXII is activated by its binding on the negatively charged surface. Subsequently, FXIIa catalyzes the conversion of prekallikrein to active kallikrein (Witt I and Dörner K. 2006). By proteolysis kallikrein together with high-molecular-weight kininogen activates FXII. Due to high-molecular-weight kininogen bradykinin gets released. By kallikrein activated factor XII leads to an increased conversion of prekallikrein to kallikrein. This amplifier loop is the basic precondition for a quick and sufficient activation of the plasmatic coagulation process.

At the same time factor XIIa activates factor XI, which in turn activates factor IX in the presence of calcium. Factor IXa forms together with its co-factor VIII, calcium ions and phospholipids a complex, the so-called tenase complex. Co-factor VIII gets activated by a small amount of initially synthesized thrombin. By activation of platelets calcium and phospholipids get exposed on the outer surface of thrombocytes. The formation of this complex is absolutely essential for the proteolytic cleavage of factor X – its activation by factor IXa.

In this complex factor VIIIa is the rate-limiting co-factor for factor IXa. Initially synthesized thrombin also activates factor XI. This leads to an additional increase of activation of factor IX.

1.1.3.2.2 Extrinsic System

The extrinsic pathway is initiated in vivo by tissue factor, activated endothelial cells, subendothelial smooth muscle cells, and subendothelial fibroblasts after vascular injury (Golan DE, et al. 2011). The activation starts with the release of tissue thromboplastin (tissue factor), an integral membrane protein, from damaged cells. Together with factor VIIa the tissue factor forms a complex, which activates, in the presence of calcium, factor X on the phospholipids of activated platelets (PF3). Not only factor X gets activated by the complex TF/FVIIa, but also factor IX, which forms another complex with factor VIIIa. In turn the complex FIXa/FVIIIa intensifies the FX activation (Neumann HA. 2008). On this account we here find the connection between the extrinsic and intrinsic system. Furthermore, the TF/FVIIa complex also autocatalytically activates factor VII (Brackmann HH, et al. 2003). Factor Xa itself also activates factor VII, whereby it promotes its own activation due to TF-FVIIa complex (Pötzsch B and Madlener K. 2010).

FXa alone only cleaves a small amount of prothrombin in thrombin, because its full enzymatic ability is unfolded only in complex with FVa. This small amount of generated thrombin is enough to cause a series of amplifying reactions. Thrombin activates factor V, which operates as a cofactor as well as factor VIII. Together with FXa factor Va forms an enzymatic complex, prothrombinase, which raises the activity of FXa a thousand-fold. Consequently, there is a dramatic increase in thrombin generation. The prothrombinase can only contribute in combination with phospholipids, presented due to activated platelets, and calcium ions to a proteolytic cleavage of prothrombin. Thrombin also activates the platelets. These do not only get activated by binding on exposed collagen, but also by thrombin. The activation of platelets causes a change of morphology, which enables tethering to the exposed subendothelium and to each other. It also enables efficient binding of tenase and prothrombinase enzyme substrate

complexes. This binding assures the full efficiency of these complexes. Moreover, the greater the area of provided phospholipids, the more prothrombin is converted.

Thrombin also leads to the activation of factor VIII and factor XI. They increasingly generate FXa by activation of FIX and the generation of tenase-complex. The thrombin generation is significantly accelerated by this positive feedback. The importance of factor VIII and IX becomes apparent by their absence in hemophilia A (FVIII) and B (FIX).

The activation phase is completed with the formation of enzymatically active thrombin (Estler CJ. 2000).

1.1.3.3 Coagulation- and Retraction Phase

Fibrinogen is the ultimate protein substrate of the coagulation cascade and forms the principal structure-protein of the fibrin clot. Thrombin cleaves small peptides to form a fibrin monomer. These monomers assemble into protofibrils in a half-staggered, side-to-side fashion that is stabilized by noncovalent interactions between fibrin molecules. The protofibrils laterally connect to thicker fibrin fibers and form the fibrin clot. However this clot is not stable and will ultimately come apart if not covalently cross-linked. Thrombin activates factor XIII to the trans-glutaminase enzyme factor XIIIa. Factor XIIIa, acting upon the glutamic acid and lysine side chains in the fibrin amino acid sequence, creates covalent bonds between fibrin monomer chains, creating a stable clot (Lefkowitz JB. 2008).

Because of the influence of thrombin with the participation of fibronectin, fibrin binds on the glycoprotein IIb/IIIa-receptor of the platelets in the retraction phase (Estler CJ. 2000). Furthermore the actin-myosin skeleton contracts the platelets. This results in a contraction of the wound edges. Therefore, the thrombus is additionally hardened, which in turn entails a mechanical closure of the wound (Brackmann HH, et al. 2003).

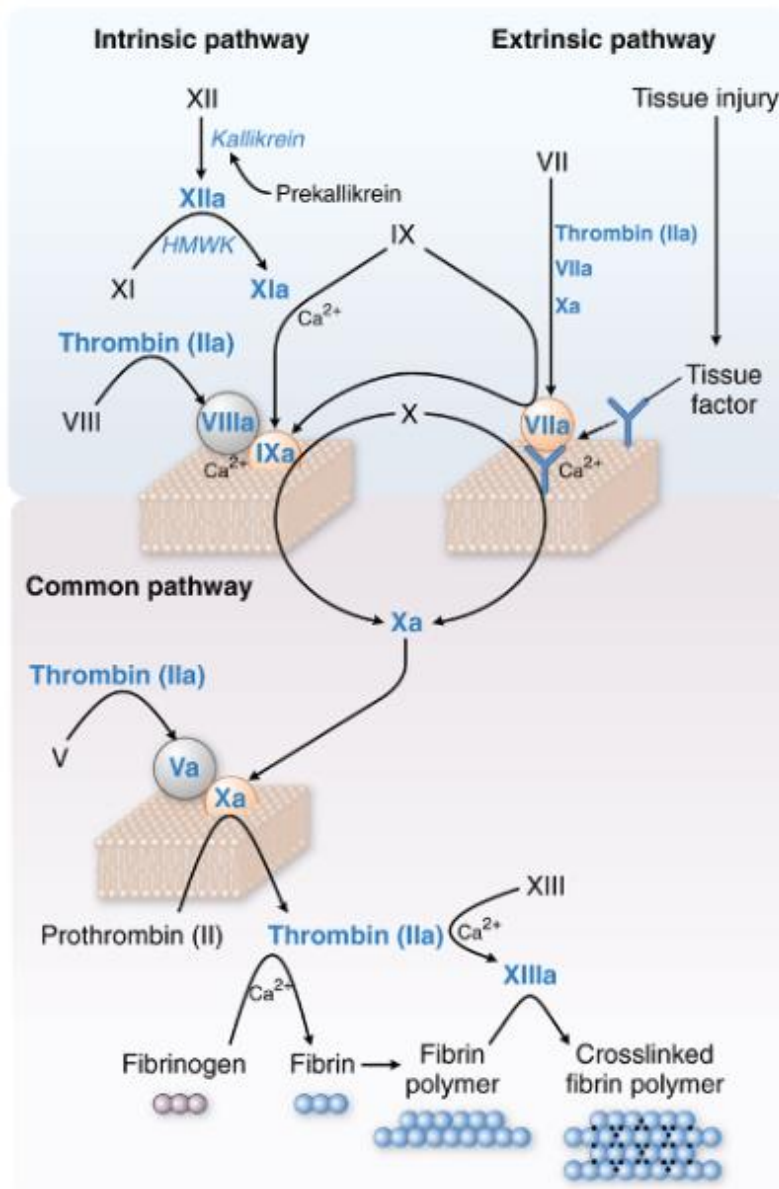


Figure 1: Coagulation cascade (Golan DE, et al. 2011)

1.1.4 Regulation of Hemostasis

When a clot is formed, there has to be some mechanism, which limits the clot to the site of an injury and ultimately leads to its removal when the injury has healed (Lefkowitz JB. 2008). In greater detail this implicates two considerations. First, hemostasis must be restricted to the local site of vascular injury. That is, activation of platelets and coagulation factors in the plasma should occur only at the site of endothelial damage, tissue factor expression and procoagulant phospholipid exposure.

Introduction

Second, the size of the primary and secondary hemostatic plugs must be restricted so that the vascular lumen remains patent.

After vascular injury, intact endothelium in the immediate vicinity of the injury becomes activated. This activated endothelium features a set of procoagulant factors that promote hemostasis at the site of injury, and anticoagulant factors that restrict propagation of the clot beyond the site of injury. The procoagulant factors, such as tissue factor and phosphatidylserine, tend to be membrane-bound and localized at the site of injury—these factors provide a surface on which the coagulation cascade can proceed. In contrast, the anticoagulant factors are generally secreted by the endothelium and are soluble in the blood. Thus, the activated endothelium maintains a balance of procoagulant and anticoagulant factors to limit hemostasis to the site of vascular injury (Golan DE, et al. 2011).

Uncontrolled coagulation could lead to thrombosis or embolism. To prevent these, deregulation of hemostasis is required. This is on the one hand performed by inhibition of the coagulation process and on the other hand by the dissolution of the fibrin clot (fibrinolysis)

The initiation of inactivation steps is indeed simultaneously with those of activation. But it runs through additional intermediate steps and depends on the concentrations of individual factors. This results in a time slot until the inhibition starts. Hence, sufficient blood coagulation is ensured. In addition, only by binding to the injured tissue and their subsequent shape change, platelets create the requirement for clotting. Therefore just when this has happened coagulation factors and also tenase- or prothrombinase-complex can bind and exert their effect. As a consequence, the coagulation remains local to the injury (Brackmann HH, et al. 2003).

After vascular injury, the endothelium surrounding the injured area participates in several separate mechanisms that limit the initiation and propagation of the hemostatic process to the immediate vicinity of the injury. These mechanisms involve prostacyclin (PGI₂), antithrombin III, proteins C and S, tissue factor pathway inhibitor (TFPI), and tissue-type plasminogen activator (t-PA).

Prostacyclin (PGI₂) is an eicosanoid (i.e., a metabolite of arachidonic acid) that is synthesized and secreted by the endothelium. By acting through G_s protein-coupled platelet-surface PGI₂ receptors, this metabolite increases cAMP levels within platelets and thereby inhibits platelet aggregation and platelet granule release. PGI₂ also has potent vasodilatory effects; this mediator induces vascular smooth muscle relaxation by increasing cAMP levels within the vascular smooth muscle cells. (Note that these mechanisms are physiologically antagonistic to those of TxA₂, which induces platelet activation and vasoconstriction by decreasing intracellular cAMP levels.) Therefore, PGI₂ both prevents platelets from adhering to the intact endothelium that surrounds the site of vascular injury and maintains vascular patency around the site of injury (Golan DE, et al. 2011).

1.1.4.1 Coagulation Inhibitors

Protein C is a vitamin K-dependent serine protease zymogen. **Protein S**, a vitamin K-dependent glycoprotein, acts as a cofactor for APC (activated protein C) (Colman RW, et al. 2005). These proteins slow the coagulation cascade by inactivating coagulation factors Va and VIIIa. Protein C and protein S are part of a feedback control mechanism, in which excess thrombin generation leads to activation of protein C, which in turn helps to prevent the enlarging fibrin clot from occluding the vascular lumen.

Specifically, the endothelial cell-surface protein **thrombomodulin** is a receptor for thrombin and protein C in the blood. (Golan DE, et al. 2011). When thrombin binds to thrombomodulin, thrombin loses its substrate specificity and is no longer able to activate fibrinogen, FV, FVIII, FXI and the platelets (Brackmann HH, et al. 2003). Thrombomodulin-bound thrombin cleaves protein C to activated protein C APC. In a reaction that requires the cofactor protein S, activated protein C then inhibits clotting by cleaving (and thereby inactivating) factors Va and VIIIa (Golan DE, et al. 2011).

Antithrombin III is a plasma proteinase inhibitor, a serpine (SERine Protease Inhibitor), which inactivates thrombin and the enzymes responsible for the generation of thrombin. This combination of properties makes ATIII a very powerful and important endogenous anticoagulant molecule (Colman RW, et al. 2005). Antithrombin III inactivates thrombin and other coagulation factors such as IXa, Xa, XIa, and XIIa (where

Introduction

“a” denotes an “activated” factor) by forming a stoichiometric complex with the coagulation factor (Golan DE, et al. 2011). The catalytic-site serine of thrombin reacts with an arginine in the active center of ATIII to form a covalent inactive complex (Colman RW, et al. 2005). These interactions are enhanced by a heparin-like molecule that is expressed at the surface of intact endothelial cells, ensuring that this mechanism is operative at all locations in the vascular tree *except* where endothelium is denuded at the site of vascular injury. (These endothelial cell surface proteoglycans are referred to as “heparin-like” because they are the physiologic equivalent of the pharmacologic agent heparin.) Heparin-like molecules on the endothelial cells bind to and activate antithrombin III, which is thus primed to form a complex with the activated coagulation factors (and thereby inactivate them) (Golan DE, et al. 2011). Once thrombin is bound to fibrin, it is resistant to ATIII and even more so to ATIII-heparin complex (Colman RW, et al. 2005).

Tissue factor pathway inhibitor (TFPI) is a Kunitz-type serine protease inhibitor, which modulates initiations of coagulation induced by TF. In a FXa-dependent feedback system, TFPI binds directly and inhibits the TF-FVII/FVIIa complex.

TFPI consists of three multivalent Kunitz-type domains with an acidic amino-terminal region and a basic carboxy-terminal end. The first domain binds to FVIIa and the second binds to and inhibits FXa. The function of the third domain may be involved in the association of TFPI with lipoproteins. The basic C-terminus of TFPI is required for binding the TFPI to cell surfaces, and cell-bound TFPI mediates the internalization and degradation of FX and the down-regulation of surface TF-FVIIa activity.

The half-life of TFPI is rather short (60-120 min). The total normal plasma TFPI concentration is about 1,0 – 2,5 nM. The average activity in normal plasma is by definition 1 U/mL.

Normal human plasma TFPI exists in both full-length and variably carboxy-terminal truncated forms. In vivo, approximately 80% of plasma TFPI circulates as a complex with plasma lipoproteins, of which the major portion is bound to LDL (low-density lipoprotein). The most predominant forms of plasma TFPI have a molecular weight of 34-41 kDa. About 5-20% of plasma TFPI circulates free, uncomplexed, containing both

full-length and c-terminal truncated forms. Lipoprotein-associated TFPI has less anticoagulant effect than free TFPI. By the separation of LDL-bound and –free TFPI from human plasma, it could be discovered, that the inhibitory activity for TF-induced coagulation was limited to the LDL-free fraction. Furthermore, TFPI truncated at the C-terminus is a poor inhibitor of blood coagulation.

TFPI is mainly produced by and bound to the vascular endothelium, possibly by glycosaminoglycans. Moreover it is pooled in plasma and platelets. TFPI is also produced by the liver and by monocytes/macrophages (Lwaleed BA and Bass PS. 2005).

Tissue factor pathway inhibitor (TFPI), as its name indicates, limits the action of tissue factor (TF). The coagulation cascade is initiated when factor VIIa complexes with TF at the site of vascular injury. The resulting VIIa:TF complex catalyzes the activation of factors IX and X. After limited quantities of factors IXa and Xa are generated, the VIIa:TF complex gets feedback inhibited by TFPI in a two-step reaction. First, TFPI binds to factor Xa and neutralizes its activity in a Ca^{2+} -independent reaction. Subsequently, the TFPI:Xa complex interacts with the VIIa:TF complex via a second domain on TFPI, so that a quaternary Xa:TFPI:VIIa:TF complex is formed. The molecular “knots” of the TFPI molecule hold the quaternary complex tightly together and thereby inactivate the VIIa:TF complex. In this manner, TFPI prevents excessive TF-mediated activation of factors IX and X (Golan DE, et al. 2011).

Thus, TFPI switches the majority of the coagulation process from TF-FVII-dependent system to the lower part of the previously known intrinsic pathway of blood coagulation. TFPI recognizes FXa most effectively when the enzyme is in the prothrombinase complex, which contains FXa, FV, calcium ions and phospholipids. The optimal inhibition of TF-FVIIa activity is obtained by the full-length TFPI molecule rather than its C-terminus truncated form, hence the higher rate formation between the full-length TFPI molecule and FXa (TFPI-FXa complex). Thus, the TFPI-FXa complex is a much more potent inhibitor of the TF-FVIIa complex than TFPI alone (Lwaleed BA and Bass PS. 2005).

1.1.4.2 Fibrinolysis

Fibrinolysis is the ultimate and last mechanism for limiting clot formation. It also constitutes a repair mechanism along with endothelial cell regrowth and vessel recanalization. The dissolution or solubilization of fibrin clot at the correct time is crucial for the orderly process of wound healing (Colman RW, et al. 2005). Fibrinolysis starts at the same moment as the activation of coagulation. This is due to the fact that there is no excessive increase of the clot.

Plasmin is the key enzyme, a serine protease, of fibrinolysis. Various activators from the precursor plasminogen form it. In addition to the physiological activator of the urokinase type, or the activators tissue-type, the t-PA (tissue plasminogen activator), the non-physiological activator, streptokinase is the most significant (Witt I and Dörner K. 2006). Plasmin proteolytically cleaves fibrin in fibrinogen. Degradation products inhibit the proteolytic activity of thrombin and also prevent the polymerization of the fibrin monomers. Fibrinolysis itself is regulated or inhibited by α_2 -antiplasmin, α_2 -macroglobulin and plasminogen activator inhibitor (PAI).

The fibrinolytic system is regulated by the thrombin-activated fibrinolytic inhibitor (TAFI). This ensures that the clot is not dissolved too early. TAFI is a zymogen (i.e. procarboxypeptidase) and is activated by thrombin in the presence of thrombomodulin. TAFIa reduces the binding sites of t-PA, plasminogen and plasmin on the fibrin surface. Thus the resistance of the clot against proteolytic cleavage by plasmin is enhanced (Dempfle CE. 2007).

1.1.5 New concept of coagulation

Modern concepts of blood coagulation assume that tissue injury causes a release of tissue factor. TF, together with platelets, is supposed to be responsible for the localization of coagulation and its control (Roberts HR, et al. 2006).

After the successful release of TF from TF-bearing cells (monocytes, fibroblasts, endothelial cells (smooth muscle cells)), TF forms together with FVIIa a complex, which results in the activation of factor X and IX. On the surface of TF-bearing cells FXa, in complex with FVa, activates small amounts of prothrombin to thrombin (Hoffman M, et al. 1995). This small amount of thrombin is not sufficient to form a first clot yet, but is able to activate the platelets and factor V, VIII and XI. This part of coagulations is called the initial phase (thrombin generation). Activated co-factors (FV and FVIII) in complex with previously activated factors (factor IX and X) lead to a greater activation of prothrombin on the surface of activated platelets. FXIa additionally boosts the generation of thrombin because of the activation of FIX. Due to this enhancement there is an explosive increase of thrombin generation. This is the second phase of thrombin generation. Thrombin now is sufficiently present to form a clot and to activate TAFI. TAFI protects the clot from early proteolysis by plasmin, because the fibrinolytic system is activated simultaneously with the tissue injury. FXIIIa (also activated by platelets) and the retraction of platelets lead to a stabilization of the clot.

After the maximum of thrombin generation has been achieved, the thrombin concentration gradually decreases by various thrombin-degradation mechanisms (see chapter 'Regulation of Hemostasis').

1.2 Hemophilia

Hemophilia is a congenital coagulopathy, which manifests itself in an increased bleeding tendency. This can be life threatening in severe cases sometimes. There are two types of this disease, hemophilia A and B.

Patients with hemophilia A suffer from a lack of factor VIII. This type is most common form and occurs with a probability of 1: 10.000. Hemophilia B occurs much less frequently. These patients suffer from a lack of factor IX. Both types of this disease are X-linked and inherited recessively. They can also occur spontaneously.

Hemophilia is caused by different defects in the factor VIII or IX genes. Therefore, the activity or the occurrence of the two factors can be restricted differently, depending on the severity of the disease.

The defect in the FVIII gene can be triggered by a variety of mutations (e.g. point mutations, deletions, insertions, (especially intron-22-) inversion). Therefore a mutation analysis can predict a value indicating the severity or the risk of inhibitors (see below). The severity is dependent on the mutation and therefore remains the same within the family and doesn't change in a with hemophilia diseased patient's life. At about 30 percent of patients the disease occurs without corresponding family history, which may be due to spontaneous mutations.

Both genes, FVIII gene as well as FIX gene, are located on the long chain of the X-chromosome. Thus, primarily males (genotype XY) are concerned with this disease as for females (genotype XX) the second X chromosome is usually unchanged. Therefore, women with hemophilia are very rare. This can only happen, if, for example, both parents have hemophilia A or the second X chromosome is defective (e.g. Turner Syndrome). Also due to lyonisation (Lyon MF. 2002) there can occur very low FVIII concentrations. However these cases are very rare. Most of the time, women with hemophilia are only the carrier (conductor) of the defective gene. Thereby, they pass

Introduction

the defective gene to their children with a 50 percent probability. Then again sons of a hemophile father are healthy, whereas daughters are potential carriers of the gene.

FVIII is an essential factor of the coagulation system and circulates in small amounts in the blood. It is very labile in its structure and is protected from proteolysis by complex formation with VWF. The close contact with VWF protects FVIII not only from its degradation, VWF leads it to the location of coagulation. During the coagulation cascade FVIII accelerates - as a co-factor of activated factor IX (FIXa) - the activation of FX and thereby subsequently the formation of thrombin. For a normal hemostasis a thrombin generation, running particularly rapid and in sufficient concentrations is essential. So it becomes clear that the primary (cellular) hemostasis takes place under normal conditions in hemophile patients, whereas the secondary (plasmatic) coagulation is disturbed. The combined activation of FX by FIXa and FVIIIa causes, from a clinical point of view, no difference between hemophilia A and B, since both factors are involved at the same stage of the cascade.

The severity of hemophilia A is determined by the concentration (or residual activity) of factor VIII. One milliliter (normal) plasma contains one unit (U) of the factor – therefore, $100\% = 1\text{E} / \text{mL}$. A FVIII concentration of less than 1 percent is referred to as “severe hemophilia”. It manifests itself by frequent spontaneous bleeding (caused by micro trauma), especially in the joints, but also in the muscles. The dangerous, but relatively rare intracranial bleeding can be lethal when untreated. The joint bleeds can lead to synovitis, which can trigger a hemophilic arthropathy (i.e. cartilage damage that might even lead to its dissolution). Most often, the knee, elbow- and ankle joints are affected.

Patients with moderate hemophilia have a concentration of one to five percent factor VIII in the plasma. Here serious bleeding after injuries or operations constitutes a greater threat than spontaneous bleeding. The latter only occurs rarely in patients with

Introduction

light form hemophilia (FVIII concentration over 5 to about 40%). Basically for those patients, the disease is hardly noticeable in everyday life. Only bad injuries and surgeries can cause serious bleeding.

Global tests of coagulation can give indication of a bleeding tendency. Quick-test, the bleeding time and fibrinogen are normal in hemophilia A and B, whereas the APTT is prolonged. The specification is then carried out by analysis of the individual factors. There is also the possibility of prenatal diagnostic. Mostly it is only carried out when there is suspicion of a severe hemophilia because of the high risk of miscarriage.

The basic treatment of hemophilia is the intravenous application of the missing factor. The factor can be genetically engineered (recombinant factors) or derived from human plasma. Regardless of the production, attempts are made to ensure the highest possible security, as, especially with plasma-derived proteins, the risk of transmission of highly infectious viruses in the past, before implementation of effective pathogen inactivation or removal methods, was very high. The corresponding factor is given until cessation of bleeding. Nowadays the preferred treatment mode is prophylaxis (e.g. prevention of joint damage especially in the infant and toddler age). As a complementary therapy, antifibrinolytic agents (e.g. tranexam acid) and desmopressin analogues may be indicated. The intake of irreversible but also reversible platelet aggregation inhibitors (acetylsalicylic acid and derivatives or NSAIDs) is contraindicated.

1.2.1 Development of inhibitors

A possible complication is the development of inhibitors as a result of the response of the immune system. The inhibitor (alloantibody) is directed against the substituted factor and appears very early in life or after a few days of exposure (Lusher JM. 2000). The risk of formation of such antibodies is the highest during the first 20 to 50 treatments and decreases with the duration of the treatment (Kempton CL, et al. 2006). Hemophilia A patients have a significantly higher risk to develop inhibitors than hemophilia B patients. However, the clinical picture is the same. The symptoms correspond with those without inhibitors, but the bleeding is much more difficult to stop. The cause of the development of inhibitors is largely unknown, but is assumed to be genetic. The days of exposure and the nature of preparation are considered to be possible reasons (Goudemand J, et al. 2006). Therefore, inspections are carried out on a regular basis to check on possible inhibitor formations. Usually inhibitors are discovered, when the normal dose of factor does not help anymore to eliminate the symptoms.

Approximately 30 percent of patients suffering from severe forms of hemophilia A, show such formations of antibodies. The larger the defect in the FVIII gene, the higher is the risk antibodies appear (Oldenburg E and Tuddenham E. 2005). Two thirds of these patients have antibodies with medium to low titer. That means, patients have an inhibitor titer less than five Bethesda Units (BU). These are referred to as "low responders" and only very rarely show an anamnestic immune response. The last third of patients develop high-titer inhibitors ("high responder" - about 5 BU). Here, a further exposure of the clotting factor leads to a large increase of the titer.

Inhibitors in mild to moderate forms of hemophilia A are formed in only about 12 percent of patients, especially after high doses of factor over a longer period (Sharathkumar A, et al. 2003) / (White B, et al. 2000). The symptoms correlate with

those of acquired inhibitors (see below) and often disappear after prolonged FVIII abstinence.

1.2.2 Acquired inhibitors

Inhibitors are referred to as acquired inhibitors, when non-hemophiliacs are developing antibodies against a blood-clotting factor. Most often inhibitors are directed against FVIII. They are autoantibodies (polyclonal immunoglobulins G (IgG)), which are directed against special epitopes of the FVIII molecule. Triggering factors can be various underlying diseases but also an idiopathic occurrence. Diseases, which can be associated with acquired inhibitors, for example are: other autoimmune diseases (rheumatoid arthritis, lupus erythematosus), postpartum onset, and malignant tumors, caused by medication. After therapy of the primary disease, but also spontaneously, these inhibitors may disappear.

This nature of the inhibitor predominantly occurs in old age and is characterized by severe bleeding into the muscles and skin and less into the joints.

The basic diagnosis of the two types of inhibitors corresponds to the diagnosis of hemophilia without antibodies. The APTT is prolonged, whereas the Quick test, the determination of fibrinogen and the platelet concentration are within the reference range. For testing of inhibitors the Bethesda assay (Kasper CK, et al. 1975), predominantly in the Nijmegen modification (Verbruggen B, et al. 1995), is used. It improves the specificity in the lower range of titer. The result of this assay is given in Bethesda Units (BU). One BU is the amount of inhibitor, which reduces the activity of FVIII in a normal plasma pool after two hours of incubation at 37°C by 50%. Those antibodies, which occur in severe hemophilia, show a linear course of reaction (type I kinetics) in Bethesda assay - complete inhibition of FVIII activity. Antibodies of mild to

Introduction

moderate hemophilia as well as acquired antibodies usually have a non-linear response curve (type II kinetics) - incomplete inhibition of FVIII activity.

For the treatment of low titer antibodies further substitution of the corresponding factor, in high-doses, may bring success. In high-titer antibodies, hemostasis is achieved by using bypass preparations. They can trigger hemostasis without the presence of FVIII or FIX. Available preparations are activated prothrombin complex concentrate (APCC) and recombinant factor VIIa (rFVIIa). While the recombinant FVIIa is a single plasma protein, the APCC is composed of several procoagulant proteins.

Another treatment option represents the inhibitor-elimination (or reduction) by means of immunological tolerance therapy (ITT) or apheresis with immunoadsorption of the inhibitor by means of animal antibodies against IgG, bound to a pillar (Jansen M, et al. 2001). After FVIII abstinence, when the inhibitor titer has reached a plateau, FVIII is given along with APCC or rFVIIa in high-doses in ITT. This is to establish an immune tolerance against FVIII.

After cross-reactivity is checked and if it is low or even absent, also porcine FVIII is indicated for high responder. In fewer than five percent of the cases this treatment results in an anamnestic response. For hemophiliacs with autoantibodies an immunosuppressive therapy often proves to be successful (e.g. with prednisolone or cyclophosphamide).

1.3 Activated Prothrombin Complex Concentrate (APCC)

A detailed investigation of this pharmaceutical product is the aim of this diploma thesis. APCC is a purified intermediate protein concentrate isolated from human plasma.

APCC has a history of more than 30 years of successful use in controlling bleeding in hemophilic patients who have developed inhibitory antibodies against factor VIII or FIX (Turecek PL, et al. 2004). APCCs are capable of inducing thrombin generation by direct activation of FX without the necessity of participation of factor FVIII or FIX (Turecek PL, et al. 1999). Therefore they are able to trigger the coagulation bypassing these two factors.

Although APCC is produced with a dedicated activation step, the protein composition still retains a mixture of prothrombin, complex zymogens and their activation products. Therefore it is standardized to a sum activity: by definition 1 unit of activity expresses the amount of APCC that shortens the APTT (activated partial thromboplastin time) of high-titer FVIII inhibitor reference plasma to 50% of its blank value (= test control without APCC).

Several studies have shown that FXa and prothrombin play a central role in the activity of APCC. Consequently, they are considered to be the key components of this product. The prothrombinase complex has been found to be the major target site for APCC (Turecek PL, et al. 2004). This complex triggers the formation of small amounts of thrombin on the surface of TF-bearing-cells and activated platelets, but only in the presence of FV (Mann KG, et al. 1981). Thrombin causes satisfying coagulation by various amplifier loops, sometimes together with other coagulant proteins of APCC, but also with those of the patient. In more detail, the following reactions take place more or less simultaneously inducing the FVIII bypassing activity of APCC:

1. Initiation of the common pathway by allowing prothrombinase complex formation and activity.
2. Initiation of the intrinsic pathway by thrombin-dependent feedback activation.
3. Initiation of the extrinsic pathway by FXa-dependent activation of FX (Turecek PL, et al. 1999).

Figure 2: Schematic model of the mechanism of action of activated prothrombin complex concentrate (APCC)

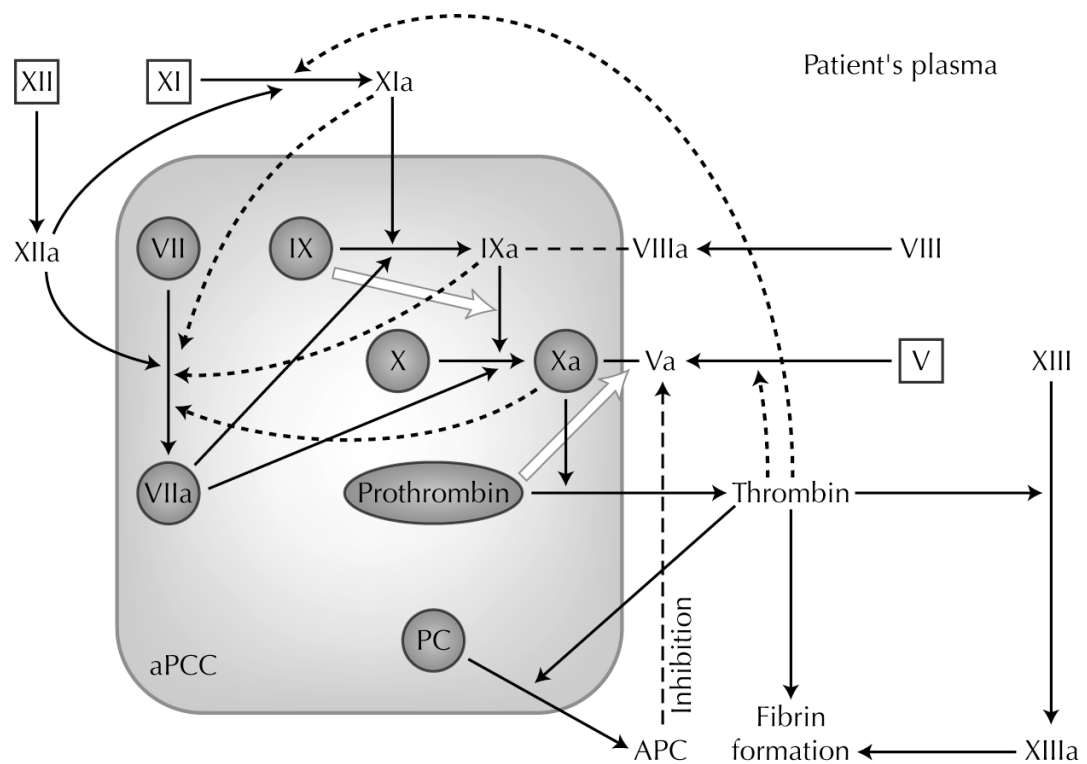


Figure 2: Procoagulant zymogens and enzymes of the prothrombin complex (circles) as they interact with factors required from the patient's plasma (squares); full arrows represent activation reactions in the coagulation cascade and dotted arrows represent feedback reactions triggering the prohemostatic process initiated by APCC. Currently, two additional possible types of feedback reactions are described by which the zymogen components prothrombin and factor IX can potentiate the prohemostatic processes. A number of proteins and coagulation enzymes inhibit the prohemostatic process by interfering at multiple stages with the reactions described in the model; only the inhibition process by activated protein C (APC) is shown (double arrow). In addition, specific (e.g., antithrombin) and unspecific coagulation inhibitors (e.g., α 2-macroglobulin) also inactivate the process when a sufficient hemostatic clot has been formed (Turecek PL, et al. 2004). According to recent studies the inhibitor TFPI may also be contained in APCC (Ogiwara K, et al. 2010)

Introduction

In previous research the APCC could be analyzed in its composition. It was found that the coagulant proteins (clotting factors) make up only a small proportion of the total amount of protein. The largest fraction of the APCC, approximately 85% - including inter α - trypsin inhibitor (IATI) and vitronectin (VN) – is made up by other plasma proteins.

The coagulant proteins are composed of the zymogens and their active forms. Activated factor VII (VIIa) is present in a relatively high concentration compared with other activated coagulation factors in APCC. FVIIa is an essential part of the exogenous activation pathway of blood coagulation and is also used in the treatment of hemophilia with inhibitors (Turecek PL, et al. 2004). Moreover, the inhibitors protein C and its cofactor protein S, the clotting influencing components, are contained in the APCC (Váradi K, et al. 2011).

Introduction

Zymogens	Factor II (FII)	1,0 U/ml (0,8 - 1,2 U/mL/ activity)
	Factor VII (FVII)	0,9 U/mL (0,3 – 1,2 U/mL/ activity)
	Factor IX (FIX)	1,6 U/mL (1,2 – 2,1 U/mL/ activity)
	Factor X (FX)	1,0 U/mL (0,7 – 1,1 U/mL/ activity)
Enzymes	Factor IIa (FIIa)	0,0004 U/mL (0,0002 – 0,0005 U/mL/ activity)
	Factor VIIa (FVIIa)	1,7 U/mL (1,5 – 2,0 U/mL/ activity)
	Factor IXa (FIXa)	0,001 U/mL (activity)
	Factor Xa (FXa)	0,0004 U/mL (0,0002 – 0,0005 U/mL/ activity)
Inhibitors	Protein C (PC)	1,3 U/mL (1,0 – 1,6 U/mL/ activity)
	Protein S (PS)	0,4 U/mL (antigen)
Co-factors	Factor V (FV)	0,66 U/mL (antigen)
	Factor VIII (FVIII)	0,075 U/mL (0,03 – 0,12 U/mL/ Antigen)
	Phospholipids (PL)	0,099 nmol (0,0003 – 0,195 nmol)
	Vitronectin (VN)	0,40 mg/mL (0,29 – 0,47 mg/mL/ Antigen)
	Inter α -trypsin inhibitor	0,69 mg/mL (0,42 – 0,84 mg/mL/ Antigen)

Table 2: Components of the APCC and their averaged concentrations of several APCC lots (minimum – maximum); the values are referred to 1 unit (U) of the APCC (Montsch P. 2010)

Additionally, recent data confirm the presence of TFPI in APCC. Furthermore, it is suggested that TFPI contained in APCC attenuates the potentials of thrombin generation in hemophilia A patients with inhibitors (Ogiwara K, et al. 2010). A detailed investigation of TFPI contained in APCC and its influence on the effect of APCC in patients was the aim of my diploma thesis.

1.4 Recombinant factor VIIa

Recombinant factor VIIa (rFVIIa) is derived from baby hamster kidney cells. It is also used for the treatment of hemophilia with inhibitor formation. Recombinant FVIIa is used as a single agent and exerts its pharmacological action only at a supra-physiological plasma level. Therefore, the usual pharmacological doses make up 10-50 nmol / L, while the physiological concentrations of FVIIa are 0.075 nmol / L. The effect of FVIIa greatly depends on tissue factor at physiological concentrations (Butenas S, et al. 2002). FVIIa activates factor X only after complex formation with TF. This represents the initial stage of the blood coagulation.

Due to the increasing concentration of FVIIa to a multiple of its physiological concentration, a TF-independent pathway is triggered (Monroe DM, et al. 1998). Recombinant FVIIa can directly bind to the phospholipid structure of activated platelets and activates, instead of the tenase complex, factor X (Monroe DM, et al. 1997) / (Hoffman M, et al. 1994). Activated FX itself activates, in complex with factor Va, prothrombin to thrombin. Thus, an activation pathway, independent of the activity of factors VIII and IX, is created. Because of the high level of FVIIa a complex formation with TF is enhanced thus triggering blood coagulation.

2 Materials and Methods

2.1 Thrombin Generation Assay

Hemophilia A patients with inhibitors are generally treated with preparations containing activated coagulation factors, which are intended to achieve hemostasis independently of factor VIII through bypassing mechanisms. Factor VIII bypassing agents, such as activated prothrombin complex concentrates (APCC), have multiple modes of action including triggering the formation of prothrombinase complex with subsequent thrombin generation, which potentiates feedback reactions mediated by thrombin and activated FX. The recombinant activated FVII preparation also has at least two models of action; FX activation dependent on tissue factor (TF) and at high doses a TF-independent reaction in which FVIIa binds to endogenous phospholipid surfaces and activates FX. Both therapeutic agents are able to bypass FVIII by enhancing thrombin formation independently of FVIII (Váradi K, et al. 2003). The information obtained from most assays to measure the hemostatic response to inhibitor-bypassing agents is limited to information about the initial phase of blood coagulation (clotting time measurement) or the formation of a solidifying clot followed by fibrinolysis (thrombelastography), and excludes the real endpoint of thrombin generation. Thrombin generation assay (TGA) measures the whole kinetics of thrombin generation even after the clot formation, and thus assess all activating and inactivating systems of coagulation (Váradi K, et al. 2004).

The basic principles of these tests are based on the TGA model developed by Hemker (Hemker HC, et al. 2000). These fundamentals were developed with a chromogenic substrate (Hemker HC, et al. 1993). For the measurement of the optical density using the chromogenic substrate, a clear solution is the precondition. When the blood clots, it becomes cloudy and opaque. Therefore, first of all fibrin must be removed or a polymerization suppressed. Again, this is not possible without the platelets being removed. The advantage of the fluorogenic substrate is that it is not dependent on the turbidity of the sample solution. This allows the use of PRP or pure blood instead of PPP when fluorogenic substrate is used.

PPP can be used to measure hypocoagulation (hemophilia observation of FVIII inhibitor bypassing therapy (Turecek PL, et al. 2003), therapy with oral anticoagulants, etc.) and hypercoagulation (protein C/S deficiency, factor V Leiden, oral contraceptives). Above all, PPP is used for the determination of the functionality of coagulation factors, regardless of the cellular components. Therefore PPP was used for the determination of APCC as the influence of coagulation factors contained in APCC and their interactions with each should be studied in this work. This plasma is normal human plasma, in which factor VIII has been depleted using an antigen- antibody reaction. In addition, this FVIII-deficient plasma is enriched with high titer human FVIII inhibitor plasma in goat plasma (FVIII inhibitor plasma). The result is a plasma, which corresponds to the patient's plasma, in which the commercial APCC is used as a medicament.

The use of PPP assumes the addition of coagulation-active phospholipids (PL). As a consequence the procoagulative activity of the phospholipids exists from the time of phospholipid addition onwards. In PRP or normal blood, this activity would gradually appear in the course of the activation process of blood clotting when platelets provide PL because of their activation.

Hemostasis is activated by the addition of a special low-concentration tissue factor-phospholipid preparation (TF/PL) (Váradi K, et al. 1999) for FVIII inhibitor plasma. The phospholipid solution is composed of 80% of PC (1-palmitoyl-2-oleoyl-phosphatidylcholine) and 20% of PS (1-palmitoyl-2-oleoyl-phosphatidyl-serine). At the same time a solution of 1mM thrombin substrate and 15mM CaCl_2 is added (the entire reaction proceeds at 37°C). As a consequence, by thrombin-mediated cleavage of the fluorophore from the substrate, the fluorescence signal begins to rise. The amount of substrate, which was implemented by thrombin during the experiment, therefore corresponds to the total enzymatic work of thrombin. The fluorescence intensity is measured with a Microplate Fluorescence Reader FL600 (Bio-TEK Instruments) with an excitation wavelength of 360nm and an emission wavelength of 460nm and calculated with a kinetic program (RFU - relative fluorescence unit) (Figure 3).

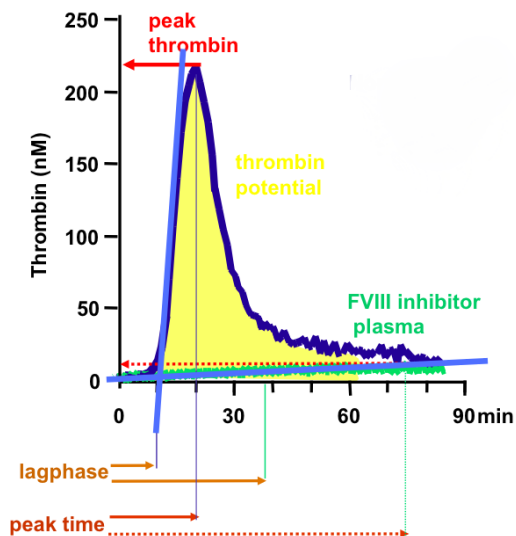


Figure 3: Thrombin generation curves of normal plasma and FVIII inhibitor plasma, and parameters of thrombin generation which can be read from the thrombin generation curve

The way thrombin gets activated is substantially dependent on the trigger factor. The TF/PL were chosen as activators of TG, because they represent the physiological pathway of coagulation activation. An excess of TF triggers the extrinsic pathway. In this case, mainly factor VII, V, X and TFPI are involved. The lower the concentration of TF is, the stronger are the factors VIII, IX, and XI included (Cawthorn KM, et al. 1998) / (Osterud B and Papaport SI. 1977). In the absence of TF the intrinsic pathway is taken, if the concentration of PL is sufficient. This pathway causes thrombin formation after about ten minutes (Hemker HC, et al. 2003) / (Chantarangkul V, et al. 2003). The triggering by low TF concentration rather reflects the physiological situation (Fareed J, et al. 1995). For phospholipids their composition as well as their concentration are crucial (Hemker HC, et al. 2003) / (de Smedt E, et al. 2008). The composition and concentration of TF and PL was chosen so that the in vivo situation largely was mimicked. These adjustments resulted in thrombin production in normal plasma and no thrombin production in FVIII inhibitor plasma (Turecek PL, et al. 2003).

The addition of calcium is essential, because it is an important binding factor during the blood coagulation (see chapter 'Plasmatic Hemostasis').

The substrate is a highly specific peptide substrate. Its properties allow a continuous measurement. The oligopeptide determines the specificity (Hemker HC and Béguin S. 1990). It has bound a fluorophore (amino-methyl-coumarin), which gets cleaved by

thrombin. For a continuous measurement, the affinity of the substrate to the enzyme, thrombin, is important to be low (Turecek PL, et al.) / (Hemker HC, et al. 1993). This low affinity results in a slow reaction with the formed thrombin (Turecek PL, et al.). Thus, the amount of the converted substrate by thrombin remains proportional to the reaction rate (Hemker HC, et al. 1993) / (Wielders S, et al. 1997).

The peptide substrate is cleaved by thrombin, and the fluorophore is liberated. The development of the fluorescence intensity at 37° C, which is an indicator of the concentration of the generated thrombin, is monitored continuously. The rate of development of fluorescence intensity was calculated for each reading (RFU/min) and converted to thrombin equivalent concentrations (nM) using a reference curve.

The thrombin reference curve is created with a known concentration of purified human α -thrombin. The thereby obtained thrombin curve, more specifically, the first derivative of the curve obtained previously, illustrates the conversion of prothrombin to thrombin. Furthermore, it shows the thrombin-induced reactions and subsequent degradation of thrombin.

There are different parameters of TG, which characterize the TG curve and therefore can be analyzed quantitatively by the following parameters: onset time, peak time, peak thrombin, thrombin potential and velocity index.

The peak thrombin signifies the highest thrombin concentration reached during the time course of thrombin formation and inhibition. The time taken to reach approximately 5 - 10% of active thrombin is characterized as onset time and shows how quickly thrombin is formed. It points out the initial thrombin formation. The lag phase shows how fast thrombin starts to generate. It's the rate of burst thrombin generation. The peak time is when the maximum thrombin activity (peak thrombin) is measured. The velocity index can be calculated with three parameters: peak thrombin, peak time and onset time. It shows the maximum speed of thrombin generation (nM/min). After the peak thrombin is reached the physiological inhibitor system is activated (ATIII/ APC-PS/ TFPI/ α 2-macroglobulin) and thrombin concentration decreases. This inactivation rate represents the efficiency of the inactivation system. The thrombin potential (expressed in FU) is the amount of thrombin that is entirely

formed within a certain period, e.g. 60 min. Its value corresponds to the area under the curve.

After all the free thrombin is inactivated, there still can be measured a residual amidolytic activity. This is caused by the α 2-macroglobulin-thrombin complex bound thrombin (Hemker HC and Béguin S. 1995). Two different processes degrade thrombin. On the one hand, antithrombin and other proteases degrade thrombin into an inactive product and on the other hand, the complex formation with α 2-macroglobulin also leads to an inactive product for blood clotting, but still has a residual amidolytic activity. Consequently, the fluorogenic thrombin substrate is implemented by two enzymes, the free thrombin and the α 2-macroglobulin-thrombin complex bound thrombin. Therefore, the rate of increase of the fluorescence signal is the amidolytic activity of both enzymes (Turecek PL, et al. 2003).

2.1.1 Reagents and Equipment

- Distilled water

Reagents: the lyophilized TGA reagents are manufactured by Technoclone

- TGA Substrate: Technoclone TGA Substrate, fluorogenic substrate/ CaCl_2 , 1mM Z-Gly-Gly-Arg-AMC/ 15mM CaCl_2 , # 8F12B00
- Tissue factor /Phospholipid Reagent: Technoclone, TGA – TF/PL-Reagent RC low #7D12B00
- Tissue factor /Phospholipid Reagent: Technoclone, TGA – TF/PL-Reagent RC high #7F12B00
- HNaBSA-buffer: Technoclone TGA-buffer, Hepes-NaCl-buffer (25mM Hepes / 175 mM NaCl , pH 7.35, 0.5% Bovine Serum Albumin (BSA)) #9P01B00
- Thrombin solution: for reference curve, dissolved in 0.5% BSA-solution, according ERL-certificate: 1000nM, #HT3832AL

Materials and Methods

Controls:

- TGA Control High, # 1B12B00, Range Peak Thrombin (nM) with RC low: 330nM (231 - 429 nM)
- TGA Control Low, # 2B02BRV, Range Peak Thrombin (nM) with RC low: 20nM (16-26 nM)
- Normal plasma, pooled, George King # 2240
- Normal plasma FACT, George King # 2150

FVIII inhibitor plasma:

- #3V1A000, #3V1B000, Baxter, immunodepleted, 50 BU/mL
- #GK1838-2468, George King, native, 50 BU/mL

Coagulation factors:

- TFPI: hum. full-length Tissue Factor Pathway Inhibitor, hTFPI positive control Nr. 4900PC, 100 µg/mL, American Diagnostic
- Human Prothrombin, #HP4040L, 100 U/mL; CoaChrom Diagnostica GmbH
- Human alpha Thrombin, #HT3832AL, 1000 NIH U/mL; CoaChrom Diagnostica GmbH
- Human Factor V, #D00015361; 300 U/mL; Calbiochem
- Human Factor VII, #HFVII3341L, 200 U/mL; CoaChrom Diagnostica GmbH
- Human Factor VIIa, Baxter int. Standard #H091R00, 28000 U/mL
- Human Factor IX, #HFIX3920AL, 100 U/mL; CoaChrom Diagnostica GmbH
- Human Factor IXa, #HFIXab3950AL, 100 U/mL; CoaChrom Diagnostica GmbH
- Human Factor X; #HFX4000AL, 100 U/mL; CoaChrom Diagnostica GmbH
- Human Factor Xa, #HFXa4040AL, 100 U/mL; CoaChrom Diagnostica GmbH
- Human Protein C, #HPC3190AL, 100 U/mL, ERL
- Human Protein S, #HPS3724AL, 100 U/mL, CoaChrom Diagnostica GmbH
- Phospholipid 80/20, Baxter produced: 06.11.2007, 1272 µM

Materials and Methods

APCCs

- 9 different APCC batches
- 1 APCC reference standard

Donor Plasma/ Patient Plasma

- Cryocheck Normal Donor Set, #ND32-020, 25 vials of plasma collected from 25 healthy normal male and female donors between 18 and 66 years of age; PrecisionBioLogic Inc.
- Patient plasma: samples of 24 patients - pre / post APCC application. Plasma was collected from subjects with hemophilia A and a high titer inhibitor who participated under informed consent in a recent clinical phase 3 study; Efficacy and Safety Study of Prophylactic Versus On-Demand Treatment With Feiba NF in Subjects With Hemophilia A or B and a High Titer Inhibitor; ClinicalTrials.gov NCT00851721 (Antunes SV, et al. 2014).

Equipment

- Microplate Fluorescence Reader, Synergy 2, software Gen. 5, Vers. 1.10. (BIO-TEK)
- Microtiter plate, 96 wells, black, NUNC Maxisorp
- Water bath with thermostat, HAAKE Mess-Technik GmbH u. Co
- Pipettes: variable piston stroke pipettes, fix volume pipettes, multichannel pipettes with fitting pipette tips, respectively
- Rotilabo-Signal-Timer, LACTAN GesmbH u. CO. KG
- Vortexer

2.1.2 Test Procedure

2.1.2.1 Preparation

The lyophilized reagents are dissolved in the volume indicated on the labels of each reagent with distilled water. The dissolved reagents should reach room temperature before use. After about 30 min reconstitution time and careful mixing (vortex), the reagents can be used.

Vials containing the TGA substrate are placed in the 37°C warm water bath immediately after dissolving and pooling.

APCC batches and various plasmas are heated for 5 minutes in a water bath before starting to work.

When working with procoagulant proteins they are adjusted with buffer to a certain concentration according to the package insert.

2.1.2.2 Procedure

The dilutions of all samples and of thrombin for the reference curve are done with HNaBSA buffer. The Fluorescence Reader is preheated to 37 ° C. After reaching this temperature, a microtiter plate is read empty, first. This is done to ensure that the microtiter plate is not broken.

Before the samples are measured, a thrombin standard curve is created separately. Thus, a serial dilution in the range of 500 nM to 5nM thrombin is done with HNaBSA buffer. Afterwards HNaBSA buffer and TGA substrates as described in the pipetting scheme listed below is added to each dilution. For each dilution double values are taken. The dilution series is applied to the column one and two on the microtiter plate (well A1 to H2), whereas the double values adjoin, respectively.

Materials and Methods

Formula for pipetting	
40 µL	Thrombin
10 µL	HNaBSA buffer
50 µL	TGA substrate

The measurement runs 10 Minutes at 37°C at a certain wavelength according to the test principle. The interval of measurement is 30 seconds.

The measurement must start right after the substrate is added, because the substrate conversion by thrombin begins immediately after its addition.

Afterwards the measurement of samples is done. The run lasts 120 minutes at 37°C and the interval is 60 seconds. Double values are taken here as well. They lie in a line in adjacent columns (well A3 & A4, B3 & B4 or A5 & A6), respectively. Again, the measurement must be started immediately after pipetting the substrate.

The preparation of the test solutions is carried out in suitable plastic test tubes. If the sample preparation is quite time consuming, it is possible to work in the ice water bath. Sample components (e.g. APCC, coagulation factors (e.g. TFPI), synthetic APCC Mix) are spiked in plasma (FVIII inhibitor plasma) in a ratio of 1:20 or 1:10. These dilutions are chosen so as not to change the properties of the plasma even slightly. According to the used combinations, the sample components are previously mixed or diluted with buffer. In the following chapter ('Results') the sample preparation is listed again at each test series in more detail. After diluting the samples they are mixed with HNaBSA buffer and TGA substrate as described in the pipetting scheme listed below and added to the microtiter plate immediately. Next, the run is started straightaway.

Formula for pipetting	
40 µL	Testing solution
10 µL	HNaBSA buffer
50 µL	TGA substrate

2.1.2.3 Validation

The analysis of data was performed using the spreadsheet program Microsoft Excel. With the fluorescence reader (RFU) measured values were converted in RFU/min. These values were converted in a thrombin-equivalent concentration (nM) with the aid of the thrombin reference curve. Therefore, the first derivative was obtained and the course of thrombin generation can be illustrated graphically. The following parameters were used for quantitative evaluation of the thrombin generation curve: onset time, peak time, peak thrombin, and velocity index.

2.2 Determination of total TFPI

The AssayMax Human TFPI ELISA kit is designed for detection of human TFPI in plasma, serum, and cell culture supernatants. In this work there is a special interest to find out more about TFPI in APCC Lots and therefore study the influence of TFPI on the activity of APCC. Consequently, the total TFPI of each of the 9 APCCs and also of the APCC standard was measured.

In this assay a quantitative sandwich enzyme immunoassay technique was used, that measures TFPI in less than 4 hours. A polyclonal antibody specific for TFPI has been pre-coated onto a 96-well microplate with removable strips. TFPI in standards and samples is sandwiched by the immobilized polyclonal antibody and biotinylated polyclonal antibody specific for TFPI, which is recognized by a streptavidin-peroxidase conjugate. All unbound material is then washed away and a peroxidase enzyme substrate is added. The color development is stopped and the intensity of the color is measured.

2.2.1 Reagents and Equipment

Reagents from AssayMax Human Tissue Factor Pathway Inhibitor (TFPI) Elisa Kit, (AssayPro, #12641107R)

- TFPI Microplate (96 well polystyrene microplate coated with a polyclonal antibody against TFPI)
- Sealing Tapes (pressure-sensitive sealing tapes, which can be cut to fit the format of the individual assay)
- TFPI standard: Human TFPI, 160ng, lyophilized, reconstituted with 4ml of MIX Diluent -> 40ng /ml standard solution
- Biotinylated TFPI Antibody
- MIX Diluent concentrate
- Wash Buffer Concentrate
- Streptavidin-Peroxidase Conjugate
- Chromogen Substrate

Materials and Methods

- Stop Solution

Samples

Plasma

- FVIII inhibitor plasma, #3V1A000, #3V1B000, Baxter, immunodepleted, 50 BU/mL
- FVIII inhibitor plasma, #GK1838-2468, George King, native, 50 BU/mL
- Normal plasma FACT, George King # 2150
- Cryocheck Normal Donor Set, #ND32-020, 25 vials of plasma collected from 25 healthy normal male and female donors between 18 and 66 years of age; PrecisionBioLogic Inc.
- Patient plasma: samples of 24 patients - pre / post APCC application. Plasma was collected from subjects with hemophilia A and a high titer inhibitor who participated under informed consent in a recent clinical phase 3 study; Efficacy and Safety Study of Prophylactic Versus On-Demand Treatment With Feiba NF in Subjects With Hemophilia A or B and a High Titer Inhibitor; ClinicalTrials.gov NCT00851721 (Antunes SV, et al. 2014).

APCCs

- 9 different APCC batches
- 1 APCC reference standard

Equipment

- Microplate reader (Benchmark, Bio-Rad)
- Pipettes (multi-channel, single pipettes, etc.) with fitting pipette tips, respectively
- distilled reagent water

2.2.2 Test Procedure

For the generation of the standard curve, the TFPI standard is diluted with the MIX diluent from 20,00 ng/mL to 0,00ng/mL TFPI (calculated concentration) in serial

Materials and Methods

dilution steps (see in table below). Before diluting, the standard is allowed to sit for 10 minutes with gentle agitation. The samples (APCC batches, plasma) are diluted with MIX diluent as well, before starting the assay. APCCs are diluted at a ratio of 1:50, 1:100, 1:200, 1:400, plasma samples of 1:5, 1:10, 1:20 and 1:40.

Standard Point	Dilution	TFPI (ng/mL)
P1	1 part Standard (40 ng/mL) + 1 part MIX Diluent	20,00
P2	1 part P1 + 1 part MIX Diluent	10,00
P3	1 part P2 + 1 part MIX Diluent	5,00
P4	1 part P3 + 1 part MIX Diluent	2,50
P5	1 part P4 + 1 part MIX Diluent	1,25
P6	1 part P5 + 1 part MIX Diluent	0,625
P7	1 part P6 + 1 part MIX Diluent	0,313
P8	MIX Diluent	0,00

Plasma from Normal Donor Set and patient plasma are diluted 1:20. Before use, all reagents and samples are brought to room temperature (20-30°C). All incubation steps are performed at this temperature. First, excess microplate strips are removed from the plate frame. 50µL of standard or samples are added per well, always in double values. The wells are covered with a sealing tape and incubated for two hours. The timer is started after the last sample addition. Afterwards, a washing step follows. The wells are washed five times with 300µL of Wash Buffer. Next, 50µL of Biotinylated Human TFPI Antibody is added to each well and incubated for one hour. A second washing step follows as described above. Subsequently, 50µL of Streptavidin-Peroxidase Conjugate is added to each well and incubated for 30 minutes. Meanwhile, the microplate reader can be turned on and the program for measuring wavelengths set up. After a third washing step, 50µL of chromogenic substrate are pipetted to each well and incubated for 8 minutes or until the sample has developed the optimal blue

color density. The reaction is stopped with the addition of 50 μ L Stop Solution per well. The color changes from blue to yellow.

The absorbance has to be read immediately on the microplate reader at a wavelength of 450 nm. To correct optical imperfections, another reading is performed at 570 nm and the results of this reading are afterwards subtracted from those at 450nm.

2.2.3 Validation

The analysis of the data was performed using the spreadsheet program Microsoft Excel. The mean of each double value was calculated for the standard as well as for the sample values. The mean value of zero standard readings (Blank) is subtracted. For the generation of the standard curve, the standard concentration of TFPI (ng/mL) was plotted on the x-axis and the corresponding mean 450 nm absorbance on the y-axis. The best fit-line was determined by using a power function. For the determination of the unknown sample concentration, the standard curve was read off and this item multiplied by the dilution factor.

2.3 Determination of TFPI Activity

The ACTICHROME[®] TFPI Activity Assay is a chromogenic assay for the measurement of tissue factor pathway inhibitor activity in human plasma. It measures the ability of TFPI to inhibit the catalytic activity of the tissue factor/FVIIa complex, which activates factor X to factor Xa. After incubation of test samples with TF/FVIIa and FX, the residual activity of the TF/FVIIa complex is measured using SPECTROZYME[®] FXa, a highly specific chromogenic substrate cleaved only by FXa generated in the assay, releasing a p-nitroaniline (pNA) chromophore. The absorbance of the pNA in the reaction solution at 405 nm is measured and compared to those values obtained from a standard curve constructed using known TFPI activity levels. This assay may be performed in either end-point or kinetic mode.

2.3.1 Reagents and Equipment

Reagents from the ACTICHROME Tissue Factor Pathway Inhibitor (TFPI) Activity Assay

American Diagnostica Inc, #111802

- Assay Buffer
- SPECTROZYME[®] FXa
- TFPI Depleted Plasma
- TFPI Reference Plasma, ca. 1 unit/mL
- Human Factor X
- Lipidated Tissue Factor
- Human Factor VIIa Reagent
- TFPI Standard, 0,2 unit/mL

Additional Reagents

- EDTA (trisodium, dihydrate)
- Distilled water
- Glacial acetic acid

Materials and Methods

Plasma

- FVIII inhibitor plasma, #3V1A000, #3V1B000, Baxter, immunodepleted, 50 BU/mL
- FVIII inhibitor plasma, #GK1838-2468, George King, native, 50 BU/mL
- Normal plasma FACT, George King # 2150
- Cryocheck Normal Donor Set, #ND32-020, 25 vials of plasma collected from 25 healthy normal male and female donors between 18 and 66 years of age; PrecisionBioLogic Inc.
- Patient plasma: samples of 24 patients - pre / post APCC application. Plasma was collected from subjects with hemophilia A and a high titer inhibitor who participated under informed consent in a recent clinical phase 3 study; Efficacy and Safety Study of Prophylactic Versus On-Demand Treatment With Feiba NF in Subjects With Hemophilia A or B and a High Titer Inhibitor; ClinicalTrials.gov NCT00851721 (Antunes SV, et al. 2014).

APCCs

- 9 different APCC batches
- 1 APCC reference standard

Equipment

- Microplate reader (Benchmark, Bio-Rad)
- Pipettes (multi-channel, single pipettes, etc.) with fitting pipette tips, respectively
- 96 microwell test plate (U. bottom Microtiter Plates Immulon 2HB #NT320111)

2.3.2 Test Procedure

All reagents are prepared for the test as the assay description says and placed on melting ice until they are used in the assay. TFPI standard concentrations of 0.2, 0.1, 0.08, 0.06, 0.04 and 0.02 unit/mL are prepared by diluting the 0.2 unit/mL TFPI Standard with TFPI Depleted Plasma. Samples are heated for 5 minutes on the water

bath. The test substances as well as the TFPI Reference Plasma are diluted at a ratio of 1:5, 1:10, 1:20 and 1:40 with TFPI Depleted Plasma.

First, 20 μ L of TFPI Standard, diluted Reference Plasma and diluted test samples are added to each well, always in double values. Second, 20 μ L TF/FVIIa complex are added to the wells. Afterwards, the micro plates are covered and incubated at 37°C for 30 minutes. Subsequently 20 μ L Human Factor X is added to each well. Again, the micro plate is covered and incubated at 37°C for 15 minutes. At last, 20 μ L of EDTA and 20 μ L of SPECTROZYME FXa substrate are added to each well. The reaction begins immediately upon addition of the substrate. The solution turns yellow as the reaction continues. After 5 minutes the reaction is stopped by adding 50 μ L of glacial acetic acid to each well. Then the absorbance of the solution can be read at a wavelength of 405 nm.

2.3.3 Validation

The analysis of data was performed using the spreadsheet program Microsoft Excel. The mean of each double value was calculated for the standard as well as for the sample values. For the generation of the standard curve, the TFPI Standard activity (unit/mL) was plotted on the x-axis and the corresponding mean 405 nm absorbance on the y-axis. The best fit-line was determined by using a power function. For the determination of the unknown sample concentration, the standard curve was read off and this item multiplied by the dilution factor.

2.4 Immunoprecipitation

Immunoprecipitation (IP) is the technique of precipitating a protein antigen out of solution using an antibody that specifically binds to that particular protein. This process can be used to isolate and concentrate a particular protein from a sample containing many thousands of different proteins. Immunoprecipitation requires that the antibody be coupled to a solid substrate at some point in the procedure.

It was an aim of this diploma thesis to immunoprecipitate Tissue Factor Pathway Inhibitor in different APCC batches to visualize TFPI in the gel electrophoresis (Western Blot). Pretests showed that without immunoprecipitation of this special protein a visualization of TFPI in different APCC lots is not possible, because there are too little TFPI and too many other proteins contained.

For the immunoprecipitation the Thermo Scientific Pierce Direct IP Kit was used. It enables highly effective and efficient antigen immunoprecipitations by directly immobilizing purified antibodies onto an agarose support. Immobilizing the antibody provides faster and easier immunoprecipitations, enables reuse of immobilized antibody, and results in purified antigen free from antibody contamination.

Immunoprecipitation is achieved using less than 10µg of antibody and a short coupling protocol. After the antibody is coupled to the Thermo Scientific AminoLink Resin, the antigen sample is incubated with the immobilized antibody to form the immune complex. The complex is washed to remove non-bound material, and a low pH elution buffer is used to dissociate the bound antigen from antibody.

In contrast to traditional methods, the direct IP kit uses an amine-reactive support that does neither contain Protein A nor Protein G and does not need a crosslinker for covalent immobilization. Furthermore, this method couples any primary amine-containing molecule, unlike methods requiring an antibody species and subclass that binds strongly to Protein A or Protein G. The kit includes optimized buffers to high antigen yield and efficient spin columns and collection tubes, which shortens the protocol by minimizing handling and mixing.

2.4.1 Reagents and Equipment

Reagents contained in the Thermo Scientific Pierce Direct IP Kit, Cat: 2614, #ME157119A

- AminoLink Plus Coupling Resin
- 20x Coupling Buffer
- Sodium Cyanoborohydride Solution
- Quenching Buffer
- Wash Solution
- IP Lysis/ Wash Buffer
- 100x Conditioning Buffer
- 20x Tris-Buffered Saline
- Elution Buffer
- Lane Marker Sample Buffer, Non-reducing, (5x)
- Pierce Spin Columns – Screw Cap
- Microcentrifuge Collection Tubes
- Microcentrifuge Sample Tubes
- Pierce Control Agarose Resin (crosslinked 4% beaded agarose)

Additional Reagents

- Ultrapure water
- Washing Buffer: 20nM Hepes-NaOH, 200mM NaCl, 0,1% Tween20 pH7,4; #2268/1-592
- Tween 20; #21000715

Antibody

- Polyclonal anti-TFPI AB: AF2974 R&D Systems, Lot: XQI0111031, #N2268/10612, Conc.: 342 µg/mL

Equipment

- Centrifuge
- Rotator

2.4.2 Test Procedure

2.4.2.1 *Coupling of Antibody to AminoLink plus Coupling Resin*

First, the AminoLink Plus Coupling Resin and the other reagents are equilibrated to room temperature. After preparing the Coupling Buffer, 100 μ L of gently swirled AminoLink Resin are added in a Pierce Spin Column. It is placed in a microcentrifuge tube and centrifuged at 1000 x *g* for 1 minute. 100 μ L of the Pierce Control Agarose Resin is taken for negative control. After centrifugation the flow-through is discarded. Third, the resin is washed twice by adding 200 μ L of Coupling Buffer and afterwards centrifuged. The flow-through is again discarded. Fourth, the bottom of the spin column is gently tapped on a paper towel to remove any excess liquid and the bottom plug is inserted. Fifth, 20 μ g of affinity-purified antibody (polyclonal anti-TFPI antibody, AF2974) is prepared for coupling by adjusting the volume to 200 μ L. Therefore sufficient ultrapure water and 20X Coupling Buffer to produce 1X Coupling Buffer can be used. This mix is pipetted directly to the resin in the spin column. Then the work is moved to a fume hood. There, 3 μ L of Sodium Cyanoborohydride Solution is added to every 200 μ L reaction volume. At this working step, one has to wear gloves and work carefully, because the solution is highly toxic. After adding the solution the reaction tubes are incubated on a rotator for 90 minutes, centrifugation follows. The resin is put into a new centrifuge tube and washed twice with 200 μ L of Coupling Buffer, respectively. In the following step, 200 μ L of Quenching Buffer are added to the column. Again the column is centrifuged. In the fume hood 200 μ L of Quenching Buffer and 3 μ L Sodium Cyanoborohydride Solution are added. The column is incubated on the rotator for 15 minutes and centrifuged. Then the resin is washed twice with 200 μ L of Coupling Buffer and centrifuged after each wash. Again it is washed, now using 150 μ L of Wash Solution and afterwards centrifuged. This step is repeated 6 times. For storing, 200 μ L of Coupling Buffer is added and the screw cap is attached. (Storage at 4°C).

2.4.2.2 Antigen Immunoprecipitation

Immunoprecipitation of TFPI out of APCC samples with antibody-coupled resin (polycl. anti-TFPI-AB (AF2974))

First, the antibody-coupled resin is washed 3 times with Washing Buffer. Then the APCC samples are diluted to 20U with Wash Buffer and 10% of Tween20 (10 μ L) to a total volume of 1000 μ L. Afterwards, the diluted samples are added to 50 μ L of antibody-coupled resin in the spin column and incubated for 90 minutes at room temperature. Thereafter, the spin columns are washed 5 times with 1000 μ L of Washing Buffer and centrifuged 1 minute at 1000 x g. For the preparation for SDS-PAGE, 100 μ L of Wash Buffer and 50 μ L of Sample Buffer are added to the protein-antigen complex. After heating the samples at 95-100°C for 5 minutes and cooling down at room temperature, the samples are ready for applying to the gel.

2.5 Electrophoresis

Gel electrophoresis is a widely known technique used to separate and identify macromolecules as DNA, RNA, or proteins based on size, form, or isoelectric point. The separation of molecules by electrophoresis is based on the fact that charged molecules migrate with different velocity through a gel matrix upon application of an electric field. This technique has become a main tool in biochemistry, molecular biology, analytical chemistry and proteomics. Gel electrophoresis is normally used for analytical purposes, but may be a preparative technique to partially purify molecules before applying other techniques, mainly mass spectroscopy to perform proteome analysis (García-Descalzo L, et al. 2012).

One of the aims of this diploma thesis was the visualization of TFPI contained in different APCC batches with gel electrophoresis. Therefore the SDS PAGE was used.

2.5.1 SDS PAGE - Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

Gel electrophoresis of proteins with a polyacrylamide matrix, commonly called polyacrylamide gel electrophoresis (PAGE) is undoubtedly one of the most widely used techniques to characterize complex protein mixtures. It is a convenient, fast and inexpensive method because it only requires quantities of protein in the order of micrograms.

If the proteins are in a medium having a pH different from their isoelectric point, they have a net electrical charge and therefore the ability to move when subjected to an electric field. The migration velocity is proportional to the ratio between the charges of the protein and its mass. The higher the charge per unit of mass the faster the migration.

Proteins do not have a predictable structure as nucleic acids, and thus their rates of migration are not similar to each other. They cannot even migrate when an electromotive force is applied (when they are in their isoelectric point). In these cases, the proteins are denatured by adding a detergent such as sodium dodecyl sulfate (SDS) to separate them exclusively according to molecular weight. SDS is a reducing agent

that breaks disulfide bonds, separating the protein into its sub-units and also gives a net negative charge, which allows them to migrate through the gel in direct relation to their size. In addition, denaturation makes them lose their tertiary structure and therefore migration velocity is proportional to the size and not to tertiary structure.

There are some noteworthy features of the polyacrylamide gel electrophoresis:

- Gels suppress the thermal convection caused by application of the electric field, and can also act as a sieving medium, retarding the passage of molecules. They can also simply serve to maintain the finished separation, so that a post electrophoresis stain can be applied.
- The polyacrylamide gels are formed by polymerization of acrylamide by the action of a cross-linking agent, the bis-acrylamide, in the presence of an initiator and a catalyst. Persulfate ion ($S_2O_8^{2-}$), that is added as ammonium persulfate (APS) is the gel solidifying initiator and a source of free radicals, while TEMED (N, N, N', N'- tetramethylethylenediamine) catalyzes the polymerization reaction by stabilizing these free radicals. In some situations, for example, isoelectric focusing the presence of persulfate can interfere with electrophoresis, so ribofavin and TEMED are used instead (García-Descalzo L, et al. 2012). Although the gels are generally more difficult to prepare and handle, involving a longer time for preparation than agarose gels, they have major advantages over agarose gels. They have a greater resolving power, can accommodate larger quantities of DNA without significant loss in resolution and the DNA recovered from polyacrylamide gels is extremely pure (Barril P and Nates S. 2012).
- The rate of polymerization is determined by the concentration of persulfate (catalyst) and TEMED (initiator).
- The acrylamide/bisacrylamide ratio as well as the total concentration of both components affects the pore size and rigidity of the final gel matrix. These, in turn, affect the range of protein sizes that can be resolved. The size of the pores created in the gel is inversely related to the amount of acrylamide used. For instance, 7% polyacrylamide gel has larger pores than 12% polyacrylamide gel. Gels with a low percentage of acrylamide are typically used to resolve large

proteins, and high percentage gels are used to resolve small proteins. "Gradient gels" are specially prepared to have low percent-acrylamide at the top and high percent-acrylamide at the bottom, enabling a broader range of protein sizes to be separated (García-Descalzo L, et al. 2012).

Anyway, it should be noted that polyacrylamide is a neurotoxin (when unpolymerized), but with proper laboratory care it is no more dangerous than various commonly used chemicals (Barril P and Nates S. 2012).

2.5.2 Western Blot

Immunodetection of proteins by western blot

Western blot is a widely used method in molecular biology and biochemistry to detect proteins in a sample of cell homogenate or extract. The proteins are transferred from the gel to a membrane, made of nitrocellulose, nylon or polyvinylidene difluoride (PVDF), where they are examined using specific antibodies of the protein. As a result, the amount of protein in a sample can be examined and it is also possible to compare levels among various analytical groups.

The method was initiated in the laboratory of George Stark at Stanford.

The most powerful method is the transference of proteins from the gel to a membrane by applying an electric field perpendicular to the gel. There are however other methods of transferring or applying a protein on the membrane. The simplest is to apply it directly as a small drop of a concentrated solution on the membrane. The absorption of the drop causes the adhesion of the protein to the membrane, leaving it as a spot or "dot" (this is the case when using the "dot blot"). There are devices that make the application of proteins directly to the membrane possible, using a suction that facilitates the penetration of the solution, and are named "dot blot" or "slot blot" on the basis that the proteins are applied as a drop or a circular line.

Working with proteins bound to a membrane has advantages compared to employment within the gel:

Materials and Methods

- Staining and discoloring are faster.
- No staining occurs to ampholytes in isoelectric focusing gels.
- Smaller amounts of proteins are detected as they are concentrated at the surface and not diluted across the thickness of the gel.
- The membranes are much easier to manipulate than the gel itself.

The principle of the test consists of 5 stages:

First there is the immobilization of proteins on the membrane either by transference (electrophoresis, suction, pressure...) or by direct application. The procedure starts piling a flat sponge on filter paper soaked in transference buffer, the gel, the membrane in direct contact with the gel plus filter paper and finally a flat sponge. This set is included between two layers of perforated plastic and placed in a tank, which is a saline solution (transference buffer) and two plate electrodes (designed to achieve a uniform field across the surface of the gel). They are disposed so that the gel is oriented toward the anode (-) and the membrane to the cathode (+).

Second, the saturation of all binding sites of proteins in the membrane not occupied to prevent nonspecific binding of antibodies, which are proteins as well, follows.

Third, there is the incubation with primary antibody against the protein of interest.

Fourth, the incubation with secondary antibodies, or reactives acting as ligands of the primary antibody bound to enzymes or other markers, follows. The proteins labeled with enzymes are visible by incubation with appropriate substrates to form insoluble coloured products in the place where the protein were. There are several possibilities:

Enzyme coupled secondary antibodies: an antibody to the specific binding antibody is conjugated to the enzyme peroxidase or alkaline phosphatase.

Another possibility is the use of an amplifying enzyme, which is part of a biotin-avidin-peroxidase complex or a complex with alkaline phosphatase.

Enhanced chemiluminescence (ECL) is another commonly used method for protein detection in western blots. ECL is based on the emission of light during the horse radish peroxides (HRP)- and hydrogen peroxide-catalyzed oxidation of luminol. The

emitted light is captured on film or by a CCD camera, for qualitative or semi-quantitative analysis. This method allows stripping and re-probing the blot with different antibodies (García-Descalzo L, et al. 2012).

In my diploma, the Thermo Scientific SuperSignal West Femto Maximum Sensitivity Substrate - an extremely sensitive enhanced chemiluminescent substrate for detecting horseradish peroxidase (HRP) on immunoblots – was used. This substrate's extremely intense signal output enables detection of low femtogram (that's mid-zeptomole!) amounts of protein deposited on nitrocellulose or PVDF membrane and probed with appropriate primary and secondary antibodies.

2.5.2.1 Reagents and Equipment for SDS PAGE

Reagents

- 5X SDS Sample Buffer non-reducing; Thermo, #MD157489
- Protein Ladder 10-200 kDa; MBI Fermentas, #SM0671
- 10X Tris/Glycine/SDS Running Buffer; BioRad, #161-0732
- Gel: Mini-Protean TGX, 12% Tris-Glycine gel, 12 Wells, 1 mm Thickness; BioRad, #400076695
- HNA-Buffer
- 0,1% Tween
- Marker
- TFPI-Standard full length c=261,51 µg/mL

Samples

- APCC 1 – IP – pellet no Antibody
- APCC 1 – IP – pellet
- APCC 2 – IP – pellet
- APCC 7 – IP – pellet
- APCC 2
- APCC 2 – supernatant – IP

Equipment

- Gel electrophoresis equipment: Mini-Protean Tetracell PB1128, BioRad

2.5.2.2 Test Procedure (SDS-PAGE)

At first the Running Buffer is prepared by diluting from 10fold to 1fold concentration. The TFPI-Standard is brought to a concentration of 1µg/mL with HNA-Buffer + 0,1% Tween after bringing it to room temperature and vortexing. Afterwards the diluted TFPI-Standard, Buffer and 5X SDS Buffer are diluted at a ratio of 1:7,5:2 and incubated for 5min at 90-100°C. The samples are centrifuged shortly. Once the gel is in the tank and the tank is filled with Running Buffer to the designated line, the gel has to be prepared for loading. Each lane of the gel is loaded with the samples as shown in Table 3 below. The gel runs at a voltage of 250 volt for 23 minutes.

Nr.	Sample	Sample (µL)	SDS Sample Buffer (µL)	Loading Volume (µL)
1	Marker	5	---	5
2	hTFPI-FL 0,5ng	4	1	5
3	hTFPI-FL 1ng	8	2	10
4	hTFPI-FL 2ng	16	4	20
5	APCC 1 – IP – pellet no Antibody (10µL)	8	2	10
6	APCC 1 – IP – pellet (10µL)	8	2	10
7	APCC 2 – IP – pellet (10µL)	8	2	10
8	APCC 7 – IP – pellet (10µL)	8	2	10
9	-----	---	---	---
10	APCC 2 5µL (0,14U)	8	2	10
11	APCC 2 – supernatant – IP 7µL (0,14U)	8	2	10
12	-----	---	---	---

Table 3: Pipetting schema

2.5.2.3 Reagents and Equipment for Western Blot

Reagents

- TBS: 10 tablets TBS, Amresco #071C190 + 1000mL distilled water
- Tween-20 BioRad Cat Nr.: 170-6531, #210008569
 - Washing Solution TBST: 800mL TBS + 800μL Tween-20
- Milk Powder #210006854
 - Blocking Solution: 100mL TBST + 0,5% Milk Powder
- Miser Antibody Extender Solution NC, Thermo 32110, #GD 96294
- SuperSignal West Femto Maximum Sensitivity Substrate, Thermo Scientific #34096
 - SuperSignal West Femto Luminol/Enhancer Solution #LL152819
 - SuperSignal West Femto stable peroxide Solution #LL152820

Antibodies for Incubation of the Blot

- primary antibodies
 - mouse anti-human TFPI-27: particularly detects the C-terminus of TFPI and is specific for TFPI full-length,
White Label, #N2268/1 0543, conc.: 1,8mg/mL, used conc.: 6,8 μg/μL,
dilution buffer (0,5% Milk Powder in TBST): volume/membrane (1-4mL):
15μL in 4mL
 - mouse anti-human TFPI-6: particularly detects Kunitze 2 domain and therefore binds all forms of TFPI,
White Label, #N2268/1 0480, conc.: 1,8mg/mL, used conc.: 4,5 μg/μL,
dilution buffer (0,5% Milk Powder in TBST): volume/membrane (1-4mL):
10μL in 4mL
- secondary antibody
 - sheep anti-mouse IgG HRP A6782, Sigma, #127K6072, conc.: 0,9mg/mL, used conc.: 1:1000, dilution buffer (0,5% Milk Powder in TBST):
volume/membrane (1-4mL): 3μL in 4mL

Equipment

- Trans Blot Turbo Blotting System, BioRad, #170-4155, PB1127, B55
- Trans-Blot Transfer Stack PVDF Membrane Midi, BioRad, #179-4157, 400069730
- SNAP i.d. Protein Detection System, Millipore, PB1632
- SNAP i.d. Single well blot holder, Cat.Nr.:WBAVDBH01, #R1SA 1999677
- CCD-Camera: Fusion FX7 Vilber Lourmat, Software: QuantBio1D

2.5.2.4 Test Procedure

First of all the cover from the Trans-Blot Stacks is removed. Then also the "Bottom Stack" is removed and placed in the center of the blot cartridge (anode). Afterwards the SDS-PAGE gel is released from the cartridge and attached to the membrane air bubble-free. Subsequently, the "Top Stack" filter papers are placed on the gel. With the scooter, air bubbles are removed from the sandwich. Next, the blot cartridge is closed with the cover (cathode) and pushed into Trans-Blot Turbo. Consequently, the Trans-Blot Turbo is turned on. One Midi gel is blotted at 2,5A and 25 V for 7 minutes. After the run, the apparatus is opened and the sandwich removed. The blot cassettes are rinsed with distilled water and dried afterwards.

Before the development of the membrane is started, the Washing Solution and the Blocking Solution have to be prepared. If TFPI-27 is used for the antibody incubation, one has to incubate with Miser Antibody Extender Solution for 10 minutes and wash with distilled water afterwards before starting the blocking. For further development of the membrane, the SNAP i.d. Protein Detection System is used. Therefore, the blot holder is held by the support layer (blue edges) and the membrane layer (white) is wet with distilled water in the wetting tray provided. The support layer should not be wet specifically. The wetted blot holder is then placed on the rolling pad. After that the blot is positioned in the center of the blot holder with the protein side down. To remove air bubbles the blot can be rolled gently. Then the blot holder is closed and the plot rolled one more time.

Materials and Methods

After opening the blot holding frame, the blot holder is flipped so that the protein side is up. Then it is placed inside the frame. A notch in the blot holder ensures correct placement in the frame. The frame is then closed and locked. After the addition of 30 mL of Blocking Solution, the frame is pressed down and the system knob is turned to apply vacuum. When the frame is completely empty, vacuum off can be turned off. Then 4 mL of primary antibody (mouse anti-human TFPI-27 or mouse anti-human TFPI-6, respectively) is applied across the surface of the blot holder. Incubation of 10 minutes at room temperature follows. The solution will be absorbed into the blot holder and surface may appear dry. It is very important, that no vacuum is applied until after the 10-minute incubation. Afterwards, the frame is pressed down and vacuum is applied. After about 5–8 seconds the frame is completely empty. With the vacuum running continuously, 30 mL of washing buffer are added. The washing step is repeated 3 more times (total of 4 washes). When the frame is completely empty, the vacuum can be turned off. Now 4 mL of secondary antibody (sheep anti-mouse AB) are applied evenly across the blot holder surface. Incubation for 10 minutes at room temperature with the vacuum off follows. Again, the solution will be absorbed into the blot holder and surface may appear dry. With vacuum running continuously, 30 mL of washing buffer is added. The washing step is repeated 3 more times. Then 3 more washing steps with 30 mL of TBS, respectively, are added. After turning the vacuum off and the blot holder can be removed from the frame. Then the blot is removed from the blot holder and is incubated with the appropriate detection reagent.

For the detection, the SuperSignal West Femto Maximum Sensitivity Substrate is used. Therefore, the two substrate components are mixed at a 1:1 ratio to prepare the substrate Working Solution. This step has to be carried out very cautiously, because exposure to the sun or any other intense light can harm the Working Solution. For best results the Working Solution is kept in a dark bottle and prolonged exposure to any intense light is avoided. Short-term exposure to laboratory lighting will not harm the Working Solution.

Next, the blot is incubated 5 minutes in SuperSignal West Substrate Working Solution.

Then excess reagent is drained and the blot covered with clear plastic wrap.

Signals from the substrate can be captured and documented using a capable CCD camera imager at an exposure time of 1 to 4 seconds.

2.5.2.5 Validation

For quantification, the optical density of each lane of the blot was measured with the program QuantBio-1D. The quantity (or density) of a spot is calculated from its volume. This is made of the sum of all pixel intensities composing the spot. In other words, the spot quantity then depends on the number of pixels inside the area of the spot and the intensities of these points. The analysis of data then was performed using the spreadsheet program Microsoft Excel.

Using the 3 values of different standard concentration of the TFPI full-length standard, a standard curve could be generated. For the determination of the unknown TFPI full-length in APCC batches, the standard curve was read off.

3 Results

3.1 Detailed examination of different APCC samples

A detailed investigation of the APCC pharmaceutical product is one of the aims of this diploma thesis. As already mentioned in previous chapters, APCC is an intermediate purified protein concentrate isolated from human plasma. It is used to control bleeding in hemophilic patients who have developed inhibitory antibodies against factor VIII or FIX (Turecek PL, et al. 2004). APCCs are capable of inducing thrombin generation by direct activation of FX without the necessity of participation of factor FVIII or FIX (Turecek PL, et al. 1999). Therefore they are able to trigger the coagulation bypassing these two factors.

APCCs remain a mixture of prothrombin complex zymogens and their activated products. Several studies showed that FXa and prothrombin play a critical role in the activity of APCC. Both factors are considered to be key components of APCC and have been found to be a major target site for APCC (Turecek PL, et al. 2004). Other components such as coagulation inhibitors (Protein C) and co-factors (Protein S, FV, FVIII) are also contained in APCC (Váradi K, et al. 2011). Additionally, recent data confirm the presence of TFPI (Ogiwara K, et al. 2010).

According to published data by Dr. Ogiwara K, et al. 2010, there are some patients with hemophilia A with inhibitors who do not respond very well to treatment with APCC. The results of the research of Dr. Ogiwara K, et al. 2010, support that TFPI contained in APCC may attenuate the potential of thrombin generation in hemophilia A patients with inhibitors.

In the following chapters different APCC lots are characterized with a special focus on TFPI and its effect on the activity of APCC. Therefore several analysis methods were used, such as the thrombin generation assay (TGA), the AssayMax Human TFPI ELISA kit, the ACTICHROME® TFPI Activity Assay and the SDS-PAGE and the Western Blot.

3.1.1 Characterization of APCC Lots

3.1.1.1 Test Substances

APCCs

- 9 different APCC batches
- 1 APCC reference standard

Plasma

FVIII inhibitor plasma

- #3V1A000, Baxter, immunodepleted, 50 BU/mL (FVIII Inh. Pl. Bax.)
- #GK1838-2468, George King, native, 50 BU/mL (FVIII Inh. Pl. GK.)

Normal plasma

- Normal plasma FACT, George King # 2150

3.1.1.2 Determination of Total TFPI

For the determination of the total TFPI content in APCC lots an ELISA assay (AssayPro, AssayMax Human Tissue Factor Pathway Inhibitor (TFPI ELISA Kit)), according to the test procedure described in chapter 'Material and Methods' ('Determination of Total TFPI'), was performed. Also the content of total TFPI in FVIII inhibitor plasma as well as in normal plasma was measured. Before testing APCC batches were diluted at a ratio of 1:50, 1:100, 1:200, 1:400, plasma samples of 1:5, 1:10, 1:20, and 1:40.

In Table 4 and Table 5 the total TFPI levels of the different APCC lots (Nr.1 - 9, APCC reference standard), of the two FVIII inhibitor plasmas and the normal plasma are listed. The values are shown graphically as well in Figure 4 and in Figure 5.

Results

Table 4: TFPI levels in different APCC Lots

			Potency	Total TFPI in 1 U/mL APCC	
Lot number	Type	Exp.	U/mL	(ng)	(nM)
1		1995	50,0	29,6	0,74
2		1997	28,1	10,3	0,26
3	SL/ US	2007	20,8	21,3	0,53
4	NF/ US	2009	25,0	21,0	0,53
5	NF/ US	2012	50,0	15,9	0,40
6	NF	2012	49,3	16,1	0,40
7	NF	2012	50,0	16,3	0,41
8	NF	2012	50,0	15,1	0,38
9	NF	2012	50,0	12,8	0,32
APCC reference standard			28,0	12,9	0,32

Table 5: TFPI levels in different FVIII inhibitor plasmas & normal plasma

		Total TFPI	
Plasma	BU/mL	(ng/mL)	(nM)
FVIII Inh. Pl. Bax.	50	66,09	1,65
FVIII Inh. Pl. GK.	50	113,42	2,84
Normal plasma FACT GK		105,13	2,63

Results

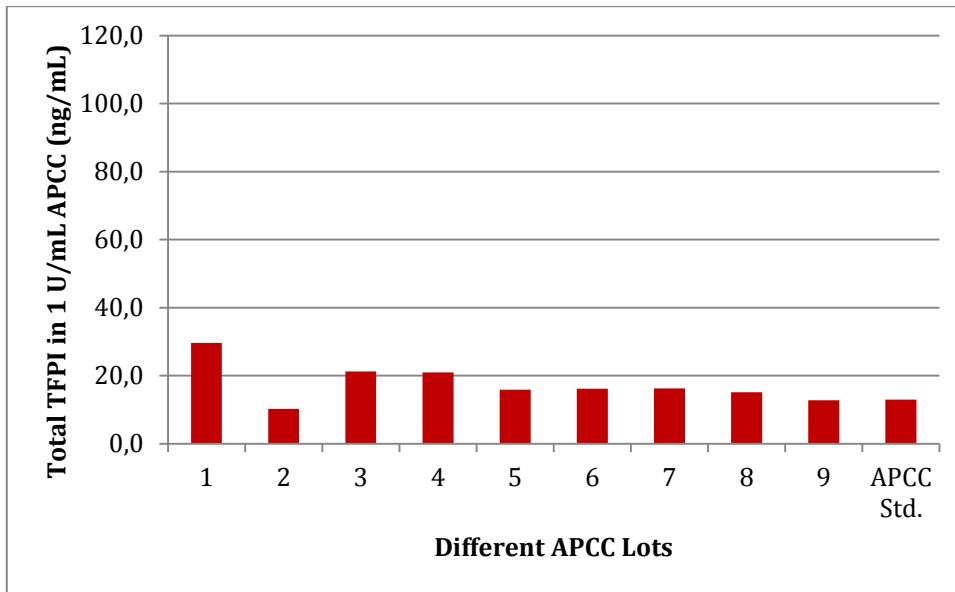


Figure 4: Total TFPI (ng/mL) in different APCC Lots measured with the Total TFPI ELISA kit (Assay Pro)

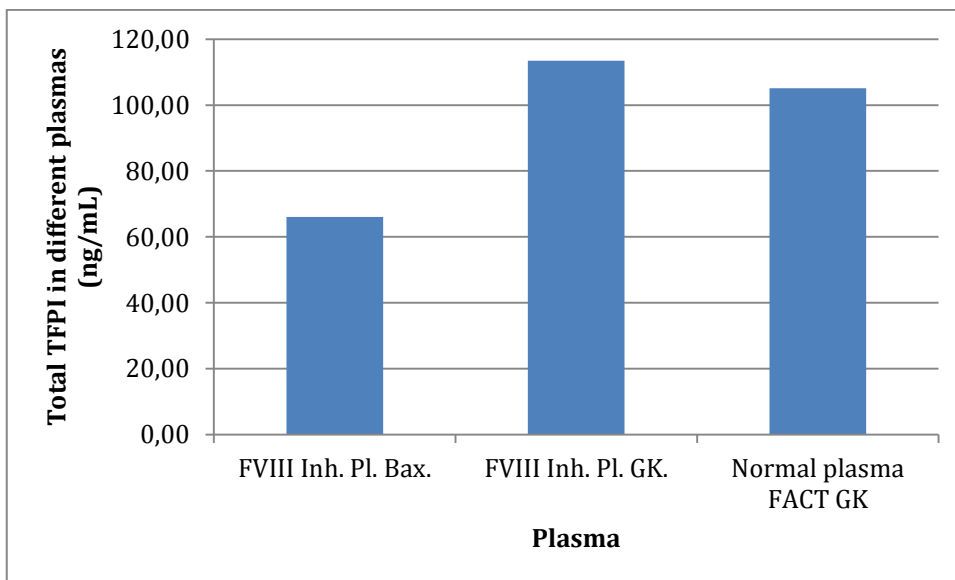


Figure 5: Total TFPI (ng/mL) levels in different FVIII inhibitor plasma & normal plasma measured with the Total TFPI ELISA kit (Assay Pro)

Results

As one can see in the tables and figures above, different APCC lots contain 12,8 – 29,6 ng total TFPI in a 1U/mL APCC solution compared to the measured 105,1 ng/mL of a pooled human normal plasma. Moreover, it should be noted, that the two FVIII inhibitor plasmas showed quite different levels of total TFPI as illustrated in Figure 5. While the value of FVIII inhibitor plasma (#3V1A000, Baxter) was about 66 ng/mL, FVIII inh. pl. (GK, #GK1838-2468) measured about 113 ng/mL. This fact played an important role for further planning of the following experiments.

3.1.1.3 Determination of TFPI Activity

The measurement of TFPI activity in APCC lots was carried out with a chromogenic assay (Actichrome TFPI Activity Assay).

Before testing all samples were diluted 1:5, 1:10, 1:20, 1:40.

Table 6: TFPI activity levels in different APCC Lots

			Potency	TFPI activity
Lot Number	Type	Exp.	U/mL	(U/ U APCC)
1		1995	50,0	n.d.
2		1997	28,1	n.d.
3	SL/ US	2007	20,8	n.d.
4	NF/ US	2009	25,0	n.d.
5	NF/ US	2012	50,0	n.d.
6	NF	2012	49,3	n.d.
7	NF	2012	50,0	n.d.
8	NF	2012	50,0	n.d.
9	NF	2012	50,0	n.d.
APCC reference standard			28,0	n.d.

n.d. not detected

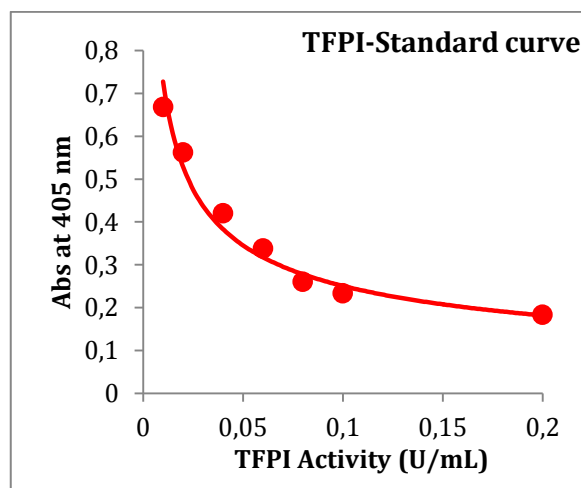
Table 7: TFPI activity levels in different FVIII inhibitor plasmas & normal plasma

Lot Number	BU/mL	TFPI activity (U/ mL)
FVIII Inh. Pl. Bax.	50	0,93
FVIII Inh. Pl. GK.	50	1,54
Normal plasma FACT GK		0,82

In Table 7 the measured values of TFPI activity in different plasmas are shown. They all ranged about 1U/mL. As shown in Table 6, TFPI activity was not detected in any of the given APCC lots. This fact led to the assumption, that the measured TFPI in all APCC lots was not active. But there still was the possibility that the assay could be disturbed. As already mentioned in chapter 'Material and Methods' ('Determination of TFPI Activity') the test principle is based on the inhibition of the activation of FX to FXa by TFPI. Due to the fact that all APCC samples themselves contain a certain proportion of FX and also FXa (Turecek PL, et al. 2004) there is some FX and therefore more FXa that TFPI cannot inhibit anymore.

Therefore the assay was carried out with negative controls in addition to the common test procedure. Instead of adding 20µL of FX, as described in assay prescription, HNaBSA Puffer was used for the negative control experiment.

The sample (APCC Nr. 2) was diluted 1:20, 1:40, 1:80, 1:160, 1:320, 1:640, 1:1280.

**Figure 6: TFPI Standard curve**

Results

Normal assay with all components				
Dilution	Absorption at 405 nm		Calculation activity U/mL	
	Value 1	Value 2	Value 1	Value 2
20	0,709	0,997	No dilution effect of APCC High endogenous FX or FXa	
40	1,128	0,828		
80	0,944	0,873		
160	0,942	0,888		
320	0,982	0,82		
640	0,94	0,887		
1280	1,02	0,901		

Table 8: Measurement of APCC Nr. 2. There was no TFPI activity detected.

Without FX				
Dilution	Absorption at 405 nm		Calculation activity U/mL	
	Value 1	Value 2	Value 1	Value 2
20	1,037	1,09	0,12	0,11
40	0,839	0,728	0,37	0,49
80	0,367	0,393	3,93	3,42
160	0,241	0,393	18,39	6,85
320	0,242	0,278	36,48	27,57
640	0,232	0,267	79,45	59,82
1280	0,253	0,273	133,39	114,38

Table 9: Measurement of APCC Nr.2 without the addition of FX as reagent. With increasing dilution of the sample, a dilution effect of APCC-FX is detectable. But again, there was no activity calculation possible.

For comparison in Figure 6 there is shown a TFPI activity standard curve and in Table 8 results of a test with using all reagents.

With this experiment the previously stated assumption was confirmed. As shown in Table 9, there was no activity calculation possible, but one can see that with each dilution FX was thinned out more and therefore less absorption was measureable. Thereby it was proven that this activity assay was not suitable for APCC, because FX and FXa contained in APCC falsified the results.

3.1.1.4 Visualization of TFPI in different APCC Lots with Gel Electrophoresis (SDS-PAGE)

For the visualization of TFPI contained in APCC lots the SDS-PAGE (Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis) was used. The samples (APCC Nr. 1, 2, 7), the standard (human TFPI full-length) and the marker were loaded on the gel and transferred to nitrocellulose for Western blot analysis. Nitrocellulose membrane was immunostained for TFPI with the indicated primary antibodies (mouse anti-human TFPI 27, mouse anti-human TFPI 6) and with antimouse conjugated to horseradish peroxidase. Anti-human TFPI 27 antibody as well as anti-human TFPI 6 are both able to bind TFPI full-length. Proteins were visualized using chemiluminescent substrate Western blotting reagents. The test procedure is described in the previous chapter 'Materials and Methods' ('Electrophoresis') in more detail.

Before performing the gel electrophoresis, an immunoprecipitation of TFPI out of the APCC lots was done. This was due to the fact that pretests showed that, without performing immunoprecipitation of this special protein, a visualization of TFPI in different APCC lots was not possible, because the amount of TFPI was too little and the amount of other proteins contained in APCC too big.

Results

Table 10 illustrates the pipetting scheme of the pretests. Therefore the APCC lot Nr. 1 and Nr. 2 were loaded on the gel with different volumes, respectively.

Nr.	Sample
1	Marker
2	hTFPI-FL 0,5ng
3	hTFPI-FL 1ng
4	hTFPI-FL 2ng
5	APCC Nr.1 - 5µL
6	APCC Nr.1 - 10µL
7	APCC Nr.1 - 20µL
8	-----
9	APCC Nr.2 - 5µL
10	APCC Nr.2 - 10µL
11	APCC Nr.2 - 20µL
12	-----

Table 10: SDS-PAGE: pipetting scheme of pretest

Results

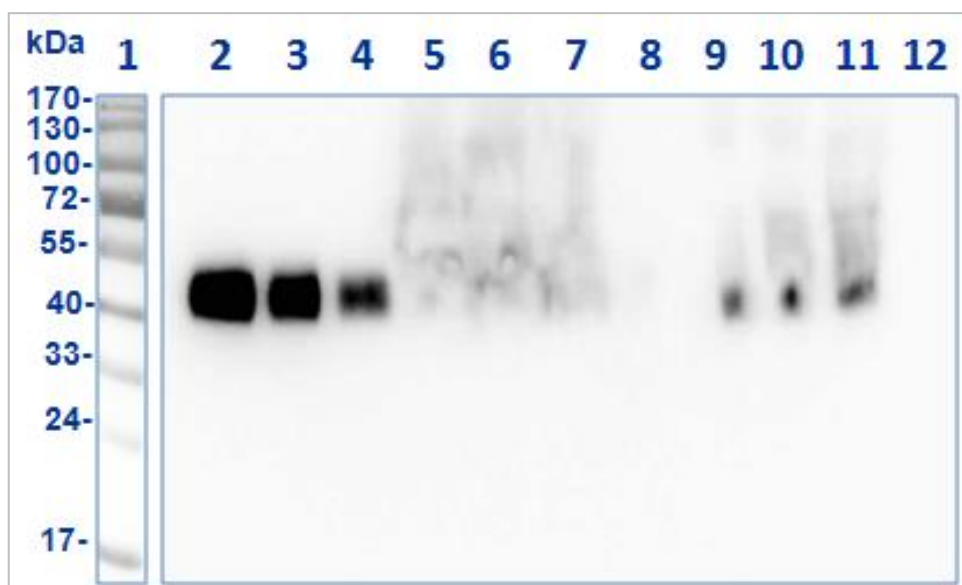


Figure 7: Western Blot (pretest) with anti-TFPI-6 (K2), 2 s

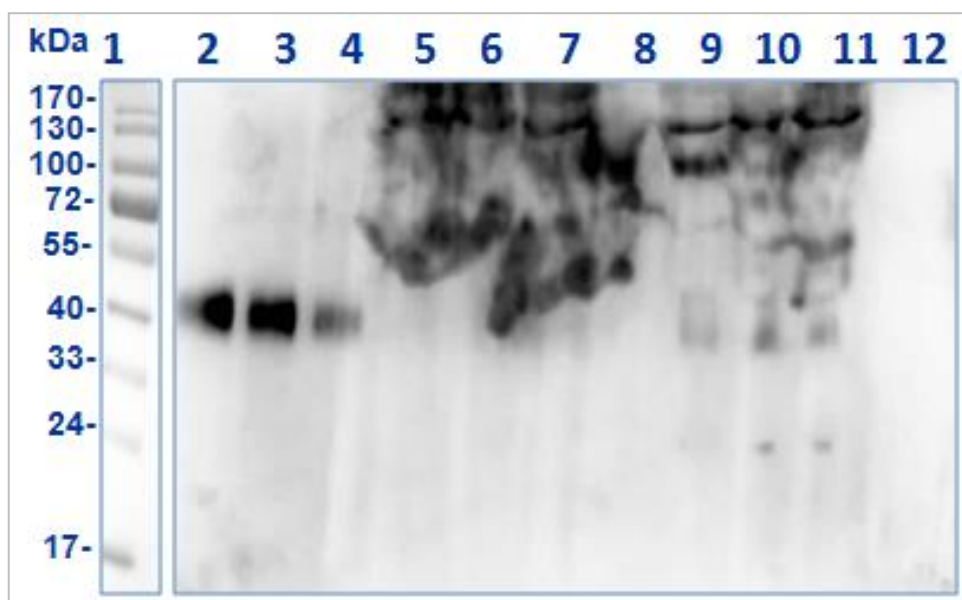


Figure 8: Western Blot (pretest) with anti-TFPI-27 (C-terminal), 32 s

Figure 7 and Figure 8 demonstrate that the used APCC samples contained too many other proteins and too little TFPI, to display TFPI in the Western Blot. Both Western Blots exemplify this assumption, because neither in lane 5 to 7 (APCC Nr.1) nor in lane 9 to 11 (APCC Nr. 2) anything can be seen clearly.

Table 11 elucidates the pipetting scheme of the experiments with previous immunoprecipitation with a polyclonal anti-TFPI antibody (polyclonal anti-TFPI AB:

Results

AF2974 R&D Systems). For the immunoprecipitation there were always used 20 Units APCC. Hence 10 μL of each APCC IP pellet (200 μL) were loaded on the gel, so the end concentration of APCC on the gel was 1 Unit, respectively.

Nr.	Sample
1	Marker
2	hTFPI-FL 0,5ng
3	hTFPI-FL 1ng
4	hTFPI-FL 2ng
5	APCC 1 – IP – pellet no Antibody (10 μL)
6	APCC 1 – IP – pellet (10 μL)
7	APCC 2 – IP – pellet (10 μL)
8	APCC 7 – IP – pellet (10 μL)
9	-----
10	APCC 2 5 μL (0,14U)
11	APCC 2 – supernatant – IP 7 μL (0,14U)
12	-----

Table 11: Pipetting scheme of tests with previous immunoprecipitation

Results



Figure 9: Western Blot of APCC-IPs with anti-TFPI-6 (K2), 1 s

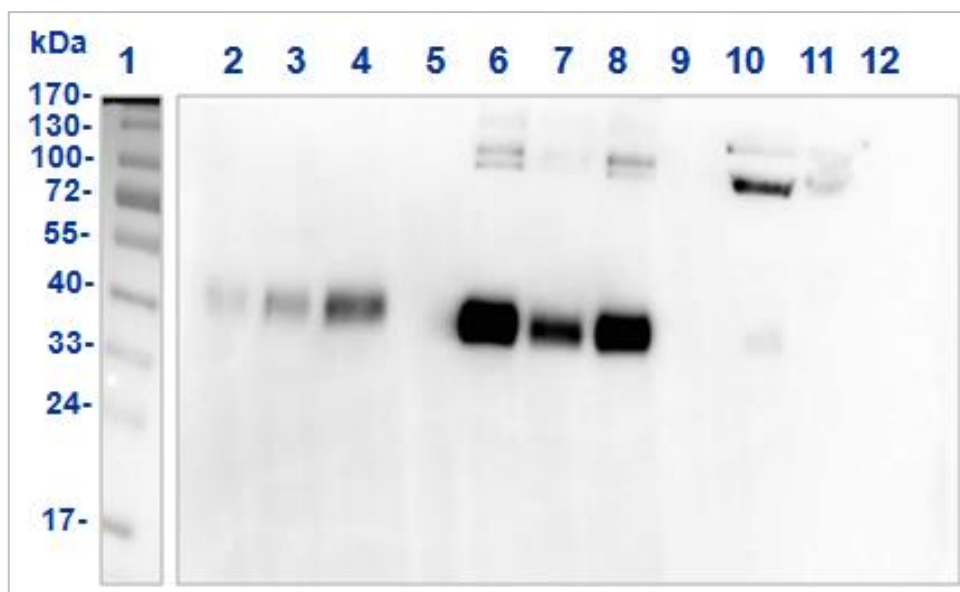


Figure 10: Western Blot of APCC-IPs with anti-TFPI-27 (C-terminal), 4 s

Results

As shown in Figure 9 and in Figure 10 it was possible to visualize TFPI full-length out of APCC lots with immunoprecipitation with a polyclonal antibody (polyclonal anti-TFPI AB: AF2974 R&D Systems). Truncated TFPI could not be detected and was probably below the detection limit of the Western Blot. For quantitative analysis of the data, measured with CCD camera, the spreadsheet program Microsoft Excel was used for calculation. With the different concentrations of full-length TFPI standard, which were applied to the gel, a standard curve (Figure 11) could be generated. For the determination of the unknown TFPI full-length in APCC batches, the standard curve was read off. As presented in Table 12 the final concentration of TFPI full-length of the APCC lots was 3,6 ng/1 Unit APCC (APCC Nr. 1), 1,8 ng/1 Unit APCC (APCC Nr.2) and 2,5 ng/1 Unit APCC (APCC Nr.7).

Samples	TFPI fl (ng)	Volume
hTFPI Standard	0,5	2889064
hTFPI Standard	1	6029059
hTFPI Standard	2	11897628
	final conc. TFPI fl (ng/1 U APCC)	
APCC 1 – IP – pellet (10 μ L)	3,6	21782560
APCC 2 – IP – pellet (10 μ L)	1,8	10572861
APCC 7 – IP – pellet (10 μ L)	2,5	15247520

Table 12: Calculation of TFPI full-length of APCC lots

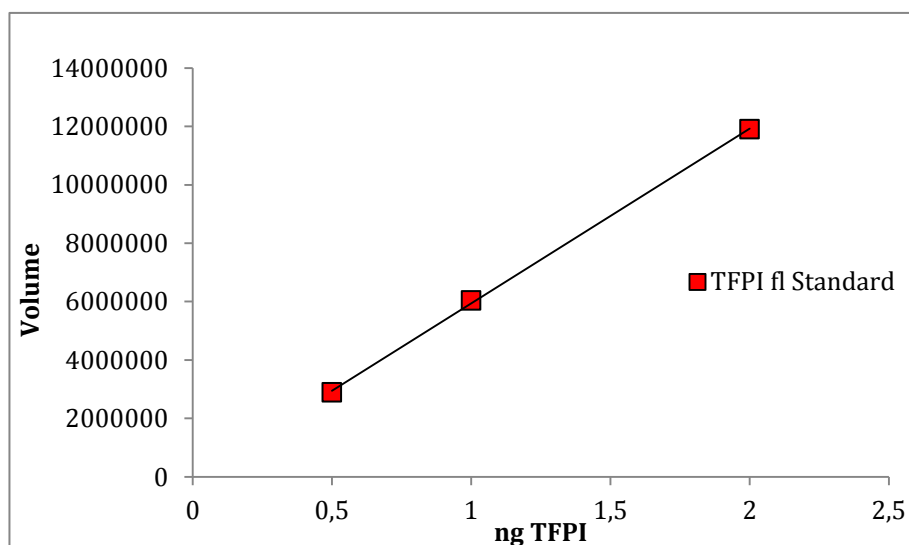


Figure 11: TFPI fl standard curve of measured values of applied TFPI standard concentrations with CCD camera

3.1.2 Influence of TFPI in plasma and TFPI in APCC on the APCC activity

3.1.2.1 Comparison of FVIII inhibitor plasma with low and high TFPI content

Before measuring APCC samples in the TGA, they were prediluted with HNaBSA buffer and afterwards spiked in a ratio of 1:20 in FVIII inhibitor plasma so the end concentrations of APCC were 0/ 0,125/ 0,25/ 0,5/ 1 Unit APCC/ml in FVIII inhibitor plasma. Two FVIII inhibitor plasmas were used: one with low TFPI content (#3V1A000, Baxter, total TFPI: 66ng/mL) and one with high TFPI content (#GK1838-2468, George King, total TFPI: 113 ng/mL). The testing solutions were pipetted with HNaBSA buffer and TGA substrate in the microtiter plate, respectively, as described in chapter 'Material and Methods' ('Thrombin Generation Assay').

Table 13: Peak Thrombin (nM) of 1 Unit APCC/mL FVIII inhibitor plasma

	FVIII inhibitor plasma	
	Bax (plasma with low TFPI)	GK (plasma with high TFPI)
TFPI (ng/mL)	66	113
APCC Lot	Peak Thrombin (nM)	
APCC Standard	218,2	152,2
1	263,2	212,7
2	206,0	124,7
3	247,2	174,3
4	254,7	170,1
5	323,4	221,0
6	219,6	193,7
7	227,3	214,4
8	219,9	187,4
9	193,0	161,2

Results

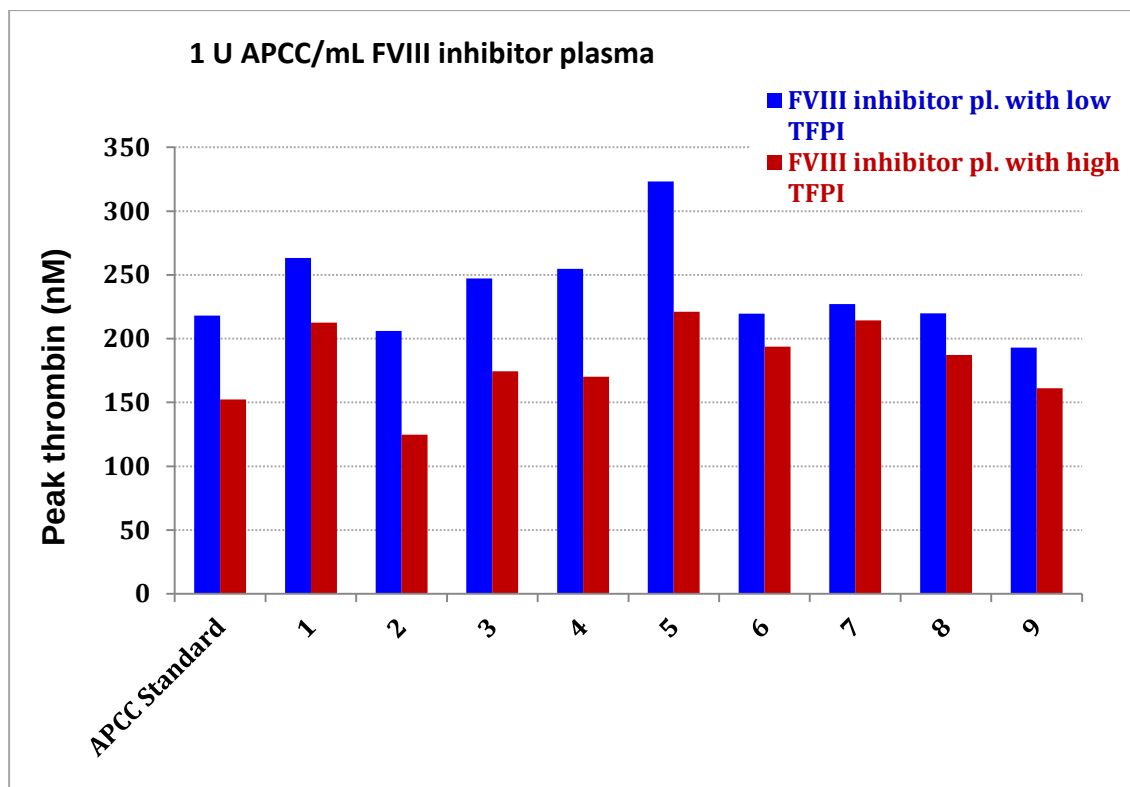


Figure 12: Peak Thrombin of APCC lots in both FVIII inhibitor plasmas at an APCC concentration of 1 U/mL

As shown in Table 13 and in Figure 12 the peak thrombin of all APCC lots of 1 U/mL APCC ranged between 193nM to 323,4nM in plasma with low TFPI content (#3V1A000, Baxter, total TFPI: 66ng/mL) and 124,7nM to 221nM in plasma with high TFPI content (#GK1838-2468, George King, total TFPI: 113 ng/mL).

Dose response of APCC in concentrations of 0/ 0,125/ 0,25/ 0,5/ 1 Unit APCC/ml in FVIII inhibitor plasma is illustrated graphically in Figure 13 and Figure 14. It should be noted that thrombin generation in plasma with high TFPI content was lower than in plasma with low TFPI content. This could be due to the fact that plasma with high endogenous TFPI content has the stronger inhibitory effect on the thrombin generation of APCC lots than plasma with lower TFPI content. Thus, it is proven that TFPI contained in plasma has an effect on the thrombin generation of APCC batches in FVIII inhibitor plasma.

Results

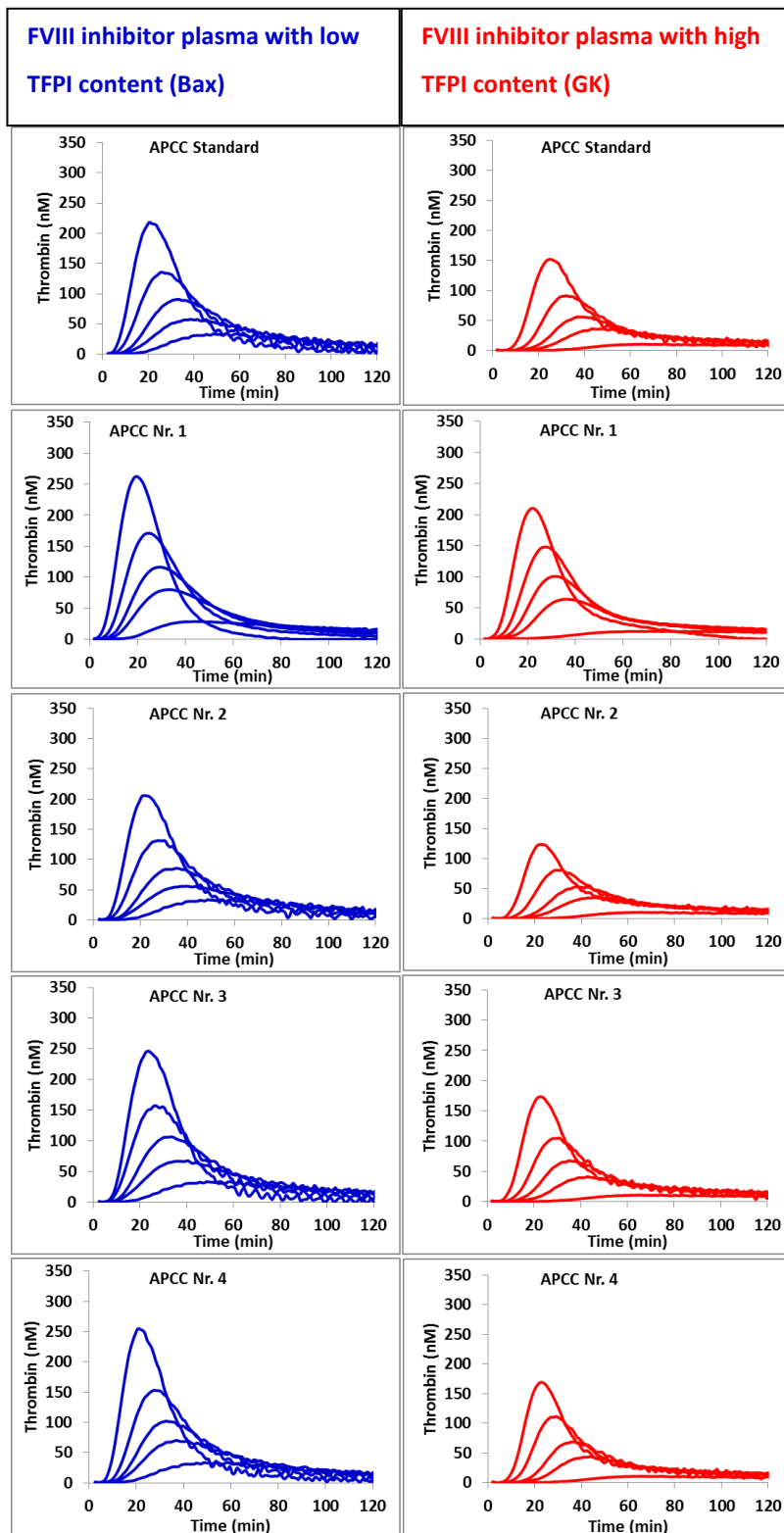


Figure 13: Dose response of APCC lots (APCC standard, APCC Nr.1 – 4). Thrombin generations curves in APCC concentrations of 0/ 0,125/ 0,25/ 0,5/ 1 Unit APCC/ml FVIII inhibitor plasma (FVIII Inh. Pl. with low TFPI content (Bax), FVIII Inh. Pl. with high TFPI content (GK)).

Results

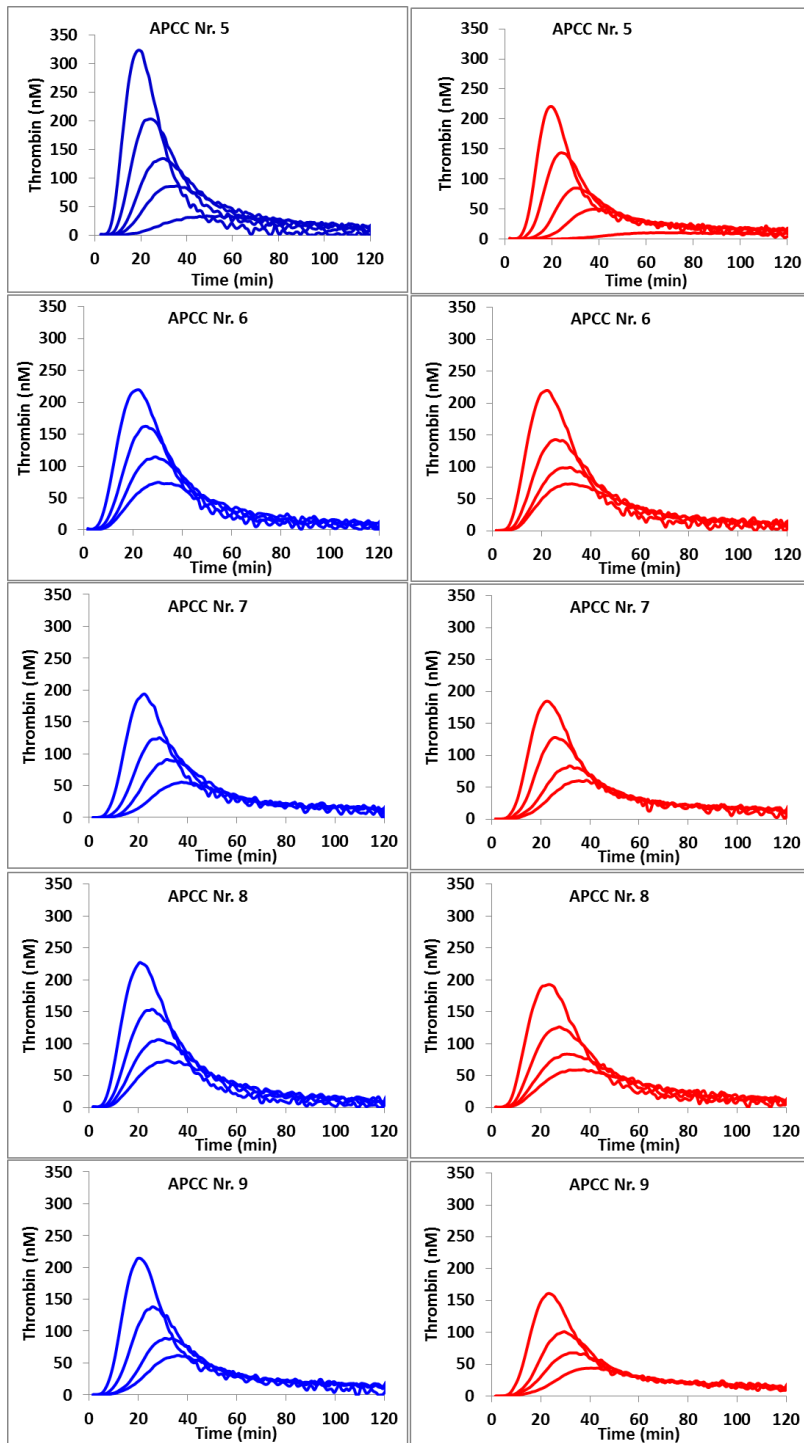


Figure 14: Dose response of APCC lots (APCC standard, APCC Nr.5 – 9). Thrombin generations curves in APCC concentrations of 0/ 0,125/ 0,25/ 0,5/ 1 Unit APCC/ml FVIII inhibitor plasma (FVIII Inh. Pl. with low TFPI content (Bax), FVIII Inh. Pl. with high TFPI content (GK)).

3.1.2.2 Total TFPI Elisa: FVIII inhibitor plasma with/ without APCC spiking

The total TFPI of samples was measured with the total TFPI ELISA kit. As samples FVIII inhibitor plasma (Baxter, #3V1B000; George King, #GK1838-2468) before and after spiking of APCC batches were taken. Each APCC lot was spiked in a ration of 1:20 in plasma, so the end concentration of APCC was 1Unit/mL. Each test solution was diluted 1:20 with the MIX diluent as described in more detail in chapter 'Materials and Methods' ('Determination of total TFPI') before performing the assay.

As shown in Table 14 and Figure 15 total TFPI increased after spiking APCC in FVIII inhibitor plasma because of the added amount of TFPI in APCC.

APCC Lot	Plasma Lot	ng/mL	nM
no APCC	3V1B000	61	1,53
1	3V1B000	86	2,16
2	3V1B000	67	1,68
3	3V1B000	78	1,96
4	3V1B000	79	1,99
5	3V1B000	76	1,89
6	3V1B000	67	1,68
7	3V1B000	84	2,10
8	3V1B000	69	1,73
9	3V1B000	76	1,91
APCC Standard	3V1B000	71	1,79
no APCC	GK1839-2468	102	2,56
1	GK1839-2468	132	3,29
2	GK1839-2468	115	2,89
3	GK1839-2468	131	3,28
4	GK1839-2468	134	3,35
5	GK1839-2468	129	3,23
6	GK1839-2468	125	3,14
7	GK1839-2468	126	3,14
8	GK1839-2468	117	2,93
9	GK1839-2468	110	2,74
APCC Standard	GK1839-2468	122	3,05

Table 14: Total TFPI content of FVIII inhibitor plasmas before and after spiking APCC

Results

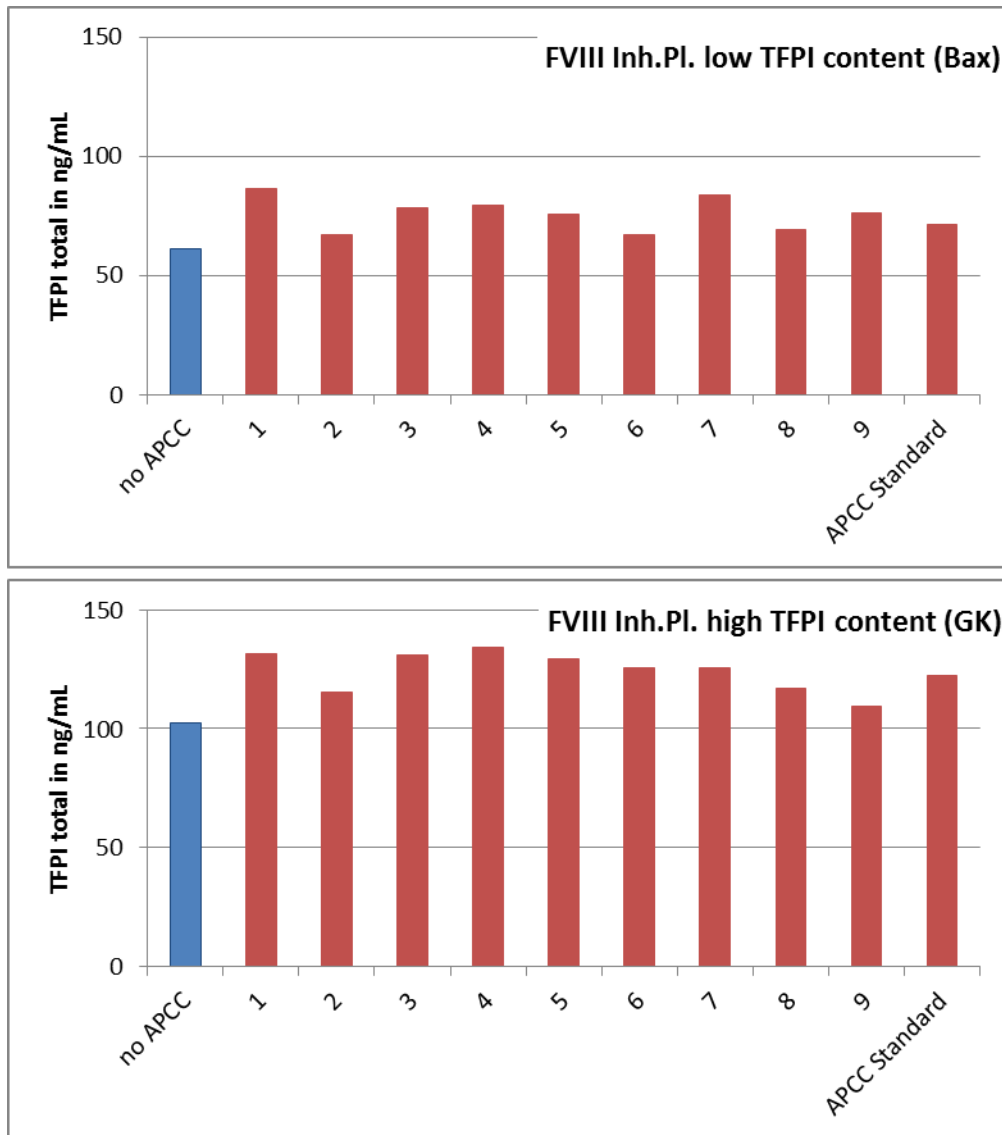


Figure 15: Comparison of total TFPI content of FVIII inhibitor plasmas before and after spiking APCC

The results of this experiment can also be compared with the results presented in Table 4, where the total TFPI content in APCC batches was measured. In Figure 16 and Figure 17 it is demonstrated that the increase of total TFPI in FVIII inhibitor plasma corresponded to the total TFPI content in APCC batches (coefficient of determination $R^2=0,5$).

Results

APCC batches	Total TFPI (ng/mL)	
	Increase in FVIII Inh. Pl. Bax	Content in APCC
1	25	30
2	6	10
3	17	21
4	18	21
5	14	16
6	6	16
7	23	16
8	8	15
9	15	13
APCC Std.	10	13

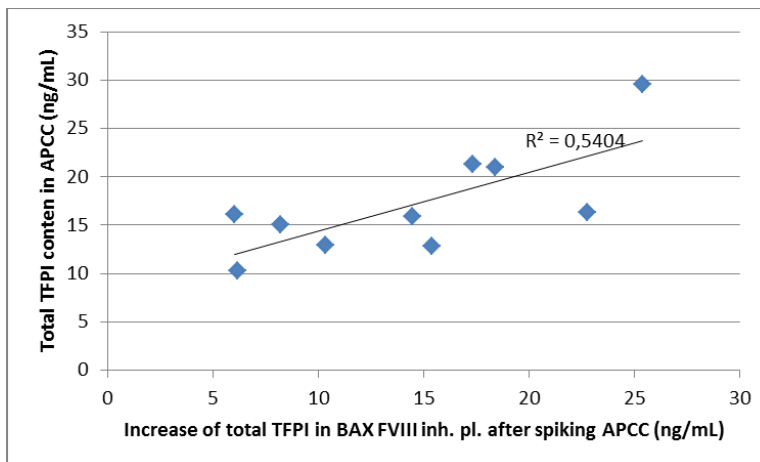
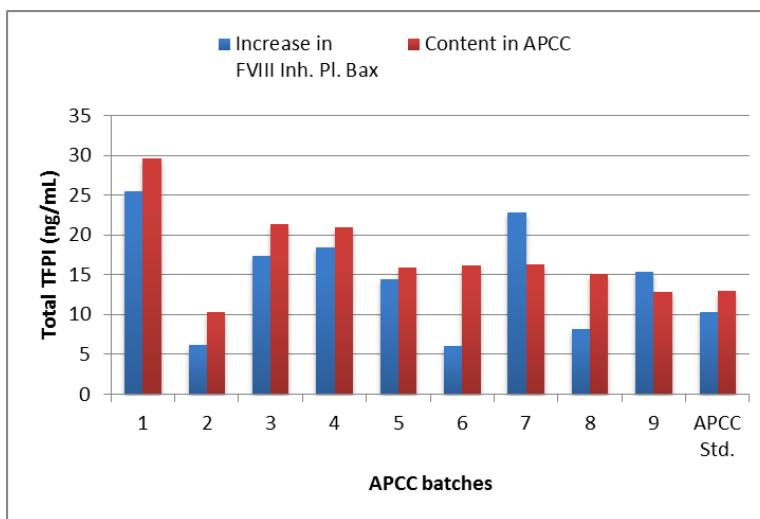


Figure 16: Correlation of increase of total TFPI in FVIII inh. pl. (Bax) (ng/mL) and of total TFPI content (ng/mL) measured in APCC batches

Results

APCC batches	Total TFPI (ng/mL)	
	Increase in FVIII Inh. Pl. GK	Content in APCC
1	29	30
2	13	10
3	29	21
4	32	21
5	27	16
6	23	16
7	23	16
8	15	15
9	7	13
APCC Std.	20	13

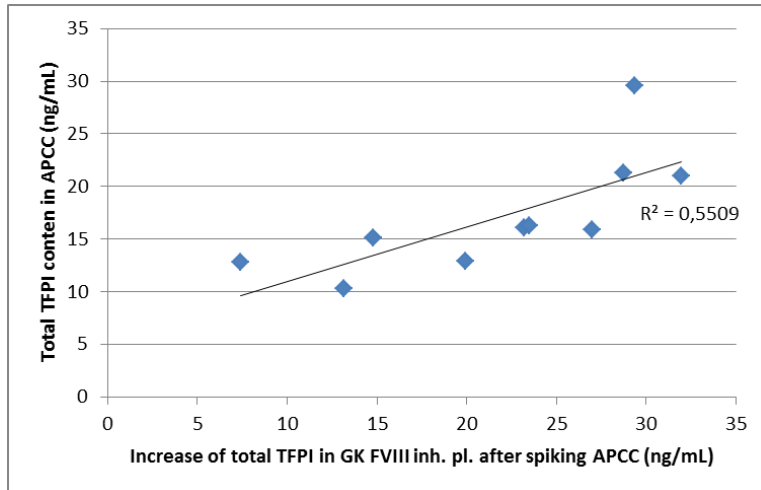
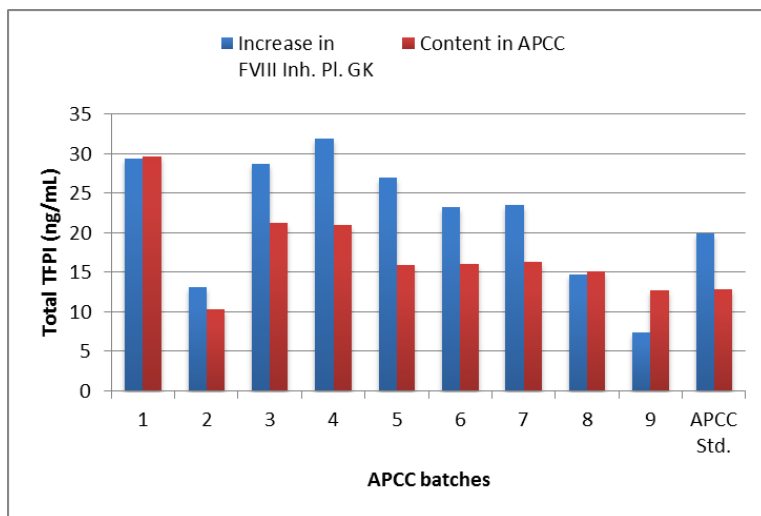


Figure 17: Correlation of increase of total TFPI in FVIII inh. pl. (GK) (ng/mL) and of total TFPI content (ng/mL) measured in APCC batches

3.1.3 TFPI spiking experiments

The aim of these experiments was to figure out the effect of added TFPI to the system (APCC + FVIII inhibitor plasma) on the APCC activity. These experiments were performed with the thrombin generation assay. TFPI was spiked in both systems, once in APCC lots and once in FVIII inhibitor plasma. TFPI was added in different concentrations (0/25/50/75/100/150/200 ng/mL). An APCC dose response was performed with concentrations of 0 to 1 U of APCC/mL FVIII inhibitor plasma. Afterwards sample preparation and test procedure was continued in the usual way as described in more detail in chapter 'Material and Methods' ('Thrombin Generation Assay').

Not alone there was the ambition to find out about the effect of TFPI on APCC activity, it was also attempted to discover if it made any differences if TFPI was spiked in APCC or in FVIII inhibitor plasma.

3.1.3.1 *Spiking TFPI in FVIII inhibitor plasma*

TFPI was added to FVIII inhibitor plasma (#3V1A000, Baxter) in 7 different concentrations (0/25/50/75/100/150/200 ng/mL plasma). Afterwards APCC Nr. 2 was serially diluted for dose response and spiked in each of these plasmas (plasma with different TFPI concentrations) in a ration of 1:20 (1 part APCC and 19 parts plasma) receiving end concentrations of 0/ 0,125/ 0,25/ 0,5/ 1 U of APCC/mL. The thrombin generation was measured in the TGA and thrombin generation curves were generated. In Figure 18 it can be seen that the thrombin generation decreases with increasing added TFPI. This phenomenon can be observed in all APCC concentrations as well as in both different FVIII inhibitor plasmas.

Results

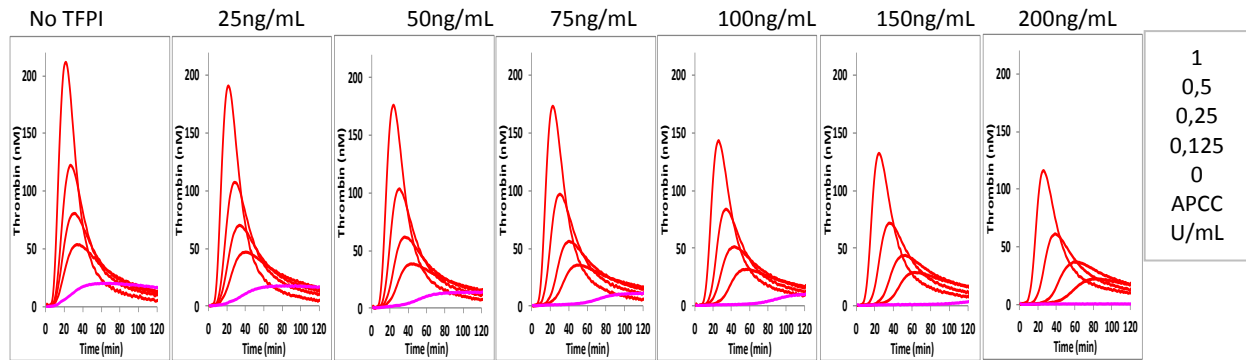


Figure 18: TFPI spiked in FVIII inhibitor plasma in different concentrations and APCC dose response. Thrombin generation decreases with increasing added TFPI.

3.1.3.2 Spiking TFPI in APCC

TFPI was added to APCC (APCC Nr.2) in 7 different concentrations. Each APCC-TFPI mix was serially diluted first and spiked in FVIII inhibitor plasma (#3V1A000, Baxter) in a ratio of 1:20 so the end concentrations of APCC were 0/ 0,125/ 0,25/ 0,5/ 1 U of APCC/mL FVIII inhibitor plasma and 0/25/50/75/100/150/200 ng/U/mL APCC of added TFPI.

Unlike in the previous experiment, here TFPI was spiked in APCC before a dose response was performed in FVIII inhibitor plasma with the whole APCC-TFPI mix. The thrombin generation was measured in the TGA.

Figure 19 proves that the thrombin generation decreased with increasing added TFPI.

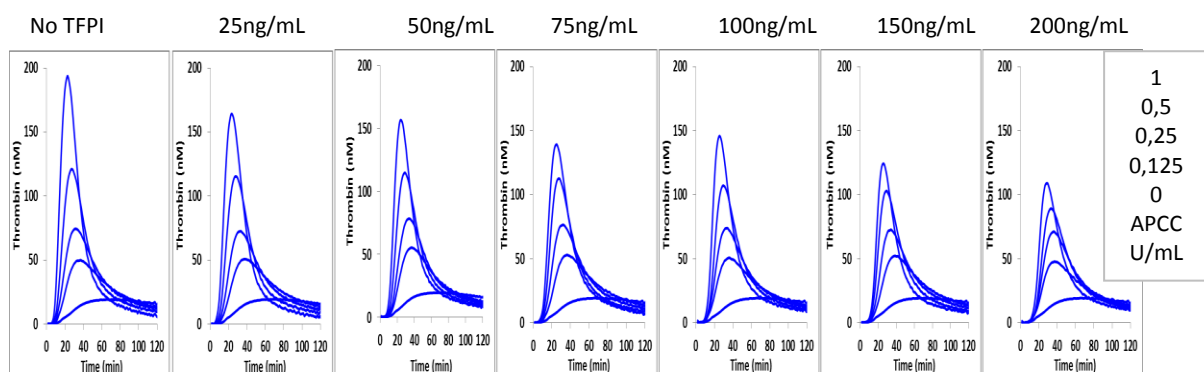


Figure 19: TFPI spiked in APCC in different concentrations and APCC dose response in FVIII inhibitor plasma. Thrombin generation decreases with increasing added TFPI.

Results

TFPI in Plasma	Peak Thrombin (% of no TFPI added)				
TFPI added (ng/mL plasma)	APCC (U/ml) in FVIII inhibitor plasma				
	0	0,125	0,25	0,5	1
200	5	42	46	50	55
150	18	54	54	59	62
100	49	59	63	68	67
75	58	68	70	79	81
50	68	73	77	84	83
25	87	88	87	88	90
0	100	100	100	100	100

Table 15: Peak Thrombin (% of no TFPI added) of APCC in FVIII inhibitor plasma, when TFPI was spiked in plasma

TFPI in APCC	Peak Thrombin (% of no TFPI added)				
TFPI added (ng/U/mL APCC)	APCC (U/ml) in FVIII inhibitor plasma				
	0	0,125	0,25	0,5	1
200	100	95	93	74	57
150	100	104	96	81	61
100	100	104	100	87	73
75	100	110	102	90	72
50	100	107	105	96	85
25	100	101	98	96	85
0	100	100	100	100	100

Table 16: Peak Thrombin (% of no TFPI added) of APCC in FVIII inhibitor plasma, when TFPI was spiked in APCC

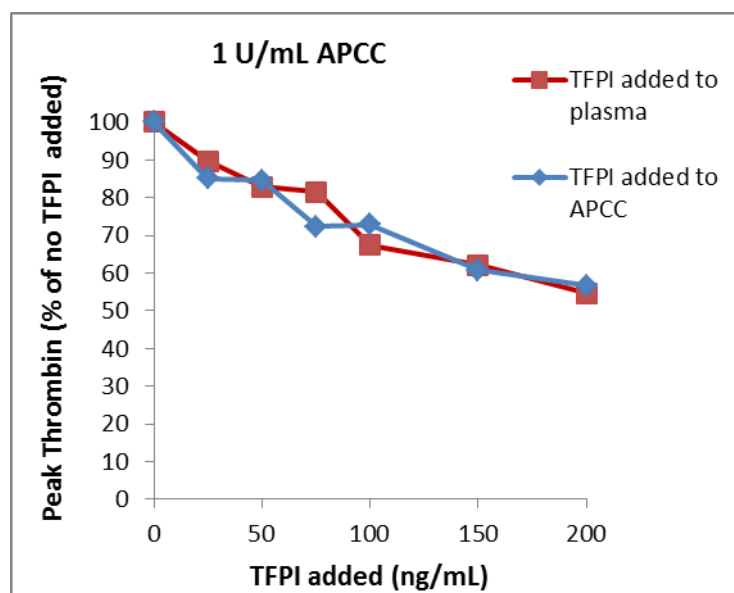


Figure 20: No difference if TFPI is added to plasma or to APCC considering that the total TFPI is identical in both systems in the end.

Results

Considering that the total TFPI was identical in both systems, they showed the same decreasing tendency of thrombin generation with increasing added TFPI, which is illustrated in Figure 20. Therefore it can be concluded, that adding TFPI to plasma or to APCC makes no difference.

Summing up, thrombin generations of APCC in FVIII inhibitor plasma depends on TFPI concentration in plasma and on TFPI concentration in APCC.

Physiological total TFPI concentrations normally range between 40 and 100 ng/mL in normal human plasma (Lwaleed BA and Bass PS. 2005). As shown in Figure 18 increasing TFPI concentrations to 200 ng/mL in plasma, meaning that 100ng/mL were added, resulted in 33% thrombin generation decrease.

Determined total TFPI concentrations in APCC batches ranged between 10 to 30 ng/mL in 1U APCC. Increasing to 75 ng/mL (50 ng/mL added) resulted in 15% thrombin generation decrease, which is elucidated in Figure 19.

Hence, this raised the question if plasma TFPI concentration had a more prominent effect in non-responding patients. This issue will be investigated in more detail later on.

3.1.4 Comparison of APCC and synthetic APCC-Mix

Now, a few facts can already be concluded.

First, thrombin generation of APCC in FVIII inhibitor plasma depends on TFPI concentration in plasma and TFPI concentration in APCC.

Second, TFPI levels in APCC approximately range between 10 and 30 ng/mL in 1U APCC.

The purpose of the following experiments was to discover the influence of TFPI on a synthetic APCC-Mix and if there can be observed the same effects compared with normal APCC.

3.1.4.1 Creation of the synthetic APCC-Mix

This "synthetic" APCC was reconstructed from its purified components. A selection of hemostatic components was chosen to achieve an APCC-effect as similar as possible. Consequently, factor II, factor Xa, factor VII, factor IX, factor FX, factor IIa, factor FIXa, factor V, Protein C, Protein S and phospholipid 80/20 were mixed together in specific proportions (Montsch P. 2010) and afterwards filled up with HNaBSA buffer to an end volume. A 20-fold working concentration of each factor had to be taken because the whole mix was spiked 1:20 in FVIII inhibitor plasma afterwards. The preparation of the samples was done freshly. Subsequently the measurement in the TGA was started. Final concentrations of each factor of the synthetic APCC-Mix are listed in Table 17.

Factors	Final Concentration U/mL plasma	20-fold Working Concentration (U/mL)
FII	1	20
FXa	0,0006	0,012
FVII	1	20
FIX	1,5	30
FX	1	20
FIIa	0,0005	0,01
FVIIa	1,5	30
FIXa	0,001	0,02
Protein C	1	20
Protein S	0,2	4
FV	0,5	10
PL 80/20	0,2	4

Table 17: Particular concentration of each component for generation of synthetic APCC mix (Montsch P. 2010)

3.1.4.2 Comparison of APCC and synthetic APCC-Mix in the TGA

Before the effect of TFPI on the APCC-Mix could be investigated, the similarity of APCC and synthetic APCC-Mix had to be proved. Therefore both samples, as usual spiked 1:20 in FVIII inhibitor plasma (#3V1B000, Bax), were measured in the TGA. APCC Nr.2 was used as representative for all APCC batches.

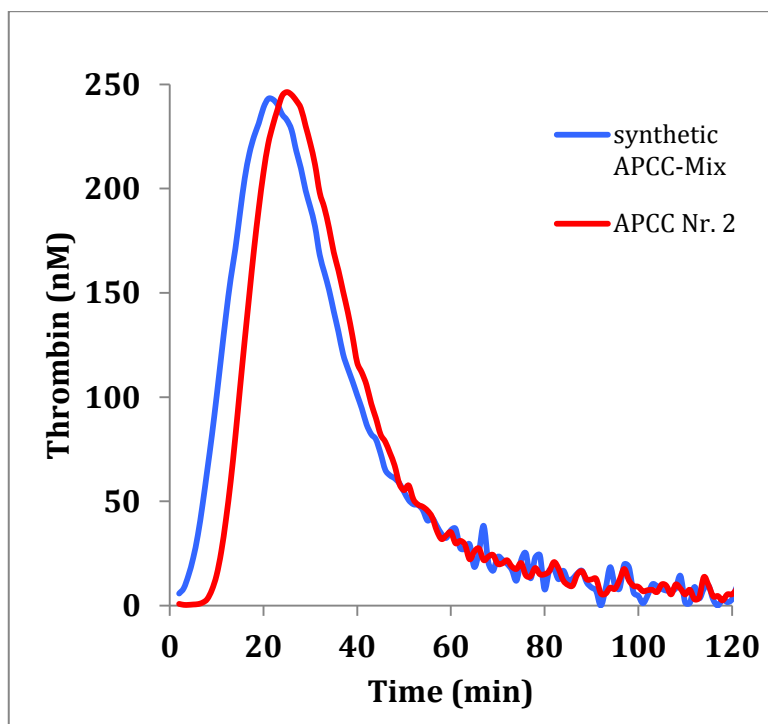


Figure 21: Thrombin generation curves of APCC Nr.2 and synthetic APCC-Mix (1 Unit/mL respectively) in FVIII inhibitor plasma (#3V1B000, Baxter).

Results

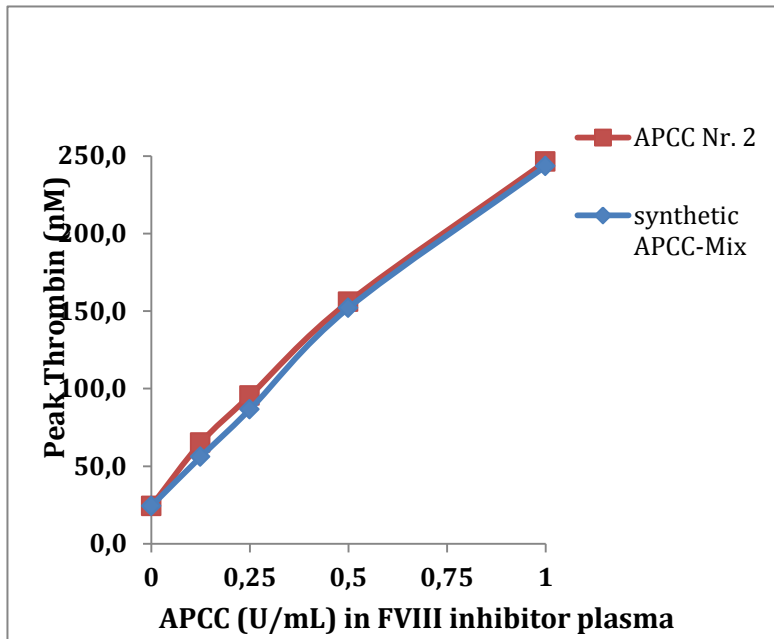


Figure 22: Comparison of dose response of APCC and synthetic APCC-Mix in FVIII inhibitor plasma (#3V1B000, Baxter).

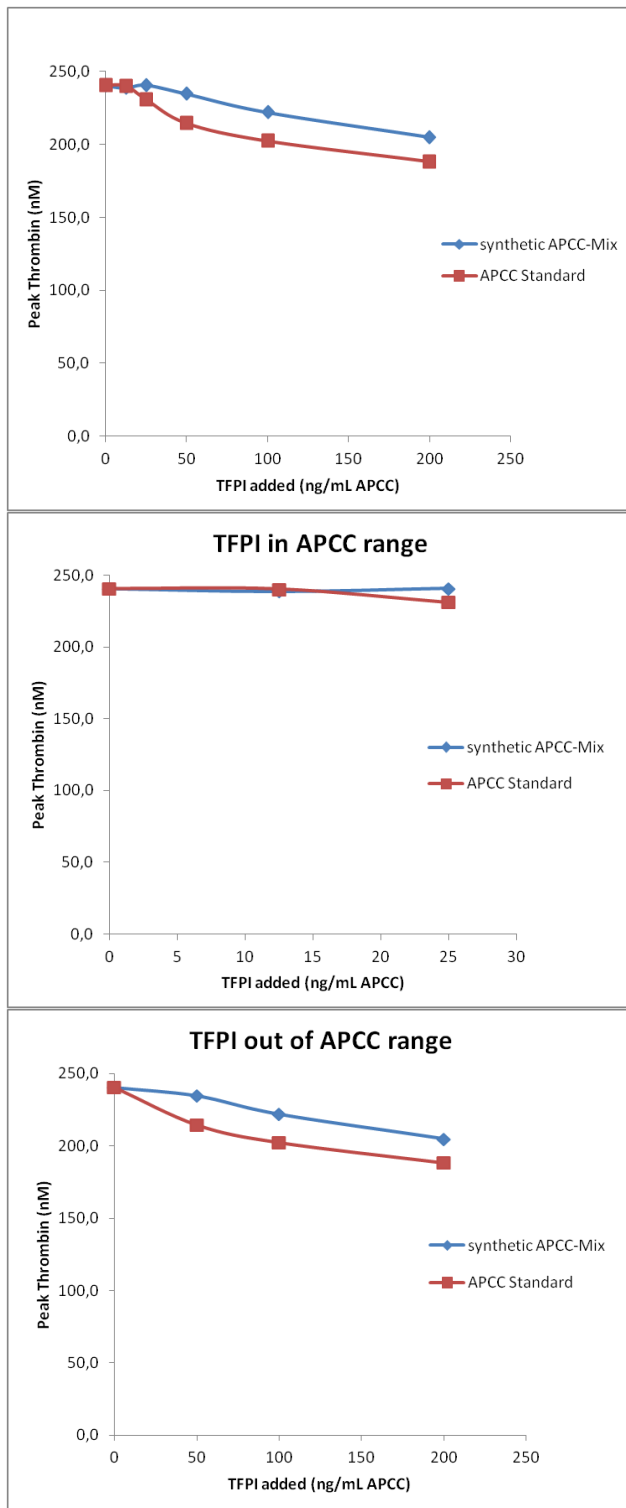
As expected from previous studies, the synthetic APCC-Mix exposed an analogous thrombin generation to APCC, which is graphically shown in Figure 21 and Figure 22.

3.1.4.3 Influence of TFPI on synthetic APCC-Mix

To observe the effect of TFPI on the synthetic APCC-Mix, TFPI was added in different concentrations (0 - 200ng/mL) in the synthetic APCC-Mix, respectively. For comparison, we also spiked TFPI in the APCC standard with same concentrations. Correspondingly, added with TFPI and afterwards spiked 1:20 in FVIII inhibitor plasma, both systems were measured in the TGA separately.

Results

Figure 23: Thrombin generation of synthetic APCC-Mix and APCC standard (1U/mL) after spiking different concentrations of TFPI



Results

In Figure 23 it can be seen that APCC and synthetic APCC-Mix had very similar thrombin generation after spiking TFPI. With increasing added TFPI the thrombin generation decreased. It should be noted, that at a TFPI concentration as it is contained in APCC batches (10 – 30ng/mL) there was just a very slight decrease of thrombin generation in APCC and in the synthetic APCC-Mix as well. It therefore appears that added TFPI, in concentrations as it is contained in APCC, shows no prominent effect on the thrombin generation. This leads to the assumption that TFPI contained in APCC does not influence the effect of APCC in patients with hemophilia A with inhibitors.

3.2 Investigation of plasma samples of healthy donors and of patients with hemophilia A with inhibitors

Another aim of the diploma thesis was to find differences in normal plasma compared with patient plasma of patients with hemophilia A with inhibitors concerning the thrombin generation, TFPI content and TFPI activity. Likewise, a detailed investigation on patient plasma before and after APCC application was done. There also were the ambitions to find correlations between the different parameters (Peak Thrombin, TFPI activity, total TFPI) for trying to make eventual predictions concerning the response of patients to APCC.

3.2.1 Comparison of normal plasma (from healthy donors) and patient plasma (patients with hemophilia A with FVIII inhibitors)

3.2.1.1 Test substances

- Cryocheck Normal Donor Set, #ND32-020, 25 vials of plasma collected from 25 healthy normal male and female donors between 18 and 66 years of age; PrecisionBioLogic Inc.
- Plasma from subjects with hemophilia A and high titer inhibitors (Patient plasma - before APCC application)

3.2.1.2 Characterization in the TGA

The thrombin generation assay was carried out as described in more detail in chapter 'Materials and Methods' ('Thrombin Generation Assay'). The thrombin generation of plasma samples, which are listed below (Table 18), was measured and is graphically shown in Figure 24.

Results

Normal plasma		Patient plasma (Hemophilia A + Inh.)	
Samples	Peak Thrombin (nM)	Patient	Peak Thrombin (nM) pre-samples
F32209	142,5	1	116,2
F32210	130,1	2	10,6
F32211	195,6	3	7,9
F32212	176,0	4	20,3
F32213	225,9	5	49,3
F32214	105,0	6	23,1
F32215	152,8	7	42,2
F32216	184,4	8	29,7
F32217	176,0	9	10,5
F32218	193,6	10	9,9
M32272	191,2	11	33,0
M32273	313,9	12	35,9
M32274	178,2	13	10,6
M32275	256,6	14	30,2
M32276	221,1	15	36,2
M32277	182,5	16	71,6
M32278	257,3	17	24,1
M32279	239,5	18	41,8
M32280	205,8	19	16,6
M32281	214,1	20	3,6
M32282	276,8	21	53,1
M32283	106,2	22	41,9
M32284	84,3	23	60,4
M32285	144,4	24	52,0
mean ± SD	189,7 56,2	mean ± SD	34,6 25,2

Table 18: Peak Thrombin (nM) of normal plasma and patient plasma samples (before APCC infusion)

Results

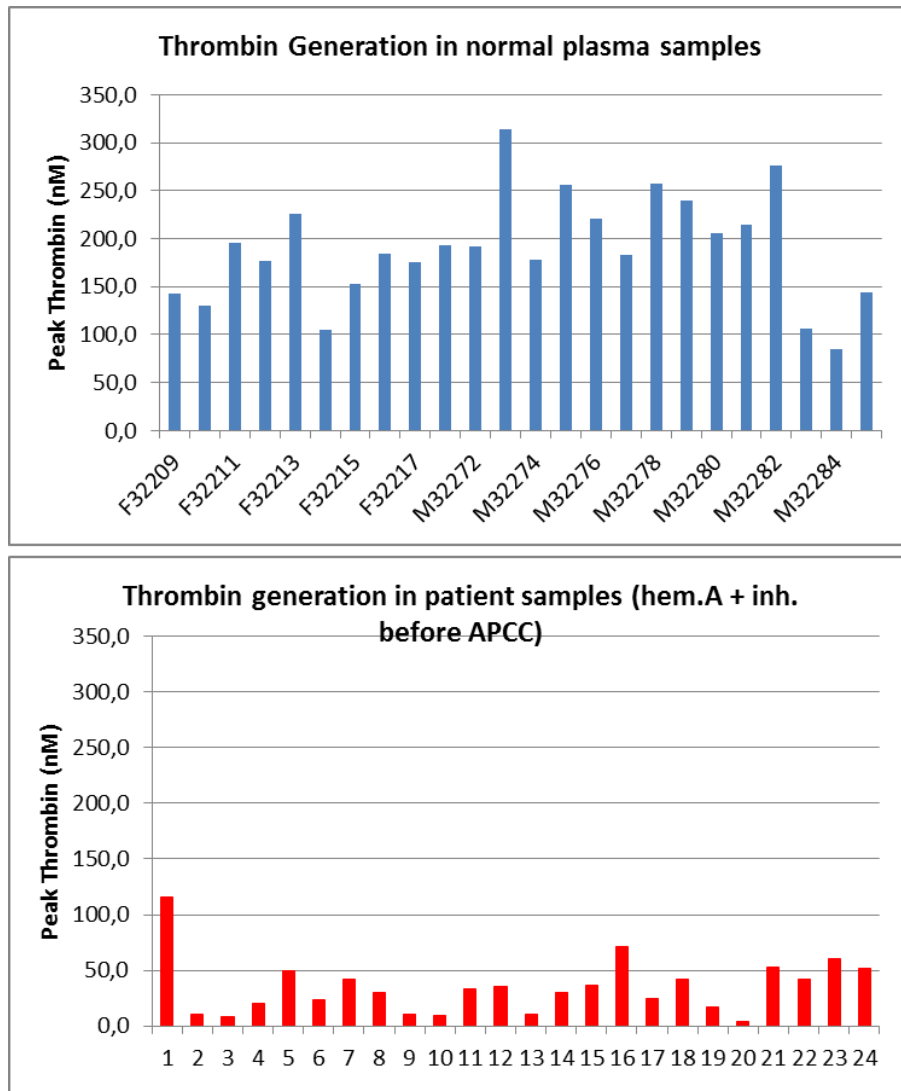


Figure 24: Thrombin generation in normal plasma and in patient plasma samples (before APCC application). TG in patient plasma was very low compared to TG in normal plasma.

As illustrated in Figure 24 thrombin generation in patients with hemophilia A with inhibitors before application of APCC was very low compared to thrombin generation in normal plasma. The mean value of peak thrombin calculated for normal plasma was about 190nM with a standard deviation of 56,2. In comparison, patient samples measured a peak thrombin of 34,6nM on average and a standard deviation of 25,2. Table 18 shows the detailed tabulation and calculation of measured values. As it can be seen there is a high variability of thrombin generation response in different plasmas of both groups.

Results

3.2.1.3 Determination of total TFPI

The total TFPI of samples was measured with the total TFPI ELISA kit. The samples were diluted in a range of 1:20 with MIX diluent. The further test procedure was performed as described in more detail in chapter 'Materials and Methods' ('Determination of total TFPI').

Normal plasma			Patient plasma (Hemophilia A + Inh.)		
Samples	Total TFPI (ng/mL)	Total TFPI (nM)	Patient	Total TFPI (ng/mL) pre	Total TFPI (nM)
F32209	204,9	5,12	1	105,8	2,65
F32210	99,0	2,48	2	123,1	3,08
F32211	235,9	5,90	3	120,2	3,01
F32212	146,8	3,67	4	107,3	2,68
F32213	116,9	2,92	5	135,3	3,38
F32214	150,9	3,77	6	176,5	4,41
F32215	114,2	2,86	7	136,3	3,41
F32216	174,5	4,36	8	128,9	3,22
F32217	158,6	3,97	9	155,6	3,89
F32218	107,8	2,69	10	162,6	4,07
M32272	179,7	4,49	11	172,6	4,31
M32273	205,5	5,14	12	164,0	4,10
M32274	167,8	4,20	13	130,4	3,26
M32275	159,7	3,99	14	207,5	5,19
M32276	130,1	3,25	15	163,2	4,08
M32277	166,3	4,16	16	129,7	3,24
M32278	172,7	4,32	17	74,6	1,86
M32279	125,2	3,13	18	82,6	2,06
M32280	147,4	3,69	19	63,8	1,60
M32281	159,8	4,00	20	78,3	1,96
M32282	90,5	2,26	21	162,8	4,07
M32283	165,4	4,13	22	59,7	1,49
M32284	129,7	3,24	23	103,6	2,59
M32285	160,6	4,01	24	81,0	2,03
mean	152,9	3,82	mean	126,1	3,2
± SD	35,0	0,9	± SD	39,8	1,0

Table 19: Total TFPI content of normal plasma and patient plasma (before APCC infusion)

Results

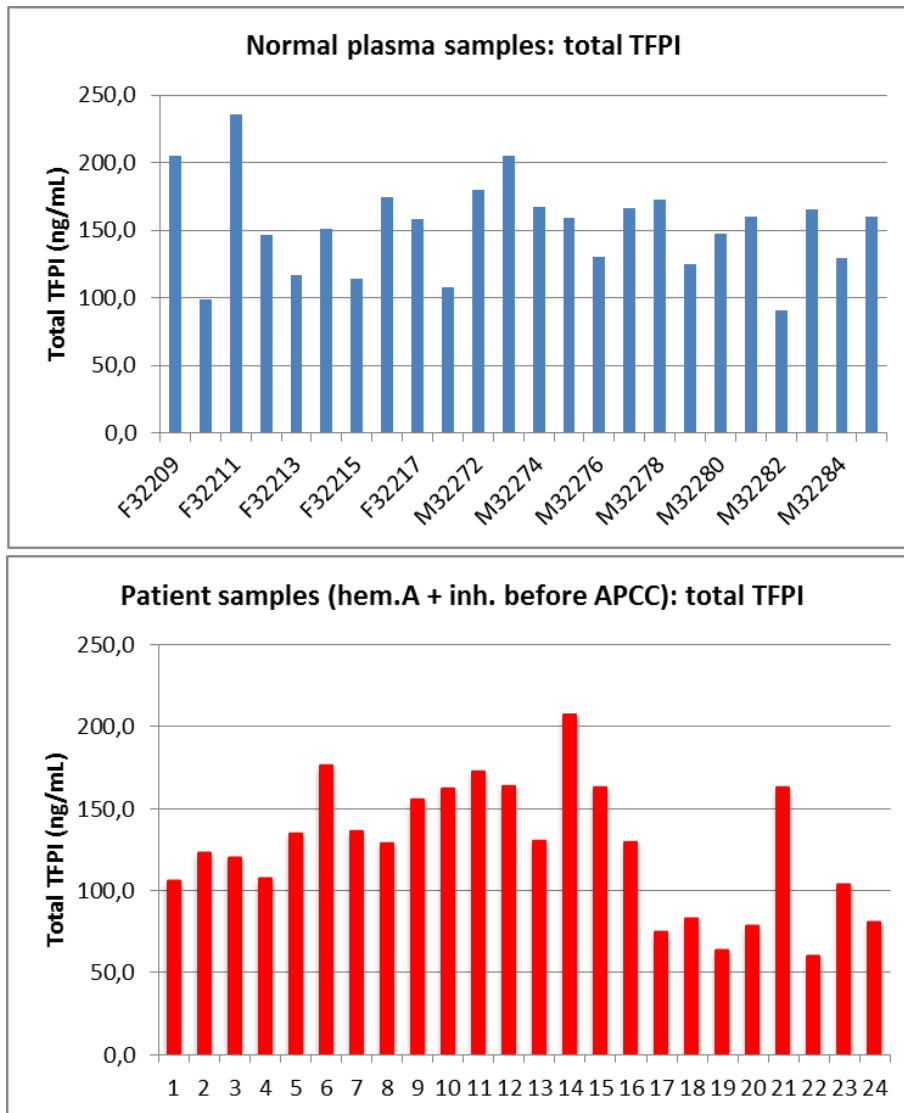


Figure 25: Total TFPI content of normal plasma and patient plasma (before APCC infusion)

Table 19 and Figure 25 demonstrate that total TFPI in patient plasma was significantly lower compared to normal plasma. While normal plasma samples valued an average total TFPI content of 152,9 ng/mL (SD = 35,0), patient plasma samples measured a mean value of 126,1 ng/mL (SD = 39,8) of total TFPI. The significance was confirmed by statistical analysis (*t*-test (Rasch B, et al. 2010)).

3.2.1.4 Determination of TFPI Activity

The measurement of TFPI activity in APCC lots was carried out with a chromogenic assay (Actichrome TFPI Activity Assay). As sample preparation, normal plasma samples were diluted 1:30 and patient plasma samples were diluted 1:20. The test procedure was afterwards performed as described in the chapter 'Material and Methods' ('Determination of TFPI Activity').

Normal plasma		Patient plasma (Hemophilia A + Inh.)	
Samples	TFPI activity (U/mL)	Patient	TFPI activity (U/mL) pre
F32209	3,1	1	1,3
F32210	2,2	2	2,1
F32211	1,9	3	1,9
F32212	2,1	4	1,7
F32213	1,1	5	1,4
F32214	3,0	6	2,6
F32215	2,0	7	1,9
F32216	4,3	8	2,1
F32217	2,9	9	2,2
F32218	1,6	10	1,7
M32272	2,5	11	2,1
M32273	3,3	12	1,8
M32274	3,6	13	1,7
M32275	2,4	14	1,5
M32276	2,6	15	2,7
M32277	3,4	16	1,6
M32278	2,1	17	1,0
M32279	1,3	18	1,0
M32280	2,8	19	1,1
M32281	1,7	20	1,8
M32282	1,4	21	1,9
M32283	2,5	22	1,1
M32284	1,8	23	1,9
M32285	4,0	24	1,5
mean ± SD	2,5 0,8	mean ± SD	1,7 0,5

Table 20: TFPI activity of normal plasma and patient plasma samples (before APCC application)

Results

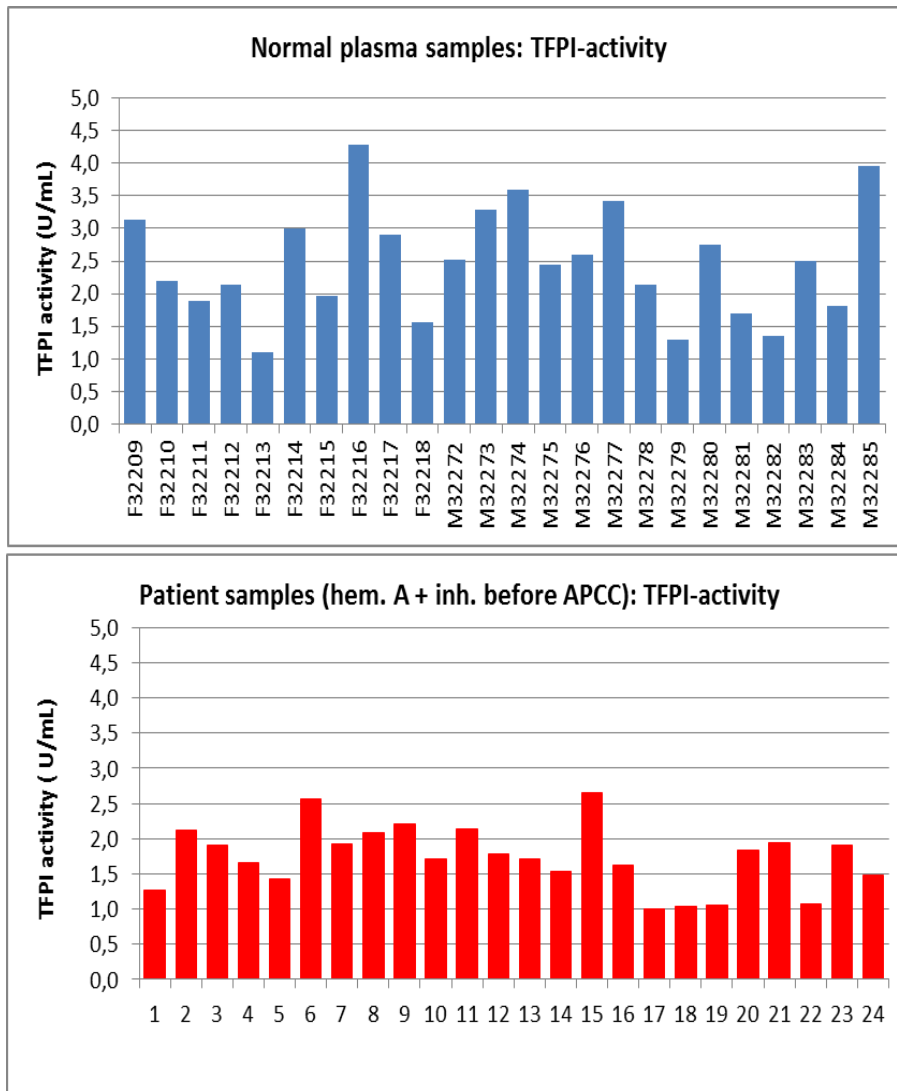


Figure 26: TFPI activity of normal plasma and patient plasma samples (before APCC application)

Figure 26 elucidates that TFPI activity in patient plasma was significantly lower compared to normal plasma. The significance was confirmed by statistical analysis (t -test (Rasch B, et al. 2010)). As shown in Table 20 the mean value of TFPI activity is 2,5 U/mL (SD = 0,8) in normal plasma samples and 1,7 U/mL (SD = 0,5) in patient plasma samples.

3.2.2 Characterization of patient plasma samples with hemophilia A with inhibitors before and after APCC application

3.2.2.1 Test substances

- Cryocheck Normal Donor Set, #ND32-020, 25 vials of plasma collected from 25 healthy normal male and female donors between 18 and 66 years of age; PrecisionBioLogic Inc.
- Plasma from subjects with hemophilia A and high titer inhibitors (Patient plasma - before and after (15 minutes) APCC application)

3.2.2.2 Characterization in the TGA

Thrombin generation of the plasma samples was measured performing the thrombin generation assay.

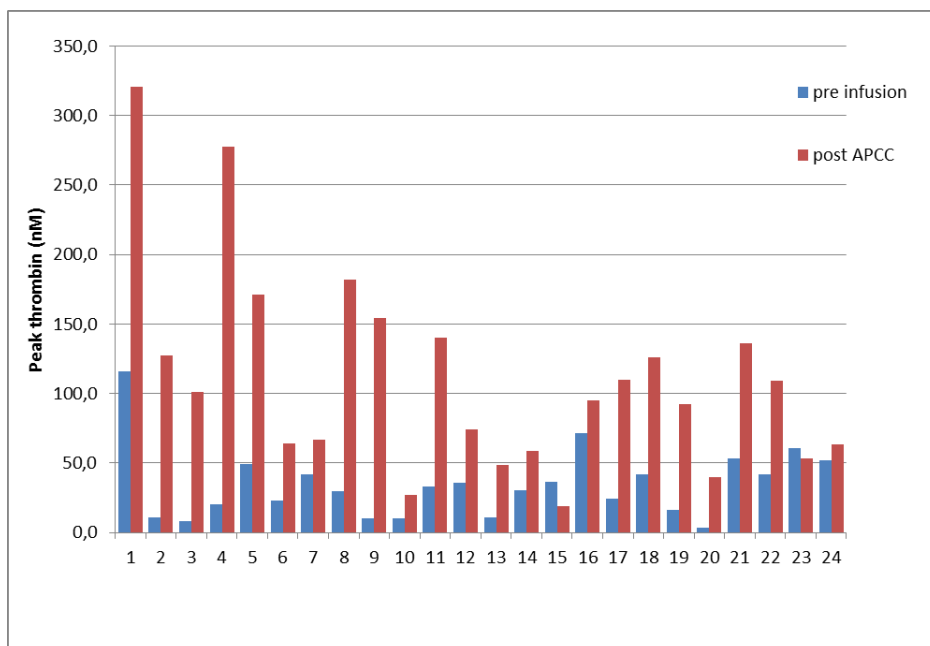


Figure 27: Thrombin generation in patient plasma before / after APCC application

In Figure 27 it is graphically shown that thrombin generation increased after APCC application in every patient. However the samples showed great differences regarding the response to APCC. Some of them showed a high response and some showed poor response.

Results

For further experiments it was tried to divide the patients in two groups. Therefore, responders were arbitrarily defined as follows. When the difference between pre-and post-infusion peak thrombin was smaller than 75 nM the patient was categorized as a poor responder. The classification is shown in Table 21 below.

Good Responder		Poor Responder	
Patient-ID	APCC-Response Difference Peak Thrombin post-pre (nM)	Patient-ID	APCC-Response Difference Peak Thrombin post-pre (nM)
1	204,3	6	40,7
2	116,7	7	24,9
3	93,0	10	16,9
4	257,4	12	38,3
5	121,7	13	38,0
8	152,5	14	28,6
9	143,8	15	-17,0
11	107,4	16	23,7
17	85,6	20	36,2
18	84,1	22	67,1
19	75,7	23	-6,8
21	83,2	24	11,6

Table 21: Classification in poor and good responder

3.2.2.3 Determination of total TFPI

The total TFPI ELISA kit was used for measuring the total TFPI of patient plasma samples. They were diluted in a range of 1:20 with MIX diluent. The further test procedure was performed as described in more detail in chapter 'Materials and Methods' ('Determination of total TFPI').

Results

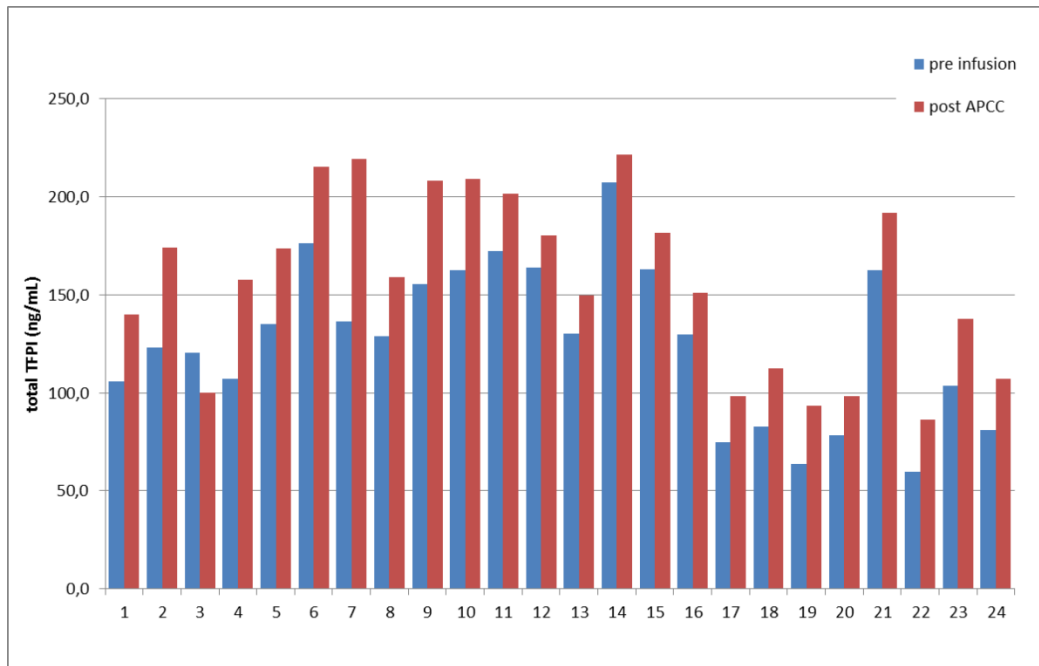


Figure 28: Total TFPI (ng/mL) in patient samples before / after APCC application

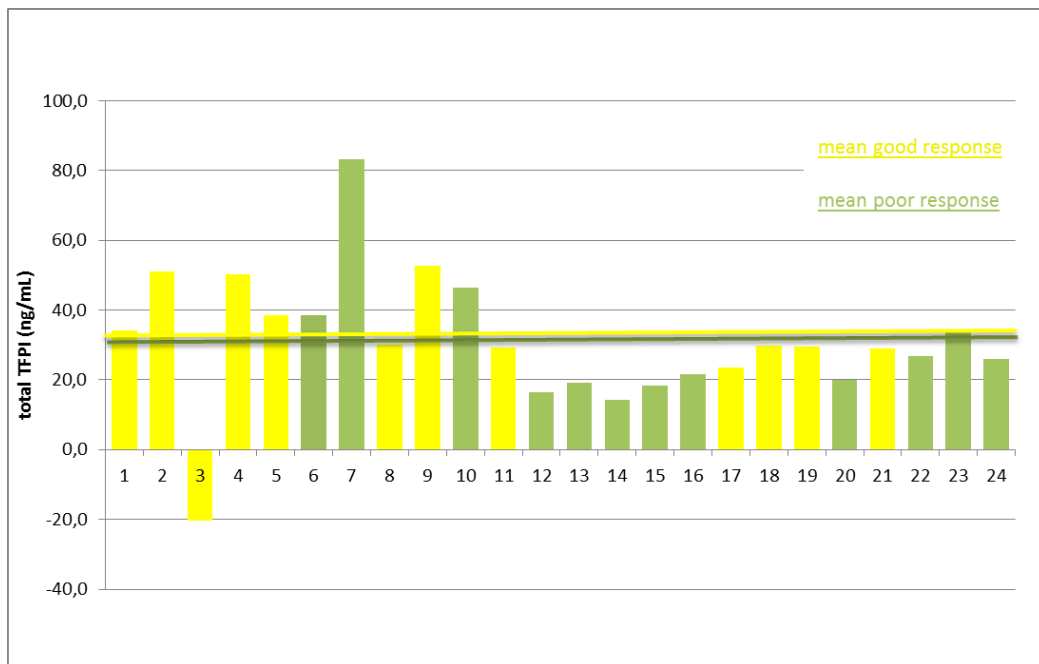


Figure 29: Difference of total TFPI (ng/mL) in patient samples before / after APCC application

	Difference Total TFPI pre - post APCC (ng/mL)	
All patients	mean $\pm$ SD	30,9 $\pm$ 18,7
	Range	(-20,2) 14,2 – 83,2
good responders	mean $\pm$ SD	31,4 $\pm$ 19,3
	Range	(-20,2) 23,5 – 52,7
poor responders	mean $\pm$ SD	30,4 $\pm$ 19,1
	Range	14,2 – 83,2

Table 22: Difference total TFPI of pre and post patient plasma samples classified in two groups: good and poor responder

Similar to the in vitro spiking experiments, the injection of APCC increased the total TFPI level in the patients, which is demonstrated in Figure 28. Furthermore, the increase corresponds to the TFPI content of the APCC batches.

Summing up, there were no differences between good responders and poor responders concerning the pre-post APCC total TFPI as it is shown graphically in Figure 29 and listed in Table 22.

3.2.2.4 TFPI content in patient plasma: Correlation between poor and good APCC response

Using the arbitrary classification of poor and good responder, it was tried to find any correlations between the measured parameters.

It was attempted to find out if there existed any connection between the TFPI activity or the total TFPI content and the peak thrombin in normal plasma samples as well as in patient plasma samples (before and after APCC application). So, if there is a correlation, then maybe there could be made predictions concerning the response to APCC.

Results

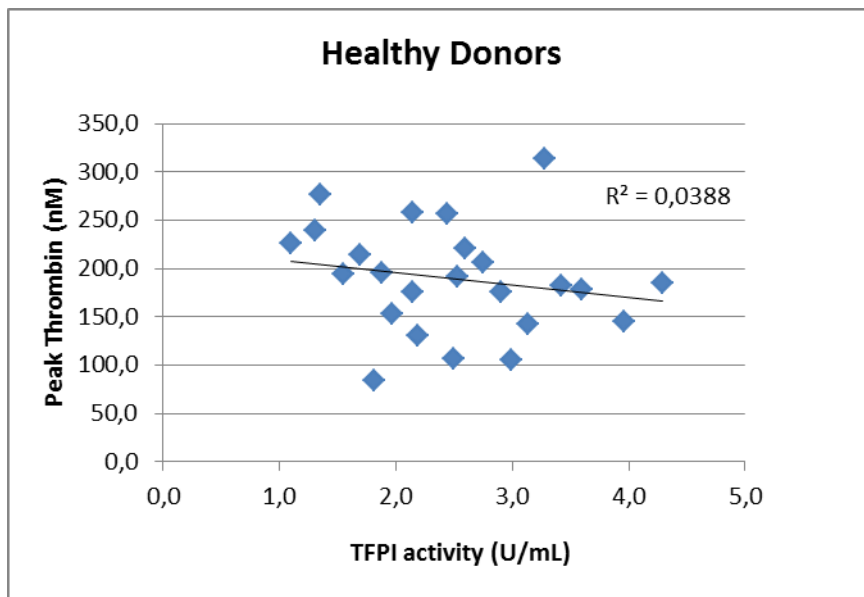


Figure 30: Healthy donors (normal plasma samples): there is no correlation between TFPI activity (U/mL) and Peak Thrombin (nM) ($R^2 = 0,0388$).

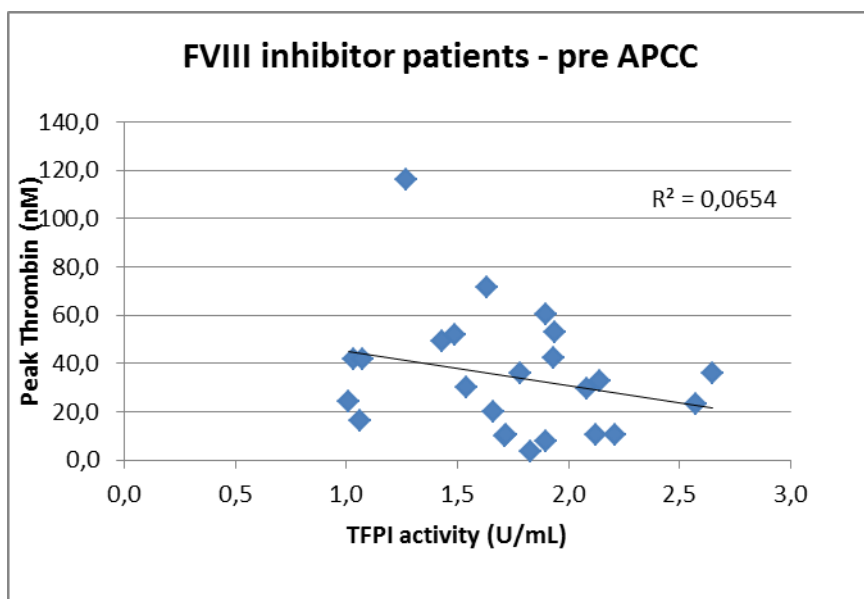


Figure 31: Hemophilia A patients with FVIII inhibitor (patient samples before APCC application): there is no correlation between TFPI activity (U/mL) and measured Peak Thrombin (nM) ($R^2 = 0,0654$).

Figure 30 graphically illustrates that there was no correlation between TFPI activity and peak thrombin in healthy individuals, as the coefficient of determination only was about 0,039.

As in Figure 31, here too no correlation between TFPI activity measured before APCC injection and peak thrombin in hemophilia A patients with FVIII inhibitor could be found. Also in this case, the coefficient of determination is very low ($R^2 = 0,065$).

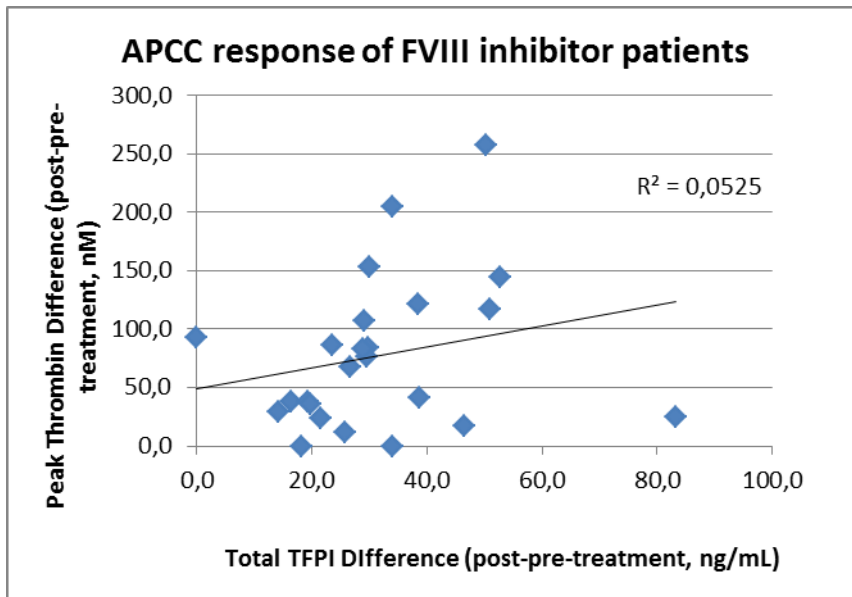


Figure 32: APCC response of patients with hemophilia A with FVIII inhibitors: there is no correlation between the Peak Thrombin Difference (post-pre-treatment, nM) and the total TFPI Difference (post-pre-treatment, ng/mL) ($R^2 = 0,0525$).

Figure 32 displays the not existing correlation between the peak thrombin difference and the total TFPI difference of patient samples. This fact is approved by a very low coefficient of determination ($R^2 = 0,052$).

Summing up, it can be said that due to the fact of no correlation between the particular parameters, no predictions concerning the response to APCC of hemophilia A patients with FVIII inhibitors can be made.

4 Conclusion and Discussion

The aim of this work was a detailed investigation of APCC (Activated Prothrombin Complex Concentrate) with special focus on the influence of TFPI, contained in APCC (as investigated recently - Ogiwara K, et al. 2010), on the in vitro procoagulant effect in patients with hemophilia A with inhibitors.

Hemophilia is a severe bleeding disorder, which can result in significant morbidity and mortality. There are two types of this disease, hemophilia A and B. Patients with hemophilia A suffer from a lack of factor VIII. Patients with hemophilia B suffer from a lack of factor IX. Hemophilia is caused by a defect in the factor VIII or IX gene.

Therefore, the activity or the occurrence of the two factors can be restricted differently, depending on the severity of the disease. Replacing the deficient factor effectively treats bleeding episodes. However, it is possible that patients develop inhibitors to factor VIII or IX, which leads to severe and expensive complications in treatment. The presence of high titer inhibitors, in particular, renders replacement therapy completely ineffective. In such cases, bypassing agents are required to treat bleeding episodes. Currently, two bypassing agents are available for treating hemophilia patients with inhibitors: activated prothrombin complex concentrates (APCC) and recombinant activated factor VII. Both bypassing agents are safe, effective treatments for bleeding episodes in hemophilia patients with inhibitors (Myung Hee Han, Young Shil Park, 2013).

By definition, activated prothrombin complex concentrates (APCCs) are capable of inducing thrombin generation by direct activation of FX without the necessity of participation of factor VIII (Turecek PL, et al. 1999). APCC is an intermediate purified protein concentrate and isolated from human plasma.

Conclusion and Discussion

The complex of activated FX (FXa) and FII (prothrombinase complex) had been identified to be the key components of this product. It has been found to be a major target site for APCC (Turecek PL, et al. 2004).

The FXa-FII complex induces immediate TG on the membrane of TF-bearing cells, and activated platelets, but only in the presence of FV (Mann KG, et al. 1981). This low amount of thrombin induces the activation of further clotting factors by feedback reactions which also involve the patient's endogenous haemostasis factors. Other protein components of APCC have amplifying roles by increasing the concentrations of the available clotting proteins. The simultaneous increase in the amount of natural inhibitors helps to balance TG, and thus may prevent thrombosis (Váradi K, et al 2011).

APCC is standardized to a sum activity: by definition, 1 unit of activity expresses the amount of APCC that shortens the APTT (activated partial thromboplastin time) of high-titer FVIII inhibitor reference plasma to 50% of blank value (= test control without APCC) (Turecek PL, et al. 2004).

For a detailed examination of TFPI contained in APCC and its influence on the APCC effect in patients with hemophilia A, there were used several assays. The thrombin generations assay (TGA), an ELISA test for the measurement of total TFPI, a TFPI activity assay for determination of TFPI activity, SDS-PAGE and Western Blot for the visualization of TFPI in APCC lots.

The thrombin generation assay (TGA) enables the pharmacodynamic and pharmacokinetic properties of by-passing therapies to be monitored, thus helping to optimize treatment (Váradi, K., et al. 2003). It was developed for monitoring the kinetic of thrombin generation in human FVIII inhibitor plasma reconstituted in vitro with activated prothrombin complex concentrate. The coagulation is triggered in citrated plasma by adding low concentration of tissue factor/phospholipid complex and CaCl_2 in the presence of fluorogenic thrombin substrate. Generated thrombin cleaves the substrate and therefore the fluorescence intensity increases. The intensity of the fluorescence signal (FU) is continuously monitored, and the rate of increase in the

Conclusion and Discussion

fluorescence signal for every time point, which reflects the actual thrombin concentrations, is calculated (Váradi K, et al. 2003).

For the determination of total TFPI in APCC samples the AssayMax Human TFPI ELISA kit was used. A polyclonal antibody specific for TFPI has been pre-coated onto a 96-well microplate with removable strips. TFPI in standards and samples is sandwiched by the immobilized polyclonal antibody and biotinylated polyclonal antibody specific for TFPI, which is recognized by a streptavidin-peroxidase conjugate. After addition of a peroxidase enzyme substrate, the color development is stopped and the intensity of the color is measured.

For the measurement of tissue factor pathway inhibitor activity in human plasma the ACTICHROME[®] TFPI Activity Assay, a chromogenic assay, is intended. It measures the ability of TFPI to inhibit the catalytic activity of the tissue factor/FVIIa complex to activate factor X to factor Xa. The residual activity of the TF/FVIIa complex is measured using SPECTROZYME[®] FXa, a highly specific chromogenic substrate cleaved only by FXa generated in the assay, releasing a p-nitroaniline (pNA) chromophore. The absorbance of the pNA in the reaction solution at 405 nm is measured.

For the visualization of TFPI in APCC samples, SDS-PAGE was used. Thereby, proteins based on the isoelectric point can be separated and identified. The separation of molecules by electrophoresis is based on the fact that charged molecules migrate through a gel matrix upon application of an electric field. The proteins have a net electrical charge if they are in a medium having a pH different from their isoelectric point and therefore have the ability to move when subjected to an electric field. The migration velocity is proportional to the ratio between the charges of the protein and its mass. The higher charge per unit of mass the faster the migration.

Afterwards the immunodetection of TFPI with Western Blot followed. This method is widely used to detect proteins in a sample of cell homogenate or extract. The proteins are transferred from the gel to a membrane, made of nitrocellulose, nylon or polyvinylidene difluoride (PVDF), where they are examined using specific antibodies to the protein. As a result, the amount of protein in a sample can be examined and it is

also possible to compare levels among various analytical groups (García-Descalzo L, et al. 2012).

Before performing gel electrophoresis, it was necessary to immunoprecipitate TFPI out of APCC samples for optimal results in the SDS-PAGE. Immunoprecipitation (IP) is the technique of precipitating a protein antigen out of solution using an antibody that specifically binds to that particular protein and concentrates a particular protein from a sample containing many thousands of different proteins, in this case TFPI out of APCC.

As already mentioned, APCC is a mixture of activated coagulation factors, prothrombin complex zymogenes, inhibitors and co-factors. Furthermore, recent data confirm the presence of TFPI in APCC (Ogiwara K, et al. 2010).

According to published data by Dr. Ogiwara K, et al. 2010, there are some patients with hemophilia A with inhibitors, who do not respond very well to treatment with APCC. The results of the research of Dr. Ogiwara K, et al. 2010, support that TFPI contained in APCC attenuates the potentials of thrombin generation in hemophilia A patients with inhibitors.

In this work, it was tried to figure out the effective influence of TFPI contained in APCC on the APCC effect in patients with hemophilia A with inhibitors. Therefore the subsequent areas of investigations and key questions are as follows:

- TFPI content in different APCC batches/ FVIII inhibitor plasma samples
- Is the TFPI in APCC active?
- Visualization of TFPI in APCC with SDS-PAGE
- Investigation of the effect of TFPI-content of APCC – in vitro modeling by TGA
- Effect of TFPI concentration in plasma/ in APCC
- Investigation on the influence of TFPI on synthetic APCC-Mix
- Determination of TFPI levels in normal plasma
- Determination of TFPI levels in plasma from patients with hemophilia A with inhibitors before and after APCC treatment

Conclusion and Discussion

In the first part of my work I investigated a detailed examination and characterization of APCC samples with special interest of TFPI and its influence on APCC activity.

By means of the ELISA assay (AssayPro, AssayMax Human Tissue Factor Pathway Inhibitor (TFPI ELISA Kit)) the content of total TFPI in 9 different APCC batches and one APCC reference standard could be measured. Moreover, two FVIII inhibitor plasmas were measured as well as pooled human normal plasma. APCC lots contained 12,8 – 29,6 ng total TFPI in a 1U/mL APCC solution compared to the measured 105,1 ng/mL of pooled human normal plasma. The value of FVIII inhibitor plasma (#3V1A000, Baxter) was about 66 ng/mL and the value of FVIII inhibitor plasma (GK, #GK1838-2468) about 113 ng/mL. So it could be noted, that the two FVIII inhibitor plasmas showed quite different levels. This fact played an important role for further planning of the following experiments.

It was also tried to measure the TFPI activity in APCC lots and the plasmas with a chromogenic assay (Actichrome TFPI Activity Assay). While the values of TFPI activity in plasma ranged about 1 U/mL (see Table 7), none was detected in any of the given APCC lots. The assay was also carried out with negative controls in addition to the common test procedure because there was the possibility that the assay could be disturbed. As a matter of fact, thereby it was proven that this activity assay was not suitable for APCC, because FX and FXa contained in APCC falsified the results.

For the visualization of TFPI contained in APCC samples the SDS-PAGE and subsequent Western Blot were performed. Before the gel electrophoresis, an immunoprecipitation of TFPI out of the APCC lots was carried out. (Pretests showed that without performing immunoprecipitation of this special protein, a visualization of TFPI in different APCC lots was not possible, because the amount of TFPI was too little and the amount of other proteins contained in APCC too big.) As a result, it was possible to visualize TFPI full-length out of APCC lots. The final concentration of TFPI full-length of the APCC lots was 3,6 ng/1 Unit APCC (APCC Nr. 1), 1,8 ng/1 Unit APCC (APCC Nr.2) and 2,5 ng/1 Unit APCC (APCC Nr.7). Truncated TFPI could not be detected because it probably was below the detection limit of the Western Blot.

Conclusion and Discussion

After the assessment of TFPI content and its visualization with the SDS-PAGE, a detailed investigation on the influence of TFPI in plasma and TFPI in APCC on the APCC activity follows.

First, APCC samples were measured in the TGA comparing their thrombin generation in FVIII inhibitor plasma with low and high TFPI content. The peak thrombin of all APCC lots of 1 U/mL APCC ranged between 193nM to 323,4nM in plasma with low TFPI content (#3V1A000, Baxter, total TFPI: 66ng/mL) and 124,7nM to 221nM in plasma with high TFPI content (#GK1838-2468, George King, total TFPI: 113 ng/mL). Therefore it could be discovered that thrombin generation in plasma with high TFPI content was lower than in plasma with low TFPI content. This led to the assumption, that plasma with high endogenous TFPI content had the stronger inhibitory effect on the thrombin generation of APCC batches than plasma with lower TFPI content. Accordingly, it was verified that TFPI contained in plasma has an effect on the thrombin generation of APCC batches in FVIII inhibitor plasma.

In the next experiment the total TFPI in FVIII inhibitor plasma was measured with and without APCC spiking. The total TFPI increased after spiking APCC in FVIII inhibitor plasma because of the added amount of TFPI in APCC. The increase of total TFPI in FVIII inhibitor plasma also corresponds to the total TFPI content in measured APCC batches in one of the first experiments (coefficient of determination $R^2=0,5$).

Subsequently, spiking experiments followed to examine the effect of added TFPI to the system (APCC + FVIII inhibitor plasma) on the APCC activity and on the thrombin generation in FVIII inhibitor plasma, respectively. On the one hand TFPI was spiked in different concentrations in APCC and this TFPI-APCC in FVIII inhibitor plasma was measured in the TGA. On the other hand TFPI was spiked in FVIII inhibitor plasma and APCC with TFPI-plasma mix was measured in the TGA. This was done to find out if it made any differences spiking TFPI in APCC or plasma. Considering the total TFPI was identical in both systems in the end, both ways showed the same decreasing tendency of thrombin generation after increasing added TFPI. Consequently, there exists no difference in TFPI added to plasma or APCC. In addition, thrombin generations of APCC

Conclusion and Discussion

in FVIII inhibitor plasma depended on TFPI concentration in plasma as well as on TFPI concentration in APCC.

The purpose of the following experiments was to determine the influence of TFPI on a synthetic APCC-Mix and if there can be discovered the same effects compared with normal APCC. The "synthetic" APCC was reconstructed from its purified components and showed analogous thrombin generation to APCC in the TGA. To monitor the influence of TFPI on the synthetic APCC-Mix, TFPI was added in different concentrations (0 - 200ng/mL) in the synthetic APCC-Mix, respectively. TFPI was also spiked in the APCC standard with the same concentrations, for comparison. APCC and synthetic APCC-Mix resulted in exhibiting very similar thrombin generation after spiking TFPI. With increasing added TFPI the thrombin generation decreased. Moreover it could be established that at a TFPI concentration as it is contained in APCC batches (10 – 30ng/mL) there was just a very slight decrease of thrombin generation in APCC and in the synthetic APCC-Mix as well. Thus, TFPI, in concentrations as it is contained in APCC, appears to show no prominent effect on the thrombin generation, which leads to the assumption that TFPI contained in APCC does not influence the effect of APCC in patients with hemophilia A with inhibitors.

The second part of this work describes the differences in normal plasma compared with patient plasma of patients with hemophilia A with inhibitors concerning the thrombin generation, TFPI content and TFPI activity. Furthermore it was approached to obtain correlations between the different parameters (Peak Thrombin, TFPI activity, total TFPI) trying to find relations that would eventually enable to make predictions concerning the response of patients to APCC.

At first, plasma samples of healthy donors were compared with samples of patients with hemophilia A with inhibitors before the application of APCC.

Plasma samples of healthy donors and of patients with hemophilia A with inhibitors were measured in the TGA. The mean value of peak thrombin calculated for normal plasma was about 190 nM with a standard deviation of 56,2. In comparison, in patient

Conclusion and Discussion

samples a peak thrombin of 34,6 nM and a standard deviation of 25,2 was measured on average. So thrombin generation in patients with hemophilia A with inhibitors before application of APCC was very low compared to thrombin generation in normal plasma. Nevertheless both groups showed a high variability of thrombin generation response in different plasmas of both groups.

Measuring the total TFPI in patients and normal plasma it showed that the total TFPI in patient plasma was significantly lower compared to normal plasma. While normal plasma samples showed an average total TFPI content of 152,9 ng/mL (SD = 35,0), patient plasma samples measured a mean value of 126,1 ng/mL (SD = 39,8) of total TFPI. The significance was confirmed by statistical analysis (*t*-test (Rasch B, et al. 2010)).

TFPI activity in patient plasma also was significantly lower compared to normal plasma. The significance was confirmed by statistical analysis (*t*-test (Rasch B, et al. 2010)). The mean value of TFPI activity was 2,5 U/mL (SD = 0,8) in normal plasma samples and 1,7 U/mL (SD = 0,5) in patient plasma samples.

The following experiments were performed to compare patients' plasma samples before and after APCC application in view of a possible influence of TFPI.

In the TGA the thrombin generation rose in every patient after APCC application. Nonetheless the samples showed great differences concerning the response to APCC. Some of them showed high response, some poor response. Therefore they very separated arbitrarily in two groups: poor and good responders. All patients having a difference pre-post infusion peak thrombin of lower than 75 nM were defined as poor responders, the one with values higher than 75 nM as good responders.

For further experiments the patients were divided into two groups as mentioned above.

Also the total TFPI increased after APCC application and it corresponded to the TFPI content of the APCC batches in addition. But no differences could be observed between good responders and poor responders regarding the pre-post APCC total TFPI as it is shown graphically in Figure 29 and listed in Table 22.

Conclusion and Discussion

Regarding possible existing correlations, there was found no correlation between TFPI activity and peak thrombin in healthy individuals, as the coefficient of determination only was about 0,039. Also no correlation existed between TFPI activity measured before APCC injection and peak thrombin in hemophilia A patients with FVIII inhibitor ($R^2 = 0,065$), nor one between the peak thrombin difference and the total TFPI difference of patient samples ($R^2 = 0,052$). Thus it can be said that due to the fact of no correlations between the particular parameters, there cannot be made any predictions concerning the response to APCC of hemophilia A patients with FVIII inhibitors.

Summing up, TFPI levels in APCC approximately range between 10 and 30 ng/mL in 1U APCC. The thrombin generation of APCC in FVIII inhibitor plasma depends on TFPI concentration in plasma and TFPI concentration in APCC. Likewise, TFPI in APCC has no prominent effect on the thrombin generation, because the concentration is very low in APCC as it could be seen in the spiking experiments as well as in the experiments with the „synthetic“ APCC-Mix. Whether TFPI in APCC is active or not could not be determined.

The thrombin generation after APCC application increases in patient samples with hemophilia A with inhibitors, this however to a different extent.

There exists no correlation between total TFPI and the response to APCC of patients with hemophilia A with inhibitors. Therefore no predictions can be made based on pre-treatment TFPI level of a patient for his response to APCC.

Finally it can be concluded that TFPI in APCC seems to have no influence on the effect of APCC in patients and the data of this work did not confirm the findings of Ogiwara et al.

5 Summary (English)

Activated prothrombin complex concentrates (APCC) are an established treatment for bleeding in patients with hemophilia who have developed inhibitory antibodies against factor VIII (FVIII) or factor IX (FIX) (Turecek PL, et al. 2004). Hemophilia is a severe bleeding disorder where patients suffer from a lack of FVIII or FIX. Primarily these patients are treated with substitution of the deficient factor. However, in some cases it is possible that patients develop inhibitors to factor VIII or IX, which requires treatment with products which are capable of inducing thrombin generation without the necessity of participation of FVIII or FIX, such as APCC or activated factor VII (FVIIa) (Myung Hee Han and Young Shil Park. 2013).

APCC is comprised of the vitamin K dependent prothrombin complex proteins in their zymogen forms. These are the procoagulant factors FII, FVII, FIX, FX, and the anticoagulant protein C in physiologically balanced ratio. It also contains small amounts of the activated forms of the procoagulant factors as well as the cofactors protein S, FV and FVIII. Multiple interactions of these components with patients' endogenous hemostatic factors restore the impaired thrombin generation of hemophilia patients who have developed inhibitors. A complex of activated FX and FII (FXa-FII) in APCC is considered to be the key active principle (Váradi K, et al. 2011). In addition, recent data show the presence of TFPI in APCC. Furthermore, it is suggested that TFPI contained in APCC attenuates the potentials of thrombin generation in hemophilia A patients with inhibitors (Ogiwara K, et al. 2010).

The aim of this work is to verify if TFPI contained in APCC actually has an influence on the APCC effect in patients with inhibitors.

Therefore, in the first part of this work 9 different APCC samples and one APCC reference standard were examined in more detail with special interest on TFPI contained in APCC. Measurement results of total TFPI levels in APCC ranged approximately between 10 and 30 ng/mL per 1U APCC (performed with total TFPI

Summary (English)

ELISA assay). Throughout analysis of the results no TFPI activity was detected (measured with TFPI Activity Assay). By means of SDS-PAGE and Western Blot, full-length TFPI was possible to visualize in APCC batches. In the thrombin generation assay (TGA), which describes the dynamics of hemostasis by measuring the kinetics of clot formation triggered by thrombin (Turecek PL, et al. 2004), it could be proved that thrombin generation of APCC in FVIII inhibitor plasma depends on the TFPI concentration in plasma and APCC. However, it was shown that TFPI, in concentrations as occur in APCC, had no prominent effect on the thrombin generation.

The second part of this work concentrates on a detailed investigation of plasma samples of patients with hemophilia A with inhibitors before and after APCC application. With aid of different parameters measured from the samples (thrombin generation, total TFPI, TFPI activity) it could be proved that there existed no correlation between total TFPI and the response to APCC of patients with hemophilia A with inhibitors, extrapolated from *in vitro* spiking studies. Therefore no predictions can be made of a patients' response to APCC based on his/her pre-treatment TFPI level.

Hence, it can be concluded that TFPI in APCC seems to have no influence on the effect of APCC in patients. The results of this work therefore did not confirm the findings of Ogiwara et al.

6 Summary (German)

Aktivierte Prothrombinkomplexkonzentrate (APCC) sind eine etablierte Behandlung bei Blutungen von Patienten mit Hämophilie, die inhibitorische Antikörper gegen Faktor VIII (FVIII) oder Faktor IX (FIX) entwickelt haben (Turecek PL, et al. 2004). Hämophilie ist eine schwere Blutgerinnungsstörung. Erkrankte leiden unter einem Mangel an FVIII oder FIX. Hauptsächlich werden diese Patienten durch Substitution des fehlenden Faktors behandelt. In einigen Fällen ist es jedoch möglich, dass Patienten Inhibitoren gegen Faktor VIII oder IX entwickeln. Diese Patienten müssen mit Medikamenten (APCC oder aktivierten Faktor VII (FVIIa)) behandelt werden, welche zur Thrombinbildung führen ohne, dass dabei FVIII oder FIX beteiligt ist (Myung Hee Han und Young Shil Park. 2013).

APCC besteht aus den zymogenen Formen der Vitamin K-abhängigen Prothrombin-Komplex- Proteinen. Diese sind die prokoagulativen Faktoren FII, FVII, FIX, FX, und das gerinnungshemmende Protein C in einem physiologisch ausgewogenen Verhältnis. APCC enthält weiters kleine Mengen aktivierte Formen der prokoagulativen Faktoren sowie der Co-Faktoren Protein S, FV und FVIII. Zahlreiche Interaktionen dieser Komponenten mit den patienteneigenen hämostatischen Faktoren können die gestörte Thrombingenerierung von Hämophiliepatienten, die Inhibitoren entwickelte haben, wiederherstellen. Ein Komplex aus aktivierten FX und FII (FXa-FII) im APCC soll das aktive Schlüsselprinzip sein (Váradi K, et al. 2011). Weiters bestätigen jüngste Daten die Präsenz von TFPI in APCC. Außerdem wird vermutet, dass TFPI, der in APCC enthalten ist, das Potential der Thrombinbildung in Hämophilie A Patienten mit Inhibitoren vermindert (Ogiwara K, et al. 2010).

Ziel dieser Arbeit ist es, zu überprüfen, ob TFPI, der in APCC enthalten ist, tatsächlich einen Einfluss auf die APCC Wirkung bei Patienten mit Inhibitoren hat.

Daher wurden, mit besonderem Augenmerk auf TFPI in APCC, im ersten Teil dieser Arbeit neun verschiedene APCC Proben und ein APCC Referenzstandard im Detail untersucht. Messergebnisse des gesamten TFPI Gehalts in APCC variierten zwischen 10

und 30 ng/ ml pro 1U APCC (durchgeführt mit total TFPI ELISA Assay). Bei der Analyse der Ergebnisse konnte keine TFPI -Aktivität nachgewiesen werden (mit TFPI – Aktivitätstest (TFPI Activity Assay)). Mittels SDS- PAGE und Western- Blot gelang es full-length TFPI in den APCC Proben zu visualisieren. Mithilfe des Thrombin Generation Assays (TGA), welcher die Dynamik der Gerinnung durch Messung der Kinetik der Bildung von Blutgerinnseln, ausgelöst durch Thrombin, beschreibt (Turecek PL, et al. 2004), konnte nachgewiesen werden, dass die Thrombinbildung von APCC in FVIII Inhibitor Plasma von der TFPI -Konzentration im Plasma und im APCC abhängt . Es wurde jedoch gezeigt, dass TFPI in Konzentrationen, wie sie in APCC auftreten, keine bedeutende Wirkung auf die Thrombinbildung hatte.

Der zweite Teil der Arbeit konzentriert sich auf eine detaillierte Untersuchung der Plasmaproben von Patienten mit Hämophilie A mit Inhibitoren, vor und nach APCC Anwendung. Basierend auf *in-vitro* Spiking Studien konnte mit Hilfe von verschiedenen, gemessenen Parametern der Proben (Thrombinbildung, Gesamt -TFPI-Gehalt, TFPI -Aktivität) nachgewiesen werden, dass keine Korrelation zwischen dem gesamten TFPI Gehalt und der Reaktion auf APCC bei Hämophilie A - Patienten mit Inhibitoren existierte. Daher können keine Vorhersagen der Reaktion eines Patienten auf APCC auf Grundlage seines / ihres Vorbehandlungs-TFPI Levels getroffen werden.

Schlussfolgernd scheint TFPI in APCC keinen Einfluss auf die Wirkung von APCC in Patienten zu haben. Die Resultate dieser Arbeit bestätigen daher nicht die Ergebnisse von Ogiwara et al.

7 Appendix

7.1 List of Tables

Table 1: List of coagulation factors ((Barthels M and Depka M. 2002)/ (Golenhofen K. 2006)/ (Pötzsch B and Madlener K. 2010)/ (Hagemann O. 2013)/ (Lefkowitz JB. 2008))	13
Table 2: Components of the APCC and their averaged concentrations of several APCC lots (minimum – maximum); the values are referred to 1 unit (U) of the APCC (Montsch P. 2010)	33
Table 3: Pipetting schema.....	61
Table 4: TFPI levels in different APCC Lots	68
Table 5: TFPI levels in different FVIII inhibitor plasmas & normal plasma	68
Table 6: TFPI activity levels in different APCC Lots.....	70
Table 7: TFPI activity levels in different FVIII inhibitor plasmas & normal plasma	71
Table 8: Measurement of APCC Nr. 2. There was no TFPI activity detected.	72
Table 9: Measurement of APCC Nr.2 without the addition of FX as reagent. With increasing dilution of the sample, a dilution effect of APCC-FX is detectable. But again, there was no activity calculation possible.	72
Table 10: SDS-PAGE: pipetting scheme of pretest	74
Table 11: Pipetting scheme of tests with previous immunoprecipitation	76
Table 12: Calculation of TFPI full-length of APCC lots	78
Table 13: Peak Thrombin (nM) of 1 Unit APCC/mL FVIII inhibitor plasma	79
Table 14: Total TFPI content of FVIII inhibitor plasmas before and after spiking APCC.	83

Appendix

Table 15: Peak Thrombin (% of no TFPI added) of APCC in FVIII inhibitor plasma, when TFPI was spiked in plasma	89
Table 16: Peak Thrombin (% of no TFPI added) of APCC in FVIII inhibitor plasma, when TFPI was spiked in APCC.....	89
Table 17: Particular concentration of each component for generation of synthetic APCC mix (Montsch P. 2010)	92
Table 18: Peak Thrombin (nM) of normal plasma and patient plasma samples (before APCC infusion)	98
Table 19: Total TFPI content of normal plasma and patient plasma (before APCC infusion).....	100
Table 20: TFPI activity of normal plasma and patient plasma samples (before APCC application).....	102
Table 21: Classification in poor and good responder.....	105
Table 22: Difference total TFPI of pre and post patient plasma samples classified in two groups: good and poor responder	107

7.2 List of Figures

Figure 1: Coagulation cascade (Golan DE, et al. 2011)	17
Figure 2: Schematic model of the mechanism of action of activated prothrombin complex concentrate (APCC)	31
Figure 3: Thrombin generation curves of normal plasma and FVIII inhibitor plasma, and parameters of thrombin generation which can be read from the thrombin generation curve	37
Figure 4: Total TFPI (ng/mL) in different APCC Lots measured with the Total TFPI ELISA kit (Assay Pro)	69
Figure 5: Total TFPI (ng/mL) levels in different FVIII inhibitor plasma & normal plasma measured with the Total TFPI	69
Figure 6: TFPI Standard curve	71
Figure 7: Western Blot (pretest) with anti-TFPI-6 (K2), 2 s	75
Figure 8: Western Blot (pretest) with anti-TFPI-27 (C-terminal), 32 s	75
Figure 9: Western Blot of APCC-IPs with anti-TFPI-6 (K2), 1 s	77
Figure 10: Western Blot of APCC-IPs with anti-TFPI-27 (C-terminal), 4 s	77
Figure 11: TFPI fl standard curve of measured values of applied TFPI standard concentrations with CCD camera	78
Figure 12: Peak Thrombin of APCC lots in both FVIII inhibitor plasmas at an APCC concentration of 1 U/mL	80
Figure 13: Dose response of APCC lots (APCC standard, APCC Nr.1 – 4). Thrombin generations curves in APCC concentrations of 0/ 0,125/ 0,25/ 0,5/ 1 Unit APCC/ml FVIII inhibitor plasma (FVIII Inh. Pl. with low TFPI content (Bax), FVIII Inh. Pl. with high TFPI content (GK))	81

Appendix

Figure 14: Dose response of APCC lots (APCC standard, APCC Nr.5 – 9). Thrombin generations curves in APCC concentrations of 0/ 0,125/ 0,25/ 0,5/ 1 Unit APCC/ml FVIII inhibitor plasma (FVIII Inh. Pl. with low TFPI content (Bax), FVIII Inh. Pl. with high TFPI content (GK)).	82
Figure 15: Comparison of total TFPI content of FVIII inhibitor plasmas before and after spiking APCC	84
Figure 16: Correlation of increase of total TFPI in FVIII inh. pl. (Bax) (ng/mL) and of total TFPI content (ng/mL) measured in APCC batches	85
Figure 17: Correlation of increase of total TFPI in FVIII inh. pl. (GK) (ng/mL) and of total TFPI content (ng/mL) measured in APCC batches	86
Figure 18: TFPI spiked in FVIII inhibitor plasma in different concentrations and APCC dose response. Thrombin generation decreases with increasing added TFPI.	88
Figure 19: TFPI spiked in APCC in different concentrations and APCC dose response in FVIII inhibitor plasma. Thrombin generation decreases with increasing added TFPI.	88
Figure 20: No difference if TFPI is added to plasma or to APCC considering that the total TFPI is identical in both systems in the end.	89
Figure 21: Thrombin generation curves of APCC Nr.2 and synthetic APCC-Mix (1 Unit/mL respectively) in FVIII inhibitor plasma (#3V1B000, Baxter).	93
Figure 22: Comparison of dose response of APCC and synthetic APCC-Mix in FVIII inhibitor plasma (#3V1B000, Baxter).	94
Figure 23: Thrombin generation of synthetic APCC-Mix and APCC standard (1U/mL) after spiking different concentrations of TFPI	95
Figure 24: Thrombin generation in normal plasma and in patient plasma samples (before APCC application). TG in patient plasma was very low compared to TG in normal plasma.	99
Figure 25: Total TFPI content of normal plasma and patient plasma (before APCC infusion).	101

Appendix

Figure 26: TFPI activity of normal plasma and patient plasma samples (before APCC application)	103
Figure 27: Thrombin generation in patient plasma before / after APCC application ..	104
Figure 28: Total TFPI (ng/mL) in patient samples before / after APCC application	106
Figure 29: Difference of total TFPI (ng/mL) in patient samples before / after APCC application	106
Figure 30: Healthy donors (normal plasma samples): there is no correlation between TFPI activity (U/mL) and Peak Thrombin (nM) ($R^2 = 0,0388$).....	108
Figure 31: Hemophilia A patients with FVIII inhibitor (patient samples before APCC application): there is no correlation between TFPI activity (U/mL) and measured Peak Thrombin (nM) ($R^2 = 0,0654$).....	108
Figure 32: APCC response of patients with hemophilia A with FVIII inhibitors: there is no correlation between the Peak Thrombin Difference (post-pre-treatment, nM) and the total TFPI Difference (post-pre-treatment, ng/mL) ($R^2 = 0,0525$).....	109

7.3 List of Abbreviations

ADP	adenosine diphosphate
APC	activated protein C
APTT	activated partial thromboplastin time
APCC	activated prothrombin complex concentrate
conc.	concentration
IATI	inter α - trypsin inhibitor
IP	immunoprecipitate
ITT	immunological tolerance therapy
Nr.	number
PAF	platelet activated factor
PAI	plasminogen activator inhibitor
PF	platelet factor
PPP	platelet-poor plasma
PRP	platelet-rich plasma
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TAFI	thrombin-activated fibrinolytic inhibitor
TF	tissue factor
TG	thrombin generation
TFPI	tissue factor pathway inhibitor
TFPI fl	tissue factor pathway inhibitor full-length
hTFPI	human tissue factor pathway inhibitor
VN	vitronectin
VWF	von-Willebrand factor

7.4 References

- Antunes SV, et al. 2014.** *Randomized comparison of prophylaxis and on-demand regimens with FEIBA NF in the treatment of haemophilia A and B with inhibitors. Haemophilia.* 2014 Jan;20(1):65-72.
- Barril P and Nates S. 2012.** *Introduction to Agarose and Polyacrylamide Gel Electrophoresis Matrices with Respect to Their Detection Sensitivities, Gel Electrophoresis - Principles and Basics.* Dr. Sameh Magdeldin (Ed.), ISBN: 978-953-51-0458-2, InTech, Available from: <http://www.intechopen.com/books/gel-electrophoresis-principles-and-basics/introduction-to-agarose-and-polyacrylamide-gel-electrophoresis-matrices-with-respect-to-their-detect>
- Barthels M and Depka M. 2002.** *Gerinnungskompndium.* Stuttgart : Thieme, 2002.
- Brackmann HH, et al. 2003.** *Klinische Anwendung plasmatischer und rekombinanter Gerinnungsfaktoren.* s.l. : uni-med, 2003.
- Butenas S, et al. 2002.** *Mechanism of factor VIIa-dependent coagulation in hemophilia blood.* s.l. : Blood;vol.99:923-930, 2002.
- Cawthorn KM, et al. 1998.** *Blood Coagulation in Hemophilia A and Hemophilia C.* s.l. : Blood by the American Society of Hematology; www.bloodjournal.org; 91: 4581-4592, 1998.
- Chantarangkul V, et al. 2003.** *Thrombin generation assessed as endogenous thrombin potential in patients with hyper- or hypocoagulability.* s.l. : heamatologica/journal of heamatology vol. 88(05): 547-554, 2003.
- Colman RW, et al. 2005.** *Hemostasis and Thrombosis: Basic Principles and Clinical Practice.* s.l. : Lippincott Williams & Wilki, 5th ed. (December 2005)
- de Smedt E, et al. 2008.** *The technique of measuring thrombin generation with fluorogenic substrates: I. Necessity of adequate calibration.* s.l. : Thromb Haemost; 100:343-349, 2008.

Dempfle CE. 2007. *The TAFI system – The new role of fibrinolysis.* Universitätsklinikum Mannheim, I. Medizinische Klinik, Mannheim : Hämostaseologie; Vol 27; p. 278-281, 2007. ISSN: 0720-9355

Estler CJ. 2000. *Pharmakologie und Toxikologie.* s.l. : Schattauer; 5. Auflage, 2000.

Fareed J, et al. 1995. *Tissue factor antigen levels in various biological fluids.* s.l. : Blood Coagul. Fibrinolysis; 6 (suppl 1):32-36, 1995.

García-Descalzo L, et al. 2012. *Gel Electrophoresis of Protein, Gel Electrophoresis - Principles and Basics.* Dr. Sameh Magdeldin (Ed.), ISBN: 978-953-51-0458-2, InTech, Available from: <http://www.intechopen.com/books/gel-electrophoresis-principles-and-basics/gel-electrophoresis-of-proteins>

Goudemand J, et al. 2006. *Influence of the type of factor VIII concentrate on the incidence of factor VIII inhibitors in previously untreated patients with severe hemophilia A.* s.l. : Blood;107: 46-51, 2006

Golan DE, et al. 2011. *Principles of Pharmacology: The Pathophysiologic Basis of Drug Therapy.* s.l. : Lippincott Williams & Wilkins; 3rd edition, 2011.

Golenhofen K. 2006. *Basislehrbuch Physiologie.* München : Elsevier GmbH; Urban & Fischer Verlag; 4 Auflage, 2006.

Hagemann O. 2013. Laborlexikon. (online, citation of December, 2013). <http://www.laborlexikon.de/Lexikon/Tabellen/45-Gerinnungsfaktoren.htm>.

Halkier T. 2009. *Mechanisms in Blood Coagulation, Fibrinolysis and the Complement System.* s.l. : Cambridge University Press; Auflage: 1, 2009.

Hemker HC and Béguin S. 1990. *Method of determining the endogenous thrombin potential of plasma and blood.* 1990.

Hemker HC and Béguin S. 1995. *Thrombin Generation in Plasma: Its Assessment Via the Endogenous Thrombin Potential.* s.l. : Thromb. Haemost.; 74(1) 134-138, 1995.

Hemker HC, et al. 1993. *Continuous Registration of Thrombin Generation in Plasma, Its Use for the Determination of the Thrombin Potential.* s.l. : Thromb. and Haemost.; 70, (4) 617-624, 1993.

Hemker HC, et al. 2000. *The Thrombogram: Monitoring Thrombin Generation in Platelet Rich Plasma.* s.l. : Thromb. Haemost.; 83: 589-591, 2000.

Hemker HC, et al. 2003. *Calibrated Automated Thrombin Generation Measurement in Clotting Plasma.* s.l. : Pathophysiol. Haemost. Thromb.; 33:4-15, 2003.

Hoffman M, et al. 1995. *Factors IXa and Xa play distinct roles in tissue factor-dependent initiation of coagulation.* s.l. : Blood; 86:1794-1801,1995.

Hoffman M, et al. 1994. *Human monocytes support factor X activation by VIIa, independent of tissue factor: Implications for the therapeutic mechanism of high dose factor VIIa in hemophilia.* s.l. : Blood;83:38-41, 1994.

Jansen M, et al. 2001. *Treatment of coagulation inhibitors with extracorporeal immunoadsorption (Ig-Therasorb).* s.l. : Brit J Haematol;112:91-97, 2001.

Kasper CK, et al. 1975. *A more uniform measurement of factor VIII inhibitors.* s.l. : Thrombos Diathes Haemorrh;34:870-872, 1975.

Kempton CL, et al. 2006. *Incidence of the inhibitors in a cohort of 838 males with hemophilia A previously treated with factor VIII concentrates.* s.l. : J Thromb Haemost;4:2576-2581, 2006.

Lefkowitz JB. 2008. *Coagulation Pathway and Physiology.* (online, citation of December, 2013)
http://www.cap.org/apps/docs/cap_press/hemostasis_testing/coagulation_pathway.pdf

Lusher JM. 2000. *Inhibitor antibodies to factor VIII and factor IX: management.* s.l. : Semin Thromb Hemost;26:179-88, 2000.

Lwaleed BA and Bass PS. 2005. *Tissue factor pathway inhibitor: structure, biology and involvement in disease.* s.l. : Journal of Pathology, 10.1002/path. 1871, 2005.

Lyon MF. 2002. *X-chromosome inactivation and human.* s.l. : Acta Paediatr Suppl; 91: 107-112,2002.

Mann KG, et al. 1981. *The role of factor V in the assembly of the prothrombinase complex.* s.l. :Ann NYAcadSci; 370:378-388, 1981.

Monroe DM, et al. 1998. *A possible mechanism of action of activated factor VII independent of tissue factor.* s.l. : Blood Coagul Fibrinolysis;Suppl1:15-20, 1998.

Monroe DM, et al. 1997. *Platelet activity of high-dose factor VIIa is independent of tissue factor.* s.l. : BrJ Haematol;99:542-547, 1997.

Montsch P. 2010. *Charakterisierung der Funktion von Proteinbestandteilen in einem aus humanem Plasma isolierten Prothrombinkomplexkonzentrat.* Diplomarbeit Universität Wien 2010.

Mutschler E, et al. 2005. *Mutschler – Arzneimittelwirkungen kompakt.* s.l. : Wissenschaftliche Verlagsgesellschaft mbH Stuttgart, 2005.

Mutschler E, et al. 2008. *Mutschler Arzneimittelwirkungen, Lehrbuch der Pharmakologie und Toxikologie.* s.l. : Wissenschaftliche Verlagsgesellschaft mbH Stuttgart; 9. vollständig neu bearbeitete und erweiterte Auflage, 2008.

Myung Hee Han and Young Shil Park. 2013. *Sequential therapy with activated prothrombin complex concentrates and recombinant activated factor VII to treat unresponsive bleeding in patients with hemophilia and inhibitors: a single center experience.* s.l. : Blood Research; 48:282-6. 2013.

Neumann HA. 2008. *Das Gerinnungssystem.* s.l. : ABW Wissenschaftsverlag GmbH, 2008.

Oldenburg E and Tuddenham E. 2005. *Inhibitor to factor VIII – molecular basis.* s.l. : Blackwell Publishing Ltd; 59-63, 2005.

Ogiwara K, et al. 2010. *Tissue Factor Pathway Inhibitor Causes Unresponsiveness to Activated Prothrombin Complex Concentrates for Hemophilia A Patients with Inhibitors.* s.l. : Poster of 53rd ASH, American Society of Hematology. 2010.

<https://ash.confex.com/ash/2010/webprogram/Paper29266.html>

Osterud B and Papaport SI. 1977. *Activation of Factor IX by the reaction product of tissue factor and Factor VII: Additional pathway for initiating blood coagulation.* s.l. : Proc. Natl. Acad. Sci. USA; Vol. 74, No. 12, pp 5260-5264, 1977.

Pötzsch B and Madlener K. 2010. *Hämostaseologie.* s.l. : Springer-Verlag GmbH; 2.Auflage, 2010.

Rasch B, et al. 2010. *Quantitative Methoden 1.Einführung in die Statistik für Psychologen und Sozialwissenschaftler.* s.l.: Springer-Verlag GmbH; Auflage: 3., erw. Aufl. 2010.

Roberts HR, et al. 2006. *A Cell-Based Model of Thrombin Generation.* s.l. : Semin. Thromb. Hemost.; 32(suppl 1): 32-38,2006. ISSN 0094-6176.

Sharathkumar A, et al. 2003. *Intensive exposure to factor VIII is a risk factor for inhibitor development in mild hemophilia A.* s.l. : J Thromb Hemost;1:1228-1236, 2003.

Turecek PL, et al. 1999. *Factor Xa and Prothrombin: Mechanism of Action of FEIBA.* s.l. : VoxSanguinis; 77 (Suppl 1), 72-79, 1999.

Turecek PL, et al. 2003. *Factor VIII Inhibitor-Bypassing Agents Act by Inducing Thrombin Generation and Can Be Monitored by a Thrombin Generation Assay.* s.l. : Pathophysiol. Hemost. Thromb.; 33:16-22, 2003.

Turecek PL, et al. 2004. *FEIBA: mode of action.* s.l. : Hemophilia, 10, (Suppl. 2), 3-9, 2004.

Turecek PL, et al. 2004. *Update on the Mechanism of Action and Future of Activated Prothrombin Complex Concentrates.* s.l. : Current Hematology Reports, 3:331-337, 2004.

Turecek PL, et al. *Thrombin Generation Assay (TGA): test for monitoring the pharmacological properties of inhibitor treatment in hemophilia.*

Váradí K, et al. 1999. *Phospholipid-bound tissue factor modulates both thrombin generation and APC-mediated factor Va inactivation.* s.l. : Thromb. Haemost.; 82:1673-1679, 1999.

Váradí K, et al. 2003. *Monitoring the bioavailability of FEIBA with a thrombin generation assay.* s.l. : Journal of Thromb. and Haemost.; 1:2374-2380, 2003.

Váradí K, et al. 2004. *Thrombin generation assay and other universal tests for monitoring haemophilia therapy.* s.l. : Haemophilia; 10, (suppl. 2), 17-21, 2004.

Váradí K, et al. 2011. *Characterization and role of hemostatic proteins in activated prothrombin complex concentrate FEIBA.* presented at 55. Jahrestagung 2011 der Gesellschaft für Thrombose- und Hämostaseforschung e. V. (GTH). 2011.

Verbruggen B, et al. 1995. *The Nijmegen modification of the Bethesda assay for factor VIII:C inhibitors: Improved specification and reliability.* s.l. : Thromb Haemost;73;247-250,1995.

White B, et al. 2000. *High responding factor VIII inhibitors in mild hemophilia - is there a link with recent changes in clinical practice.* s.l. : Haemophilia;6:113-115.2000.

Wielders S, et al. 1997. *The routine determination of endogenous thrombin potential, first results in different forms of hyper- and hypocoagulability.* s.l. : Thromb. Haemost.; 77 : 629-636, 1997.

Witt I and Dörner K. 2006. *Klinische Chemie und Hämatologie.* s.l. : Georg Thieme Verlag; 6.Auflage, 2006.

8 Curriculum Vitae

Name	Margarita Löschberger
------	-----------------------

Berufserfahrung und Praktika

April 2012 – Juli 2012	Baxter Innovations GmbH, Wien
Oktober 2012 –	Diplomarbeit
November 2012	<i>Vollzeit Beschäftigung</i>
Oktober 2011 – Jänner 2014	Bachapotheke, Wien
	<i>Geringfügige Beschäftigung</i>
September 2010 –	Salvia Apotheke, Gr. Enzersdorf
September 2011	<i>Geringfügige Beschäftigung</i>
September 2006 –	Österreichische Agentur für Gesundheit und Ernährungssicherheit GmbH, Wien
September 2010	Abt.: AGES-Akademie, Unternehmenskommunikation
	<i>Geringfügige Beschäftigung</i>
August 2011	Baxter Innovations GmbH, Orth/Donau
August 2010	Abt.: Analytical Development
August 2009	<i>Ferialpraxis</i>
August 2008	
August 2007	
August 2006	

August 2005

Glorit Bausysteme GmbH, Gr. Enzersdorf

Ferialpraxis

Juli 2005

Gemeindeamt Orth an der Donau

Ferialpraxis

Ausbildung

2006 - 2014

Universität Wien

Pharmaziestudium

1998 – 2006

Konrad Lorenz BG/ BRG Gänserndorf

Realgymnasium mit ergänzendem Unterricht in Biologie, Umweltkunde, Physik und Chemie

Abschluss mit Reifeprüfung

1994 – 1998

Volksschule Orth/Donau