universität wien

DIPLOMARBEIT

Investigation of platelet adhesion properties of Von-Willebrand-Factor drug candidates

angestrebter akademischer Grad

Magistra der Pharmazie (Mag.pharm.)

Verfasserin / Verfasser:	Karoline Maria Sindelar
Matrikel-Nummer:	0100964
Studienrichtung /Studienzweig (lt. Studienblatt):	A449 Pharmazie
Betreuerin / Betreuer:	Doz. Dr. Peter L. Turecek

Wien, im November 2008

Contents

1. Abstract

The von Willebrand factor (VWF) is a key factor in the blood coagulation system. It binds platelets to collagen fibres of ruptured vessel walls. Patients with von Willebrand desease have a decreased VWF level in their blood or mutations in their VWF protein leading to dysfunction. For these patients, it is vital to receive VWF substitutes.

This diploma thesis investigates the platelet adhesion properties of five different VWF substitutes: (a) plasma-derived VWF/factor VIII concentrate (Immunate#09HZ2000I), (b) Pro-recombinant VWF 298, (c) mature recombinant VWF 158, (d) plasma-derived VWF 09/47, (e) plasma-derived VWF 07 42/3. These concentrates were tested in their pure version and with addition of recombinant and plasma-derived factor VIII.
The experiments were carried out in a flow chamber system, simulating the blood flow through a ruptured blood vessel. A solution containing erythrocytes, platelets, buffer, and the VWF concentrate was used as blood imitation. Collagen-coated glass plates functioned as the ruptured vessel wall, while a pump ensured a proper wall shear stress rate. Clots of platelets on the glass plates were subsequently visually detected and counted by a computer programme.

The results indicate that the plasma-derived VWF/factor VIII concentrate (Immunate#09HZ2000I) and the plasma-derived VWF 09/47 performed best. The addition of factor VIII did not noticeably influence the platelet adhesion properties. In the light of the scattering results and the influence of individual blood samples from unknown donors, the results are unsteady and need to be verified.

Zusammenfassung

Der Von Willebrand Faktor (VWF) ist einer der wichtigsten Faktoren des Blutgerinnungssystems. Er bindet Thrombozyten an die Kollagenfasern von rupturierten Gefäßwänden. Patienten, die unter der Von Willebrand Krankheit leiden, haben entweder zu geringe VWF Konzentrationen im Blut oder durch Mutationen veränderte VWF-Funktionen. Für diese Patienten ist VWF Substitution lebensnotwendig.

Diese Diplomarbeit untersucht die Thrombozytenadhäsion von fünf verschiedenen VWF Konzentraten: (a) plasmagereinigtes VWF/Faktor VIII Konzentrat (Immunate#09HZ2000I), (b) Pro-rekombinater VWF 298, (c) reifer rekombinanter VWF 158, (d) plasmagereinigter VWF 09/47, und (e) plasmagereinigter VWF 07 42/3. Diese Konznetrate wurden ohne und mit Zusatz von rekombinantem und plasmagereinigtem Faktor VIII auf ihre Thrombozytenbindung hin untersucht.
Die Experimente wurden mit Hilfe eines Flusskammersystems getestet, welches den Blutfluss durch ein verletztes Blutgefäß simulierte. Als Untersuchungslösung wurden Erythrozyten, Thrombozyten, eine Pufferlösung und das jeweilige VWF Konzentrat gemischt. Diese Mischung diente als Blutersatz. Glasplättchen wurden mit Kollagen beschichtet um die zerstörte Gefäßwand zu imitieren, während eine Pumpe für die richtige Scherspannung eines rupturierten Gefäßes sorgte. Anschließend wurden die kollagenbeschichteten Glasplättchen gefärbt und die gebildeten Thromben wurden mittels eines Bildverarbeitungsprogramms gezählt und ausgewertet.

Die Ergebnisse zeigen, dass das plasmagereinigte VWF/Faktor VIII Konzentrat (Immunate#09HZ2000I) und der plasmagereinigte VWF 09/47 die beste Thrombozytenadhäsion erzielen. Die manuelle Zugabe von Faktor VIII hat die Thrombozytenadhäsionseigenschaften der Konzentrate nicht wesentlich beeinflusst. Die Ergebnisse sind aber nicht eindeutig und unterliegen teils hohen Schwankungen. Diese stammen unter anderem von den verschiedenen Blutproben, die von anonymen Spendern kamen.

2. Introduction

The aim of this thesis was to investigate different Von Willebrand factor concentrates concerning their platelet adhesion abilities. Patients suffering from von Willebrand disease need such concentrates to balance their von Willebrand factor levels, which are decreased because of von Willebrand factor dysfunction or severely decreased levels of von Willebrand factor in their blood system. In this work five differently produced von Willebrand factor concentrates, both, plasma derived, including one commercially available drug product, and recombinant ones, were tested and compared. These von Willebrand factor concentrates were all produced by *Baxter BioScience*.

In order to test the platelet adhesion ability of the different VWF samples a flow chamber was used. The glass plate inserted into the flow chamber was coated with inactivated collagen to imitate the ruptured blood-vessel wall. A solution, containing erythrocytes, platelets, buffer and the von Willebrand factor sample, was mixed and functioned as a blood imitation. This blood was then pumped through the flow chamber, where the connected pump was programmed to imitate the shear stress of a ruptured vessel. After the experiment, photos of the glass plates were made and the formed blood clots were detected by a computer programme that calculated the blood-clot-covered surface of the glass plates.

Due to the fact that most of the patients suffering from von Willebrand disease also have decreased factor VIII levels, the tested von Willebrand factor concentrates were mixed with factor VIII concentrates to investigate eventual additive or subtractive effects in the von Willebrand factor platelet adhesion ability. Finally, two different von Willebrand factor concentrates were mixed and tested.

This diploma thesis is organised as follows: Section 3 provides an overview of the theory of haemostasis, which describes the background of the experiments. In Section 4, the materials and the flow-chamber method are described in detail. Section 5 summarises the obtained results and compares the different von Willebrand factor concentrates. In Section 6 the results are discussed and conclusions are drawn.

3. Theory

In this section, the physical and biological process of haemostasis are explained, which is simulated in the experiments carried out for this thesis. The two investigated clotting factors that are important for haemostasis are the von Willebrand factor (VWF) and factor VIII, which are both described in detail in this chapter.

3.1. Haemostasis

"Blood coagulation and platelet-mediated primary haemostasis have evolved as important defence mechanisms against bleeding. The coagulation system is triggered in response to rupture of endothelium, which allows exposure of blood to the extravascular tissue. The responses of the coagulation system are coordinated with the formation of the platelet plug that initially occludes the vascular lesion. Anticoagulant mechanisms ensure careful control of coagulation and, under normal conditions, they prevail over the procoagulant forces. Disturbances of the natural balance between the procoagulant and anticoagulant systems due to generic or acquired factors may result in bleeding or thrombotic diseases." [1]

Factor I	Fibrinogen
Factor II	Prothrombin
(Factor III)	Tissue Thromboplastin
(Factor IV)	Calcium ions
Factor V	Labile Factor
Factor VII	Stable Factor
Factor VIII	Antihemophilic Factor
Factor IX	Christmas Factor, or Plasma Thromboplastin Component (PTC)
Factor X	Stuart-Prower Factor
Factor XI	Plasma Thromboplastin Antecedent (PTA)
Factor XII	Hageman Factor
Factor XIII	Fibrin Stabilizing Factor

Figure 1. List of clotting factors involved in the blood coagulation

The process of blood coagulation is very complex. According to the specific reactions the process of haemostasis can be subdivided into four main phases: (a) the activation of clotting, followed by (b) the process of clotting, divided into the intrinsic and extrinsic pathway, then (c) the regulation of clotting and finally (d) the dissolution of the clot, the fibrinolysis.

The initial event activating the coagulation system is the damage of endothelial cells, where matrix proteins, such as collagen and fibronectin are exposed to the blood system. This is followed by vascular constriction to limit the flow of blood in the area of injury. In order to build a clot, platelets must first adhere to the exposed collagen, then release the contents of their granules, and finally, aggregate. The mediator between platelets and the endothelial surface is the von Willebrand's factor (VWF), which acts as a bridge between these two. Parallel to platelet adhesion the clotting cascades are activated.

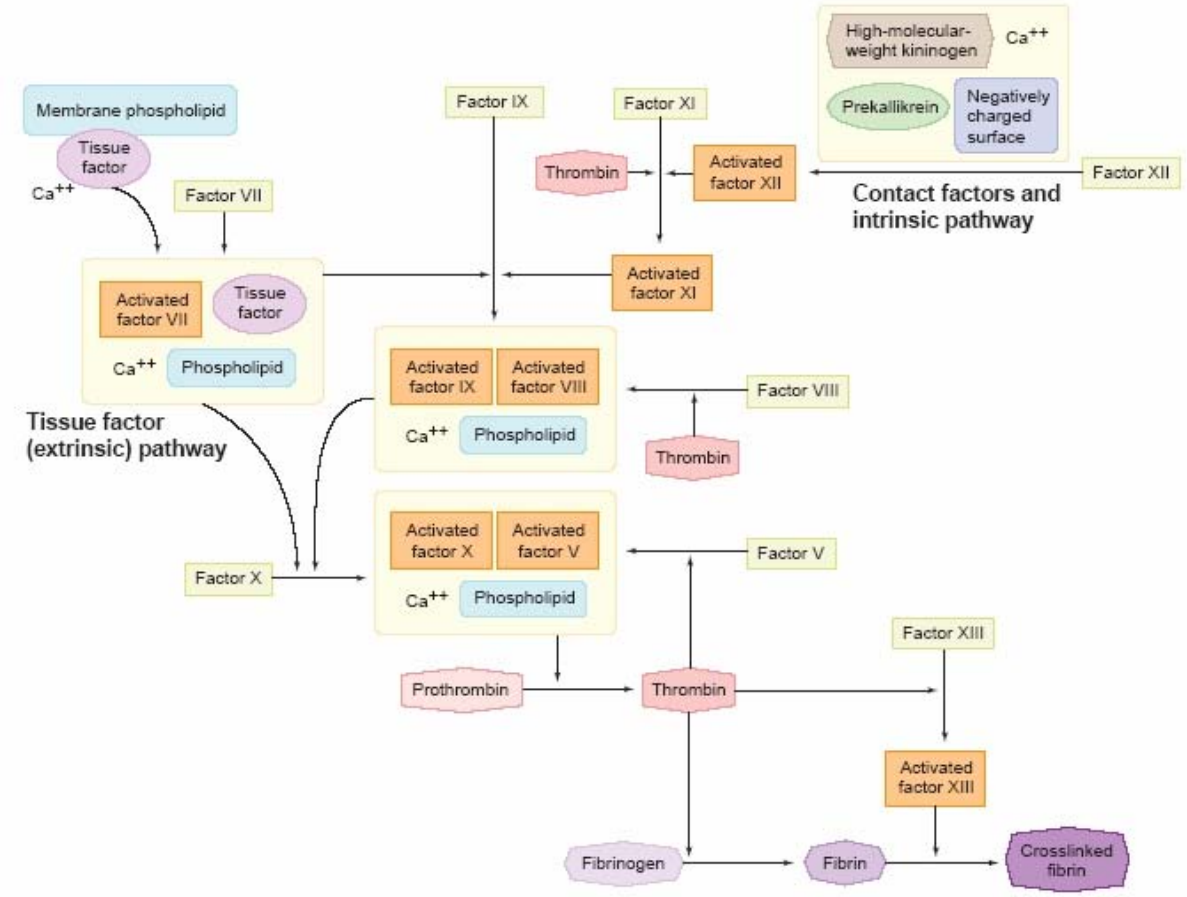


Figure 2. Reaction cascade of blood coagulation

The *intrinsic pathway* leads to fibrin formation based on the activation of factor XII, resulting in the activation of factor X, to translate prothrombin into thrombin. Meanwhile, the *extrinsic pathway* also activates factor X after the formation of an activated tissue factor-factor VIIa complex leading to the production of fibrin.

3.1.1. Intrinsic pathway

As shown in Figure 2, the intrinsic pathway is initiated when prekallikrein, high-molecular-weight kininogen, factor XI, and factor XII are exposed to a negatively charged surface. This results in the conversion of prekallikrein to kallikrein, which then activates factor XII to factor XIIa. Furthermore, a reaction cascade is activated leading to the activation of factor XI and the release of bradykinin from high-molecular-weight kininogen. Factor XIa converts factor IX into its activated form in the presence of Ca^{2+}. Factor VIII circulates bound to the von Willebrand factor. After activation, factor VIIIa dislocates and acts as a receptor for the factors IXa and X on the surface of activated platelets. Again, the presence of Ca^{2+} is required to activate factor X into factor Xa. This is where the intrinsic and the extrinsic pathway converge.

3.1.2. Extrinsic pathway

The activation of the extrinsic pathway starts with the release of factor III and the tissue factor. Then factor III converts factor VII into its activated form. In the presence of Ca^{2+} factor VIIa activates factor X. At the activated factor X, the two pathways meet. An additional link between the extrinsic and the intrinsic pathway exists trough the ability of tissue factor and factor VIIa to activate factor IX.
The main point in these both pathways is the translation of factor X into factor Xa, which activates thrombin from prothrombin. To initiate the building of fibrin, the protease thrombin cleaves the A and B chains of fibrinogen.

The fibrin monomers finally build long strands, which leads to the formation of an unstable clot. Factor XIIIa, activated by thrombin, is a transglutaminase that stabilises the clot by covalent crosslinking of fibrin.

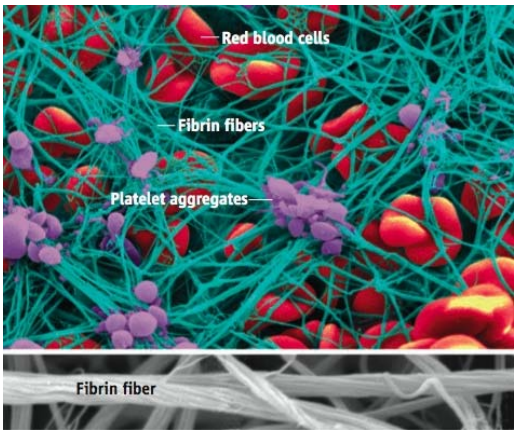


Figure 3. Blood clot

The fibrin monomers finally build long strands, which leads to the formation of an unstable clot. Factor XIIIa, activated by thrombin, is a transglutaminase that stabilises the clot by covalent crosslinking of fibrin.
Plasmin, a serine protease that circulates as the inactive proenzyme plasminogen, is responsible for the degradation of fibrin clots.

3.1.3. Haemophilia

Haemophilia, one of the most common inherited bleeding disorders, is caused by defects in the process of haemostasis. There are two forms of haemophilia, haemophilia A and B.

Haemophilia A, or classic haemophilia, is a bleeding disorder resulting from a deficiency in factor VIII. Depending on the remaining level of factor VIII in the blood system, there are the severe, the moderate, and the mild forms of haemophilia A. Patients with deficiencies of factor VIII suffer joint and muscle haemorrhage, easy bruising and prolonged bleeding from wounds. Treatment of

haemophilia A is accomplished by infusion of factor VIII concentrates prepared either from human plasma or from recombinant DNA technology.
Haemophilia B on the contrary results from deficiencies in factor IX. The patients also suffer from decreased factor IX clotting activity and prolonged coagulation time.

3.2. Platelets

Platelets are the smallest, but the most numerous cells in blood among the other two major types of blood cells (erythrocytes and lymphocytes). Platelets are small cytoplasmic bodies with a diameter of 1.5-4µm and a thickness of 0.5-2µm. Platelets have, similar to red blood cells, no nucleus. The normal platelet count is 160,000-300,000 per µl of blood [2], but numbers differ slightly according to the source of literature. Since platelets are so small, they make up just a tiny fraction of the whole blood volume.

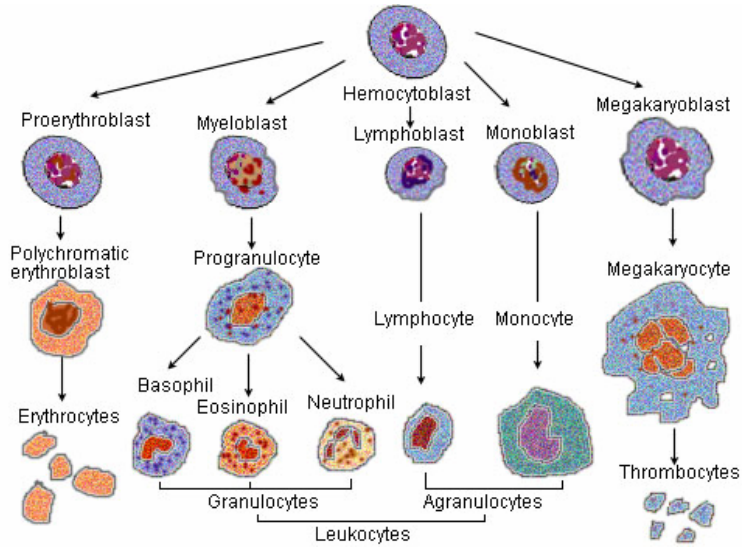


Figure 4. Genesis of platelets

Platelets are produced from megakaryocytes in the bone marrow as shown in Figure 4. These megakaryocytes undergo a process of fragmentation that results

in the release of over 1,000 platelets per megakaryocyte. The lifespan of platelets is approximately up to two weeks in a healthy organism.

Inside, platelets contain numerous granules, which are, according to their function, divided into three main granules (a) α-granules, (b) dense granules and (c) lysosomes. Inside α-granules there are proteins, especially fibrinogen and fibronectin, growth factors, such as platelet-derived growth factor (PDGF) and transforming growth factor (TGF-β), besides various clotting factors. The dense granules mainly contain low-molecular substances, such as nucleotides, metal ions and serotonin. Lysosomes contain acid hydrolases.

Platelet activation results in spilling of the contents of the granules into the blood. As mentioned above, platelets are essential for haemostasis and blood coagulation.

Platelet adhesion is triggered by the simultaneous binding of (a) the adhesion protein of VWF to the glycoprotein-Ib/IX-complex of platelets and (b) to the exposed collagen fibrils. When the platelts bin to the collagenous surface, the platelets form a clot, which is stabilised by fibrinogen. The clot finally closes the wound and due to platelet contraction the wound contracts.

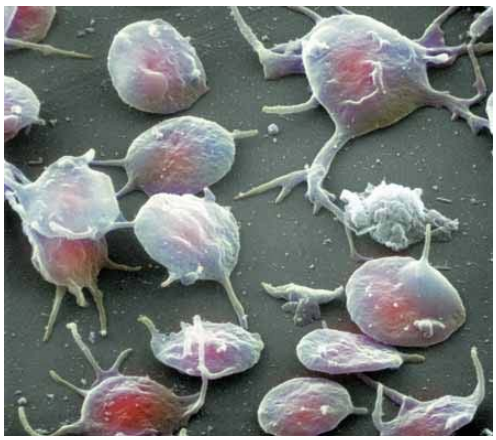


Figure 5. Activated platelets

Generally, low platelet counts increase bleeding risks and high platelet counts may lead to thrombosis. In some cases the platelet count is within a healthy range, but the platelets are dysfunctional. For instance, various pharmaceutical drugs, such as aspirin, irreversibly disrupt platelet function by inhibiting cycolooxygenase-1 (COX 1) and hence the normal haemostasis.

3.3. Von Willebrand factor

The von Willebrand factor was discovered by Dr. Erich von Willebrand (1870-1949) in 1926. He was a doctor in Finland, who first described a hereditary bleeding disorder in families from the Åland islands, who had a tendency for mucosal bleeding, which he named "hereditary pseudo-haemophilia". In the 1970s, the VWF was purified for the first time.

"Von Willebrand factor (VWF) is a blood glycoprotein that is required for normal haemostasis, and deficiency of VWF, or von Willebrand disease (VWD), is the most common inherited bleeding disorder. VWF mediates the adhesion of platelets to sites of vascular damage by binding to specific platelet membrane glycoproteins and to constituents of exposed connective tissue. These activities appear to be regulated by allosteric mechanisms and possibly by hydrodynamic shear forces. VWF also is a carrier protein for blood clotting factor VIII, and this interaction is required for normal factor VIII survival in the circulation." [3]

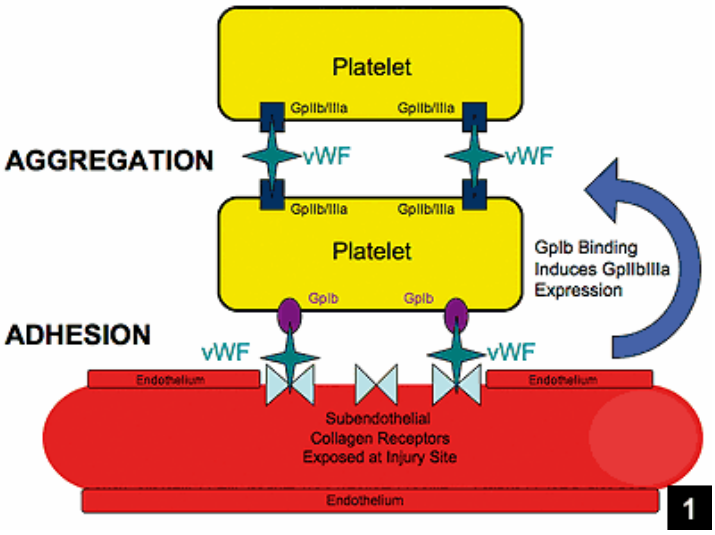


Figure 6. Von Willebrand factor binding platelets to collagen

3.3.1. Biosynthesis and structure of von Willebrand factor

According to [3-5], VWF is synthesised in endothelial cells and is stored in intracellular organelles and the Weibel-Palade bodies, or secreted. Additionally, the VWF is also synthesised in the megakaryocytes, where it is stored in the α-granules. Endothelial cells release the VWF as large multimers, which then circulate in the plasma as a series of high-molecular-weight multimers. These mulitmers are generated by proteolysis and shear stress enhances the sensitivity to proteolytic cleavage.

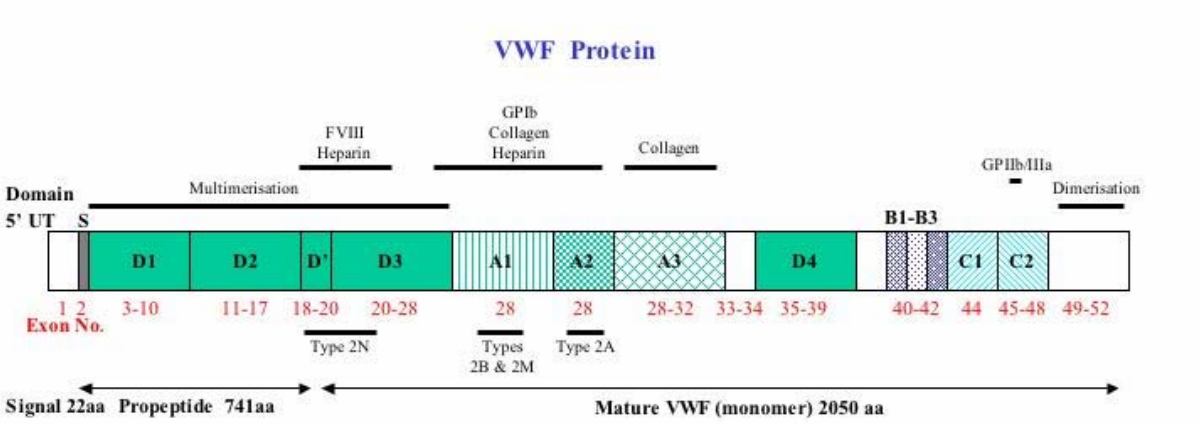
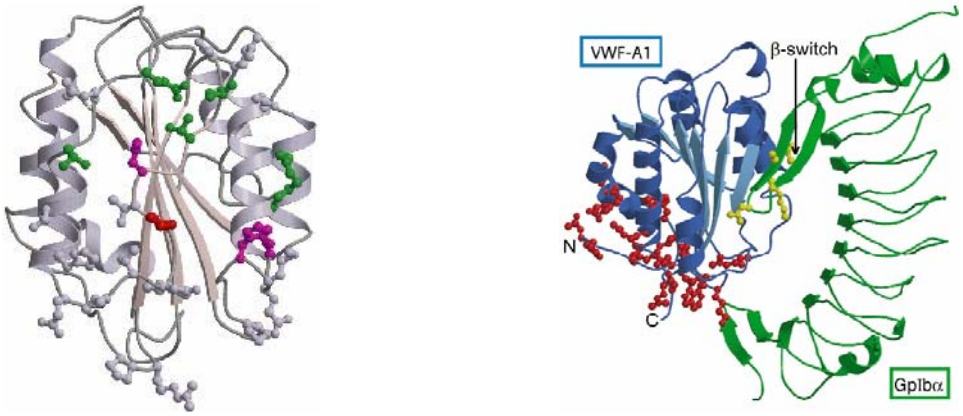


Figure 7. Structure of the von Willebrand factor

As shown in the Figure 7, each VWF subunit shows a characteristic pattern of homologous A, B, C and D domains, which are independent building blocks in many other proteins [6]. The propeptide contains a D1 and D2 domain. The mature subunit consists of D'-D3-A1-A2-A3-D4-B1-B2-C1-C2 domains and a C-terminal part of 151 amino acids that has no internal homology. The subunit apparently contains both a rod domain and a globular domain and corresponds to one half of the protomer. The protomer consists of two identical monomer subunits linked by disulfide bonds at the C terminus. The disulfide bonds in the amino-terminal globular ends link promoters to form VWF multimers. The A3 subunit of the VWF is responsible for the collagen binding during haemostasis, whereas the VWF A1 domain binds to the glycoprotein-Ib-alpha (Gp-Ibα) domain on the surface of platelets. These two regions are relevant for the VWF adhesion properties investigated in this diploma thesis. The total structure of the VWF

protein is yet unknown, but the three-dimensional structures of the VWF-A3 and the VWF-A1 domains are shown in Figure 8.



(a) (b)

Figure 8. Ribbon drawings of (a) the crystal structure of the collagen binding VWF-A3 domain, (b) Schematic representation of the complex of GpIbα (green) and the VWF-A1 domain (blue).

3.3.2. Function of von Willebrand factor

At high wall shear stress rates, platelet adhesion is mediated almost completely by the VWF, whereas at low wall shear stress rates platelet adhesion is independent of the VWF. The role of the VWF is mainly to both form a bridge between its A1 domain and platelets, and to bind to the collagenous surface through its A3 binding domain. Additionally, the VWF contains binding sites for heparin, ristocetin, botrocetin and sulfatides. Furthermore, the VWF serves as carrier protein for factor VIII. Bound to the VWF, factor VIII is protected against binding to membrane surfaces and to proteolytic attack by various serine proteases such as activated protein C. Due to its essential role in haemostasis, mutations or deficiencies of the VWF lead to severe malfunctions in the human platelet adhesion and coagulation system.

3.3.3. Von Willebrand factor and factor VIII

Factor VIII is an essential clotting factor, which acts as cofactor for factor IXa. In the presence of Ca^{2+} and phospholipids, factor VIIIa and factor IXa form a complex to activate factor X. In [6], the authors discuss that haemophilia A can be cured in factor VIII-deficient dogs and humans by liver transplantation, which suggests that the liver is the major site of factor VIII synthesis. Factor VIII contains three distinct domains arranged in the order A1-A2-B-A3-C1-C2 as shown in Figure 9. The C domains are capable of binding negatively charged phospholipids, whereas the B domain is essential for VWF binding. Factor VIII also contains two acidic regions. The first one, located between A1 and A2, is important for the procoagulant activity. The second acidic region, which is between B and A3, is required for the association of factor VIII with VWF.

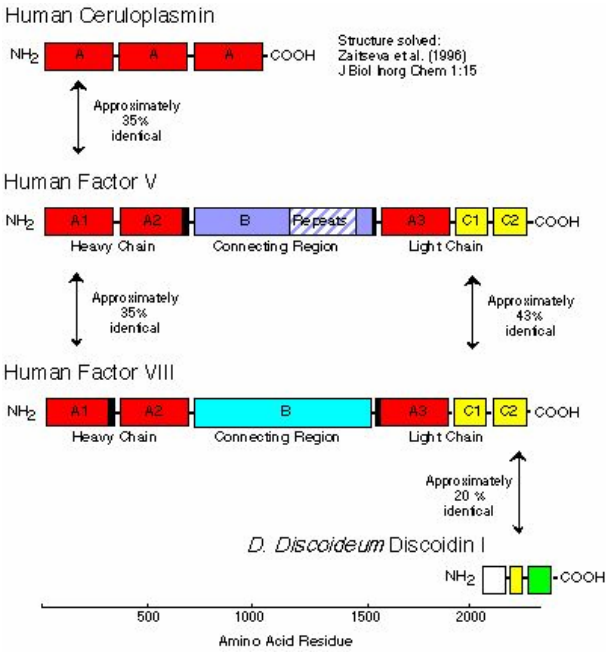


Figure 9. Resemblance of factor VIII and factor V

In plasma, factor VIII is stabilised and protected from degradation because of its association with the VWF molecule. The light chain of factor VIII interacts noncovalently with the N-terminal region of the VWF protein. The half-life of

factor VIII in the presence of normal VWF is approximately 12 hours, whereas its half-life is reduced to 2 hours in the absence of VWF. [7]

3.3.4. Von Willebrand Disease

The Von Willebrand Disease is a genetic disease that can be inherited from either parent. It affects males and females equally. A man or woman with VWD has a 50% chance of passing the gene on to his or her child. As discussed in [8], hereditary bleeding disorders such as the VWD are more common in countries with a high rate of consanguinity and a large number of births per family. A family history of a bleeding disorder is the primary risk factor. Approximately one out of 100 people are affected with VWD, which makes it the most common bleeding disorder. VWD results from a quantitative or qualitative deficiency of VWF. The importance of specific regions of VWF, which interact with other proteins of the coagulation cascade, has led to the classification of three subtypes of VWD: (a) based upon quantitative deficiencies (type 1 and 3), and (b) based upon qualitative deficiencies (type 2). Type 1 is the mildest and most common form of VWD. Patients have low levels of VWF and may also have deficiencies in factor VIII as well. Type 2 is characterised by mutations in the VWF protein, which lead to malfunction. Of the five known subtypes of type 2 (A, B, C, M, N), type 2A is the most common one and is characterised by a relative reduction in intermediate and high-molecular-weight multimer complexes. The most severe form of VWD is Type 3, where patients suffer from a total lack of VWF, and the factor VIII levels are often less than 10% resulting in secondary haemophilia. People suffering from VWD bruise easily, have recurrent nosebleeds and bleed after tooth extraction, tonsillectomy and other surgery. One possible treatment is desmopressin, which causes a temporary increase in the VWF levels. As pointed out in [9], patients with VWD who cannot be treated with desmopressin exclusively most frequently require substitution of both VWF and factor VIII.

4. Materials and Method

The aim of this diploma thesis was to observe the different platelet adhesion abilities of various VWF concentrates. For that a flow-chamber system was used.

This flow-chamber experiment was an *ex-vivo* blood coagulation study, where the flow chamber simulated a blood vessel and the attached pump imitated the normal blood flow under shear stress. An erythrocyte-platelet-buffer solution was used to imitate blood, and was mixed directly before the flow-chamber experiment was started. Herein, various VWF concentrates were tested in order to evaluate their platelet adhesion properties in the blood flow. To carry out the flow-chamber experiment the following equipment and reagents were needed.

4.1. Equipment

- Flow Chamber System, Cat: 303 0000, Oligene
- Silicon gasket (0.3mm), Cat: 303 0541, Oligene
- Cover slips, diameter 25mm, Cat: 303 0522, Oligene
- Clip on spring, Cat: 303 0510, Oligene
- Pump, type: Ismatec IPC, Cat: ISM935, #03763-00097
- Centrifuge Sorvall RC3B, Rotor H4000
- Cellcounter Sysmex, #pocH-100i
- Microscope Olympus BX60
- Digitrace, IMATEC elektro. Bildanalyse GmbH (1998)

4.2. Reagents

Table 1 shows the used reagents and their preparation. The buffers were prepared one day in advance, except for the 4% HSA-Krebs Ringer buffer, the Acetylsalicylicacid-buffer (ASA-buffer) and the Glutaraldehyde fixation buffer, which were prepared on the day of the experiment.

Acetic acid 0.1M	
Kollagenreagens Horm 1mg, Nycomed, # 10261165 (collagen reagent)	
Krebs Ringer buffer pH 7.35	for 1l:
107mM NaCl, Merck p.a. #K34684504520	6.25 g
20mM NaHCO3, Merck p.a. #K22116029 543	1.68 g
4mM KCl, Merck p.a. #821 TA499533	0.30 g
2mM Na2SO4, Merck p.a. #007 TA777649	0.28 g
19mM Tri-Na-Citrat.2H2O, Merck p.a. #A570148 442	5.59 g
2.5 mM CaCl2.2H2O, Merck p.a. #912 TA642982	0.37 g
0.5 % Glucose.H2O, Merck for biochem #620 K1261442	5 g
HEPES/NaCl bufferpH 7.35	for 1l:
10mM HEPES, Merck buffer substance #K91648610 941	2.38 g
150mM NaCl, Merck p.a. #K31900304 314	8.77 g
4% HSA-Krebs Ringer buffer	
Krebs Ringer pH 7.35 + 20% HSA	
Krebs Ringer buffer pH 6.5, without calcium	for 1l:
107mM NaCl, Merck p.a. #K34684504520	6.25 g
20mM NaHCO3, Merck p.a. #K22116029 543	1.68 g
4mM KCl, Merck p.a. #821 TA499533	0.30 g
2mM Na2SO4, Merck p.a. #007 TA777649	0.28 g
19mM Tri-Na-Citrat.2H2O, Merck p.a. #A570148 442	5.59 g
0.5 % Glucose.H2O, Merck for biochem #620 K1261442	5 g
TBS pH 7.4	for 1l:
150mM NaCl, Merck p.a. #K31900304 314	8.77 g
20mM Tris, Merck buffer substance #239 K17260582	2.43 g
100mM Acetylsalicylicacid (ASA-buffer)	for 1ml EtOH:
Acetylsalicylicacid 100mM in Ethanol	18mg ASA
Glutaraldehyde #126H1444, Cat: G-6257, Sigma	
Fixation buffer pH 7.2: Buffer tablets PH 7.2 TP221168, Cat: 9468 dissolve 1 tablet in 1 litre a.d.	
Human Albumin 20%, Baxter, #0107400H	
Acetylsalicylicacid, #55H0294, Sigma, Cat:A-5376	
Ethanol 96%, #K34220471 503, Merck, Cat:1.00971.2500	
May-Grünwald staining solution dissolve 0.25 g dye #L194952 Merck 1352 in 100ml Methanol Merck K25094709817 at +60°C for 60min-> filtrate 2 x after 24 hrs.	

Table 1. List of reagents and buffer preparation

4.3. Test articles

- Plasma derived VWF/factor VIII- Concentrate, Immunate#09HZ2000I [10]
- Pro-recombinant-VWF 298 (Pro-rVWF 298)
- Mature recombinant VWF 158 (mature-rVWF 158)
- Plasma-derived VWF 09 (pd-VWF 09)
- Plasma-derived VWF 07 42/3 (pd-VWF 07 42/3)
- VWF SEC 06

4.4. Procedure

The procedure was always carried out in the same way, following exactly the same protocol for each experiment. The experiment was prepared as follows: First, the buffer solutions were prepared, and the glass plates were coated with collagen one day in advance. Then, the platelet and the erythrocyte concentrates were diluted, washed, and mixed to create the blood imitation. Afterwards the samples of different VWF concentrates were prepared and added to the blood imitation, and the flow chamber perfusion experiment was started. Subsequently, the glass plates were stained and then left to dry over night. Finally the stained glass plates were detected under the microscope using a calculation program on a computer. The individual steps of this experiment are detailed in the following paragraphs.

4.4.1. Coating

The glass plates were coated with collagen reagent (0.5mg collagen reagent + 0.05M acetic acid) one day before the actual experiment was carried out. Therefore 150µl of the collagen reagent was pipetted on each glass plate. Then, the coated glass plates were left to dry in the refrigerator at +4 °C for at least 12 hours.

4.4.2. Preparation of platelets

The platelets used were provided by The Red Cross, Austria. First, the platelets were filled into a special glass for centrifugation, then the Krebs Ringer buffer w/o calcium, pH=6.5, was added in equal parts. The ASA stock solution was diluted 1:100 with the Krebs Ringer buffer, pH=6.5. From this 1mM ASA buffer about 1% (related to the platelet solution) was added to the platelet solution. Afterwards the platelet-buffer-solution was centrifuged at 2200 rpm for 10 minutes at 10°C without break. Then the supernatant was discarded and the remaining pellet of platelets was dissolved in Krebs Ringer buffer pH=7.35. Finally, the platelet number was counted. The platelet number should lie between approximately $1000x10^3$ and $2000x10^3$. During the experiment, unused platelets were kept cool in an ice bath.

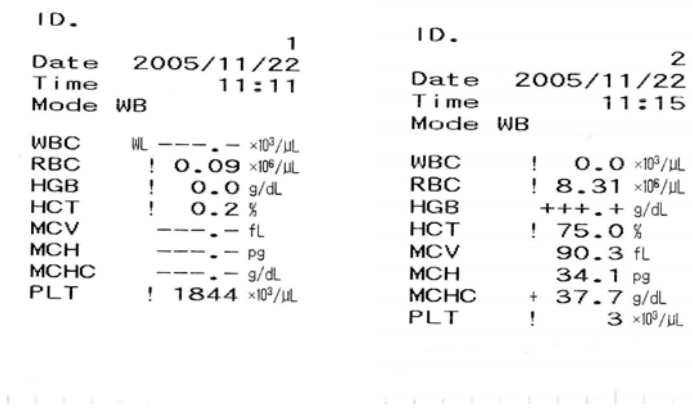


Figure 10. Haematocrit and Platelet count

In Figure 10 two typical prints of the platelet counts and the haematocrit are shown, using the cell counter Sysmex #pocH-100i.

4.4.3. Preparation of erythrocytes

The erythrocytes were also provided by The Red Cross, Austria. First, the erythrocytes were filled into a specific glass made for centrifugation and then

diluted with the HEPES/NaCl buffer, pH=7.35, in equal parts. Afterwards the solution was centrifuged at 2000rpm for 10 minutes at 10°C without break. The supernatant was discarded and the remaining erythrocytes were washed in the Krebs Ringer buffer, pH=7.35. Then, the gained solution was again centrifuged, this time at 2200rpm for 10 minutes at 10°C without break. Finally, the haematocrit (HCT) was measured. The HCT should be over 60%. During the experiment, unused erythrocytes were cooled in an ice bath.

4.4.4. Preparation of samples for testing

The calculated amount of erythrocytes, platelets, the 4% HAS-Krebs Ringer buffer solution and the sample are mixed and incubated for 10 minutes at 37°C. In this study, three concentrations of each sample were tested: (a) 0.5 U/ml, (b) 1 U/ml, and (c) 2 U/ml. The exact amount of the sample was calculated as follows:

$$\text{volume of platelets} = \frac{\text{total volume of sample solution}}{(\text{platelets per } \mu\text{l} / 250000)}$$

$$\text{volume of erythrocytes} = \frac{\text{total volume of sample solution}}{(\text{HCT in \%} / 35.0)}$$

$$\text{volume of sample} = \frac{\text{total volume of sample solution}}{(\text{VWF: Ag in U/ml} / \text{concentration})}$$

The total sample mix was 1.5ml. The amount of platelets depended on the platelet count of the concentrate. The amount of erythrocytes was calculated in proportion to the measured HCT. The correct shear rate, γ, was calculated as follows:

$$\gamma = \frac{6Q}{ab^2} \cdot 1.03,$$

where Q denotes the flow rate in ml/s, $a = 0.2\,\text{cm}$ and $b = 0.03\,\text{cm}$ denote the slight width and height, respectively. Table 2 shows a typical experiment calculation file.

Flow chamber experiment, 06.08.2006

MIX:	1,5 ml total			
	MIX (ml)	MIX Soll		
Platelets	0,22	250000	platelets/µl	1703000
Erythrocytes	0,75	35,0	HCT %	69,9
Sample	0,53			

Samples		VWF:Ag U/ml	Concentration (AgU/ml) 8 sample ml	HSA-KR ml	4 sample ml	HSA-KR ml
rVWF 299		15,7	**0,764**	0,236	**0,382**	0,147
rVWF 343		36,8	**0,326**	0,203	**0,163**	0,366
rVWF 158		67,6	**0,178**	0,351	**0,089**	0,440
VWF pd 07 42/3	Eluat 1 n. Dial. + MT	58	0,207	0,322	0,103	0,425
Immunate #09HZ2000I		115	0,104	0,424	0,052	0,477

Samples		VWF:Ag U/ml	Concentration (AgU/ml) 3 sample ml	HSA-KR ml	2,5 sample ml	HSA-KR ml	2 sample ml	HSA-KR ml	1 sample ml	HSA-KR ml	0,5 sample ml	HSA-KR ml
rVWF 299		15,7	0,287	0,242	0,239	0,290	0,191	0,338	0,096	0,433	0,048	0,481
rVWF 298		35,7	**0,122**	0,406	**0,102**	0,427	**0,082**	0,447	**0,041**	0,488	**0,020**	0,508
rVWF 158		67,6	**0,067**	0,462	**0,055**	0,473	**0,044**	0,484	**0,022**	0,507	**0,011**	0,518
VWF pd 07 42/3	Eluat 1 n. Dial. + MT	58	**0,078**	0,451	**0,065**	0,464	**0,052**	0,477	**0,026**	0,503	**0,013**	0,516
Immunate #09HZ2000I		115	**0,039**	0,490	**0,033**	0,496	**0,026**	0,503	**0,013**	0,516	**0,007**	0,522

Calculation of shear rate:

Shear rate ≅= $6Q/(ab^2) * 1.03$
Q (flow rate, ml/sec)
a (slight width, cm) a = 0.2 cm
b (slight height, cm) b = 0.03 cm

Table 2. Example of a calculation file for the volume and concentration of samples

4.4.5. Perfusion in the flow chamber

Before the actual perfusion experiment could be started, the water bath was filled and heated up to 37°C to imitate body temperature. The pump was programmed to imitate the correct shear stress of a ruptured blood vessel. Then the collagen coated glass plates were put into the flow chamber, layered with a silicon gasket, and fixed with a clip on a spring as shown in Figure 11.

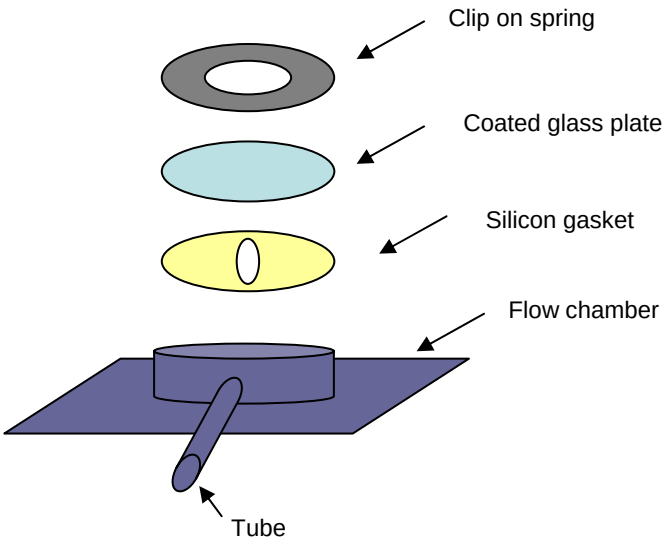


Figure 11. Preparation of the flow chamber

Every experiment was carried out as a double determination, where two flow chambers were used in parallel in the same system. Some of these experiments were also carried out multiple times. The setup of the experiment is shown in Figure 12. The flow chambers, readily equipped with the coated glass plates, were put into the water bath. Then, Tube A was put into the sample vial and connected to flow chamber 1, Tube B connected the two flow chambers, and Tube C went from flow chamber 2 to the pump. Finally Tube D came from the pump and went back into the sample vial. Simultaneously, the timer was set, and the pump was started. The sample solution circulated for 4 minutes.

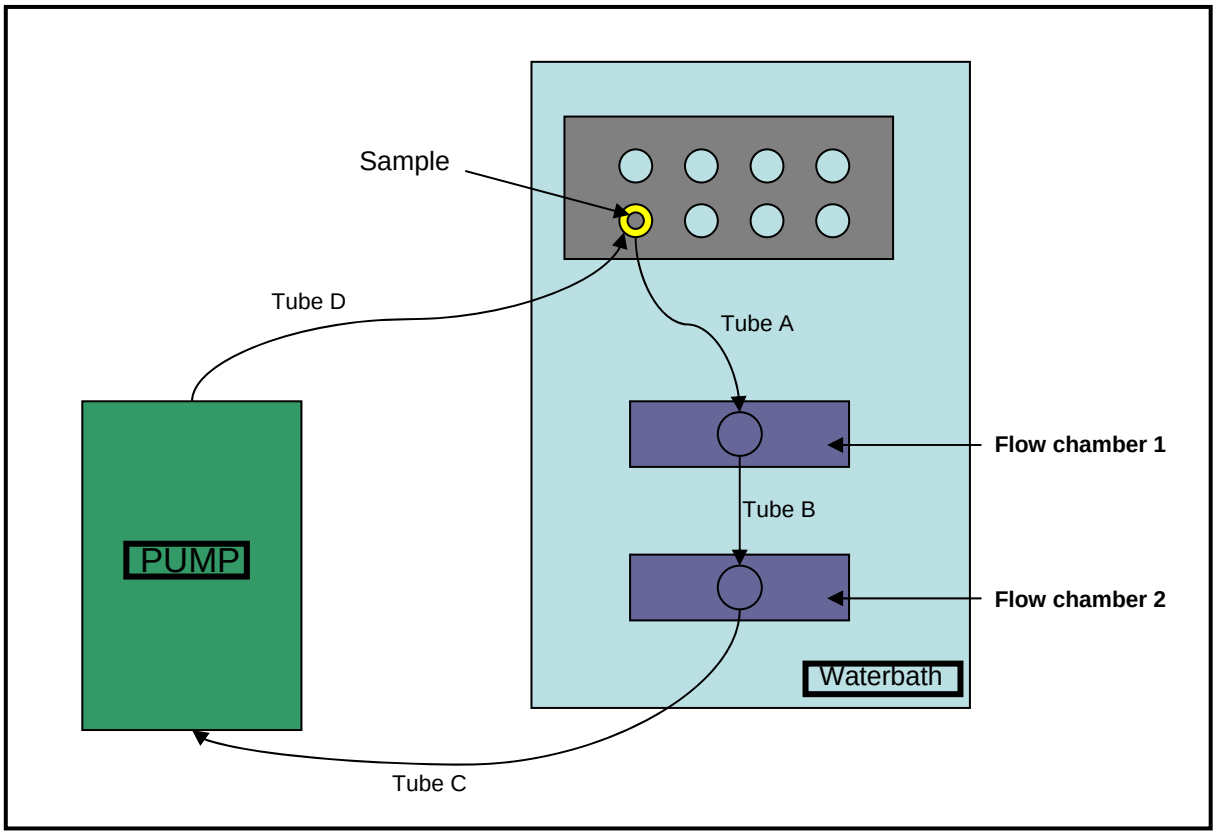


Figure 12. Set up of the flow chamber experiment

4.4.6. Staining

After four minutes of blood circulation, the pump was stopped and the glass plates were taken out of the flow chamber. Then they were put into the glutaraldehyde buffer (0.5% glutaraldehyde diluted in TBS buffer) for 20 minutes, to fix the clots on the surface. Afterwards the glass plates were laid out on paper and left to dry over night, for at least 12 hours at room temperature. After drying, 150µl of the May-Grünwald staining solution were pipetted on each glass plate and left there to react for exactly one minute at room temperature. If the solution was left on the surface for longer than one minute, the staining got too dark for proper detection. After staining, 150µl of the fixation buffer solution, pH=7.2, were added on each plate to dilute the staining solution. The mixture was left on the plates for two minutes. After that the glass plates were washed with buffer solution and left to dry at room temperature.

4.4.7. Detection

For detection, the stained glass plates were put under the microscope. The programme “Digitrace” (IMATEC elektro. Bildanalyse GmbH) counted the clots on the collagen-coated surface of the glass plates. Twelve pictures of each glass plate were taken, at which the segmentation parameters were set to 0/117 in the programme. The light intensity was set to 10.5. After the pictures were taken, the programme counted the clotted surface and calculated the percentage of the clotted area of the glass plate. The mean value of these twelve pictures was taken and the percentage of plate coverage was used to compare the binding abilities of the different samples. The result was blank-value corrected and stored for further evaluation.

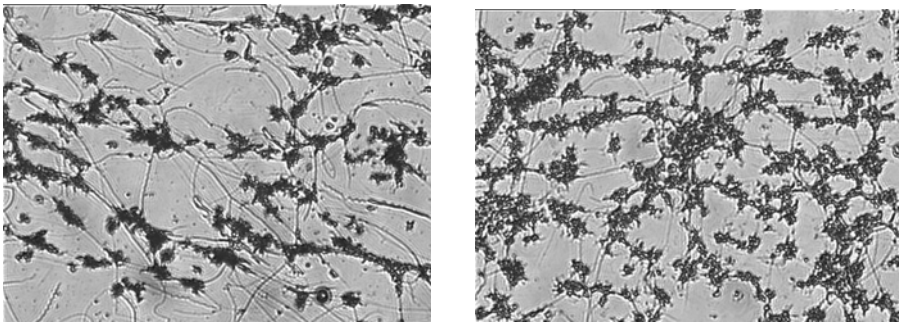


Figure 13. Blood clots on the collagen coated surface of glass plates after staining

5. Results and discussion

In this study, various VWF concentrates were tested under the same conditions. In this experiment the coated glass plates simulated the ruptured vessel wall of a human body. The flow chamber itself built the blood vessel and the pump simulated the shear stress of a ruptured vessel wall. The samples consisted of erythrocytes, platelets, buffer solution and the VWF sample to simulate human blood at the physiologic pH value of 7.35. The following results show that different types of VWF bind differently to the collagenous surface.

In the following figures the experiments were named after the date of the blood donation, such as bd060831, where “bd” stands for blood donor and “060831” stands for August 31, 2008. The %-Coverage refers to the clot-covered glass plate surface values calculated with the image processing computer programme (Digitrace).

5.1. Differences in VWF bindings

All the following results are blank corrected and averaged. In the graphs, each point shows the mean value of various experiments, which were carried out on different days using different erythrocytes- and platelet concentrates from different donors. The experiments were always carried out in the same way for all samples.

5.1.1. VWF/factor VIII-Concentrate (Immunate#09HZ2000I)

Immunate#09HZ2000I is a sterile, lyophilised, double-virus-inactivated plasma-derived high-purity human VWF/factor VIII complex. Immunate#09HZ2000I is manufactured from pooled human plasma. Each individual plasma donation is screened with approved tests for Hepatitis B surface antigen (HBsAg), and for antibodies to HIV-1/HIV-2, and HCV, to prevent later infections using the Immunate#09HZ2000I concentrate [10]. The experiment was carried out as described in Section 4.4. Three concentrations of the VWF/factor VIII-

concentrate were tested: (a) 0.5 U/ml, (b) 1 U/ml, and (c) 2 U/ml. The tables containing the numeric results for the tested VWF/factor VIII-concentrate can be found in Chapter 7.1.

Figure 14 summarises the results of all experiments with the VWF/factor VIII-concentrate. All data was blank corrected and averaged. The graph shows that the platelet adhesion properties of the VWF/factor VIII-concentrate at the concentration of 0.5 U/ml are mainly between 10% and 15%, whereas at a concentration of 1.0 U/ml, the platelet adhesion property is between 17% and 20%. Finally, at the concentration of 2.0 U/ml the platelet adhesion property is between 20% and 27%. Two experiments (bd060831 and bd060808) differed significantly from the others, which may be due to the fact that the blood donors were anonymous. Hence, any blood coagulation inhibiting drugs, such as aspirin or others, influence the experiment and the herewith tested platelet adhesion ability of the samples under test. These problems could be avoided in future by establishing an adequate group of donors, where their anamneses are known. Also, the experiment bd060822 showed an unusual drop at the concentration of 1.0 U/ml, which could be due to leakage of the flow chamber during the experiment, or, as mentioned above, individual differences in the blood concentrate. All in all, it was observed that the platelet adhesion ability of the VWF/factor VIII-concentrate Immunate#09HZ2000I increased with higher concentration.

Although Immunate#09HZ2000I is not a single VWF concentrate, but also contains factor VIII, it was used as reference for all other experiments, because of its relatively high platelet adhesion properties.

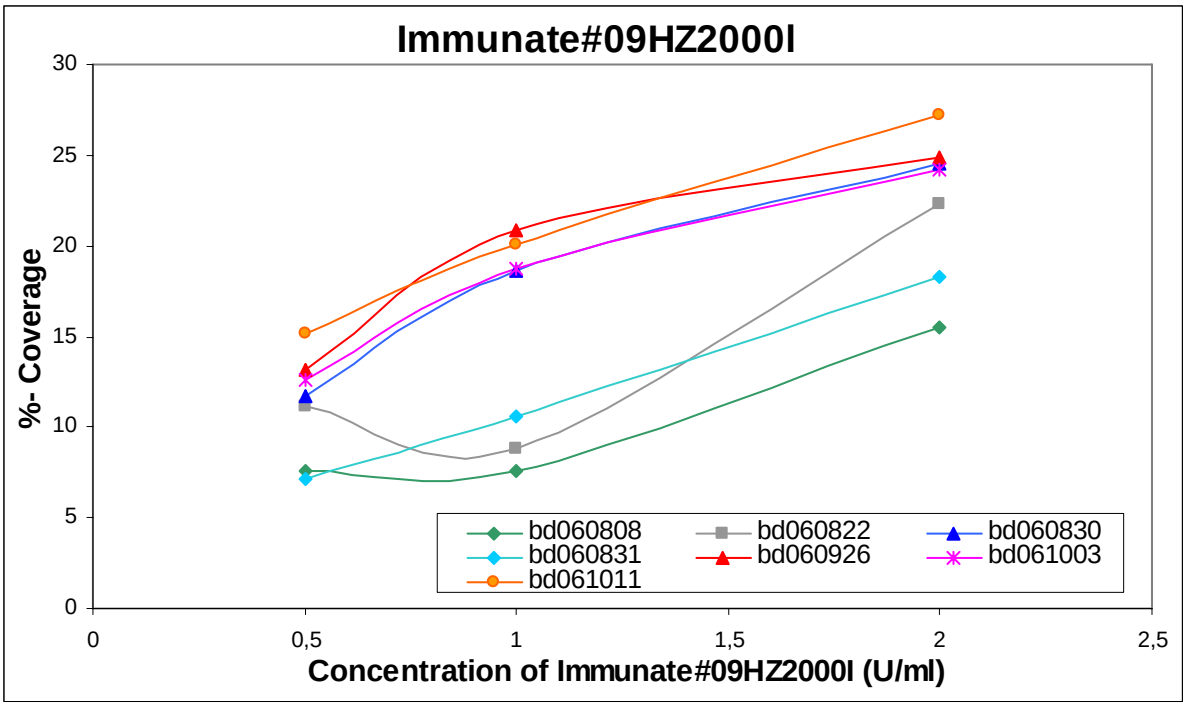


Figure 14. Results for Immunate#09HZ2000I

5.1.2. Pro-recombinant VWF 298 (Pro-rVWF 298)

The VWF 298 is a recombinant Pro-VWF molecule. The recombinant Pro-VWF 298 (Pro-rVWF 298) was produced using a proprietary blood-free technology and was subsequently purified.

Figure 15 summarises the results of all experiments using the Pro-rVWF 298 concentrate. At a concentration of 0.5 U/ml, the platelet adhesion ability of the Pro-rVWF lies in-between 4% and 6.5%, whereas at the concentration of 1.0 U/ml all the results are approximately 8%. Finally, at the concentration of 2.0 U/ml the results are almost equally at 10%. It could also be observed that at the concentration of 0.5 U/ml, the results are more spread than at the other two concentrations. The curve bd061011 showed an uncharacteristically low result, which may be due to the individual differences of the blood donors.

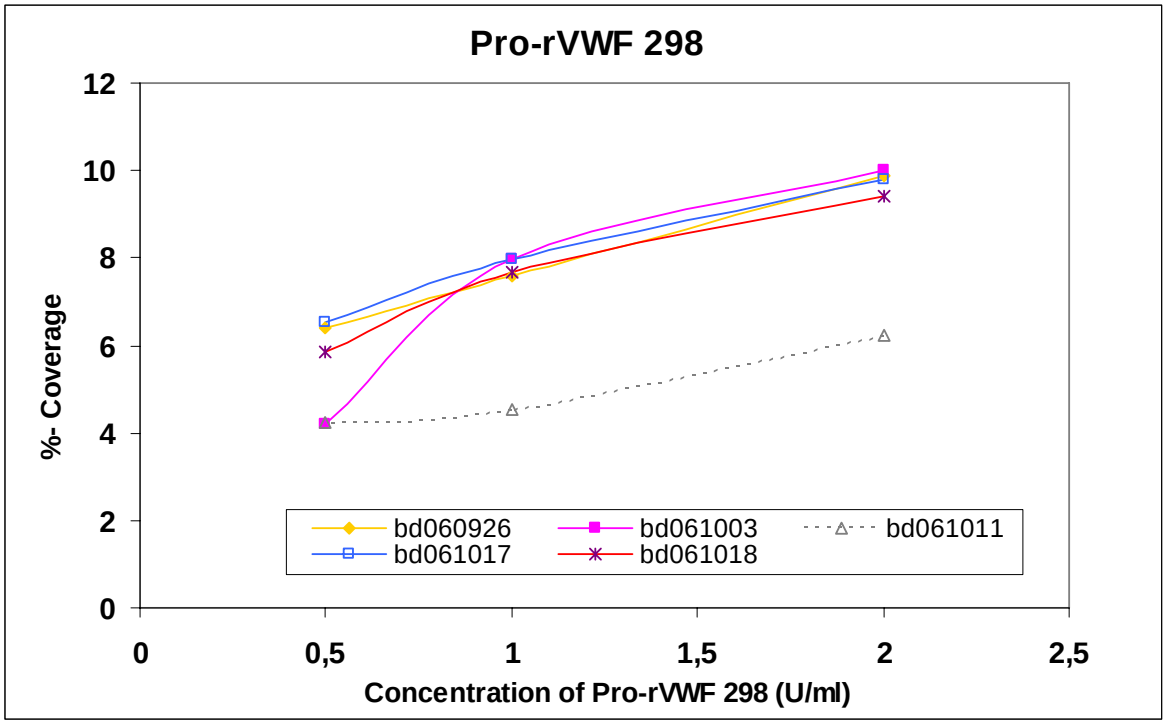


Figure 15. Results for Pro-rVWF 298

5.1.3. Mature recombinant VWF 158 (mature-rVWF 158)

The VWF 158 is a mature, recombinantly processed VWF [12]. The mature subunits contain the binding sites for factor VIII, collagen, platelets and heparin. The recombinant mature-VWF 158 was also processed without the use of human blood and then purified.

In Figure 16 the results for the mature-rVWF 158 are shown. At the concentrations of 0.5 U/ml and 1.0 U/ml, the curves bd060919 and bd060927 show similar results, whereas at the concentration of 2.0 U/ml, the result range lies between 12% and 16%. The results of curve bd060808 are clearly lower than the other two, which again may be due to the fact that the blood donors were anonymous and the results my have been influenced by blood coagulation inhibiting drugs.

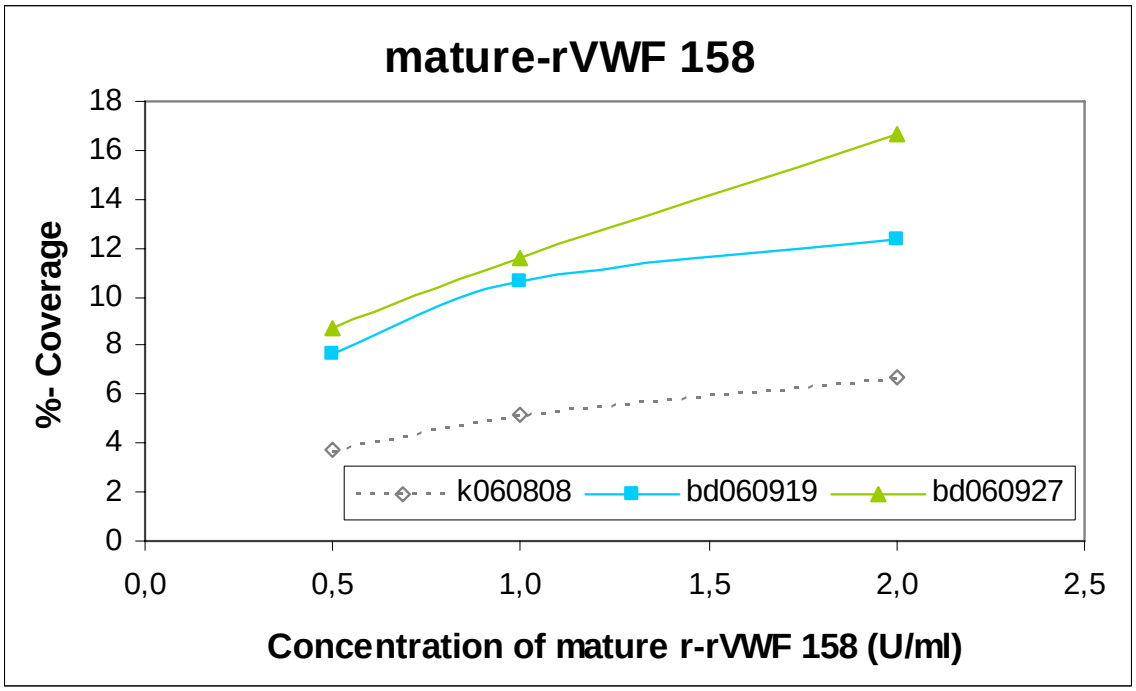


Figure 16. Results for mature-rVWF 158

5.1.4. Plasma-derived VWF 09 (pd-VWF 09)

The VWF 09 was plasma-derived from human plasma and subsequently virally inactivated. The results for pd-VWF 09 at the concentration of 0.5 U/ml are at 10%, whereas at the concentration of 1.0 U/ml of pd-VWF 09, the results are at 14%. At the concentration of 2.0 U/ml the platelet adhesion abilities of the pd-VWF 09 are at 20%, which is shown in Figure 17.

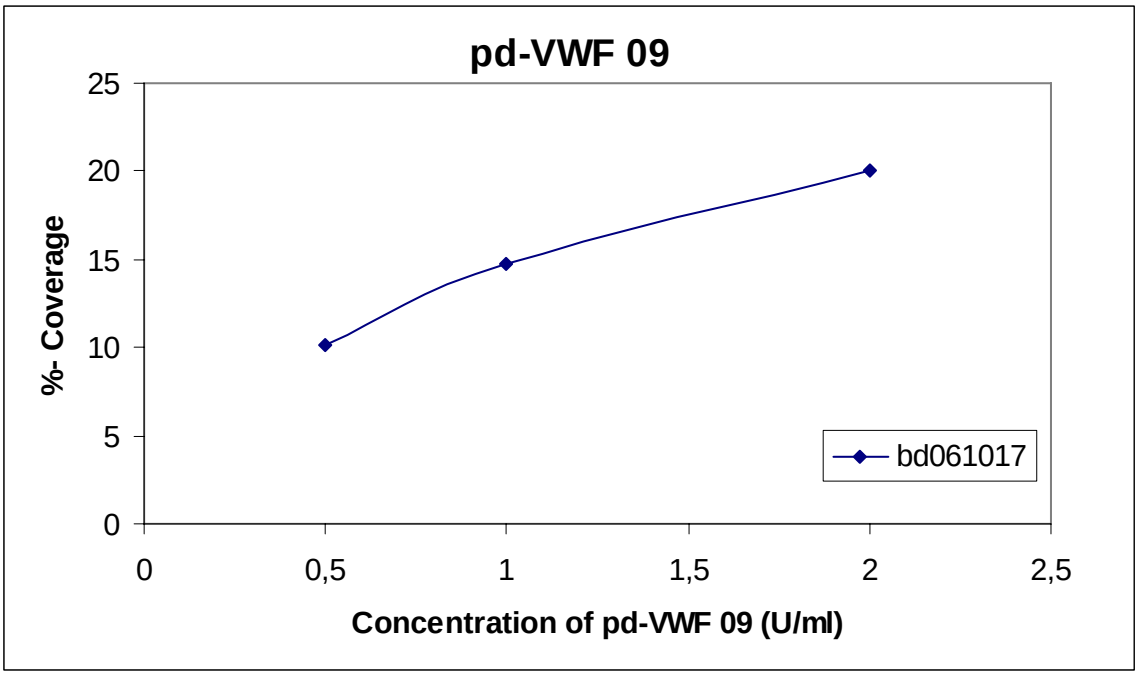


Figure 17. Results for the pd-VWF 09

5.1.5. Plasma-derived VWF 07 42/3 (pd-VWF 07 42/3)

The VWF 07 42/3 is a plasma-derived and virally inactivated human VWF. As Figure 18 shows, the results for the platelet adhesion ability of the pd-VWF 07 42/3 at the concentration of 0.5 U/ml are between 5% and 7%. At the concentration of 1.0U/ml only the results of the experiments bd060919 and bd060927 are quite similar and lie at 7%. These two also compare at the concentration of 2.0 U/ml at approximately 10%.The curve bd060808 shows an uncharacteristic drop at the concentration of 1.0 U/ml and is also far below the other curves at the concentration of 2.0 U/ml, which again could be due to the anonymous blood donors. However, the curve bd060822 shows an uncharacteristically high platelet adhesion value of 19% at the concentration of 2.0 U/ml.

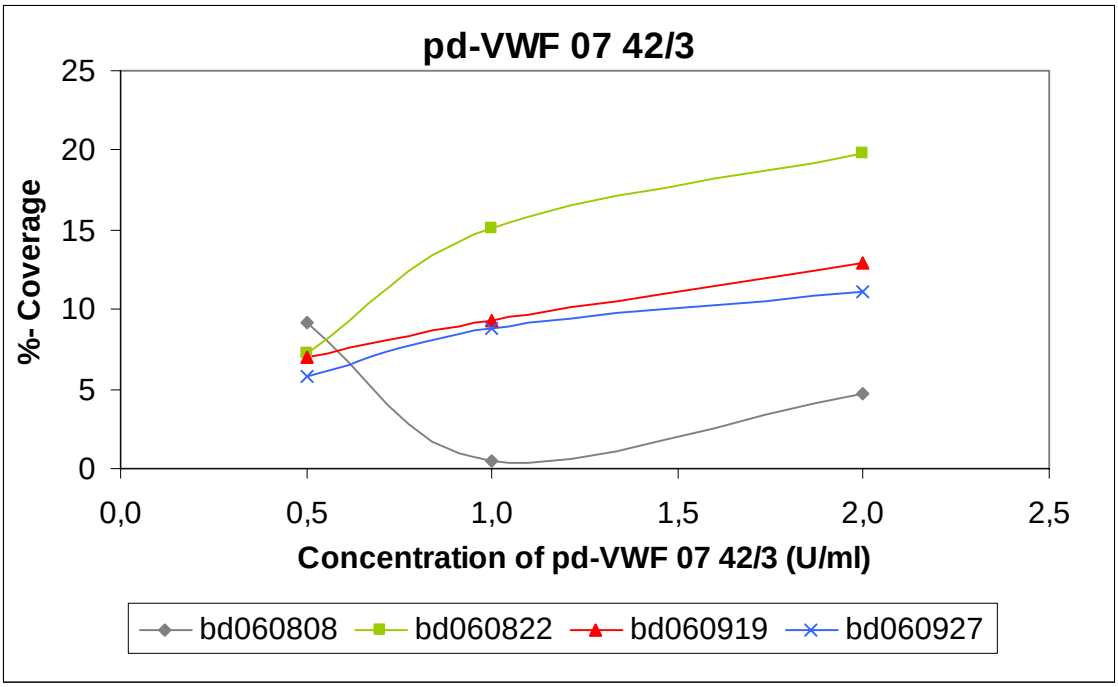


Figure 18. Results of pd-VWF 07 42/3

5.2. Influence of factor VIII in VWF binding

The motivation for these experiments was to determine if clot forming is increased or diminished when factor VIII is added. For these experiments, VWF concentrates as well as factor VIII concentrates were mixed and the flow chamber experiment was carried out as described in 4.4.5.

5.2.1. Pro-rVWF 298 and factor VIII

In these experiments the Pro-rVWF 298 was either mixed with a plasma-derived factor VIII concentrate, or with a recombinant factor VIII concentrate. As Figure 19 shows, the platelet adhesion properties of the Pro-rVWF 298 were marginally influenced by the addition of the factor VIII concentrates. The curves bd060926, bd061017 and bd061018 show the results for the Pro-rVWF 298 without the addition of factor VIII as described in 5.1.2. They show approximately equal results at each of the three concentrations used. The Pro-rVWF 298 was the mixed with a plasma-derived factor VIII (pd-factor VIII) concentrate (1:1) and

the results for this experiment are shown in the curve bd061129pdFVIII. The values for the platelet adhesion properties at 0.5 U/ml and 1.0 U/ml are somewhat lower than the ones without the pd-factor VIII. In contrast, the recombinant factor VIII/Pro-rVWF 298 mixture showed a slightly higher platelet adhesion property than the Pro-rVWF 298 concentrate alone as exemplified by curve bd061018rFVIII. Note that the results from this experiment have a large variance due to various influences, such as anonymous blood donors.

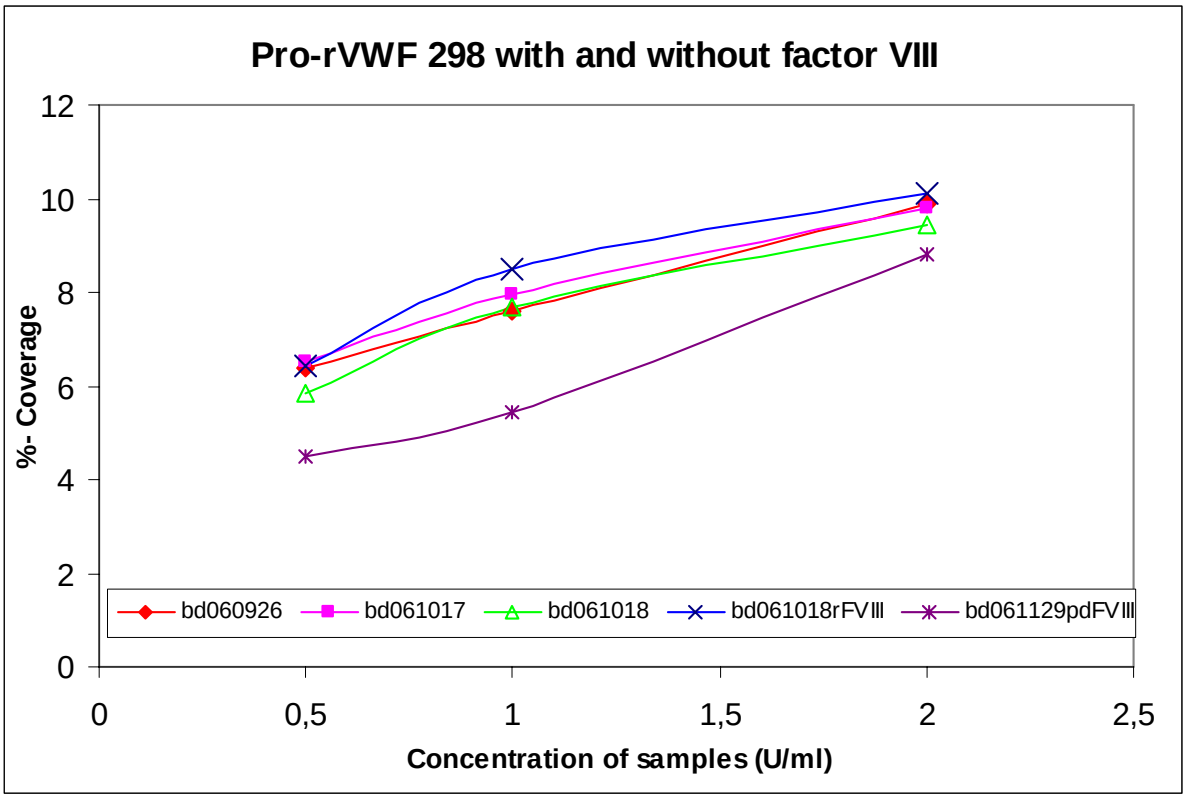


Figure 19. Results of Pro-rVWF with and without different factor VIII concentrates

5.2.2. Mature-rVWF 158 and factor VIII

The mature-rVWF 158 was tested with a plasma-derived factor VIII and also a recombinant factor VIII. As Figure 20 shows, the platelet adhesion properties of the mature-rVWF 158 were not significantly influenced by the addition of neither the plasma-derived factor VIII nor the recombinant factor VIII as shown in curves bd061128pdFVIII and bd061128rFVIII.

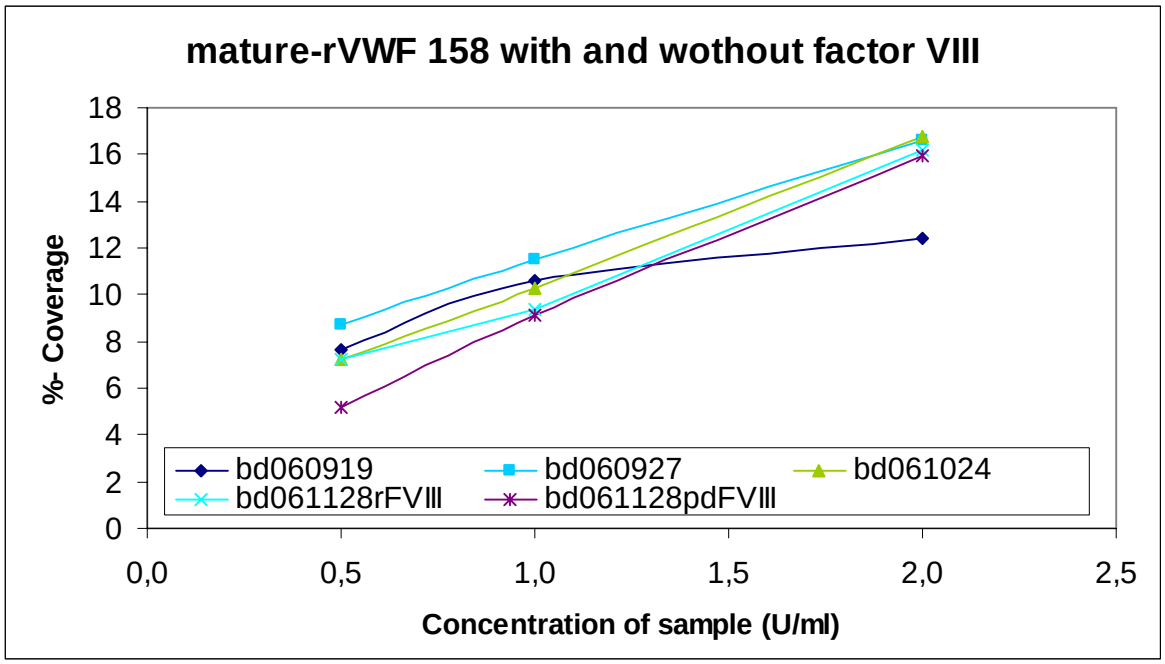


Figure 20. Results for the mature-rVWF 158 with and without factor VIII

5.2.3. Plasma-derived VWF 09 and factor VIII

In Figure 21 the results for the plasma-derived VWF 09 with and without the addition of either recombinant factor VIII or plasma-derived factor VIII are shown. In the curves, no effect of factor VIII can be seen. All results are in the same fluctuation range. Thus, the results of this test are not conclusive regarding the influence of factor VIII in the plasma-derived VWF 09 platelet adhesion properties.

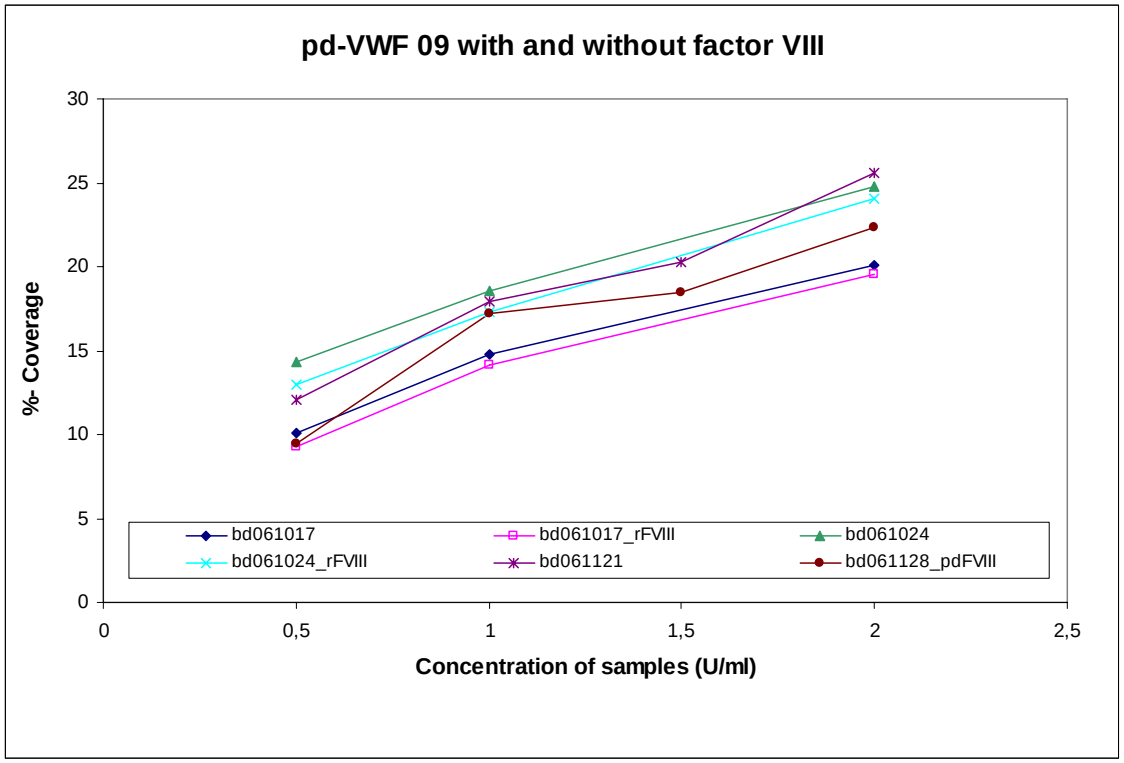


Figure 21. Results for the plasma-derived VWF 09

5.3. Competition experiment

The aim of this experiment was to investigate subtractive or additive effects in the platelet adhesion abilities, when two different VWF concentrates were mixed. For this experiment the plasma-derived VWF 09 and the VWF SEC 06 were used. Three different mixtures were tested: (a) 50% (V/V) of pdVWF 09 and 50% (V/V) of VWF SEC 06, (b) 25% (V/V) and 75% (V/V), respectively, and (c) 75% (V/V) and 25% (V/V), respectively. In Figure 22 the results of this study are summarised. In comparison to the VWF SEC 06 alone, the mixtures showed a significantly higher platelet adhesion property, aside from the concentration 0.5 U/ml, where the 50% : 50% mixture showed the lowest values. All in all, it turns out that the combination of different VWF concentrates may increase the platelet adhesion properties of the mixture.

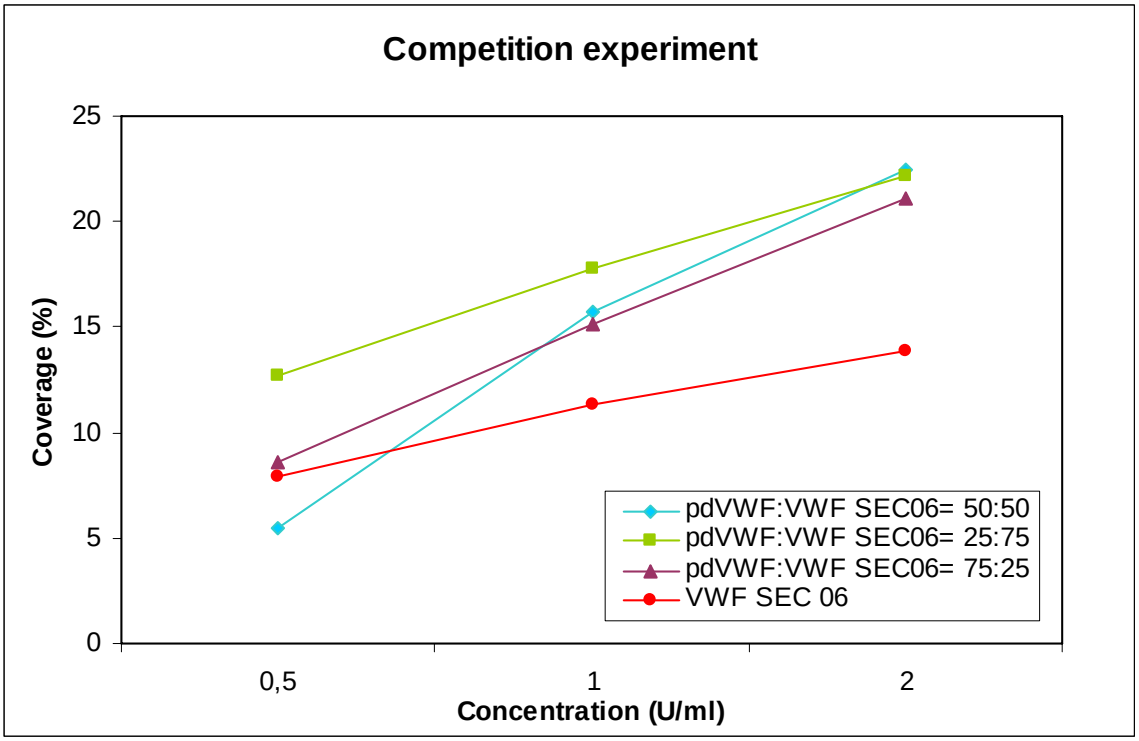


Figure 22. Results of the competition experiment

5.4. Comparison and discussion

The following results were obtained by the method described in Section 4.3. In Figure 23 all the VWF concentrates are summarised. The plasma-derived VWF/factor VIII concentrate (Immunate#09HZ2000I) and the plasma-derived VWF 09 (pd-VWF 09) performed best; their platelet adhesion properties at a concentration of 2.0 U/ml are at 25% and at 20%. The fact that the Immunate concentrate performed best was to be expected, because in [9] the authors have already found out the Immunate concentrate showed remarkable results in clinical trials.

In contrast, the pro-recombinant VWF 298 (pro-rVWF 298) lead to the lowest results in binding to the collagen-coated surface. The fact that the Pro-rVWF concentrates showed the lowest platelet adhesion properties, may be due to a diminished Pro-VWF/factor VIII binding. As pointed out in [13] the Pro-VWF molecule showed lower binding affinity for factor VIII, which may be due to either not accessible binding sites on the VWF protein, or an altered affinity to these specific binding sites. In [11] the authors discuss that the Pro-VWF protein failed to mediate platelet adhesion. They also pointed out that multimerisation is required for VWF to attain functional GPIb binding. The presence of uncleaved VWF propeptide inhibits both factor VIII binding and its stabilisation.

In [14] the author described the advantages of recombinant VWF in comparison to the plasma–derived VWF. Several limitations connected with the plasma-derived VWF, such as proteolytic degradation during the manufacturing process or variation in multimer composition and also donor dependence could be overcome by recombinant processing. Also the risk of infection with HIV or Hepatitis can be excluded using recombinant VWF for therapy. In contrast to the findings in [14], the recombinant VWF concentrates used in these experiments did not perform nearly as well as the plasma-derived ones. In [14] the author also discussed that the platelet adhesion properties of VWF are dependent on the degree of multimerisation of the VWF protein. The pro-rVWF is mostly a dimer having a low multimerisation degree. In contrast to the pro-rVWF, the plasma-derived VWF concentrates intrinsically have a high multimerisation degree which is supposed to lead to higher platelet adhesion properties. The multimerisation degree of the mature-rVWF protein is yet unknown, but it is believed to be in the

same range as the plasma-derived VWF concentrates. This is reflected in the results presented in this diploma thesis, where the mature-rVWF showed better platelet adhesion properties than the pro-rVWF (see Figure 23).

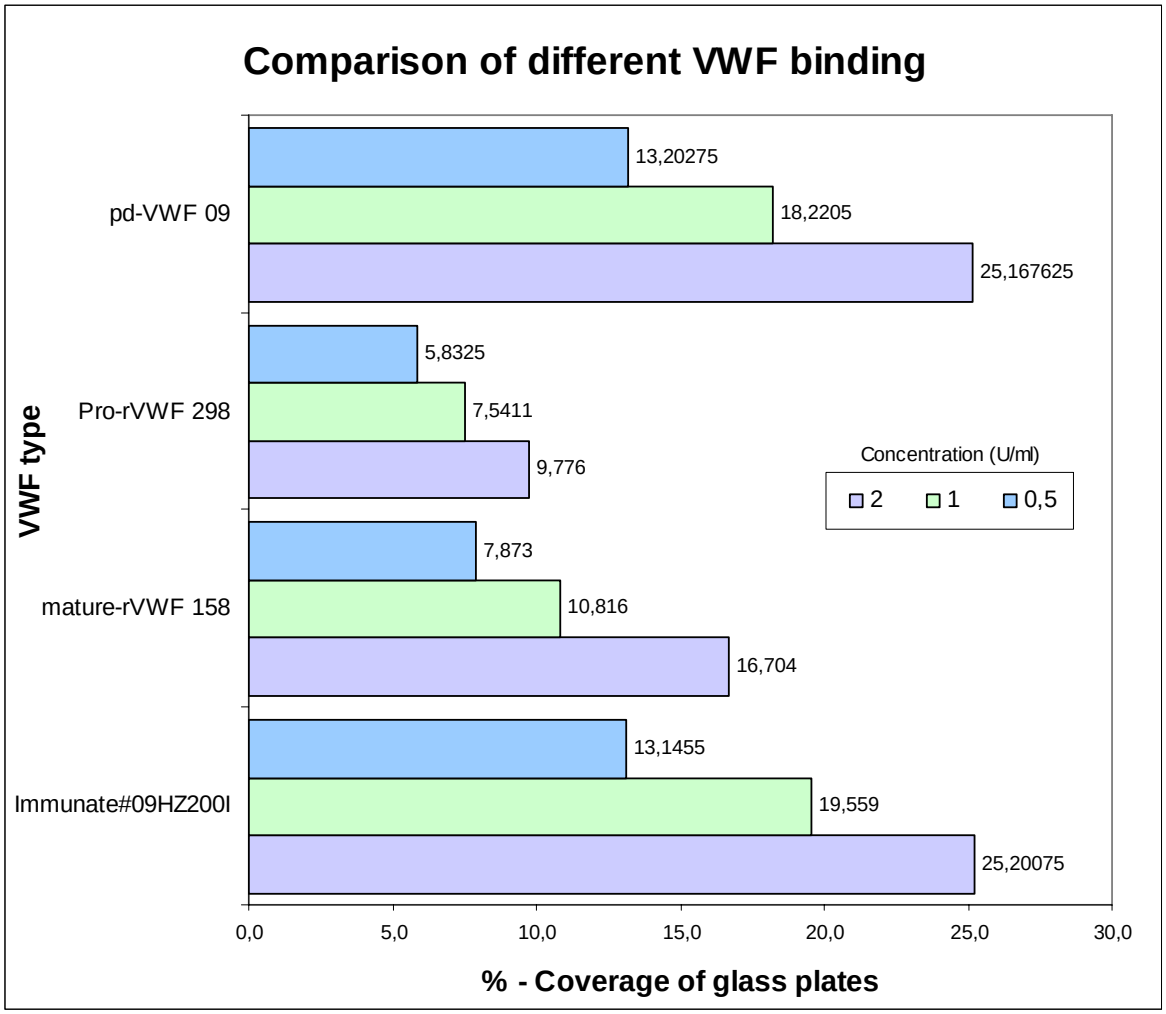


Figure 23. Comparison of all VWF-samples

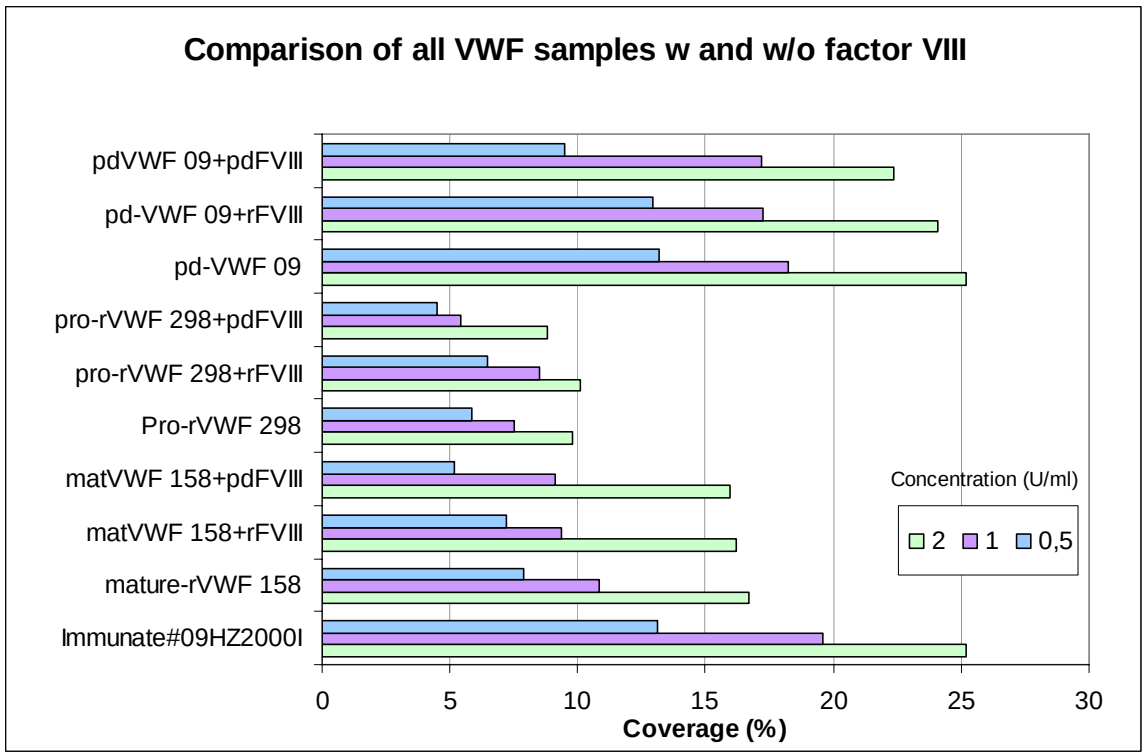


Figure 24. Comparison of platelet binding properties of all VWF samples in presence and absence of recombinant or plasma-derived factor VIII

In Figure 24 all the results regarding all combinations of VWF concentrates (plasma-derived and recombinant) and factor VIII addition (plasma-derived and recombinant) are summarised. Interestingly, all the VWF/factor VIII mixtures showed lower platelet adhesion properties than the respective VWF concentrates alone, except for the Pro-recombinant VWF 298 (pro-rVWF 298). There, the mean values for the pro-rVWF 298 in mixture with the recombinant factor VIII are slightly higher than the VWF concentrate alone or in mixture with the plasma-derived factor VIII. Due to few problems concerning the flow chamber and the calculation programme, which are summarised in Section 5.5, this result may not be significant.

Another problem that may play a major role regarding the scattered results is the fact that the blood used, came from different donors. The donors were also anonymous, which means that their anamneses were also unknown. So, the influence of different drugs, probably taken before the blood donation, could not be investigated in these experiments. It must be said that blood coagulation inhibitors or various cyclooxygenase inhibitors, such as Aspirin, also affected the

results severely. So for further investigation and to verify the results, a specific group of donors would be beneficial.

All in all, it turns out that the best platelet adhesion properties can be expected using the pd-VWF/factor VIII concentrate (Immunate#09HZ2000I) or the pd-VWF 09.

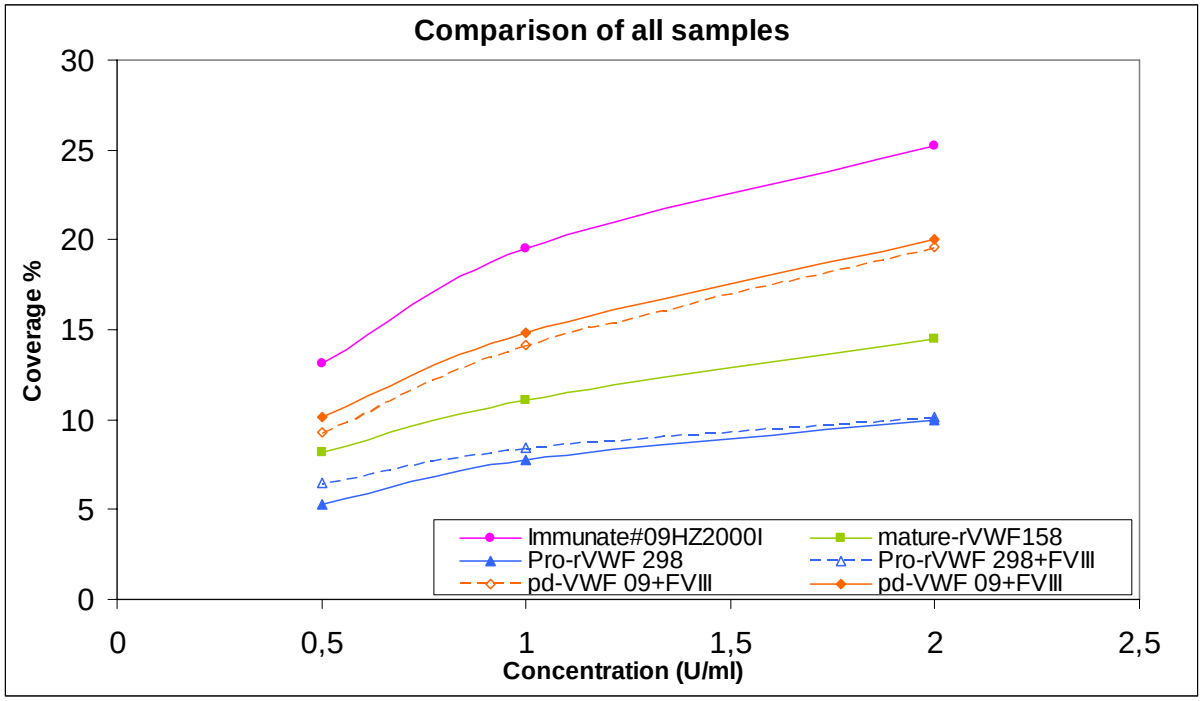


Figure 25. Comparison of all VWF samples with and without factor VIII (mean value)

Figure 25 shows the mean results obtained with different VWF concentrates alone in comparison to the mean results obtained with the VWF/factor VIII concentrates. In this graph, the origin of the factor VIII concentrates was not accounted for. The plasma-derived and the recombinant factor VIII concentrate values were combined and averaged. Again, it can be clearly seen that the pd-VWF concentrate Immunate#09HZ2000I performed best. However, the addition of factor VIII to the VWF concentrates showed no specific increase of the platelet adhesion properties of the concentrates. This leads to the conclusion that the addition of factor VIII does not significantly influence the VWF-induced platelet adhesion properties. Thus, for patients suffering from both VWD and decreased

factor VIII levels, factor VIII can be added to VWF concentrates, without compromising the platelet adhesion assisted by the VWF.

5.5. Difficulties concerning the flow-chamber experiment

The problem interpreting these results is that the blank values often ranged from 8% to 12% of the collagen-coated-glass-plate coverage. This means that the unspecific binding of the platelets to the collagenous surface without the addition of any VWF was relatively high. This is particularly problematic for small values of the platelet adhesion property, since a high blank correction leads to questionable results. Also, by slight unpredictable leakage of the flow chamber, the programmed shear stress rates slightly differed. This effect led to a lower platelet adhesion.

Furthermore, the clots on the collagenous surface of the glass plates were not always uniformly distributed, which means that some parts were heavily covered with blood clots, while others were not. Due to that fact, the evaluation was uncertain at some points because the computer was programmed to take photos every couple of millimetres. Therefore, some pictures contained less clots than others and the programme calculated too low glass plate coverage. For that reason some of the results differed strongly.

6. Conclusion

In this diploma thesis, the platelet adhesion properties of five different VWF concentrates produced by *Baxter Bioscience* were tested. Also, the effect of factor VIII addition to the VWF concentrates was investigated.
It turned out that the plasma-derived VWF/factor VIII concentrate (Immunate#09HZ2000I) showed the highest platelet adhesion properties. This concentrate was also rates as excellent or good results in clinical efficacy tests [9]. Only for patients with additional factor XIII deficiency the concentrate failed to perform well.

Concentrates where recombinant or plasma-derived factor VIII was manually added to the VWF solution did not perform as well.
The worst performance was observed with Pro-rVWF 298, independent of the addition of factor VIII, which is consistent to the results presented in [11], where the authors discussed that deletion of the vWF propeptide produced a dimeric VWF molecule that failed to mediate platelet agglutination, suggesting that multimerization is required for vWF to attain functional GPIb binding. They eventually found that the presence of uncleaved VWF propeptide inhibits both factor VIII binding and stabilization.
Also generally, it was observed that the addition of factor VIII did not significantly influence the results obtained by pure VWF concentrates. However, this result might be compromised by the large variation in the blood samples.

A number of difficulties were encountered in the experiments:
Every experiment was conducted with blood from different donors. The fact that the anamneses of the blood donors were unknown led to widely scattered results. For future experiments it is necessary to select a suitable group of donors with known anamneses. In this way one could systematically avoid drug interactions with blood coagulation in the experiment, leading to more reproducible results.
During the experiments, also a few shortcomings of the test system were encountered, which sometimes led to questionable results. To improve the significance of the results, both the flow chamber and the detection method need to be improved.

The flow chamber can be made leak proof by using a screw-top, which would be better suited than the clip-on spring. To avoid the glass plates from breaking, plastic plates are suggested.

To solve the problem of the counting of the clots, a different detection method should be considered. One possibility is radio labelling the platelets or the VWF molecules and detecting the clots on the glass plates via a gamma-counter. Another method would be to label the platelets or the VWF molecules with fluorescent substances and detect the clots with a fluorescence detector. These methods exclude the problem of taking photos from non-uniformly covered areas.

7. Appendix

7.1. Detailed results

The next pages present the detailed results from all experiments.

Immunate#09HZ2000I - VWF- Factor VIII- Concentrate

Date	Filenumber	Concentration	Platenumber	%-Coverage	Mean	Blank	Blank_corrected
						9,846	
08.08.2006	bd060808	2	6	25,861			
		2	7	24,845	25,353		15,507
		1	16	17,455	17,455		7,609
		0,5	21	17,406	17,406		7,560
						11,781	
22.08.2006	bd060822	2	67	34,369			
		2	68	33,709	34,039		22,258
		1	72	21,496			
		1	75	19,788	20,642		8,861
		0,5	16	22,527			
		0,5	17	23,325	22,926		11,145
						8,596	
30.08.2006	bd060830	2	9	33,549			
		2	10	33,595			
		2	11	34,064			
		2	13	31,163	33,093		24,497
		1	15	26,843			
		1	30	25,964			
		1	31	28,886	27,231		18,635
		0,5	21	19,264			
		0,5	23	17,652			
		0,5	25	20,842			
		0,5	37	23,336	20,274		11,678
						15,826	
31.08.2006	bd060831	2	40	36,767			
		2	42	31,268			
		2	44	34,408	34,148		18,322
		1	25	27,111			
		1	24	24,107			
		1	29	28,100	26,439		10,613
		0,5	20	22,836			
		0,5	26	24,967			
		0,5	27	21,123	22,975		7,149
						10,138	
26.09.2006	bd060926	2	5	34,204			
		2	6	35,818	35,011		24,873
		1	7	32,421			
		1	8	29,587	31,004		20,866
		0,5	12	23,260	23,26		13,122
						10,123	
03.10.2006	bd061003	2	26	33,770			
		2	27	34,954	34,362		24,239
		1	14	28,663			
		1	10	28,948	28,8055		18,683
		0,5	18	22,513			
		0,5	22	22,965	22,739		12,616

						9,951	
11.10.2006	bd061011	2	71	37,145	37,145		27,194
		1	67	30,003	30,003		20,052
		0,5	64	25,117	25,117		15,166

Pro-rVWF 298

Date	Filenumber	Concentration	Platenumber	%-Coverage	Mean	Blank	Blank_corrected
						10,138	
26.09.2006	bd060926	2	29	22,654			
		2	30	18,363			
		2	20	19,000			
		2	21	16,344	20,006		9,868
		1	22	17,791			
		1	24	18,358			
		1	31	16,992			
		1	32	20,079	17,714		7,576
		0,5	27	17,534			
		0,5	28	16,209			
		0,5	33	16,865	16,537		6,399
						10,123	
03.10.2006	bd061003	2	35	22,124			
		2	36	18,116			
		2	38		20,120		9,997
		1	39	18,374			
		1	40	17,776			
		1	41		18,075		7,952
		0,5	43	13,758			
		0,5	44	14,894			
		0,5	45				
		0,5	46		14,326		4,203
						9,951	
11.10.2006	bd061011	2	57	15,985			
		2	58	16,418	16,202		6,251
		1	51	14,154			
		1	56	14,858	14,506		4,555
		0,5	46	14,218			
		0,5	47	14,124	14,171		4,220
		4	52	18,421	18,421		8,470
		8	55	16,721			
						9,739	
17.10.2006	bd061017	2	63	22,174			
		2	64	16,918	19,546		9,807
		1	65	15,895			
		1	67	19,503	17,699		7,960
		0,5	68	15,692			
		0,5	69	16,832	16,262		6,523
						7,377	
18.10.2006	bd061018	2	95	14,283			
		2	96	16,809	16,809		9,432
		1	97	14,789			
		1	98	15,354	15,072		7,695
		0,5	101	13,257			
		0,5	102	13,162	13,210		5,833

Pro-rVWF 298 & r-factor VIII

						7,377	
18.10.2006	bd061018FVIII	2	11	15,176			
		2	12	17,481	17,481		10,104
		1	14	15,849	15,849		8,472
		0,5	99	13,928			
		0,5	100	13,715	13,822		6,445

Pro-rVWF298 & pd-factor VIII

						6,739	
29.11.2006	bd061129	2	28	15,787			
		2	30	15,351	15,569		8,830
		1,5	31	13,819			
		1,5	32	12,670	13,245		6,506
		1	34	11,971			
		1	35	12,399	12,185		5,446
		0,5	36	11,414			
		0,5	38	11,062	11,238		4,499

mature-rVWF 158

Date	Filenumber	Concentration	Platenumber	%-Coverage	Mean	Blank	Blank_corrected
						9,846	
08.08.2006	bd060808	2	46	15,919			
		2	47	17,258	16,589		6,743
		1	50	14,329			
		1	49	15,762	15,046		5,200
		0,5	52	14,297			
		0,5	51	12,913	13,605		3,759
						10,202	
19.09.2006	bd060919	2	11	23,272			
		2	11	24,747			
		2	13	21,190			
		2	13	19,517	22,600		12,398
		1	16	20,327			
		1	16	22,277			
		1	17	19,360			
		1	17	21,224	20,797		10,595
		0,5	24	15,156			
		0,5	24	18,578			
		0,5	25	20,276			
		0,5	25	17,533	17,886		7,684
						9,844	
27.09.2006	bd060927	2	37	27,087			
		2	38	26,955			
		2	39	25,416	26,486		16,642
		1	42	21,209			
		1	51	20,552			
		1	52	22,388	21,383		11,539
		0,5	48	17,740			
		0,5	49	18,181			
		0,5	50	19,731	18,551		8,707
						6,546	
24.10.2006	bd061024	2	34	23,312			
		2	36	17,666	23,312		16,766
		1	37	17,790			
		1	38	15,930	16,860		10,314
		0,5	39	15,016			
		0,5	40	12,532	13,774		7,228

mature-rVWF 158 & r-factor VIII							
						6,739	
28.11.2006	bd061128	2	10	22,758			
		2	11	23,079	22,919		16,180
		1	13	17,372			
		1	14	14,790	16,081		9,342
		0,5	15	14,633			
		0,5	17	13,311	13,972		7,233
mature-rVWF 158 & pd-factor VIII							
						6,739	
28.11.2006	k061128	2	41	22,689			
		2	40	broken	22,689		15,950
		1,5	45	19,129			
		1,5	46	18,450	18,790		12,051
		1	47	15,702			
		1	48	16,030	15,866		9,127
		0,5	49	13,322			
		0,5	50	10,500	11,911		5,172

Plasma-derived VWF 09

Date	Filenumber	Concentration	Platenumber	%-Coverage	Mean	Blank	Blank_corrected
						9,739	
17.10.2006	bd061017	2	48	29,318			
		2	49	28,058			
		2	50	34,391			
		2	51	27,415	29,796		20,057
		1	52	26,094			
		1	53	23,677			
		1	54	24,472			
		1	55	23,869	24,528		14,789
		0,5	56	21,298			
		0,5	57	17,096			
		0,5	61	22,867			
		0,5	62	18,196	19,864		10,125
						6,546	
24.10.2006	bd061024	2	3	31,885			
		2	4	30,730			
		2	6	30,910			
		2	7	31,652	31,294		24,748
		1	8	26,788			
		1	9	23,777			
		1	10	24,747			
		1	11	29,692	25,104		18,558
		0,5	12	21,043			
		0,5	13	22,169			
		0,5	14	21,664			
		0,5	15	18,466	20,836		14,290
						6,080	
21.11.2006	bd061121	2	43	32,670			
		2	44	30,664	31,667		25,587
		1,5	41	26,564			
		1,5	42	26,203	26,384		20,304
		1	39	23,997			
		1	40	23,929	23,963		17,883
		0,5	38	18,196	18,196		12,116

pd-VWF09 and r-factor VIII							
						9,739	
17.10.2006	bd061017	2	73	30,908			
		2	76	27,434			
		2	83	31,082			
		2	84	27,848	29,318		19,579
		1	79	24,685			
		1	86	23,898			
		1	87	22,977	23,853		14,114
		0,5	80	18,882			
		0,5	82	18,265			
		0,5	85	18,752			
		0,5	89	20,109	19,002		9,263
						6,546	
24.10.2006	bd061024	2	16	30,603			
		2	17	30,673	30,638		24,092
		1	19	24,458			
		1	20	23,183	23,821		17,275
		0,5	21	19,474			
		0,5	22	16,310	19,474		12,928

pd-VWF09 and pd-factor VIII							
						6,739	
28.11.2006	bd061128	2	18	30,002			
		2	19	28,222	29,112		22,373
		1,5	22	25,176			
		1,5	23	25,150	25,163		18,424
		1	24	24,231			
		1	25	23,681	23,956		17,217
		0,5	26	16,222			
		0,5	27	13,417	16,222		9,483

Plasma-derived VWF 07 42/3

Date	Filenumber	Concentration	Platenumber	%-Coverage	Mean	Blank	Blank_corrected
						9,846	
08.08.2006	bd060808	2	23	14,578			
					14,578		4,732
		1	25	10,301			
					10,301		0,455
		0,5	29	18,901			
		0,5	30	19,117	19,009		9,163
						11,781	
22.08.2006	bd060822	2	18	30,879			
		2	19	32,300	31,590		19,809
		1	20	27,282			
		1	21	26,546	26,914		15,133
		0,5	24	19,182			
		0,5	30	18,786	18,984		7,203
						10,202	
19.09.2006	bd060919	2	46	25,071			
		2	46	24,842			
		2	48	21,306			
		2	48	19,702	23,131		12,929
		1	49	20,554			
		1	49	17,672			
		1	50	18,258			
		1	50	18,810	19,544		9,342
		0,5	51	16,851			
		0,5	51	18,008			
		0,5	53	17,797			
		0,5	53	16,393	17,262		7,060
						9,844	
27.09.2006	bd060927	2	58	21,010	21,010		11,166
		1	55	18,556			
		1	62	18,767	18,662		8,818
		0,5	66	18,778			
		0,5	69	15,629	15,629		5,785

7.2. Figure sources

Figure 1. List of clotting factors involved in the blood coagulation (http://www.uta.edu/biology/henry/classnotes/2458/Clotting%20Factors.jpg)

Figure 2. Reaction cascade of blood coagulation (http://medicaldictionary.thefreedictionary.com/_/viewer.aspx?path=dorland&name=pathway_coagulation.jpg)

Figure 3. Blood clot (http://dept.physics.upenn.edu/~aebrown/biocurious/clot-and-fiber.jpg)

Figure 4. Genesis of platelets (http://training.seer.cancer.gov/ss_module08_lymph_leuk/images/illu_blood_cell_lineage.jpg)

Figure 5. Activated platelets (http://www.sciencehelpdesk.com/img/bg3_2/Platelets1.jpg)

Figure 6. VWF binding platelets to collagen (http://www.orthosupersite.com/images/content/obj/0803/kroonen_fig1.gif)

Figure 7. Structure of the von Willebrand factor (http://www.VWF.group.shef.ac.uk/graphics/protein.jpg)

Figure 8. Ribbon drawings of (a) the crystal structure of the collagen binding VWF-A3 domain; (b) Schematic representation of the complex of GpIb□ (green) and the VWF-A1 domain (blue). (http://www.crystal.chem.uu.nl/group-huizinga/a3-nolab.jpg, http://www.esrf.eu/UsersAndScience/Publications/Highlights/2002/MX/MX3/fig005)

Figure 9. Resemblance of factor VIII and factor V, (http://blood.uvm.edu/lab/domains.jpg)

Figure 10. Haematocrit and Platelet count

Figure 11. Preparation of the flow chamber

Figure 12. Set up of the flow chamber experiment

Figure 13. Blood clots on the collagen coated surface of glass plates after staining

7.3. Abbreviations

VWF	von Willebrand factor
VWD	von Willebrand disease
FVIII	factor VIII
pd	plasma-derived
DNA	deoxyribonucleic acid
Ca^{2+}	Calcium
Na/Cl	sodium chloride
HCT	haematocrit
COX-1	Cycolooxygenase-1
U/ml	(Antigen-) Units per millilitre
ml	millilitre
µl	microlitre
mM	millimolar
rpm	rotations per minute
°C	degree centigrade
w/o	without

7.4. Literature

[1] B. Dahlbäck, Blood Coagulation, The Lancet, Vol. 355:1627-32, 05.2000

[2] E.Mutschler, Lehrbuch d. Pharmakologie und Toxikologie, Wissenschaftliche Verlagsgesellschaft, mbH, 2001

[3] J.E. Sadler, Biochemistry and genetics of von Willebrand factor, Annual Review of Biochemistry, Vol. 67: 395-424, 1998

[4] W.E. Fowler, L.J. Fretto, K.K. Hamilton, H.P. Erickson, P.A. McKee, Substructure of human von Willebrand factor, Clin. Invest. 76(4):1491-500, 1985

[5] M.W. Chopek, J.P. Girma, K. Fujikawa, E.W. Davie, K. Titani, Human von Willebrand factor: a multivalent protein composed of identical subunits, Biochemistry, 25(11):3146-55, 1986

[6] A.Vlot, J. Koppelman, B.N. Bouma, J.J. Sixma, Factor VIII and von Willebrand factor, Thromb Haemost., 79:456-65, 1998

[7] R.A. Schwartz, E. Klujszo, R. McKenna, Factor VIII, [Online] http://www.emedicine.com/med/TOPIC981.HTM, Nov. 4, 2008

[8] H.A. Al-Mondhiry, Inherited bleeding syndromes in Iraq, Thromb Haemost., 37(3):549-55, 1977

[9] G. Auerswald, B. Eberspächer, W. Engl, C. Günther, M. Koksch, W. Kreuz, A. Nimtz, G. Pindur, H. Scheel, J.D. Schreiber, J. Siekmann, P.L. Turecek, H.H. Wolf, Successful Treatment of Patients with von Willebrand Disease using a High-Purity Double-Virus Inactivated Factor VIII/von Willebrand Factor Concentrate (Immunate©), Seminars in Thrombosis and Hemostasis, 28(2):203-1428, 2002

[10] P.L. Turecek, W. Schönhofer, T.R. Kreil, A. Weber, K. Váradi, H. Gritsch, J. Siekmann, L. Pichler, B.M. Reipert, B. Abbühl, H.P. Schwarz, IMMUNATE S/D-A new Factor VIII-von-Willebrand Factor Complex Concentrate, 33rd Hemophilia Symposium Hamburg 2002, Eds: Scharrer I, Schramm W. 117-34.

[11] R.J. Wise, A.J. Dorner, M. Krane, D.D. Pittman, R.J. Kaufman, The Role of von Willebrand Factor Multimers and Propeptide Cleavage in Binding and Stabilisation of Factor VIII, Journal of Biological Chemistry, 266(15):21948-21955, 1991

[12] B.E. Fischer, U. Schlokat, A. Mitterer, M. Reiter, W. Mundt, P.L. Turecek, H.P. Schwarz, F. Dorner, Structural analysis of recombinant von Willebrand factor produced at industrial scale fermentation of transformed CHO cells co-expressing recombinant furin, FEBS Letters 375: 259-262, 1995

[13] A.V. Bendetowicz, J.A. Morris, R.J. Wise, G.E. Gilbert, R.J. Kaufman, Binding of Factor VIII to von Willebrand Factor is enabled by Cleavage of the von Willebrand Factor Propeptide and enhanced by Formation of Disulfide- linked Multimers, Blood, 92: 529-538, 1998

[14] B.E. Fischer, Recombinant von Willebrand factor: potential therapeutic use, Thromb Thrombolysis, 8(3):197-205, 1999

General Literature:

[15] P.L. Turecek, K. Váradi, U. Schlokat, L. Pichler, F. Dorner, H.P. Schwarz, In vivo and in vitro Processing of recombinant Pro-Von Willebrand Factor, Histochem Cell Biol., 117:123-129, 2002

[16] P.L. Turecek, L. Pichler, W. Auer, G. Eder, K. Váradi, A. Mitterer, W. Mundt, U. Schlokat, F. Dorner, L.O. Drouet, J. Roussi, J.A. van Mourik, H.P. Schwarz, Evidence for Extracellular Processing of Pro-von Willebrand Factor After Infusion in Animals With and Without Severe von Willebrand Disease, Blood, 94: 1637-1647, 1999

[17] H.P.Schwarz, U. Schlokat, A. Mitterer, K. Váradi, H. Gritsch, E.M. Muchitsch, W. Auer, L. Pichler, F. Dorner, P.L. Turecek, Recombinant von Willebrand factor-insight into structure and function through infusion studies in animals with severe von Willebrand disease, Semin Thromb Hemost,28(2):215-26, 2002

[18] J. Batlle, M.S. Noya, P. Giangrande, M.F. Lopez-Fernandez, Advances in the therapy of von Willebrand disease, Haemophilia, 8(3):301-7, 2002

[19] Matthias F. Schneider, Stefan W. Schneider, Der Von-Willebrand Faktor: Ein intelligenter Gefäßkleber, BioSpektrum, 02.08., 134-139

[20] A.B. Federici, Management of von Willebrand disease with factor VIII/von Willebrand factor concentrates: results from current studies and surveys, Blood Coagul. Fibrinolysis, 16 Suppl 1:S17-21, 2005

[21] B. Plaimauer, U. Schlokat, P.L. Turecek, A. Mitterer, W. Mundt, W. Auer, L. Pichler, H. Gritsch, H.P. Schwarz, Recombinant von Willebrand factor: preclinical development, Semin. Thromb Hemost., 27(4):395-403, 2001

[22] E.G. Huizinga, S. Tsuji, R.A.P. Romijn, M.E. Schiphorst, Ph.G. de Groot, J.J. Sixma, P. Gros, Crystal Structure Reveals Blood Platelet Adhesion Complex, Science, 297:1176-1179, 2002

[23] G. Löffler, Basiswissen Biochemie mit Pathobiochemie, Springer, 5. Auflage, 2003

[24] S. Silbernagl, F. Lang, Taschenatlas der Pathophysiologie, Thieme, 1998

[25] B. Kemkes-Matthes, G. Oehler, Blutgerinnung und Thrombose, Thieme, 3. Auflage, 2001

[26] H. Brackmann, R. Schwaab, J. Oldenburg, W. Schramm, Klinische Anwendung plasmatischer und rekombinanter Gerinnungsfaktoren, 1. Auflage, 2003

[27] M. Kalafatis, J.O. Egan, C. van't Veer, K.M. Cawthern, K.G. Mann, The Regulation of Clotting Factors, Critical Reviews in Eukaryotic Gene Expression, 7 (3): 241-280, 1997

[28] K.G. Mann, P.B. Tracy, S. Krishnaswamy, R.J. Jenny, B.H. Odegaard, M.E. Nesheim, Platelets and Coagulation, Thrombosis and Haemostasis, 505-523, 1987

[29] B. Furie, B.C. Furie, The Molecular Basis of Blood Coagulation, Cell, 53: 505-518, 1988

[30] R.W. Farndale, J.J. Sixma, M.J. Barnes, G. de Groot, The Role of collagen in thrombosis and haemostasis, Thrombosis and Haemostasis, 2:1-13, 2004

[31] A.v. Bendetowicz, R.J. Wise, G.E. Gilbert, Collagen-bound von Willebrand Factor has Reduced Affinity for Factor VIII, Biological Chemistry, 274:12300-12307, 1999

[32] A.J. Vlot, S.J. Koppelmann, J.C.M. Meijers, C. Damas, H.M. van den Berg, B.N. Bouma, J.J. Sixma, G.M. Willems, Kinetics of Factor VIII-von Willebrand Factor Association, Blood, 87: 1809-1816, 1996

[33] A.J. Vlot, S.J. Koppelman, M.H. van den Berg, B.N. Bouma, J.J. Sixma, The Affinity and Stoichiometry of Binding of Human Factor VIII to von Willebrand factor, Blood, 85: 3150-3157, 1995

[34] K.S. Sakariassen, R. Muggli, H.R. Baumgartner, Meassurements of Platelet Interaction with Components of the Vessel wall in Flowing Blood, Methods in Enzymology, Vol. 169, 1989

[35] S. Butenas, B. Parhami-Seren, K.G.Mann, The influence of von Willebrand factor on factor VIII activity measurements, to appear in Thromb Haemost, 2008

[36] A.B. Federici, P.M. Mannucci, Optimizing therapy with factor VIII/von Willebrand factor concentrates in von Willebrand disease, Haemophilia, 4(3): 7-10, 1998

[37] P.M. Mannucci, P.M.Tenconi, G. Castaman, F. Rodeghiero, Comparison of four virus-inactivated plasma concentrates for treatment of severe von Willebrand disease: a cross-over randomized trial, Blood, 79(12):3130-3137, 1992

Lebenslauf

Persönliche Daten:

Karoline Maria Sindelar
Johannagasse 4/2
1050 Wien
Tel.: 0664 5586613
E-Mail: karoline.sindelar@inode.at

geboren am 01.02.1983 in Wien

Schulausbildung:

Sept. 1989 – Juni 1993	Volksschule St. Marien, Liniengasse 21, 1060 Wien
Sept. 1993- 2001	Gymnasium Mater Salvatoris, Kenyongasse 4 – 12, 1070 Wien
seit Okt. 2001	Universität Wien Studienrichtung Pharmazie Abschluss des 1. Abschnitts: 18.5.2004

Berufserfahrung:

Seit November 2006	Forschungsassistentin im Allgemeinen Krankenhaus Wien, Nuklearmedizin
August 2006 - Oktober 2006	Praktikantin bei Baxter BioScience (im Zuge der Diplomarbeit)
Oktober 2003 - Juni 2006	Technische Universität Wien Laborassistentin am Institut für Grundlagen und Theorie der Elektrotechnik
Oktober 2005 - April 2006	geringfügig beschäftigt in der Apotheke „Zum römischen Kaiser“
September 2004	Praktikantin in der „Marienapotheke“
August 2004	Praktikantin in der Apotheke „Zur Kaiserkrone“
August 2002	Trainer für Englisch Action und Fun Camp, STUDIENKREIS
2001 - 2002	Trainer für Deutsch, Latein, Englisch, Chemie, Biologie, STUDIENKREIS Bildungsmanagement GmbH
1999 - 2001	Praktikantin, Bank Austria, Mariahilferstraße 90, 1070 Wien

Publikationen:

[1] W. Wadsak, L.-K. Mien, K. Shanab, D. E. Ettlinger, D. Haeusler, **K. Sindelar**, R. R. Lanzenberger, H. Spreitzer, H. Viernstein, B. K. Keppler, R. Dudczak, K. Kletter, M. Mitterhauser, „Preparation and first evaluation of [18F]FE@SUPPY: a new PET tracer for the adenosine A3 receptor“, Nucl Med Biol., 35(1):61-66, Jan. 2008

[2] W. Wadsak, L.-K. Mien, D.E. Ettlinger, H. Eidherr, D. Haeusler, **K.-M. Sindelar**, B.K. Keppler, R. Dudczak, K. Kletter, M. Mitterhauser, "18F fluoroethylations: different strategies for the rapid translation of 11C-methylated radiotracers", Nucl Med Biol., 34(8):1019-28., Nov. 2007

[3] D.E. Ettlinger, D. Häusler, W. Wadsak, F. Girschele, **K.M. Sindelar**, L.-K. Mien, J. Ungersböck, H. Viernstein, K. Kletter, R. Dudczak, M. Mitterhauser, "Metabolism and autoradiographic evaluation of [(18)F]FE@CIT: a Comparison with [(123)I]beta-CIT and [(123)I]FP-CIT", Nucl Med Biol., 35(4):475-9, May 2008

[4] **K.M. Sindelar**, L.-K. Mien, D. Haeusler, D.E. Ettlinger, H. Viernstein, R. Dudczak, K. Kletter, M. Mitterhauser, W. Wadsak, „An improved approach to measure log P for various PET-Tracers”, poster presentation at ÖGN Conference, Bad Hofgastein, Jan 2008

[5] **K.M. Sindelar**, K.L. Mien, D. Haeusler, H. Viernstein, R. Dudczak, K. Kletter, M. Mitterhauser, W. Wadsak, „The Dilemma Using Log P to Define Lipophilicity of PET Tracers”, poster presentation at EANM Conference, Munich, Oct 2008

[6] D.E. Ettlinger, W. Wadsak, **K. Sindelar** (presenter), L.K. Mien, D. Haeusler, R. Lanzenberger, R. Dudczak, K. Kletter, M. Mitterhauser, „[18F]Fluoroethylated radiotracers: Metabolic considerations“, oral presentation at ÖGN Conference, Bad Hofgastein, Jan 2008

Besondere Qualifikationen:

Laborerfahrung:	Blut-Hirn-Schranken-Modelle (Log P Modelle) HPLC Analysen (Metaboliten, Log P) Herstellung von PET Tracern Dielektrophorese von Bakterien und Zellen Flusskammer
Fremdsprachen:	Cambridge Certificate of Proficiency 4 Sprachendiplom bei Berlitz (2008): - Englisch (perfekt in Wort und Schrift) - Russisch (Grundkenntnisse) - Französisch (sehr gut in Wort und Schrift) - Italienisch (gut in Wort und Schrift) Spanisch (Grundkenntnisse)
Führerschein:	Klasse B seit Oktober 2001
Computerkenntnisse:	Microsoft Windows und Office Kenntnisse Grundlegende PC-Hardwarekenntnisse