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Cornelia Bonstingl BSc

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Table of Contents

Abbreviations

µl..... microlitre

µm..... micrometre

º C..... degrees Celsius

ADP ..... adenosine diphosphate

$CaCl_2$ ..... calcium chloride

CLP..... common lymphoid progenitors

CMP..... common myeloid progenitors

CO_2 ..... carbon dioxide

CT..... clotting time

DMSO..... dimethyl sulfoxide

DNA..... deoxyribonucleic acid

EC ..... endothelial cells

EDTA.....ethylene-diamine-tetraacetic acid

EGM-2 ..... endothelial cell growth medium 2

etc. ..... et cetera

FBS..... Fetal Bovine Serum

FCS..... Fetal Calf Serum

FSC..... forward scatter

GMP ..... granulocyte and macrophage progenitor

h..... hours

HCO_3^- ..... hydrogen carbonate

HSC..... hematopoietic stem cells

HUVEC..... Human Umbilical Endothelial Cells

Ig ..... immunoglobulin

KCl..... potassium chloride

KH_2PO_4..... potassium dihydrogen phosphate

LED ..... light emitting diode

Log..... logarithmic

LPS ..... lipopolysaccharides

MEP ..... erythrocyte progenitors

min..... minutes

miRNA..... micro RNA

ml ..... millilitre

mm ..... millimetre

MP..... microparticle

MPP..... multipotent progenitors

Na_2HPO_4..... sodium hydrogen phosphate

NaCl ..... sodium chloride

NATEM..... native TEM

NK-cell ..... natural killer cells

nm..... nanometre

NO ..... nitric oxide

O_2 ..... oxygen

OEC..... Outgrowth Endothelial Cells

PG..... prostaglandin

P100 ..... pellet formed after 100 000 xg centrifugation-step

P14..... pellet formed after 14 000 xg centrifugation-step

PAR..... protease-activated receptor

PBS..... phosphate saline buffer

PE ..... phycoerytrin

PG..... prostaglandin

PS ..... phosphatidylserine

RNA..... ribonucleic acid

ROTEM..... rotational thromboelastometry

S0.5..... original cell supernatant after 500 xg centrifugation-step

S100 ..... remaining cell supernatant after 100 000 xg centrifugation-step

sec..... seconds

SSC ..... side scatter

T25..... cell culture flask with 25 cm^2 area to grow cells

T75..... cell culture flask with 75 cm^2 area to grow cells

TF..... tissue factor

TNF-α ..... tumour-necrosis factor alpha

t-PA ..... tissue-type plasminogen activator

u-PA ..... urokinase type plasminogen activator

UZ ..... ultracentrifugation

wb ..... whole blood

XA_2 ..... thromboxane A_2

xg ..... times gravitational force

1 Introduction

1.1 The human blood

As already Mephisto in Goethe' s Faust once famously said, '' Blood is very special juice". And indeed it is for a great variety of reasons. Most important it supplies us with oxygen and nutrients, essential for surviving. [1] Human beings have around 5 liters of blood running through their circulation (arteries, veins and capillaries). [2] 55 % of blood consists of fluid called plasma, whereas 45 % of blood is made out of cells, which can be separated via centrifugation (Fig. 1).

Plasma is made out of water, ions such as HCO_3^-, Na^+, Ca^{2+}, Mg^{2+}, Cl^- etc. and proteins such as albumins, globulins or clotting factors, nutrients such as glucose and apart from metabolic waste products it is also a carrier of hormones.[1][3] The most abundant plasma proteins are albumin (65 %), globulins (35 %) and fibrinogen (4 %). Albumins are the major contributors to maintaining the osmotic pressure. They also bind non-soluble substances like lipids or hormones and transport them through the body. Globulins also function as transport proteins but also a very important class of globulins, the immunoglobulins (IgA, IgM, IgD, IgE and IgG) can be found among them. Fibrinogen is an essential substance needed for blood coagulation, whereas clotting factors and enzymes in general do not make up more than 1 % of plasma proteins.[4]

The other 45 % of blood are cells. The vast majority of those cells are erythrocytes also named red blood cells. Erythrocytes are cells that contain 4 hemoglobin proteins, all of them having an iron-containing hem-group in their centre capable of binding oxygen. Erythrocytes do not have cell nuclei. Their main purpose is the oxygen/carbon dioxide transport and exchange throughout the whole body. [1] Apart from red blood cells

leukocytes, also named white blood cells, can be found in blood. Their main purpose is building a defense line against pathogens. Differently shaped leukocytes can be found in the blood, all of them having a cell nucleus: Neutrophils have as their main targets bacteria and fungi, eosinophils mainly attack larger parasites and they are involved in inflammatory and allergic responses, basophils release histamines for inflammatory response and monocytes migrate from the blood stream into tissue to become phagocytic macrophages, but also lymphocytes and neutrophil can enter tissue via leukocyte rolling. [5] Other leukocytes are lymphocytes such as B and T cells but also natural killer cells (NK-cells). Apart from red and white blood cells a small part of the cellular components of blood is made up by platelets also named thrombocytes playing a very important role in blood coagulation. Platelets do not have cell nuclei. [1] [6]

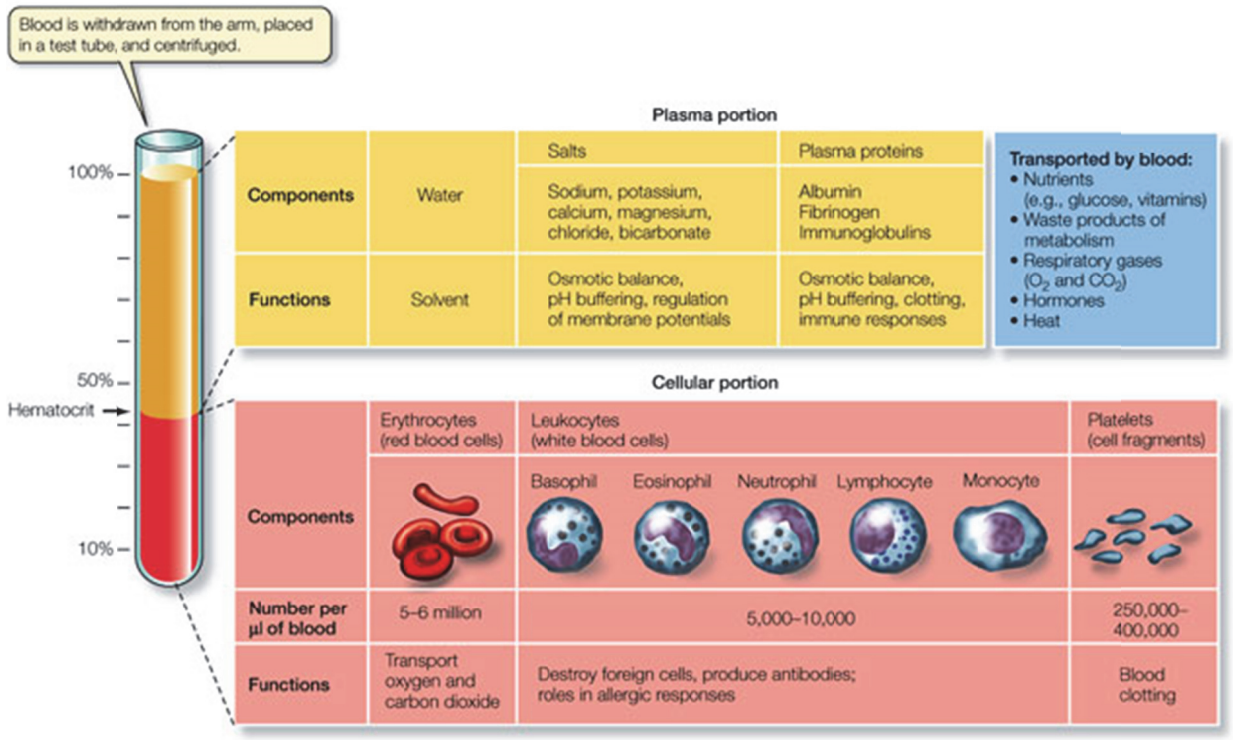


Fig. 1. Components of blood. Blood consists of plasma and cellular components easily separated via centrifugation. In plasma mainly water, salts and plasma proteins are present. Nutrients, metabolic waste products, O_2 as CO_2 and hormones are also found in plasma. The cellular components can be divided in erythrocytes, leukocytes such as basophils, eosinophils, neutrophils, lymphocytes and monocytes and platelets. [7]

Blood cells originate from pluripotent hematopoietic stem cells (HSC), which are formed in the bone marrow. The process of the formation of the cellular components of blood, called haematopoiesis, is described in Fig. 2. HSC give rise to multipotent progenitors (MPP), of which the myeloid and lymphoid lineages originate from. A myeloid progenitor can differentiate into a megakaryocyte, which produces platelets. Apart from a megakaryocyte the myeloid progenitor can alternatively differentiate into erythrocytes, mast cells or myoblast. Myoblasts can later give rise to basophils, neutrophils, eosinophils or monocytes, which can become macrophages after migration from blood into tissue. On the other hand there are lymphoid progenitors, which can give rise to natural killer cells, T-lymphocytes or B-lymphocytes. [8][9]

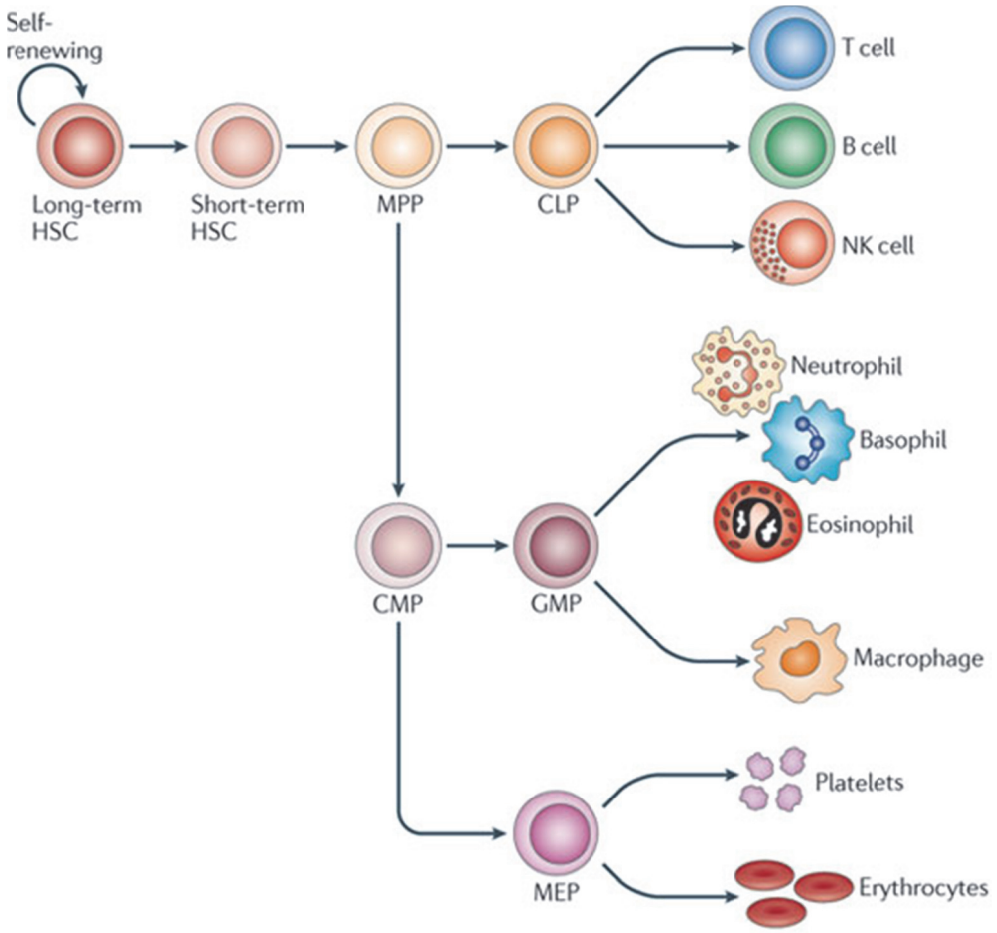


Fig. 2. Haematopoiesis. Long term haematopoietic stem cells (HSC) have the capacity of self-renew during the whole life-spam. Short-term HSC do not have ability anymore and give rise to multipotent progenitors (MPP). MPP differentiate into common lymphoid progenitors (CLP), which give rise to T-cells, B-cells and NK-cell, and into common myeloid progenitors (CMP). CMP differentiate into granulocyte and macrophage progenitor (GMP), which gives rise to neutrophils, basophils, eosinophils and macrophages, and megakaryocyte and erythrocyte **progenitors (MEP)**, which give rise to **platelets** and **erythrocytes**. [9]

1.2 Blood Coagulation

Blood coagulation is not only important to prevent exsanguination, even from small injuries, but is also crucial for wound healing. [10] Blood coagulation or clotting is divided in primary and secondary haemostasis both happening simultaneously. [2] During primary haemostasis platelets bind due to injury to exposed subendothelium, resulting in the formation of a plug in order to seal the wound. At the same time secondary haemostasis happens, also trigged by an injury, but this time the plasmatic coagulation cascade is activated, ultimately leading to the formation of fibrin to make a stable clot in order to make sure that the wound is sealed and healing ad integrum. [1][11]

1.2.1 Primary Haemostasis and platelet activation

Von Willebrand factor is a protein mostly formed by endothelial cells being released into the blood. If the subendothelial matrix gets in contact with blood during an injury, von Willebrand factor binds to collagen allowing the glycoprotein receptor GpIb bind to the subendothelium (Fig. 3). Then GpIIb-IIIa helps the platelets to bind among themselves with the use of fibrinogen. During and after binding to von Willebrand factor platelets get activated by collagen, ADP or thromboxane A_2 (TXA_2). The consequence of this is a change of shape of the platelets, transforming from a round-shaped cell to a star-like sticky version of the original one. Also as part of activation dense and alpha granules are released mainly containing ADP or thrombin and consequently promote their own platelet activation. [12][13]

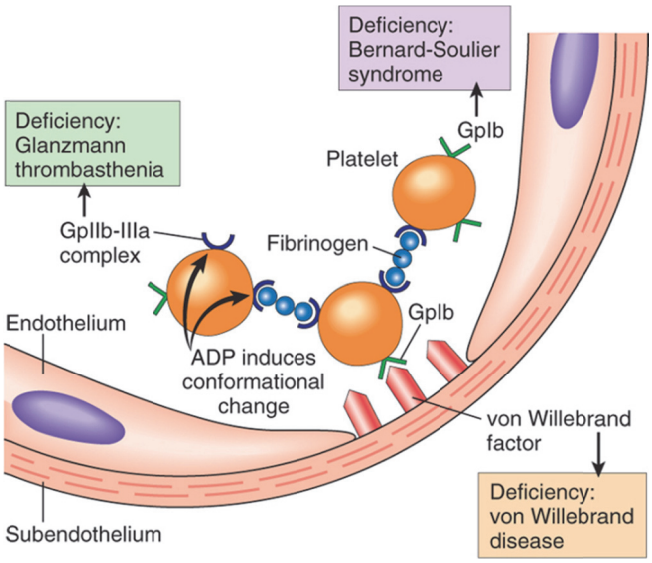


Fig 3. Platelet attachment to subendothelium. Soluble von Willebrand factor binds to the subendothelium due to an injury and binds platelets via GpIb. Platelets get attached to each other via GpIIb-IIIa and also activated. ADP release is a consequence of platelet activation and leads do conformational changes. [14]

Activated platelets can form great aggregates and their thrombin generation also helps secondary haemostasis enhancing fibrin formation. The platelet plug formed by primary haemostasis must be further stabilised by fibrin, which is formed during secondary haemostasis.

1.2.2 Secondary Haemostasis

The endothelium is modulating blood coagulation by producing coagulatory and anti-coagulatory factors without interruption in order to maintain the very sensitive balance between blood coagulation and fluidity. [15][16] In the classic coagulation model there are two pathways, which are both capable of inducing coagulation: the intrinsic and extrinsic pathway (Fig. 4). Both pathways end up in the common pathway and ultimately lead to fibrin formation. It helps to bear in mind that the principle behind the clotting cascades of all pathways is mainly transforming inactivated serine proteases into

activated, which can then cleave other enzymes in order to activate them, forming a cascade which leads to a stable fibrin clot, necessary to build a stable platelet plug. In such plugs apart from platelets also erythrocytes can be found, which are trapped in a network of fibrin (Fig 5). The individual enzymes involved these two pathways are in more detail in Table 1 described.

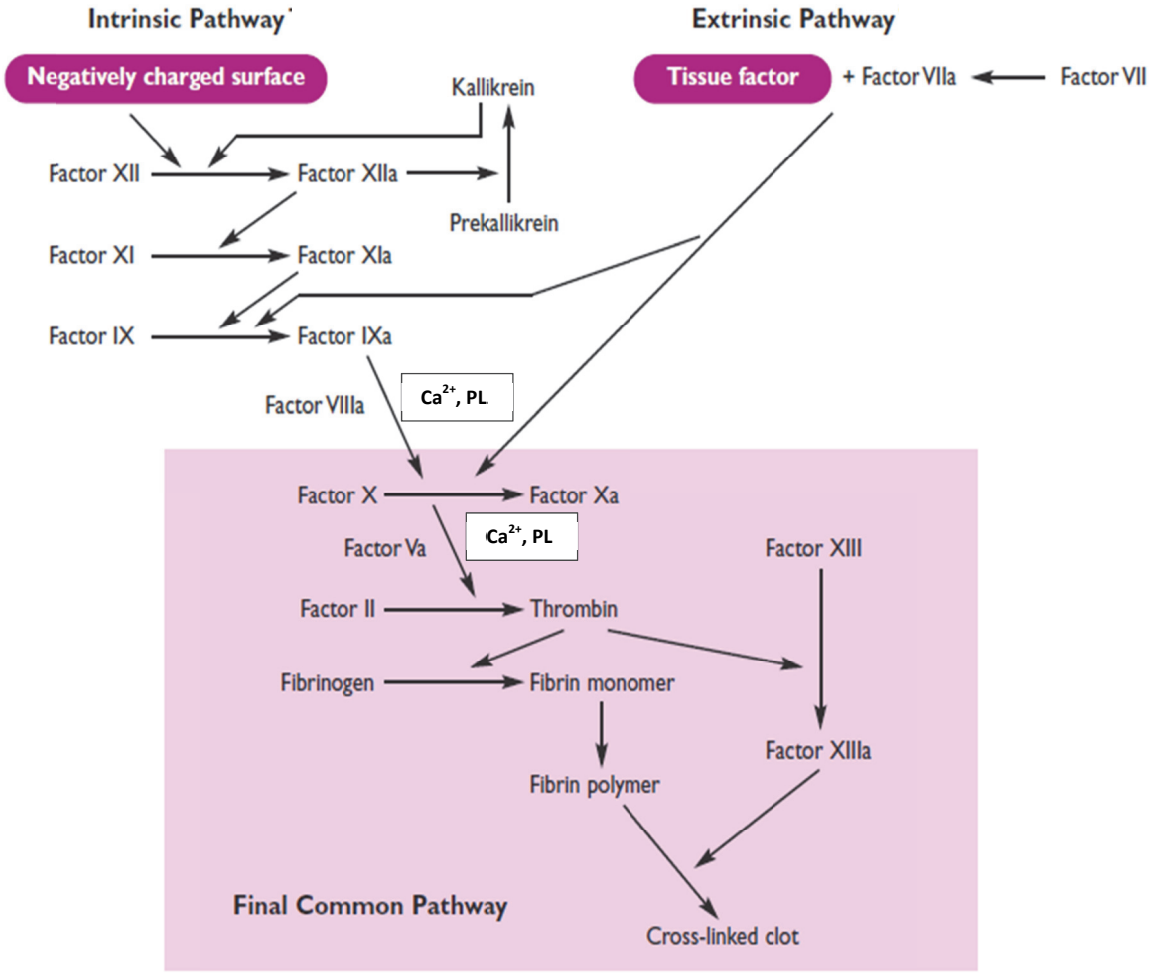


Fig. 4. Classic model for secondary haemostasis. In the figure the romanic number followed by an "a" refers to the activated version of this coagulation factor: e.g. factor VII and VIIa. The intrinsic pathway is triggered by a negative charged surface (e.g. collagen) and after activation of factor XII, XI and IX ends up in common pathway. The extrinsic pathway starts with an injury causing tissue factor release which activates factor X together with activated factor VII and thereby entering the common pathway. In the common pathway factor II also named prothrombin is converted to thrombin, which activates fibrinogen polymerisation ultimately leading to a fibrin clot. Calcium (Ca^{2+}) and phospholipids (PL) are essential for clot formation. [15][17]

The intrinsic pathway needs to get in contact with a negatively charged surface, which can be found when endothelial cells are somehow removed from the inner blood vessels giving negative charged substances in the matrix space to get in contact with factor XII. Factor XII is thereby activated, activating itself factor IX. Activated factor IX together with activated factor VIII, phospholipids (PL) and calcium cleave prothrombin into thrombin. Thrombin is one of the major key players of coagulation. In the common pathway thrombin cleaves fibrinogen which can then form a fibrin network via polymerisation. In order to build a stable cross-linked meshwork of fibrin factor VIII is needed.

The extrinsic pathway on the other hand starts with the exposure of tissue factor mainly produced by fibroblasts or leukocytes due to tissue damage. Tissue factor can then form a complex with activated factor VII, which activates factor X, also leading to the common pathway.[2][18]

Name	Description	Function
Fibrinogen (Factor I)	Molecular Weight (MW) = 340,000 daltons (Da); glycoprotein	Adhesive protein that forms the fibrin clot
Prothrombin (Factor II)	MW = 72,000 Da; vitamin K-dependent serine protease	Activated form is main enzyme of coagulation
Tissue factor (factor III)	MW = 37,000 Da; also known as thromboplastin	Lipoprotein initiator of extrinsic pathway
Calcium ions (factor IV)	Necessity of Ca++ ions for coagulation reactions described in 19th century	Metal cation necessary for coagulation reactions
Factor V (Labile factor)	MW = 330,000 Da	Cofactor for activation of prothrombin to thrombin
Factor VII (Proconvertin)	MW = 50,000 Da; vitamin K-dependent serine protease	With tissue factor, initiates extrinsic pathway
Factor VIII (Antihemophilic factor)	MW = 330,000 Da	Cofactor for intrinsic activation of factor X
Factor IX (Christmas factor)	MW = 55,000 Da; vitamin K-dependent serine protease	Activated form is enzyme for intrinsic activation of factor X
Factor X (Stuart-Prower factor)	MW = 58,900 Da; vitamin K-dependent serine protease	Activated form is enzyme for final common pathway activation of prothrombin
Factor XI (Plasma thromboplastin antecedent)	MW = 160,000 Da; serine protease	Activated form is intrinsic activator of factor IX
Factor XII (Hageman factor)	MW = 80,000 Da; serine protease	Factor that nominally starts aPTT-based intrinsic pathway
Factor XIII (Fibrin stabilizing factor)	MW = 320,000 Da	Transamidase that cross-links fibrin clot
High-molecular-weight kininogen (Fitzgerald, Flaujeac, or William factor)	MW = 110,000 Da; circulates in a complex with factor XI	Cofactor
Prekallikrein (Fletcher factor)	MW = 85,000 Da; serine protease	Activated form that participates at beginning of aPTT-based intrinsic pathway

Table 1. Coagulation factors. Coagulation factors and their molecular weight and function. [15]

In 2001 Henry and Monroe published a cell-base model of haemostasis. They proposed that coagulation does not occur in a classic "cascade" but rather in 3 overlapping steps: 1) initiation, which occurs on cells that bear tissue factor, 2) amplification, in which platelets and coagulation factors are activated and 3) propagation, during which large amounts of thrombin are produced by platelets. [17] But fibrin clot formation can also take place in plasma without any cells or platelets. [19] [20] The true model is most likely a combination between the classic and cell-based model: clotting factors and the presence of cells for further phospholipid and factor delivery are both involved in coagulation.

A much more modern access to secondary haemostasis is to consider thrombin as the complete centre of coagulation, being inhibited or its formation catalysed by a variety of feedback loops leading into a huge thrombin burst and consequently fibrin formation. For example, it is well known that thrombin activates factor V, VIII and XI. [21][22] Thrombin itself in general induces also its own feedback inhibition by forming a complex with thrombomodulin, which then can activates Protein C. Activated Protein C inactivates a various number of clotting factors, leading to the dissolving of the clotting plug, a process called fibrinolysis, necessary for healing (Fig 6). [15] Also plasminogen produced in the liver is secreted into blood.[23] Plasminogen can be converted into its active form plasmin by specific activators. Two physiological plasminogen activators are well known: t-PA (tissue-type plasminogen activator) and u-PA (urokinase type plasminogen activator in the urine). [24] Plasmin as a serine protease can degrade fibrin and dissolve the clot.[25]

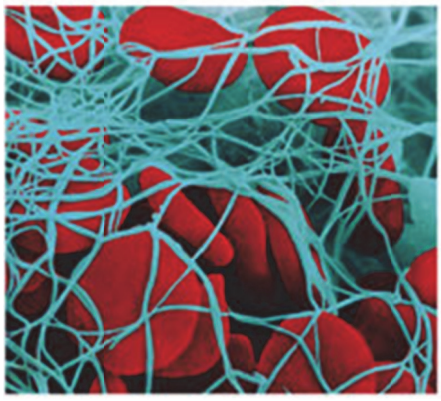


Fig. 5. Fibrin clot with erythrocytes. Fibrin is coloured in blue and red blood cells in red. In a fibrin clot are not only the smaller platelets but also erythrocytes enclosed. [7]

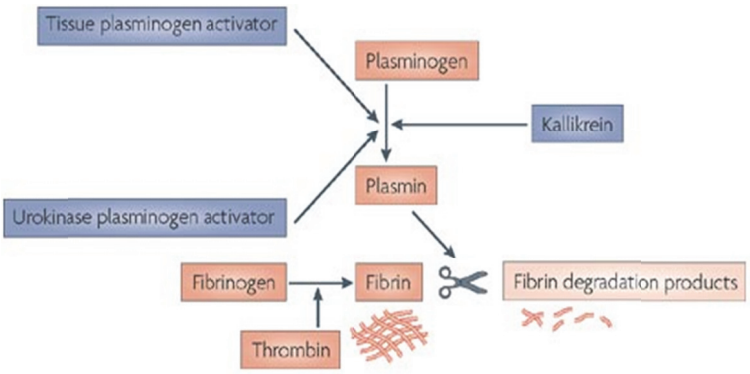


Fig. 6. Plasminogen in fibrinolysis. Plasminogen in transferred into plasmin upon activation of tissue plasminogen activator (t-PA), urokinase plasminogen activator (u-PA) or kallikrein. Plasmin cleaves fibrin and thereby initiates fibrinolysis important for wound healing. [26]

1.2.3 Thromobelastometry as a Measurement Method in Blood Coagulation

One way to measure blood coagulation is thromboelastometry. Thromboelastometry is a viscoelastometric method for testing the haemostatic capacity of whole blood by measuring the shear elastic modulus of clot forming between a cuvette and a cylindrical and moving pin, in more detail explained in the "Material and Method" section. [27]

1.2.4 Endothelium and haemostasis

Endothelial cells form the inner layer of blood vessels, which act as an important barrier between tissue and blood. Additionally endothelial cells are involved in neoangiogenesis, control leukocyte rolling, are involved in inflammation and are very important for haemostasis. [5] Blood vessel walls form a selective barrier for the transport of molecules between blood and tissue. Endothelial cells form a continuous monolayer and make up around 350 m^2 of the total vascular interface in a human being. [28] The process of forming new vessels is called neoangiogenesis and is very important during embryogenesis and tumour development, since tumours need access to the blood system to get enough oxygen for survival. Vascular endothelial growth factor (VEGF) is a very important signal protein in that context to guarantee cell proliferation. Endothelial cells respond to inflammatory stimuli such as lipopolysaccharides (LPS) or cytokines by getting activated, producing cytokines by themselves, and upregulating selectins or tissue factor expression. [5]

With the help of selectins endothelial cells can also induce leukocyte rolling upon activation and therefore supporting the immune system. They also express tissue factor, which induces blood coagulation. [5] Under physiological conditions the vascular

endothelium facilitates vascular dilatation by e.g. releasing nitric oxide (NO), prostaglandin (PG) or adenosine formation. The endothelium also prevents platelet adhesion and activation by e.g. NO and PG production. Endothelial cells also modulate coagulation by upregulation of thrombomodulin and start to fibrinolysis via t-PA production. T-PA catalyses the reaction from plasminogen to plasmin, which cleaves fibrin. [29] When the endothelium is injured, or functionally perturbed by atherosclerosis or inflammation coagulation is rather enhanced. The vessels are now constricted due to reduced production of diluting factors, but increase the expression factors, which can be procoagulatory, like tissue factor. This way blood coagulation is facilitated. [16][30]

1.2.5 Tissue Factor

Tissue factor is a 47 kDa sized transmembrane glycoprotein. Its primary function is to activate the blood clotting cascade via factor VII activation, that complex can then in return activate factor X, initiating the common pathway of secondary haemostasis and forming its final product fibrin.[31][32] The complex of TF and VIIa also activates cells via cleavage of the G-protein coupled protease-activated receptor (PAR2) 2 described in Fig. 7. [31] It also associated with other members of the PAR family, like PAR 1. [32] PARs are G-protein coupled transmembrane receptors that trigger cell response upon binding of a ligand. There are seven PARs known so far. [33] Via PAR2 activation cell signalling can lead to different processes such as atherosclerosis or tumour angiogenesis. TF-dependent activation of blood clotting is rather thought to contribute to metastasis, since the thrombin generation leads to enhanced cell migration of melanoma cells.[32]

Apart from a transmembrane TF, called full-length TF (flTF), there is also a soluble isoform of TF (asTF). The alternative spiced tissue factor lack a transmembrane domain and has

an altered C-terminus. [34] Whereas flTF is translated from 6 exons, for asTF there are only 5 exons. [35] Tissue factor is constitutively expressed in subendothelial cells such as vascular smooth muscles, where it can be found in its inactivated encrypted form. Upon activation it changes into its decrypted form. This guarantees a rapid initiation of coagulation upon vessel damage. In contrast, endothelial cells and monocytes normally express tissue factors upon stimulation only. [36] For example, tumour necrosis factor alpha (TNF-α) is well known for initiating TF-expression on endothelial cells. [37] The activation of tissue factor is not well understood. Most likely oxidation of cysteine-residues is required triggered by differently suggested molecules such a complement proteins.[34]

So far, studies could clearly prove the involvement of flTF in coagulation, but for asTF it is still quite unclear. Some publications claim to have proven a coagulatory activity of asTF, others proposed that asTF did not have any effect on coagulation. When it is about atherosclerosis though, both flTF and asTF were found in atherosclerotic plaques, both isoforms are probably contributing to plaque instability. Apart from atherosclerosis both TF-isoforms contribute to cancer development. TF-concentration is upregulated up to 1000-fold in cancer patients. Tissue factor can trigger angiogenesis providing the tumour with oxygen, but not only primary tumour growth is promoted by tissue factor, also metastasis. Firstly there exists some sort of interplay between flTF and PAR1 and secondly thrombin generation play a role in metastasis. Thrombin raises the potential of cell migration and facilitates fibrin formation, which can bind around

metastatic cells to protect them from natural kill cells (NK-cells).[32] In the bloodstream flTF is normally found microparticle-bound, whereas asTF is soluble anyways.[38] Microparticles are formed via shedding from cells and are vesicles with defined size between 100-1000nm. [39] Microparticles belong to extracellular vesicles, which will be discussed in further detail in the upcoming chapters.

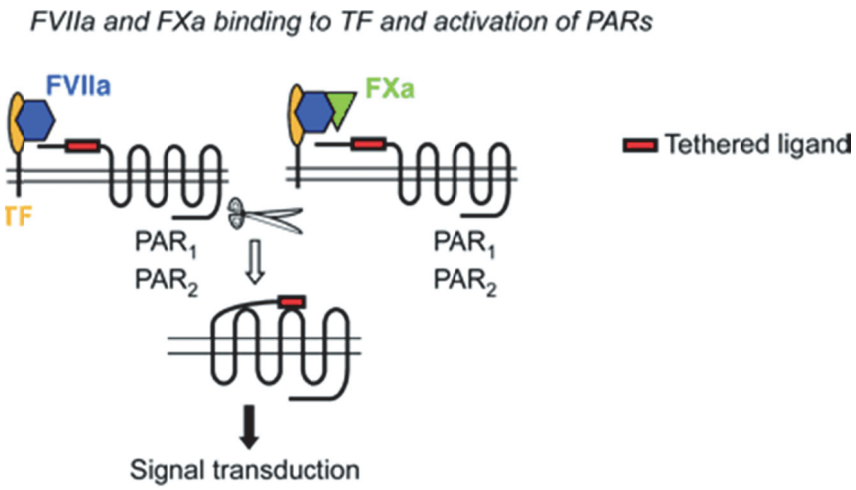


Fig. 7. Tissue factor activates PARs. Tissue factor together with factor VIIa and/-or Xa can lead to signal transduction via PAR-activation. [40]

1.2.6 The Connection between Tissue Factor and Inflammation

As already mentioned in detail TF plays an important role in blood coagulation. Its expression is upregulated by TNF-α. [41] Tumour necrosis factor alpha is an important cytokine for the immune system mostly released by macrophages. It is involved in a huge variety of processes as inflammation, apoptosis, cell proliferation, cell differentiation or triggering fever. [42] Macrophages have Toll-like receptors that induce cytokine expression such as TNF-α [43]. Toll-like receptor can recognize bacterial components and react with specific gene expression. [43] This is part of the process of

inflammation. After sensing an inflammatory stimuli, like a bacterial infection, resident macrophages in tissue produce chemokines (such as interleukin-8) attracting leukocytes to migrate into tissue, where the infection is located (Fig. 8). Macrophages release cytokines (such as TNF-α).

TNF-α stimulates the upregulation of the expression of proteins necessary for leukocyte adhesion to the endothelial cell. Therefore leukocytes are able to enter tissue.[44] [45]

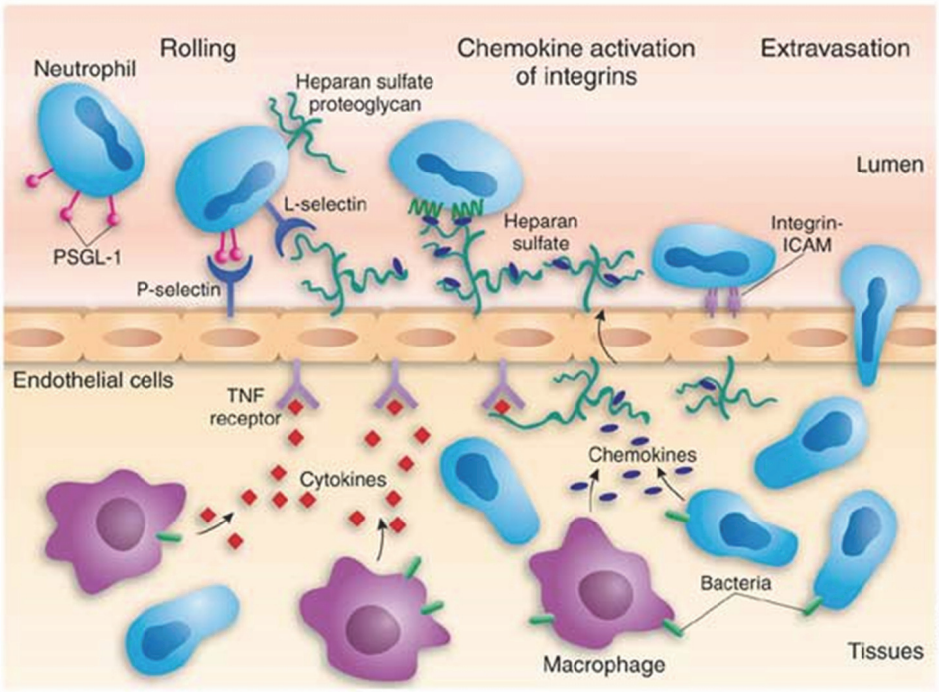


Fig. 8. Leukocyte Rolling and Transmigration. When bacteria (green) enter tissue chemokines and cytokines are released by macrophages as a response within the process of inflammation. Endothelial cell receptors react in response to cytokine presence and upregulate integrin expression. With the help of integrins leucocytes (e.g. neutrophils) can enter the infected tissue and phagocyte pathogens. [44]

Considering that after an injury that underlying tissue is prone to a bacterial infection, it would be reasonable to have a close relationship between coagulation and inflammation. Indeed it could be shown that coagulation activates inflammation and

vice versa. Inflammation activates coagulation by mainly three mechanisms: cytokine induce of TF-expression, inflammation-induced down regulation of protein C (anticoagulatory acting protein) and inhibition of fibrinolysis. On the other hand coagulation activates inflammation, since the TF/VIIa complex, Xa, and thrombin influence inflammatory cell response by changing (cytokine-)expression patterns of affected cells. [41]

Interestingly, there is also a connection between coagulation and the complement system of immune system. The complement system consists of proteins attaching to bacteria in order to destroy them, forming a first defence line against pathogens. [46] For example, it is known that thrombin can activate the complement proteins C3 and C5. [47]

1.3 Extracellular Vesicles

Extracellular vesicles are released by prokaryotic and eukaryotic cells (Fig. 9). For eukaryotic cells it is possible to differentiate between three types of extracellular vesicles, shown in Fig. 10: exosomes, microparticles (also named microvesicles) and the third kind are apoptotic bodies. Exosomes are the smallest among them, having a size range between 30 and 100 nm. The size of microparticles varies between 100 nm and 1000 nm, whereas the apoptotic bodies, are considered to be around 1 – 5 µm. [48]

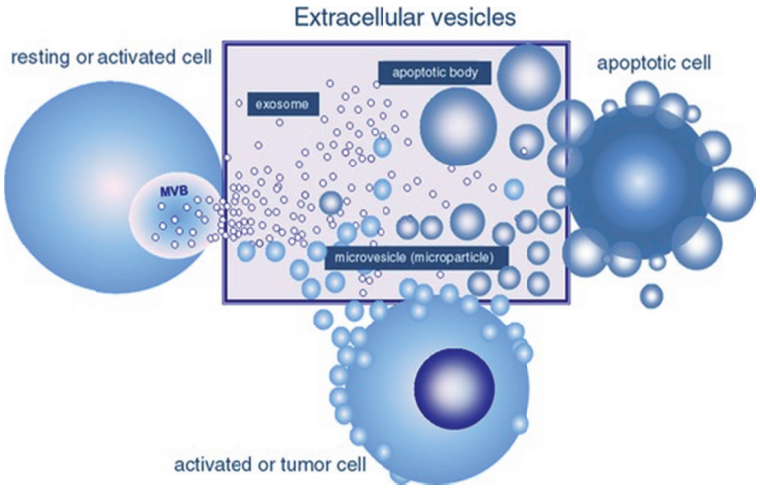


Fig. 9. Types of extracellular vesicles. There are three kind of extracellular vesicles: exosomes released physiologically via multivesicular bodies, microparticles released via cell budding upon activation and apoptotic bodies formed during the process of apoptosis.

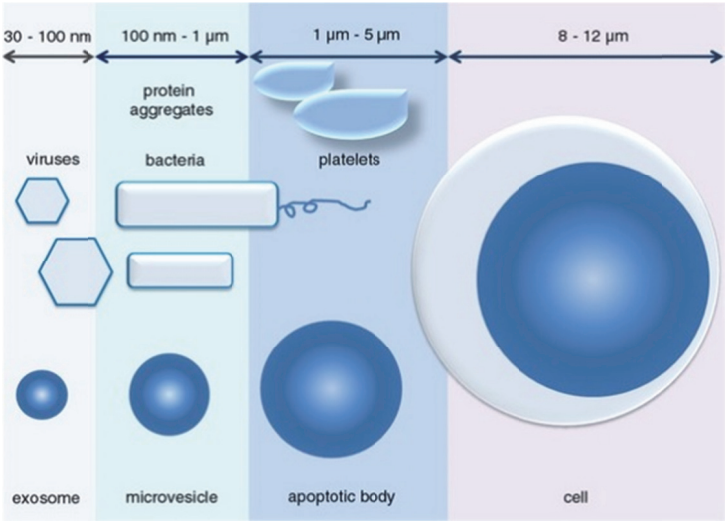


Fig. 10. Their size range varies for exosomes between 30 - 100 nm, for microparticles between 100 - 1000 nm and for apoptotic bodies between 1 - 5 µm. [48]

Exosomes are secreted by cells under normal and pathological conditions. [49] They are released via exocytosis from multivesicular bodies. Microparticles are instead formed via budding from the cell membrane. [48] They are only released upon stimulation such as shear stress, complement attack or proapoptotic triggers. [50] Monocytes, endothelial cells or hepatocytes are known for the release of microparticles in the presence of lipopolysaccharides (LPS) or cytokines such as TNF-α or interleukin-1 (IL-1).[51]

For that reason, it is not surprising that microparticles are not only involved in physiological processes, but also pathophysiological processes such as thrombosis,

inflammation or cancer. [50] When microparticles are released via budding they have cell markers (surface proteins) of the original cell they stem from. Interestingly both, exosomes and microparticles, have vesicle content of: RNA, DNA, miRNA or proteins to transfer information, but also proteins found in the vesicle membrane can trigger certain biological events. [52]

In contrast to exosomes, phosphatidyl serine (PS) is found in the membrane of microparticles to a certain extent. In blood coagulation the negative charge of PS provides an important surface for the assembly of enzyme-cofactor complexes of the coagulation cascade. [53] Apoptotic bodies are well known for having PS in their cell membrane. [54] So far there are no standard methods established to investigate on microparticles, due to the fact that the small size of them makes detection difficult in general. Flow cytometry has been established, but dynamic light scattering or atomic force microscopy can be also useful methods for the detection of microparticles. [39]

1.4 Aim of the study

The production of pro- and anticoagulatory factors of endothelial cell play a very important role in maintaining the balance of haemostasis. [55] Recent studies in the laboratory of the Ludwig Boltzmann Institute of Experimental Traumatology could show that procoagulatory mediators were released in the cell culture supernatant from Human Umbilical Endothelial Cells (HUVEC) and Outgrowth Endothelial Cells (OEC). The procoagulatory pattern was shown in enhanced clotting time in thromboelastometry, a method that allow to follow the dynamics of blood clotting formation. Tissue-Factor antibodies could partly revert enhanced clotting time, making the involvement of tissue factor highly likely. [56]

With the current follow up research project we aimed to investigate this hypothesis further in detail, whether tissue factor was released by endothelial cells, soluble or particle/bound.

To answer that question the techniques of differential centrifugation, rotational thromboelastometry, enzyme antibody inhibition and flow cytometry were carried out. With differential centrifugation microparticles should be isolated from endothelial cell culture cell supernatants. To investigate their effect on blood clotting thromboelastometry should be used. Furthermore, flow cytometry should serve as an additional method to analyse isolated microparticles.

2 Material and Methods

2.1 Antibodies

For tissue factor detection the following antibodies were used: anti-tissue factor antibody -PE labelled (e-Bioscience, Vienna Austria), anti-tissue factor antibody -PE labelled (BD, Franklin Lakes, USA) and mouse anti-human tissue factor antibody (American Diagnostica, Lexington, USA) in combination with a secondary antibody, anti-mouse antibody labelled with Alexa 488 (Invitrogen, Carlsbad, USA) or Alexa 594 (Invitrogen, Carlsbad, USA).

2.2 Reagents

The phosphate buffer saline (PBS) purchased from PAA, Pasching, Austria, had the following composition: KCl 0.2g/l, KH_2PO_4 0.2 g/l, NaCl 8.0 g/l and Na_2HPO_4 anhydrous 1.15 g/l with a pH between 7.0 and 7.5. Trypsin/EDTA 10 x (PAA, Pasching, Austria) consisted of: trypsin 5 mg/ml, EDTA 2.2 mg/ml diluted in PBS. Distilled water was used from Fresenius Kabi, Graz, Austria and following reagents were all purchased from Sigma Aldrich, St. Louis, USA: Trypan blue (0.2 %), dimethyl sulfoxide (DMSO), accutase and bovine serum albumin (BSA).

2.3 Consumables and Common Instruments

An incubator Binder (VWR, Vienna, Austria) and the Laminar HERASAFE (Thermo Scientific, Darmstadt, Germany) were used to treat cells under optimal conditions.

6-well well plates (TPP, Trasadingen, Switzerland) as well as the flasks T25 (TPP, Trasadingen, Switzerland) and T75 (TPP, Trasadingen, Switzerland) were used to grow cells. Pipettes of 5 ml, 10ml and 25 ml (Dr. Bertoni, Vienna Austria) were used in combination with pipette controllers (Hirschmann, Eberstadt, Germany) for various experiments. 15 ml and 50 ml tubes (Greiner, Kremsmünster, Austria) as well as 20 µl, 200 µl and 1000 µl pipettes (Gilson, Middleton, USA) and 1.5 ml tubes (Eppendorf, Hamburg, Germany) for normal centrifugation and Safelock tubes (Eppendorf, Hamburg, Germany) for ultracentrifugation were used for the different experiments.

2.4 Endothelial Cell Culture

Two kind of primary endothelial cells were used in order to investigate the microparticles in endothelial cell culture supernatants: Human Umbilical Vein Endothelial Cells (HUVEC) and Outgrowth Endothelial Cells (OEC).

2.4.1 Medium Change

To ensure cell growth under optimised circumstances, cell medium was changed every 2-3 days and cells were always hold in an incubator at 37° C with 5 % CO_2.

As growth medium 5 % Fetal Bovine Serum (FBS) in Endothelial Basall Medium (EGM-2) was used from purchased from Lonza, Visp, Switzerland. The content of the EGM-2 BulletKit was: hEGF, Hydrocortisone, VEGF, hFGF-B, R3-IGF-1, Ascorbic Acid, Heparin, GA-1000 (antibiotics Gentamicin and Amphotericin-B) and FBS as already mentioned.

2.4.2 Passaging Cells

In order to transfer cells from a flask or well-plate to another one cell passaging was applied: First cell medium was removed and then the cells were covered in 1x PBS, which was later removed in order to get rid of all the medium (= washing the cells).

Depending on the percentage of cells covering the flask/well and the size of the flask/well trypsin was added to the cells. Trypsin is a mixture of several isoforms of trypsin, which can be found in the digestive system to cut proteins. It breaks down extracellular proteins, which guarantee cell-matrix contact (Hemidesmosomen) and cell-cell contact (Desmosomen). [57] Ethylene-diamine-tetraacetic acid (EDTA) is often added to cells together with trypsin, since it binds metal-ions and therefore breaking down protein-protein interactions shared via cadherins, which secure cell attachment to each other and the matrix. [58] Incubation of cells with trypsin/EDTA normally took around 3 minutes at 37° C. The cells had to be detached from each other and the matrix, observed under a Zeiss microscope model Axio Observer.A1. When cells had detached, medium was added to the cells in order to inactivate trypsin. The cells were carefully suspended and immediately transferred into a tube or a flask with medium if the exact cell number was not needed. If the exact cell number had to be determined, the following protocol was normally applied for cell counting: 10 µl of cell suspension were mixed with 10 µl trypan blue (0.2 %). Then 10 µl of this suspension were added to a Neubauer chamber and put under the microscope to count 4 squares and take the mean value of them, which was then multiplied with the dilution factors. It has to be kept in mind that one of those four squares gave space to 0.1 µl. Calculation for cells per ml: mean value cells x 2 x 10 x 1000

After (or before) cell number determination the cells were centrifuged at 95 xg for 5 min with a Hettrich Zentrifuge – Universal 320 to collect them at the bottom of the tube in a pellet. The supernatant was then removed and the cells suspended in the small amount of medium left. In case the cell number was known before that step, a specific amount of medium was added to obtain the wished cell concentration. If the cell number was unknown though, the cells were diluted in medium and then the cell number was determined. Either way a specific number of cells was then transferred to a flask or a 6-well plate. The cells were then incubated at 37° C again

2.4.3 Freezing Cells

First a storage solution is prepared: 100 µl DMSO (0.22 µM filtrated) and 400 µl FCS were pipetted into a cryotube and then 500 µl of cell suspension were added, which was normally gained from a flask T25 (25 cm^2) or T75 (75 cm^2). Then the cryotube was put into a freezing container filled with propanol and taken into a -80° C fridge, which guaranteed slow cell freezing and helped to avoid cell breaking and degradation. Around 24 hours later the cells were stored in liquid nitrogen at – 196° C.

2.4.4 Thawing Cells

Depending on how many cells were thawed the appropriate flask with medium was prepared: When cells of T25 flask were used, those cells were put into a T25 flask assuming not too many cells had died and the same was applicable for the T75 cell flasks. The frozen cells were put into a 37° C water bath and thawed until only a small ice-clot was left, then immediately the cells were transferred into medium. After 4 hours the medium was exchanged to get rid of the DMSO. Cells were put back to the incubator to grow at 37° C.

2.4.5 Stimulation of Cells

The cells were stimulated or not (control) with TNF-α. TNF-α was diluted 1: 1000 with medium to reach a final TNF-α concentration of 10 µg/ml. The medium with/without TNF-α was put on the cells for approximately 24 hours. The cell culture supernatants were then further processed by differential centrifugation to obtain microparticles.

2.5 Differential Centrifugation of Cell Culture Supernatants to obtain Microparticles

The tool of differential centrifugation is very useful, because it allows to separate different sizes of cellular components. A standard protocol for cell compartment isolation is described as follows: After cell homogenisation the cell lysate is centrifuged at 1300 xg for 5 min, resulting in a pellet enriched with nuclei and cellular debris. The supernatant is centrifuged again at 17 000 xg for 15 min to collect mitochondria. At the end of another centrifugation at 80 000 xg for 60 min of the supernatant plasma membrane can be found in the pellet and at the end of another centrifugation of the remaining supernatant at

100 000 xg the pellet is enriched with ribosomes. Proteins should still be in the remaining supernatant fluid. [59]

For microparticle isolation also differential centrifugation was applied. An overview of this protocol can be found in Fig. 11: Endothelial cell culture supernatants were centrifuged at 500 xg to get rid of cells and debris. The supernatant was then centrifuged at

14 000 xg to collect larger microparticles and at 100 000 xg to isolate smaller microparticles. The remaining cell free supernatant served as a control.

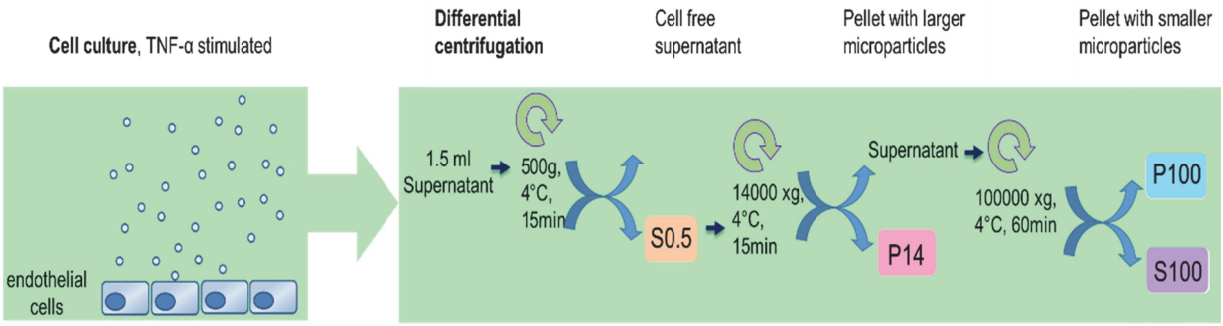


Fig. 11. Microparticle Isolation from endothelial cell culture supernatant. Endothelial cells were stimulated with TNF-α, then cell culture supernatant was subjected to differential centrifugation to concentrate microparticles. S0.5 = cell free supernatant containing microparticles; P14 = pellet from a 14 000 xg centrifugation diluted in PBS bearing "larger" microparticles, cell debris and apoptotic bodies; P100 = Pellet diluted in PBS from the 100 000 xg centrifugation, containing "smaller" microparticles and exosomes; S100 = remaining supernatant after the last step of differential centrifugation at 100 000 xg mostly particle-free.

Endothelial cells were grown in a 6-well plate until around 90 % of the surface was covered with cells (= 90 % confluency). Then the cells were stimulated with 10 ng/ml TNF-α in medium or for the controls, the medium was exchanged. Following an incubation of 24 hours cell culture supernatants of TNF-α stimulated and unstimulated endothelial cells were collected. The supernatants were centrifuged at 500 xg for 15 min at 4° C by an Eppendorf Centrifuge 5415 R. Any living or dead cells should have been collected in a pellet and therefore removed. That supernatant, S0.5, was then centrifuged at 14 000 xg for 15 min at 4° C again by an Eppendorf Centrifuge 5415 R to gain a pellet P14 diluted in 50 µl medium or PBS, which should have contained mainly larger microparticles but also cell debris and apoptotic bodies. The remaining supernatant was transferred into Safelock tubes 1.5 ml by Eppendorf. Then the supernatant was centrifuged at 100 000 xg for 1h at 4° C by a centrifuge Thermo Scientific Sorvall Discovery M150 SE in order to get the pellet P100 with smaller microparticles and exosomes, which was then diluted in 50 µl medium or PBS. The last remaining supernatant S100 should not have contained any particles and served as an important control.

2.6 Flow Cytometry

For cell and microparticle detection a flow cytometer FC 500 (Beckmann Coulter, Krefeld, Germany) was used. Flow Cytometry is a very sophisticated method to analyse very small particles in liquid suspension. It can be used to detect cells or particles, to have a closer look at cell or particle surfaces but also the inside of cells or particles, for example their internal complexity. To which extent information can extracted from a single particle or cell depends of the limit of each flow cytometer. A flow cytometer is made up of three main systems: fluidics, optics and electronics. The fluidic system transports particles in a fluid stream to the laser. The optics include the laser and components to detect light signals and the electronic subsystem converts light signals into electronic signals, which can be evaluated via a computer. [60] These 3 components work very well together to produce a complex information about a particle sample.

More detailed explanation about flow cytometry:

Firstly, the particles in suspension are injected into the fluidic system of the flow cytometer. To get information about one individual and not about many particles stuck together, the particles are put in line. The creation of that string of particles, which separates them individually, is accomplished via the sheath liquid of the fluidic system. After the hydrodynamic focussing each particle passes through one or several beams of light. In our case it was only one beam with a wavelengths at 488 nm.

When the light meets the particle a forward scatter and side scatter are created. In addition, if the particles are fluorescent, florescence is generated.

The forward scatter, as described in Fig. 12, is measured in a straight line behind the particle. The side scatter for the fluorescence are detected at a 90 degree angle to the excitation line. Different filters make sure that only light of a specific wavelength can

reach the detector, therefore making detection of different fluorescent wavelengths with different detectors possible. [61]

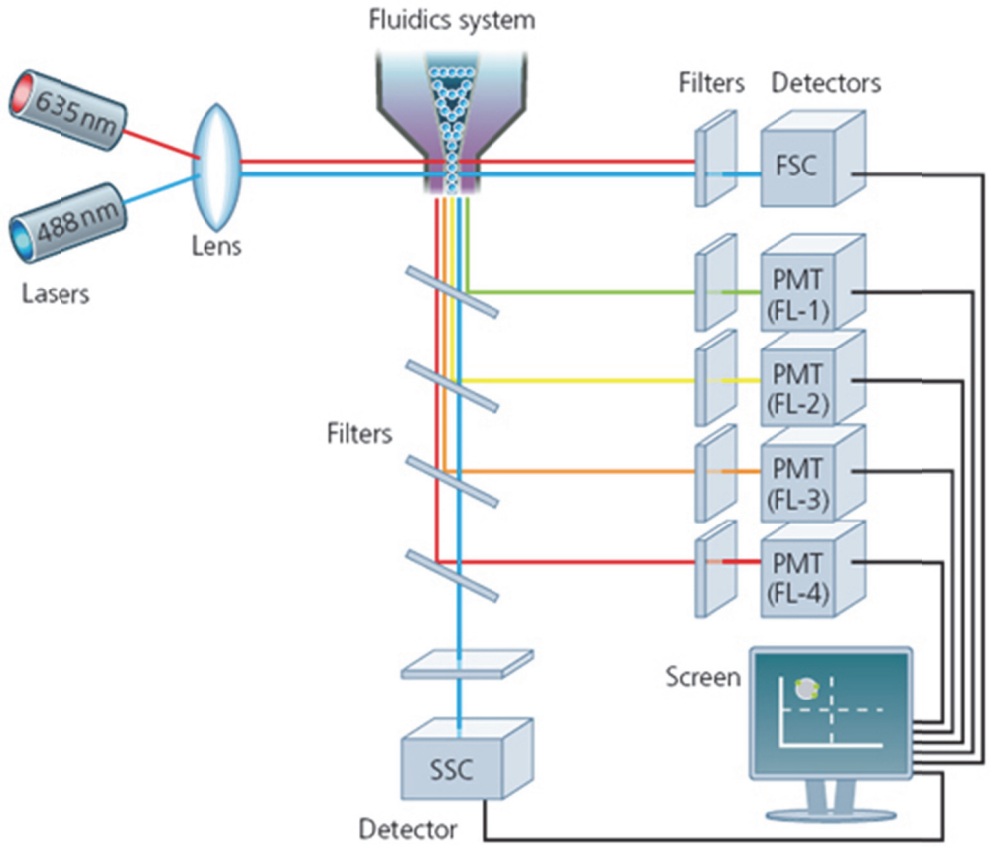


Fig. 12. Overview of Flow Cytometer. Particles are separated individually through the fluidic system and then encounter laser light, which leads to forward scattering (FSC) and side scattering (SSC) depending on particle size and granularity measured by two different detectors. If the particles had been labelled with fluorophores the laser can generate fluorescence measured by a set of detectors. Filters help to separate the different ranges of emission from each other. [61]

The forward scatter (FS) correlates with the particle size and refractive index. The larger the particle, the greater is the forward scatter signal. Refractive index is a physical property that indicates how light is transmitted and changes light velocity, while it passes through different material. The larger the difference of refractive index between the fluid and the particles is, the greater is the forward scatter.

The side scatter (SSC) correlates with the granularity of a particle. The more granular the cell the more SSC signal is created therefore providing information of the internal complexity of the particle. [62] Cells show in general autofluorescence. Detection of specific biological structures as lipid membranes, proteins or DNA can be detected via the creation of additional fluorescence using fluorochromes. [63] Cells have specific cell markers, which are surface proteins, that can identified via antibody-bound fluorochromes and therefore a distinction e.g. between T-cells from other blood cells is possible. [62]

2.6.1 Annexin V and Tissue Factor Detection of Microparticles in Pellet P14 via Flow Cytometry

Annexin V is a common marker for the detection of microparticles. [64] Annexin V is a protein that binds to the membrane component, phosphatidylserine, which is a phospholipid and normally found in the inner cytosolic side of the cell membranes. Only during apoptosis, but seemingly also during microparticle formation, flippase changes the position of phosphatidylserine and exposes it to the cells surface. Annexin V, which is normally bound to a fluorophore, can bind to phosphatidylserine on surface and PS-positive cells or particles can be detected. [65] A high number of particles was detected in the pellet P14 formed after 14 000 xg centrifugation of cell culture supernatant and was used for further flow cytometry experiments in order to detect tissue-factor bearing microparticles.

The pellet P14 f of cell culture supernatant of TNF-α stimulated endothelial cells was diluted in 50 µl Annexin V binding Buffer. The pellet was then diluted with 1150 µl Annexin V binding buffer and distributed among tubes, 300 µl per tube. One sample

was without any fluorophore, the other with 15 µl Annexin V-FITC, the third with 2 µl of anti-tissue factor antibody from American Diagnostica and the fourth with 15 µl Annexin V-FITC and with 2 µl of anti-tissue factor antibody from American Diagnostica. Also 1.5µ secondary anti-mouse antibody conjugated to Alexa 594 antibody was added in 1:200 dilution to all samples with anti-tissue-factor antibody it.

As a control the addition of Annexin V, anti-tissue factor antibody and secondary antibody labelled with Alexa 594 happened in the same way only instead of diluting a pellet in Annexin V Binding Buffer, Annexin V Binding Buffer only was used with 2 µl of anti-tissue factor antibody from American Diagnostica.

All samples were analysed via flow cytometry by a Gallios apparatus (Beckman Coulter, Krefeld, Germany). The Megamix beads (Biocytex, Marseille, France) was used to generate a measuring gate < 0.9 µm.

2.6.2 Tissue-Factor Detection on Endothelial Cells via Flow Cytometry

For the hypothesis that tissue-factor-bearing microparticles were coming from endothelial cells, we firstly aimed to detect tissue factor on endothelial cells.

The following procedure was applied: HUVEC cells were grown in 4 wells on a 6-well plate until 90 per cent confluency was reached, half of all wells were then stimulated with TNF-

α (10ng/ml) in medium for 24 h, whereas the rest was not stimulated and standard medium change was performed. When the time of stimulation passed the medium of all cells was removed and cells were covered with 1x PBS, which was immediately removed to get rid of all the medium. In addition 500 µl accutase (Sigma-Aldrich, St. Louis, USA)

was added to cells and they were put onto 37° C to detach from the surface and each other. Accutase is an enzyme mixture supposed to work in milder way when it comes to protein cleavage compared to trypsin. Only after it was made sure that the cells were detached, two wells of TNF-α stimulate cells were up taken via 10m PBS/BSA (1 %) into a 50 ml tube. The same was done with the unstimulated HUVEC cells.

The cell suspensions were centrifuged at 300 xg for 5 min at 4° C by the Thermo Scientific Haraeus Multifuge 1 S-R, the supernatant was removed and each pellet was diluted in 550 µl PBS/BSA (1 %). 100 µl of that cell suspension were transferred into flow cytometry tubes by the company Sigma Aldrich. All samples were kept on ice.

The following protocol was applied in order to make cellular components visible via the addition of an antibody-bound fluorophore measured by flow cytometry:

100 µl of the PBS/BSA (1 %) cell suspension were incubated with 2 µl anti-tissue-factor antibody except of the controls, which were incubated with the equal amount of isotype antibody control.

The anti-tissue-factor antibody was used from the following three companies:

1) e-Bioscience: anti-tissue-factor PE (phycoerytrin) labelled

2) BD: anti-tissue-factor PE (phycoerytrin) labelled

3) American Diagnostica: anti-tissue-factor unlabelled, secondary antibody conjugated to Alexa 488 (emission maximum at 488 nm)

As isotype controls for the anti-tissue factor antibodies were the following chosen:

For the antibodies from e-Bioscience and BD 2 µl of an isotype-PE were used and for the antibody from the company American Diagnostica 2 µl of Alexa 488 conjugated secondary antibody was used as an isotype control. The first step of antibody incubation

performed with the anti-tissue-factor antibodies as with the isotype-controls took place in the dark for 30 minutes. Then 1 ml of PBS/BSA (1 %) was added to each sample. The samples were then centrifuged at 300 xg for 5 min at 4° C. The supernatant was then discarded in order to get rid of all unbound antibodies. Only samples incubated with the antibody from the company American Diagnostica needed a second step of antibody incubation:

0.5 µl of secondary antibody Alexa 488 was added to the around 100 µl of fluid left after the PBS/BSA (1 %) discard. The incubation happened in the dark on ice for half an hour.

In addition to the incubation 1 ml of PBS/BSA (1 %) was added and centrifugation took place at 300 xg for 5 min at 4° C. The supernatant was then discarded to get yet again rid of the unbound antibody and 300 µl of PBS/BSA (1 %) were added to the cells.

Samples with an anti-tissue-factor antibody not from American Diagnostica did not need a secondary antibody, since those antibodies were already coupled directly to a fluorophore. When the supernatant had been discarded 300 µl of PBS/BSA (1 %) was directly added to the samples. All samples were then used for analysis via flow cytometry. Firstly the isotype controls were measured to calibrate the machine and then the appropriate samples. For the American Diagnostica antibody, Alexa 488 conjugated secondary antibody was used as an isotype control, for the other samples it was isotype-PE. The isotype control was necessary to exclude unspecific bindings and compensate unspecific fluorescent background. After every antibody addition and before direct use for flow cytometry measurement the samples were always very well mixed via a vortex mixer.

2.6.3 Tissue Factor Detection on Microparticles Isolated from Pellet 14 via Flow Cytometry

HUVEC were grown on 6-well plates to a point where around 90 per cent of the surface was covered with cells. Then the cells were incubated with TNF-α (10ng/ml) in medium for 24 h whereas the rest was not stimulated and standard medium change was performed, these samples were always referred to as without TNF-α or w/o TNF-α. The cell culture supernatants were then centrifuged as described in "Differential Centrifugation of Cell Culture Supernatants to Obtain Microparticles".

150 µl of PBS/BSA (1 %) were added to the microparticle pellet, formed from 14 000 xg centrifugation and diluted in 50 µl of PBS. That solution was transferred into flow cytometry tubes, 100 µl each. Those 100 µl were incubated with 2 µl of anti-tissue factor antibody of the company American Diagnostica. The incubation took place on ice for half an hour in the dark. After antibody incubation 1 ml PBS/BSA (1 %) was added to the solution, which was then centrifuged at 14 000 xg for 20 min at 4° C in order to collect the microparticles again in the pellet but leave the unbound antibody in the supernatant, which was discarded. To the remaining solution was then 1 µl Alexa 488 conjugated secondary antibody or Alexa 494 conjugated secondary antibody for each tube pipetted. The solution was again incubated for 30 min in the dark on ice and then 0.5 ml of PBS/BSA (1 %) were added. The particles were centrifuged at 14 000 xg for 20 min at 4° C another time and supernatant was discarded to remove unbound antibody.

250 µl of PBS/BSA (1 %) were added to the remaining solution, with which flow cytometry was performed.

For the isotype control during the first step of antibody incubation no antibody was used at all and during the second step of incubation 1 µl of secondary antibody labelled with Alexa 488 or Alexa 594 was used. The rest was equally done as described for the anti-tissue-factor antibody binding to the microparticles found in pellet P14.

The Flow Cytometry was performed with a FC 500. In order to set a 500 nm-gate, within the microparticles should be found, latex beads with a size of 500 nm were used. As a control distilled water (Fresenius Kabi, Graz, Austria) was used.

2.7 Rotational Thromboelastometry (ROTEM)

Thromboelastometry is a viscoelastometric method, which is very useful to analyse the dynamic behaviour of blood clot formation. For the analysis in that thesis the viscoelastic device ROTEM (TEM, Munich, Germany) was used.

The method is visualised in Fig. 13: A whole blood sample of 300 µl is placed into a cuvette preheated in a holder preheated at 37° C. A cylindrical cuvette is immersed into this blood sample. The pin is rotating from left to right with a fixed angle of 4.75° within with 9 seconds measuring the shear elastic modulus of clotting forming between cuvette and the moving pin. The process is detectable via a mirror, which constantly reflects light coming from a LED source towards a detector transferring photo energy into electrical. When the pin movement is restricted due to clot formation this can be detected via diminished light movement in the area of the detector. Those signals being generated are further processed in a computer for several viscoelastic coagulation parameters. These parameters of blood clotting can be observed via ROTEM, as described in Fig. 14. [27]

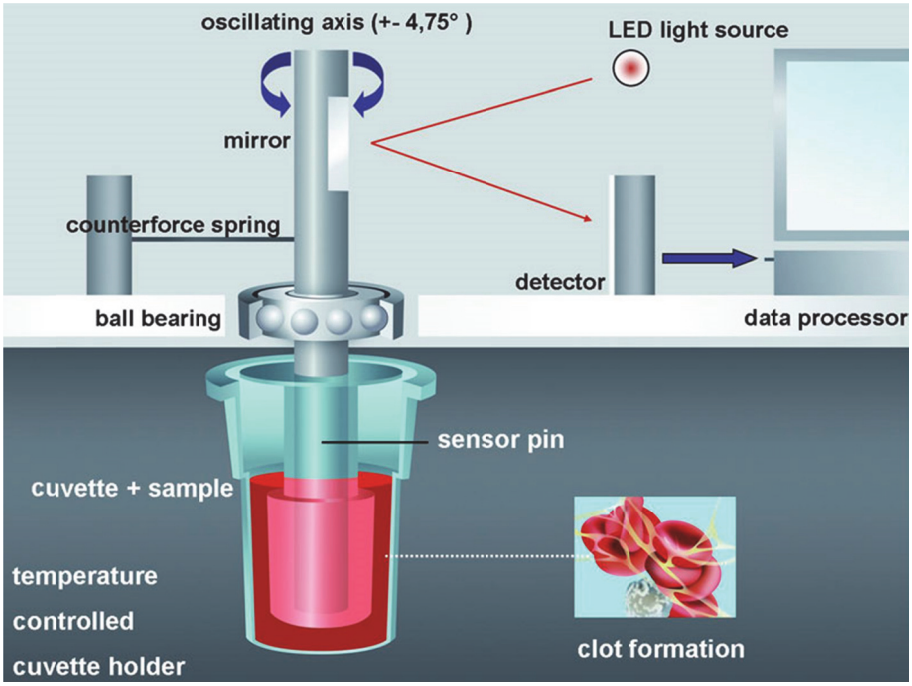


Fig. 13. Working principle of ROTEM. A whole blood sample is placed into a cuvette and sensor pin is plunged into it. The pin is rotating from left to right with a fixed angle of 4.75° within 9 seconds measuring the shear elastic modulus of a clot forming between a cuvette and the moving pin. With the start of blood coagulation the pin´s movement is getting restricted, which is measured by a detector. In fact a beam of light is reflected by a mirror attached to the pin, and when the pin´s movement is reduced the same happens to the mirror resulting in a smaller amplitude of light recorded by the detector and processed by a computer. [27]

There are different assays available for this device. However, the one used for all experiments in this thesis was the so-called (native) NATEM assay. In general, citrated blood is used. A volume of 2.7 ml freshly taken blood of different donors was diluted with 0.3 ml of 3.2 % trisodium citrated in order to prevent blood clotting. Citrate binds calcium essential blood coagulation.

To start coagulation in the NATEM assay 20 µl $CaCl_2$ (200 mM/l) is added to 300 µl of whole blood in order to make coagulation possible again. [27]

For the purpose of the current study only 50 µl of cell culture supernatant sample were added to recalcified blood in cuvette immediately before immersing the into the whole blood sample. The supernatant samples were obtained from differential centrifugation

of cell culture supernatants of TNF-α stimulated/unstimulated HUVEC: cell free supernatant due the 500 xg centrifugation (S0.5), pellet with larger microparticles thanks to a 14000 xg centrifugation (P14), the pellet with smaller microparticles obtained from a 100 000 xg centrifugation (P100) and the mainly particle free supernatant (S100).

The clotting time after the addition of above mentioned supernatant samples was recorded. In the experiments described in this thesis only Clotting Time (CT) only was used, which is the time from initiation of the assay until the blood clot forms detected by and amplitude of 2 mm in the ROTEM tracing. Only the CT parameter was evaluated, since it is supposed to be the most sensitive to activation with tissue factor. [56]

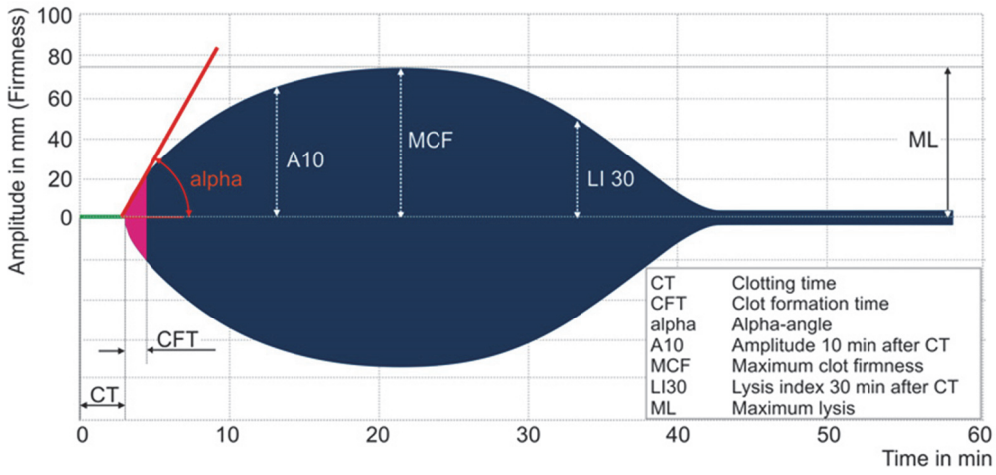


Fig. 14. Result of blood clotting assay measured via ROTEM. What is presented on the screen after data processing via the computer is an ellipse-shaped presenter of blood clotting. In contrast to standard clotting assays ROTEM give a whole subset of data as clotting time, clot formation, clot stability and lysis, which inform about the whole kinetics of haemostasis. [27]

2.7.1 Anti-Tissue Factor Antibody Inhibition of centrifuged samples

Every sample was incubated with 2 µl of anti-tissue factor antibody (conc. 0.5 mg/ml) of American Diagnostica. The samples were: 200 µl supernatant from the 500 xg centrifugation (S0.5), 50 µl of the pellet with larger microparticles thanks to the 14000 xg centrifugation (P14), 50 µl of the pellet with smaller microparticles obtained from 100 000 xg centrifugation (P100) and the 1.3 ml of the mainly particle free supernatant S100. The incubation took place at 37° C for half an hour. The antibody was supposed to have an inhibitory effect on tissue factor. For control the same samples were used with no antibody but with the same incubation time and temperature. When the incubation had been finished, 50 µl each sample were used as described above to perform a NATEM assay on the ROTEM.

3 Results

Fig. 15.describs the differential centrifugation protocol for obtaining microparticles from cell culture supernatant, which will be a useful reference for the following results.

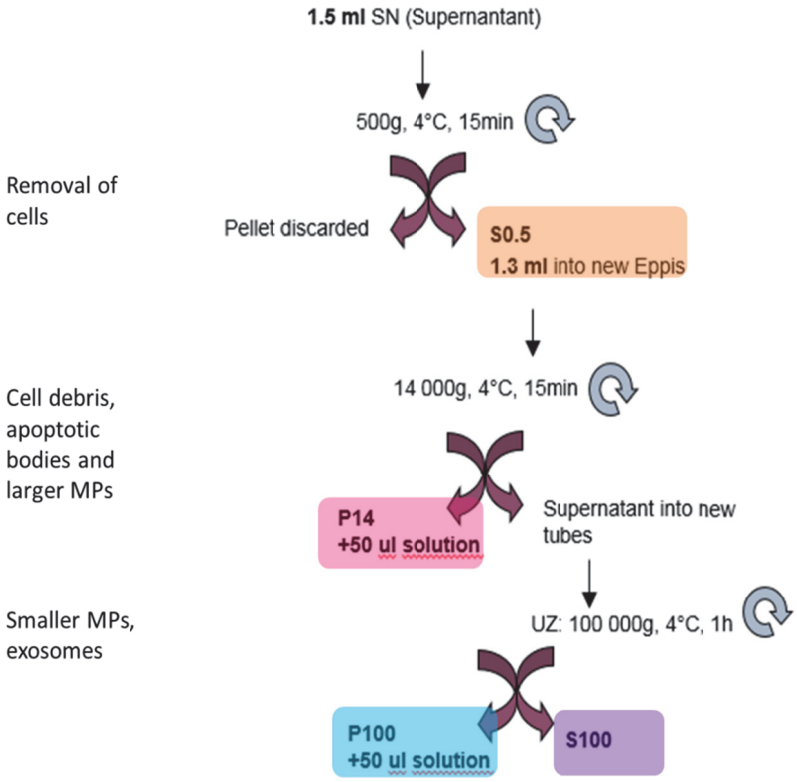


Fig. 15. Differential centrifugation of endothelial cell culture supernatant. The supernatant was centrifuged at 500 xg for cell removal, the then remaining supernatant S0.5 was centrifuged at 14 000 xg to collect the pellet P14, which mainly contained larger microparticles but also apoptotic bodies and cell debris. After another centrifugation at 100 000 xg smaller microparticles and exosomes were concentrated in the pellet P100. The remaining supernatant S100 was supposed to be mostly particle-free. Both pellets, P14 and P100 were either diluted in PBS or medium.

3.1 Rotational Thromboelastometry

3.1.1 Cell Culture Supernatants from TNF-α -stimulated Endothelial Cells Reduce Clotting Time

To determine be capable if a correlation existed between the duration of TNF-α stimulation and the mediators released in the supernatant, endothelial cells were stimulated for varying durations with TNF-α. Whole blood (wb) of various donors was used and coagulation behaviour analysed via ROTEM. The values of blood clotting time in the native NATEM assay are known to have quite a broad range, usually from around 600 to 1000 seconds or even longer.

After adding samples of the supernatant S0.5 into the NATEM assay, a clotting time reduction could be observed. The longer the TNF-α stimulation was applied, the stronger the effect on the clotting time (Figure 16).

Consequently a clear correlation between reduction of CT and time of TNF-α stimulation could be shown, indicating that the number of mediators facilitating blood coagulation increases with the time of stimulation.

For further experiments a TNF-α stimulation of 24 hours was used to get a relatively high number of CT-facilitating mediators, supposedly microparticles.

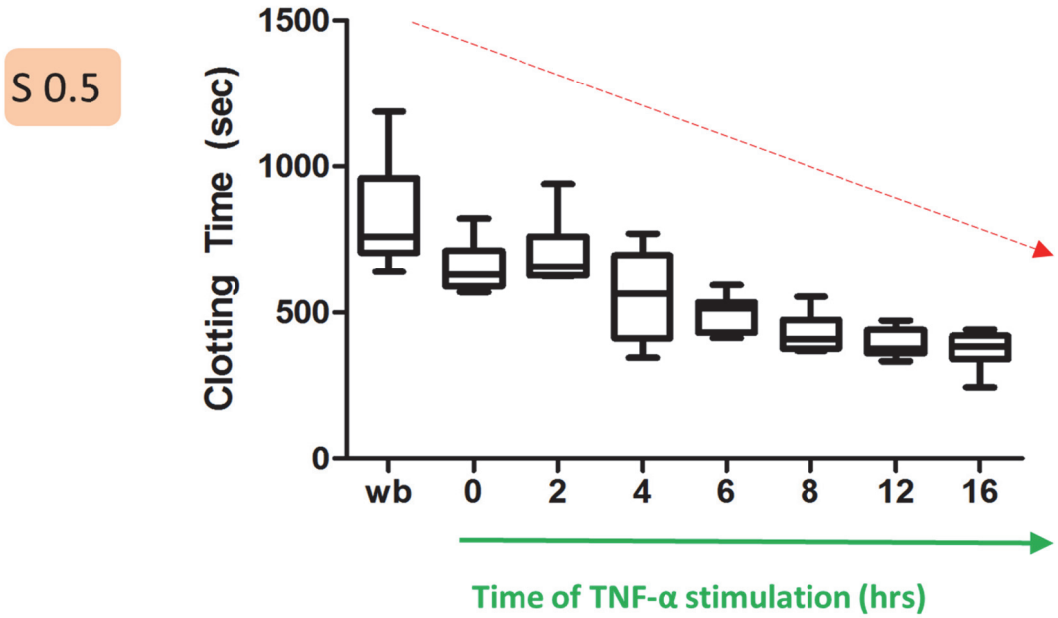


Fig. 16. Correlation between hours of TNF-α (10 ng/ml) stimulation and ROTEM Clotting Time. Endothelial cells were stimulated with TNF-α for various hours and then the cell culture supernatant was centrifuged at 500 xg to make it cell-free, called supernatant S 0.5. The clotting time showed a clear correlation between CT reduction and duration of TNF-α stimulation. As a reference whole blood (wb) and unstimulated medium (0) were used. Box-and-whiskers plots (min, max, 25th and 75th percentile, median) of absolute CT measurements in seconds (sec).

3.1.2 Reduction of Clotting Time (CT) by different (ultra-)centrifuged fractions

Endothelial cells had been stimulated for 24 hours with TNF-α and their supernatants were used for differential centrifugation in order to obtain microparticles. The impact of different centrifugation fractions on clotting time was analysed via ROTEM (Fig. 17).

The results showed a significant CT reduction with addition of S0.5 (the cell-free supernatant) compared to the control (PBS). The in PBS diluted pellet P14, which mainly contained larger microparticles, was facilitated blood coagulation as well as the P100 pellet diluted in PBS. P100 was supposed to mainly contain smaller

microparticles. On the other hand the particle free cell culture supernatant S100 did not have a significant impact on clotting time. This suggests that endothelial-cell derived mediators, mainly particles concentrated with centrifugation were involved in the clotting time reduction and facilitation of blood coagulation.

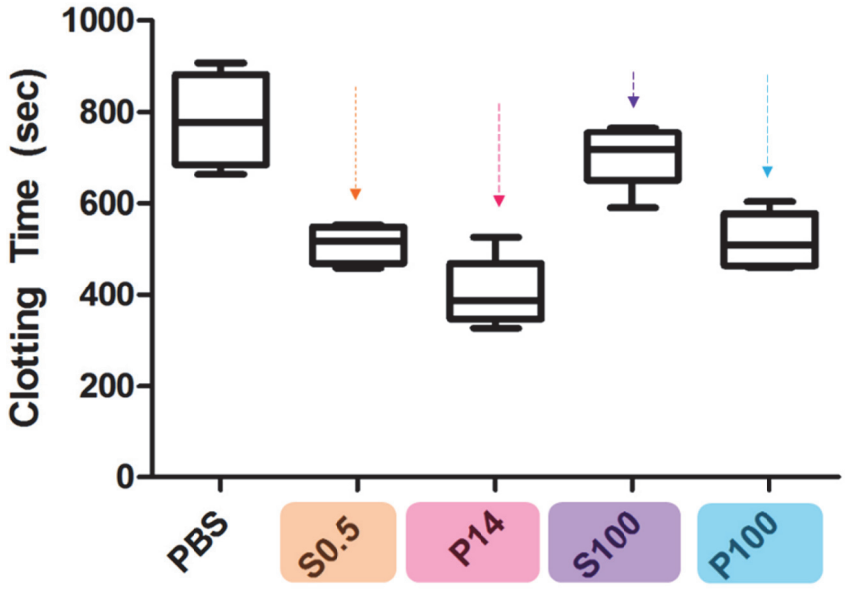


Fig. 17. Impact of different fractions of differential cell culture supernatant centrifugation on ROTEM clotting time. Endothelial cells had been stimulated for 24 hours with TNF-α. PBS was used as control. Apart from the CT reduction by the original cell culture supernatant S0.5, also the microparticle-pellets P14 and P100 showed a significant CT reduction. The particle-free S100 did not reduce CT. Box-and-whiskers plots (min, max, 25th and 75th percentile, median) of absolute CT measurements in seconds (sec).

3.1.3 No significant CT-reduction in differently (ultra-) centrifuged EGM-2 medium fractions

To determine if the medium itself was contained particles which were contributing to the clotting time reduction, the medium EGM-2 was fractionised by differential centrifugation. The fractions were analysed via ROTEM. The results in Fig. 18 showed that EGM-2 produced median clotting time values of around 500 – 600 sec. The pellet P14 from the 14 000 xg centrifugation was diluted in 50 µl EGM-2 and did not show any significant CT reduction compared to the non-centrifuged EGM-2. Similar results were obtained for the pellet P100, which was created with a 100 000 xg centrifugation and was diluted in 50 µl EGM-2. The supernatant S100 formed after the final centrifugation step at 100 000 xg also showed no significant clotting time reduction compared to the non-centrifuged EGM-2. Therefore a significant clotting time reduction coming from medium itself was excluded.

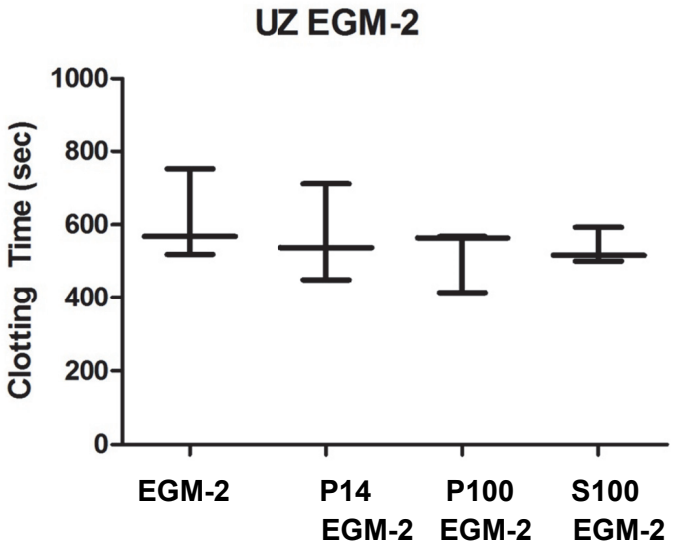


Fig. 18. Influence of differently (ultra-)centrifuged EGM-2 medium fractions on ROTEM clotting time. Non-centrifuged EGM-2 showed a median clotting time of around 500 – 600 sec. Similar results were obtained from the pellets P14 from medium only collected from a 14 000 xg centrifugation step or P100 produced after 100 000 xg centrifugation. Also the remaining supernatant S100 did not influence clotting time as compared to the non-centrifuged EGM-2.

3.1.4 The impact of anti-tissue factor antibodies on different (ultra-)centrifuged cell culture supernatant fractions

Endothelial cells were stimulated with TNF-α (10 ng/ml) for 24 hours and then the differential centrifugation was applied. The different (ultra-)centrifugation samples were incubated with anti-tissue-factor antibody and then used for ROTEM measurement and clotting time analysis. Anti-tissue factor antibody served as the inhibitory component blocking physiological tissue factor. As a control, the pure medium and PBS were used, both also incubated with anti-tissue factor antibody and CT measured via ROTEM (Fig. 19). PBS and EGM-2 with and without anti-tissue factor incubation showed a similar median clotting time of around 600 – 700 seconds. There were no significant differences between samples with or without anti-tissue factor incubation and therefore an involvement of tissue factor in either of the controls PBS and EGM-2 could be excluded.

The supernatant formed due to the 500 xg centrifugation (S0.5) clearly showed a reduction of clotting time measured via ROTEM which was partly reversed after the addition of anti-tissue factor antibody. This demonstrates the involvement of tissue-factor in the process of blood clotting. The pellet P14, collected from a 14 000 xg centrifugation, was supposed to contain larger microparticles, but also cell debris and apoptotic bodies. It showed a clear CT reduction to around 200 sec, which could be partly reversed up to a CT-value of around 400 seconds after the addition of tissue factor antibody. Similar results could be produced by the same experiment for the pellet P100 collected from a 100 000 xg centrifugation, consisted mainly of smaller microparticles. P100 displayed a clear CT reduction to around 250 seconds. This effect

could also be reversed to CT-value around 500 seconds following the tissue-factor inhibition upon antibody binding. In combination with results of P14, this presents strong evidence that tissue factor was particle-bound in those pellets and was a main contributor to clotting time reduction. S100, the remaining supernatant of all centrifugation steps, should have been mainly particle-free. There was no difference in clotting time reduction of the controls PBS and EGM-2. However, after anti-tissue-

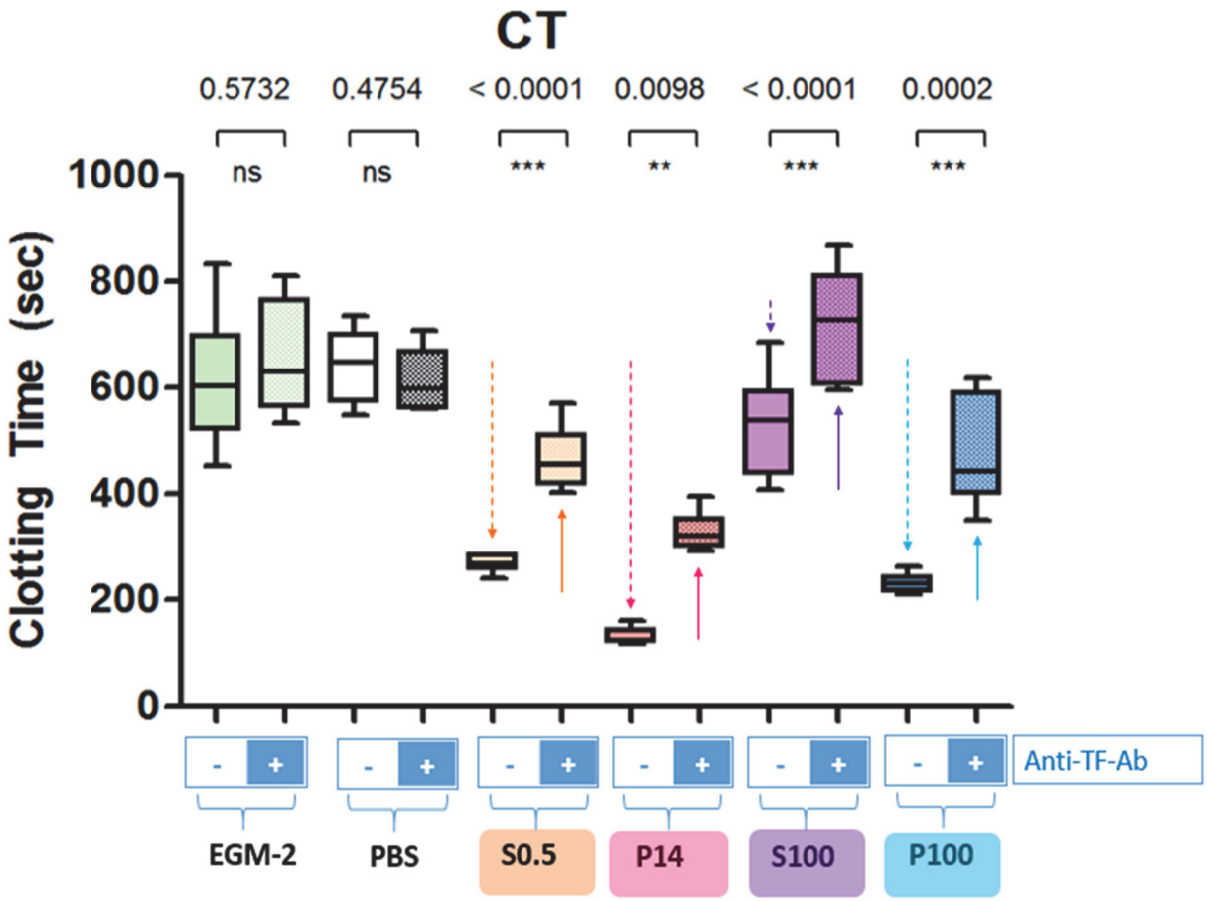


Fig. 19. Impact of anti-tissue factor antibody incubated and differently centrifuged cell culture supernatant fractions on ROTEM clotting time. As control served EGM-2 and PBS, which were both incubated with and without anti-tissue antibody. There were no significant differences between the controls before and after anti-tissue factor incubation. The cell-free supernatant S0.5 showed a clear clotting time reduction, which could be partly reversed after antibody addition. The pellet P14 mainly containing larger microparticles displayed a clear clotting time reduction, which was lifted after anti-tissue factor antibody addition. The pellet P100 with smaller microparticles produced similar results, the reduction of clotting time could equally be reversed after antibody addition. The mostly particle-free S100 did not show a clotting time reduction compared to the controls PBS and EGM-2, but CT-reduction after antibody addition was measured. Box-and-whiskers plots (min, max, 25th and 75th percentile, median) of absolute CT measurements in seconds (sec).

The results suggest that there was a strong involvement of microparticle-bound tissue factor in clotting time reduction. To get an even closer look at what exists in the cell culture supernatant of differently centrifuged fractions flow cytometry was applied.

3.2 Flow Cytometry

3.2.1 EGM-2 bears a high number of particles measured via flow cytometry

In order to answer question if an increased number of microparticles could be detected after TNF-α stimulation the flow cytometer Gallios was used. To detect microparticles the flow cytometer needed beads for calibration. The Megamix - a mix of beads with diameters 0.5 µm, 0.9 µm and 3 µm - was used.

Utilising the beads and setting the right voltage corrections at the detectors, a gate smaller than 0.9 µm and around 0.5 µm could be set. Within that gate the number of events were counted and interpreted. Distilled water, PBS and EGM-2 were measured via flow cytometry as a control. The other samples which were analysed were formed from differential centrifugation of cell culture supernatant: endothelial cells had been stimulated with TNF-α (10 ng/ml) for 24 hours and their controls were not stimulated. Their supernatants were then used for centrifugation. The sample S0.5 was due to the 500 xg centrifugation cell-free, the microparticle-containing pellets P14 from the 14 000 xg centrifugation and P100 of the 100 000 xg centrifugation were diluted in PBS. S100 was the remaining supposedly free supernatant. All samples were analysed

via flow cytometry (Fig. 20). It is important to keep in mind that for every flow cytometer there is a detection limit. For the Gallios that would be at a 300 nm.

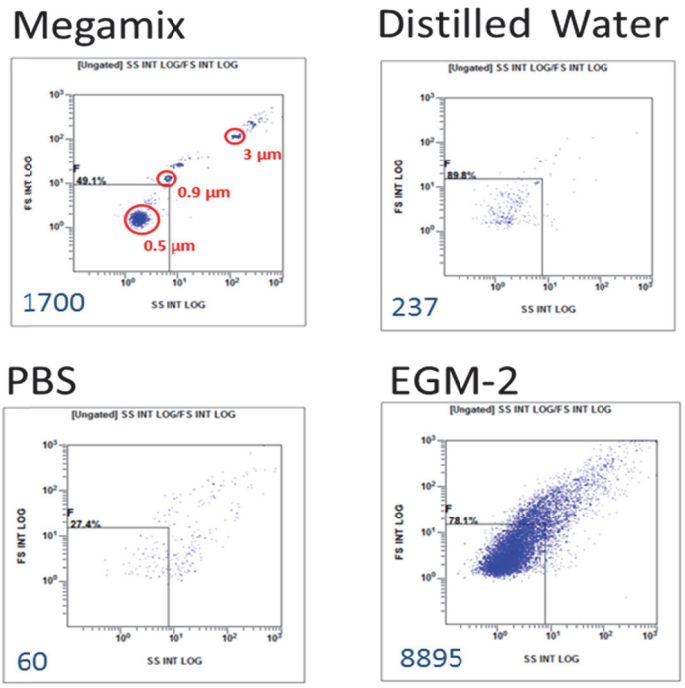


Fig. 20. Calibration of flow cytometer via Megamix and controls. The flow cytometer was calibrated with the Megamix containing beads of 0.5 µm, 0.9 µm and 3 µm in size. A gate was set around 0.5 µm. The number on the left of every graph stands for the number of events measured via the instrument. As controls distilled water, PBS and EGM-2 were used. The number on the left stands for the events detected within the gate having been set. As expected a very low number of events was detected in PBS and distilled, however a very high number of events was detected in EGM-2 only.

Fig. 20 describes the number of events, which could be detected within the gate where microparticles are found. These values were very low for the two controls, distilled water and PBS. This is as expected as microparticles should be present in these controls.

EGM-2, on the other, displayed a very high number of events within the set gate. This was not expected and desired at all, since that means, that EGM-2 itself produces a very high background noise. The particles measured by flow cytometry in EGM-2

were most likely from FCS. The attempts to dilute any pellets obtained from differential centrifugation in EGM-2 were completely discarded from that point on. To try to gain valid information from in EGM-2 diluted pellets would have been pointless since EGM-2 would have added so many additional microparticles to the microparticle pellets collected from a 14 000 xg centrifugation and 100 000 xg centrifugation, therefore, it would have been useless to try analyse those samples with flow cytometry. All pellets were diluted in PBS instead.

3.2.2 TNF-α enhances the release of microparticles by endothelial cells

As already mentioned, the supernatant of unstimulated and TNF-α (10 ng/m) stimulated endothelial cells were centrifuged to collect different fractions of (ultra-) centrifugation. All samples were then analysed via flow cytometry after setting a microparticle gate < 0.9 µm, a size within microparticles should be found. Fig. 21 shows the results of these measurements. The cell-free supernatant S0.5 showed a strong increase of detected events from 1570 to 8354 upon TNF-α stimulation. The pellet P14 formed from a 14 000 xg centrifugation showed much higher number of events without TNF-α stimulation compared to S0.5. P14 also displayed a very strong increase of events upon TNF-α stimulation. The supposedly particle free supernatant S100, which was remaining due to the 100 000 xg centrifugation, showed around the same number of events without TNF-α stimulation as S0.5 but showed no increase of events upon TNF-α stimulation. The pellet formed from a 100 000 xg centrifugation showed similar high number event as P14 without TNF-α stimulation and was significantly higher compared to the cell-free supernatant S0.5 without TNF-α stimulation. The number of events detected via flow cytometry was much higher after

TNF-α stimulation. These results strongly suggest that microparticles could be detected in both pellets P14 and P100. Another proof of the existence of microparticles is that their number increases upon stimulation. [66]

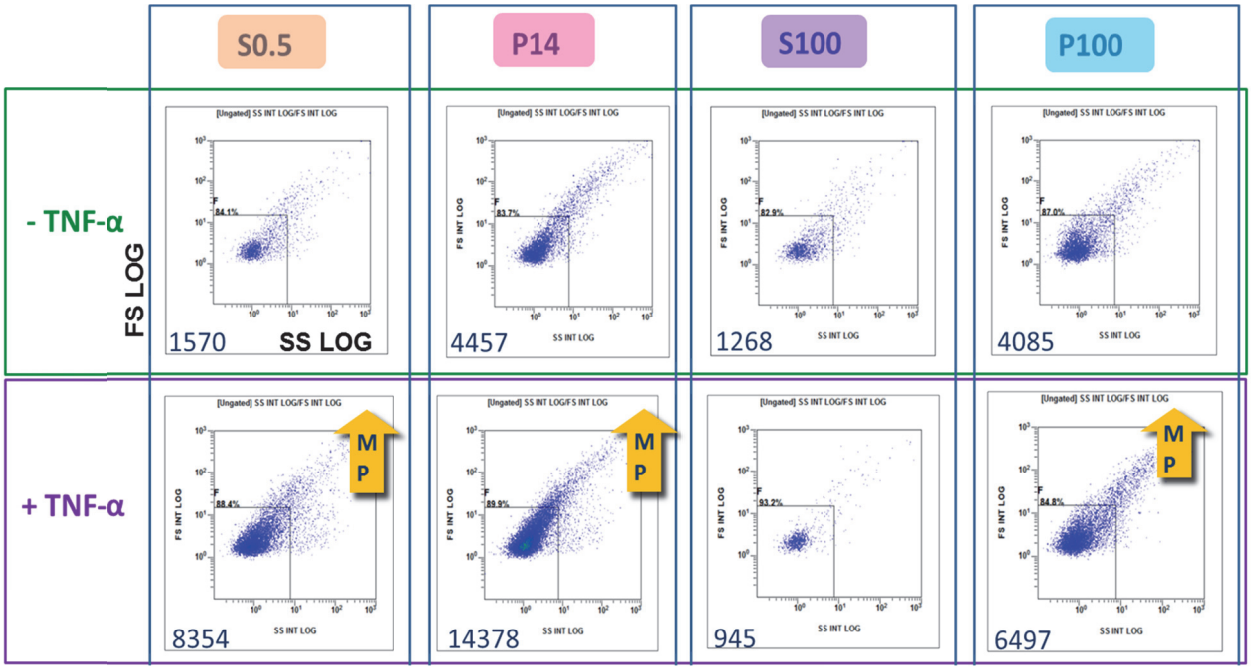


Fig. 21. TNF-α enhances the microparticles release by endothelial cells measured via flow cytometry. On the left the number of events detected within the set microparticle gate (< 0.9 µm) is given. The upper row represents the differently centrifuged cell culture supernatants collected from unstimulated endothelial cells. The lower row shows the differently centrifuged cell culture supernatants collected from TNF-α stimulated endothelial cells. The cell free supernatant S0.5 showed an increased number of events upon TNF-α stimulation. The number of events was also increased for P14 supposedly containing larger microparticles and P100 supposedly containing smaller microparticles. However, the as particle-free considered supernatant S100 displayed a much lower number of events and no raise of detected events upon TNF-α stimulation.

3.2.3 Successful Annexin-V detection on pellet P14

Endothelial cells had been stimulated with TNF-α (10 ng/ml) for 24 hours. The supernatant was then collected and centrifuged at 14 000 xg to gain a pellet mainly containing microparticles, apoptotic bodies and cell debris. The pellet was diluted in Annexin V Binding Buffer and depending on the sample, Annexin-FITC, anti-tissue-factor antibody and Alexa 594 conjugated secondary antibody were added. As a control to the pellet Binding Buffer only was used and incubated with the fluorochromes in the same manner as for P14. In Fig. 22 the results are shown: P14 incubated without any fluorochromes did not show any fluorescence although particle were visible. In the binding buffer control nearly no particles were observed via flow cytometry. When the pellet P14 was incubated with Annexin-V-FITC a clear fluorescent signal was observed. The vast majority of particles were indeed Annexin-V positive. In the control, the binding buffer only, nearly no events were detected with flow cytometry. The pellet P14, to which anti-tissue-factor antibody and secondary antibody Alexa 594 conjugated secondary antibody were added, did not show any florescence. No fluorescence could be neither detected in the binding buffer control, but a cloud of particles was appearing, these were probably antibody-aggregates.

The pellet P14 with both, Annexin V-FIT and anti-tissue-factor antibody supposedly bound to secondary antibody labelled with secondary antibody conjugated to Alexa 594, only showed a clear fluorescent signal for Annexin-V positive particles and none for tissue-factor. In the control nearly no events could be measured.

Therefore, Annexin V, a typical microparticle marker [64] was binding to particles isolated from the 14 000 xg centrifugation pellet. These particles, containing phosphateidylserine, could be microparticles or apoptotic bodies. Apoptotic bodies

are usually Annexin-V positive too. [67] No tissue factor could be detected in the experiment. This could be for several reasons. In another attempt to duplicate the experiment a different secondary antibody was used and the pellets were centrifuged after antibody addition.

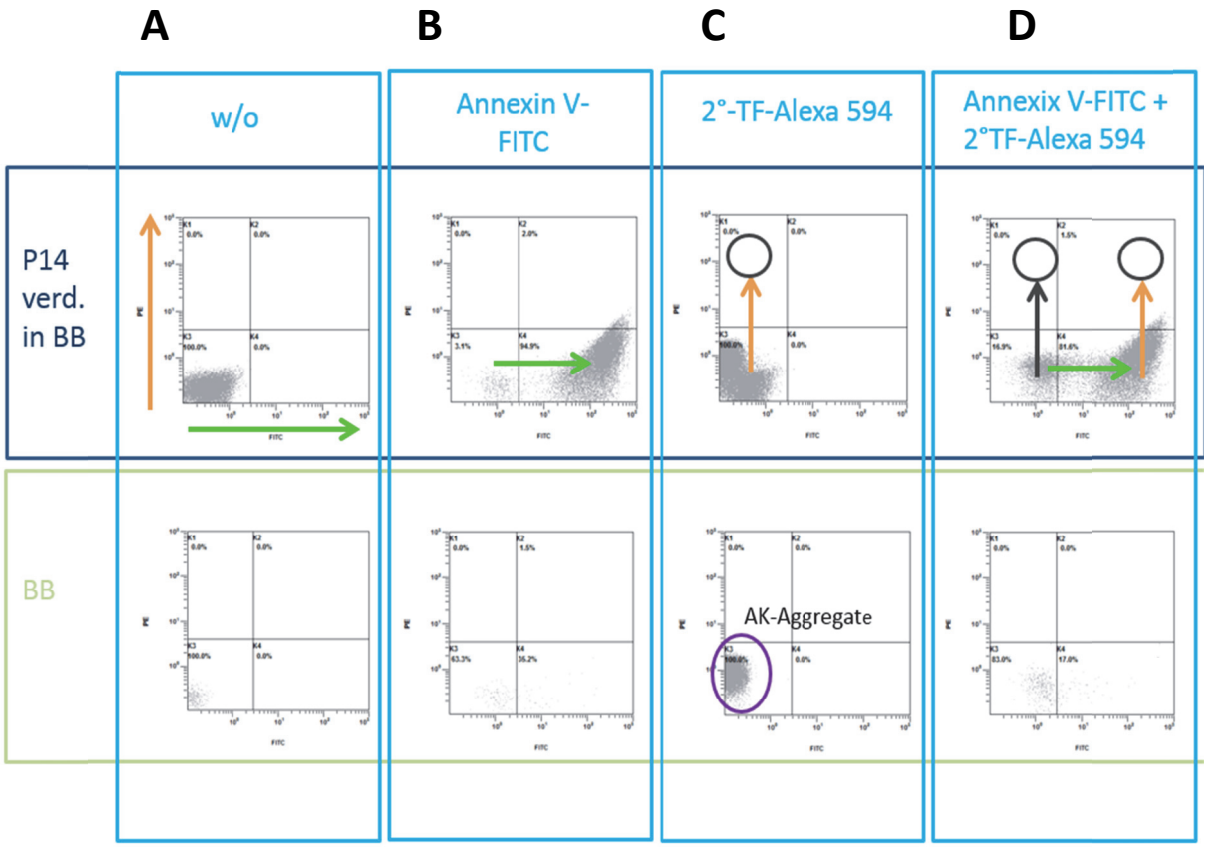
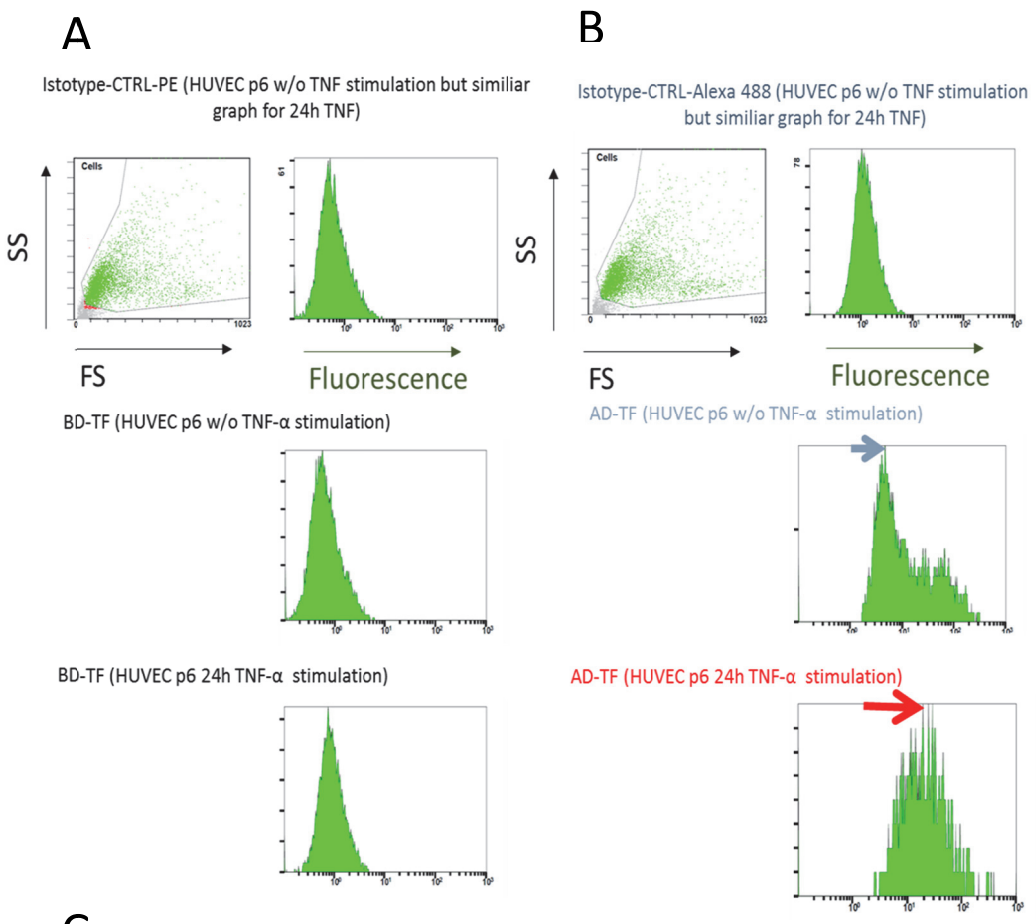


Fig. 22. Analysis of the pellet P14 (mainly larger microparticles) gained from TNF-α stimulated endothelial cells by flow cytometry. The upper row shows the pellet incubated with various fluorochromes, the lower shows the binding buffer (BB) incubated with the same fluorochromes. (A) No fluorescent particles are detected in the control without fluorochromes. (B) Annexin V-FITC fluorochromes bound particles are shown, none in the BB control. (C) No particles which anti-tissue factor antibody bound to Alexa 594 conjugated secondary antibody is visible via flow cytometry, in the control presumably antibody aggregates are shown. (D) In a mix of both Annexin-V-FITC and anti-tissue factor antibody and Alexa 594 conjugated secondary antibody were incubated with P14, only Annexin-V bound to particles is visible. The control is mainly particle free.

3.2.4 Testing of Anti-Tissue-Factor Antibody of Different Companies on Stimulated and Non-Stimulated HUVEC

HUVEC were incubated for 24 h with or without (w/o) TNF-α (10 ng/ml) and then the cells were used to determine if any of the anti-tissue-factor antibodies from various companies bind to them detected via flow cytometry. Anti-tissue factor antibodies of the following companies were used: e-Bioscience PE-coupled, BD PE-coupled and anti-tissue factor antibody of the company American Diagnostica together with a secondary antibodies conjugated to Alexa 488. The results are shown in detail in Fig. 23. After endothelial cells had been incubated with the anti-tissue factor antibody from e-Bioscience and BD the fluorescence was measured via flow cytometry. There was no fluorescence increase neither for the TNF-α stimulated nor non-stimulated endothelial cells compared to the Isotype-PE control. Consequently the antibodies from e-Bioscience and BD could not be used for further experiments. However, when endothelial cells had not been stimulated with TNF-α, but then incubated with anti-tissue factor antibody from American Diagnostica first and then secondary antibody labelled with Alexa 488 conjugated secondary antibody, a fluorescent shift compared to the isotype control could be observed. As isotype control secondary antibody labelled with Alexa 488 only was used. The increase of fluorescence was even stronger when TNF-α stimulated HUVEC were used for measuring. This indicated that first, the combination of anti-tissue factor antibody from American Diagnostica and Alexa 488 conjugated secondary antibody worked well but also that TNF-α stimulated HUVEC express a higher amount of tissue on their cell surface compared to non-stimulated HUVEC.



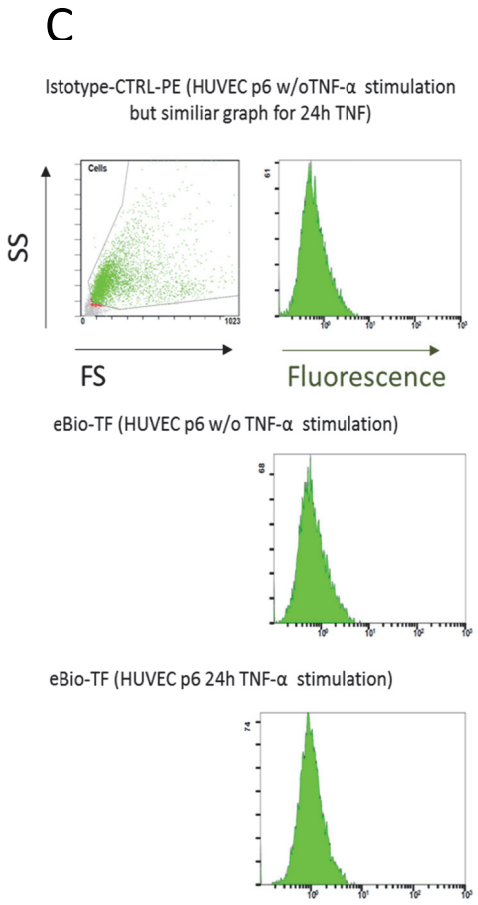


Fig. 23. Testing of various anti-tissue factor antibodies on stimulated and non-stimulated HUVEC via flow cytometry. Anti-tissue factor antibodies of BD, e-Bioscience and American Diagnostica were used for flow cytometry. (A) Compared to the isotype-PE control neither TNF-α stimulated nor non-stimulated HUVEC show an increase of fluorescence when the PE coupled anti-tissue factor antibody of BD has been used. (B) When using the anti-tissue factor antibody of American Diagnostica together with the secondary antibody labelled with Alexa 488 conjugated secondary antibody for unstimulated HUVEC an increase of fluorescence is visible compared the isotype control Alexa 488 conjugated secondary antibody only. This increase is even stronger when 24 h TNF-α stimulated HUVEC are used. (C) There is no fluorescence increase detectable when stimulated and non-stimulated HUVEC have been incubated with PE-coupled anti-tissue factor antibody compared the isotype-PE control.

3.2.5 Detection of TF on Microparticles Isolated from the Pellet P14 via Flow Cytometry

The pellet P14 from a 14 000 xg was chosen for tissue factor detection, since P14 showed a very high number of particles in flow cytometry. When HUVEC had been incubated with and without TNF-α (10 ng/ml) for 24 hours, the cell culture supernatant was removed and particles isolated via differential centrifugation. The pellet of the

14 000 xg centrifugation step contained mainly larger microparticles but also apoptotic bodies and cell debris. The pellet was diluted in PBS and then used for flow cytometry measurements following incubation with anti-tissue factor antibody from American Diagnostica with two different secondary antibodies, labelled with Alexa 488 and 594. In order to set up a gate for the detection of microparticles latex beads of 500 nm size were used: A gate around 500 nm was set, within the fluorescent particles were measured. Nearly no particles, as expected, could be detected in distilled water. The detailed results are shown in Fig. 24 and Table 2. The fluorescent intensity signal (0.3) of the latex beads and distilled water was very small. There was a clear fluorescent signal for particles isolated from TNF-α stimulated HUVEC supernatant thanks to

14 000 xg centrifugation when Alexa 488 conjugated secondary antibody was used. The mean fluorescent intensity was 582.3 compared to 0.3 of the isotype-PE control. P14 of the unstimulated HUVEC supernatant showed also a clear fluorescent signal with mean fluorescent intensity of 103.6 compared to the isotype-PE control of 1.1 when Alexa 488 conjugated secondary antibody was used. The signal of the pellets collected from the stimulated endothelial cell culture supernatants was therefore

around five times higher than the one from the non-stimulated cell culture supernatants. On the other hand when the secondary antibody labelled with Alexa 594 was used to detect microparticles, isolated from TNF-α stimulated HUVEC supernatant from a 14 000 xg centrifugation, no significant fluorescent signal could be detected. The mean fluorescent intensity was 0.3, which was the same value measured for the isotype-PE control. This is especially strange since a pellet from stimulated HUVEC supernatants was used for flow cytometry. The pellet showed a very strong signal measured with Alexa 488 conjugated antibodies that was even higher than for non-stimulated cells. These results though would very well explain why the first attempt to detect tissue factor on the surface of microparticles failed: The antibody 594 had not been binding to the antigen anymore or the fluorophore had stopped to generate fluorescence. Either way it could not be used anymore.

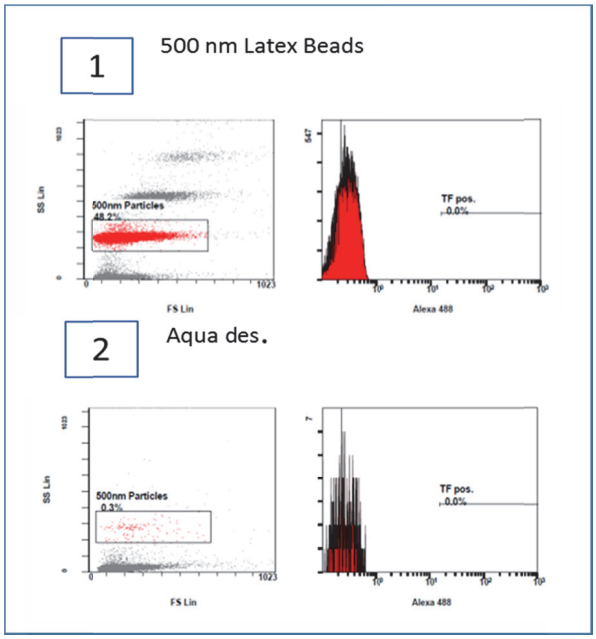
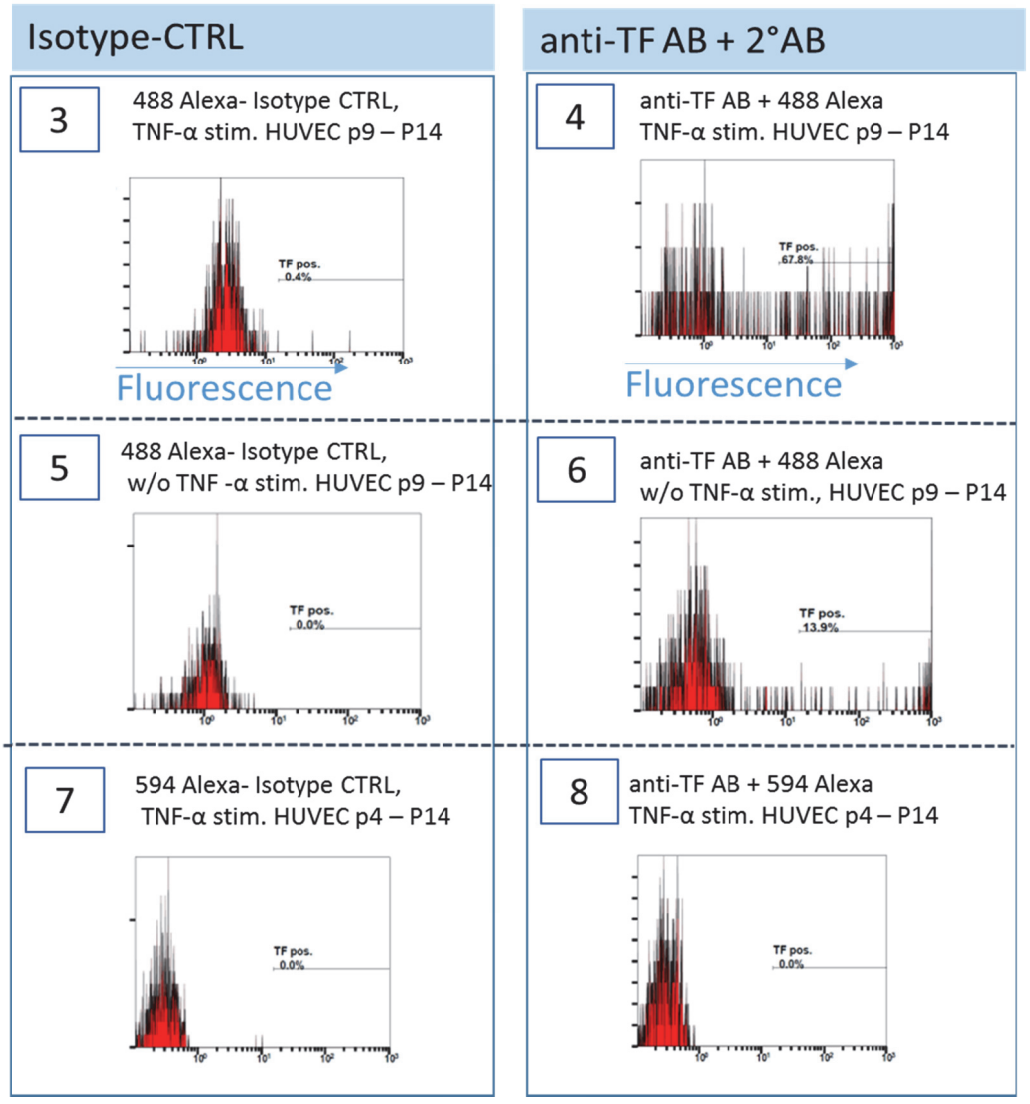


Fig. 24. The detection of tissue factor bearing particles in the pellet P14 collected after 14 000 xg centrifugation via flow cytometry. To set up a detection gate within 500 nm latex beads are used (1), nearly no particles are found in distilled water (2). Particles gained from TNF-α stimulated HUVEC supernatants show a higher fluorescence (4) compared to the control (3) than unstimulated (6) compared to the control if primary anti-tissue factor antibody and secondary antibody Alexa 488 conjugated secondary antibody is used. In contrast to that no fluorescence is detected for P14 of stimulated HUVEC supernatants using the secondary antibody conjugated to Alexa 594 (8) compared to the control (7).



SAMPLE NUMBER	SAMPLE	anti-tissue factor antibody from American Diagnostics	Secondary antibody labelled with Alexa 488 or 594	Mean fluorescence intensity (gated particles)
1	Latex beads	-	-	0.3
2	Aqua des	-	-	0.3
3	24h HUVEC p9 – P14 (Isotype CTRL)	-	+ 2°AB: Alexa 488 (1ul)	3.4
4	24h TNF HUVEC p9 – P14	+ 1° AB: AD-TF (2ul)	+ 2°AB: Alexa 488 (1ul)	582.3
5	w/o TNF HUVEC p9 – P14 (Isotype CTRL)	-	+ 2°AB: Alexa 488 (1ul)	1.1
6	w/o TNF HUVEC p9 – P14	+ 1° AB: AD-TF (2ul)	+ 2°AB: Alexa 488 (1ul)	103.6
7	24h HUVEC p4 – P14 (Isotype CTRL)	-	+ 2°AB: Alexa 594 (1ul)	0.3
8	24h HUVEC p4 – P14	+ 1° AB: AD-TF (2ul)	+ 2°AB: Alexa 594 (1ul)	0.3

Table 2. The detection of tissue factor bearing particles in the pellet P14 collected after 14 000 xg centrifugation via flow cytometry. The mean fluorescence of latex beads (1) is measured around 0.3 as it is for distilled water (2). However, the pellets collected from TNF-α stimulated HUVEC (4) have a mean fluorescent intensity of 582.3 much higher compared to the isotope control (3) at 3.4. The fluorescence is actually nearly fivefold stronger that measured for pellets produced for non-stimulated cell culture supernatants (6). Their mean fluorescent intensity is 103.6, clearly much higher than the isotope control (5) at 1.1. The samples 3, 4, 5 and 6 were all incubated with primary anti-tissue factor antibody and secondary antibody conjugated to Alexa 488. Samples 7 and 8 use secondary antibody labelled with Alexa 594 though. No clear fluorescent signal is even detectable for P14 from stimulated HUVEC supernatants, the mean fluorescent intensity 0.3, which is the same as measured for the isotype control.

4 Discussion

Endothelial cell derived microparticles are currently of high interest in the fields of cardiovascular and haematological research. A recent study at the Ludwig Boltzmann Institute of Experimental Traumatology could show the release of mediators facilitating blood coagulation by endothelial cells and it was suggested that tissue factor is likely to be involved in that process. [56] With the current follow up research project we aimed to investigate, whether tissue factor was released by endothelial cells, soluble or particle/bound.

The results showed, that microparticles derived from endothelial cells can be isolated via differential centrifugation of cell culture supernatant. This way concentrated microparticles have been shown to accelerate whole blood coagulation measured with thromboelastometry. The procoagulatory effect of endothelial cell derived microparticles was partly reversed with an antibody against tissue factor, suggesting the presence of a particle-bound tissue factor. Furthermore, corresponding the functional impact on accelerating blood coagulation, the presence of tissue-factor bearing microparticles could be confirmed by flow cytometry.

The question is: why do microparticles facilitate coagulation in general?

Cells communicate with other cells not only via direct cell-cell contact and the production of signalling molecules, but also through the release of microparticles. [68] These microparticles (MPs) are small vesicles which are potentially released from all types of cell. [68] Their release is triggered by cell activation or apoptosis. [69]. The size of microparticles is around 100 nm – 1000 nm. [70] The interest of research in

microparticles has particularly grown in the last years, because they are associated with

many diseases as vascular diseases such as cancer, diabetes or inflammation. [71] Microparticles can contain DNA, mRNA, and proteins but also their surface and integral proteins contribute to transmission of information to other cells. [72] A typical marker for microparticles is phosphatidylserine. Upon the release of microparticles, negative charged phosphatidylserine and phosphatidylethanolamine are exposed to their surface. [73] Phosphatidylserine (PS) on the surface of microparticles facilitates blood coagulation: it enhances activation of coagulation proteins, tissue factor and platelet aggregation. [72] It is of interest, that also cancer cells release TF-bearing microparticles. The pro-coagulant particles increase thrombosis and are most likely related to a higher mortality of patients with cancer. [74]

Discussion of stimulation of endothelial cells with TNF-α

Endothelial cells maintain the fragile balance pro- and anticoagulation by production of their production of haemostatic mediators. [75] During activation or apoptosis endothelial cells shed also microparticles. [76] One way of endothelial cell activation can be achieved is via tumour-necrosis factor alpha. [77] Endothelial cell derived microparticles contain not only phosphateidylserine on their surface, but also tissue factor, which both contribute to blood coagulation and were detected via flow cytometry.[78]

In order to find a significant influence of endothelial cell culture supernatant on coagulation the supernatants were harvested from various time points after incubation with TNF-α to stimulate cells. These supernatants were then measured

with rotational thromboelastometry (ROTEM) and the results showed correlation between the time of TNF-α stimulation and the clotting time reductions: This suggested the depending on duration of cells stimulation more coagulatory mediators were released.

For the hypothesis of the study it was assumed that released mediators are actually microparticles, because their release was shown to be triggered upon such activation. [69] [77] Based on the results above and for the purpose of this study it was decided to stimulate HUVEC for 24 hours with TNF-α.

Discussion of differential centrifugation

The cell culture supernatants were processed with differential centrifugation for various reasons. The original cell culture supernatant was centrifuged at 500 xg in order to remove dead cell resulting in the so-called supernatant S0.5 supposed to contain all procoagulatory mediators released by endothelial cells. In a next step this supernatant (S0.5) was centrifuged at 14 000 xg for 15 min at 4º C to receive a pellet (P14) assumed to contain larger particles, cell debris and apoptotic bodies and was diluted in diluted in 50 µl solution (first EGM-2, later PBS). The remaining supernatant was again centrifuged again at 100 000 xg for 1h at 4º C to collect a pellet (P100) suggested to contain mainly smaller microparticles and exosomes, that was also again diluted in

50 µl solution (first EGM-2, later PBS). The supposedly particle-free supernatants after 100 000 xg centrifugation was also harvested to serve as important control.

Sample	Content
S0.5	Cell free supernatant
P14	**Larger MPs**, cell debris, apoptotic bodies
S100	Particle-free cell culture supernatant
P100	**Smaller MPs**, exosomes

Table 3. Overview of the differential centrifugation samples. S0.5 is the cell free supernatant, P14 and P100 are the pellets containing microparticles and S100 is the remaining particle-free supernatant.

The results confirmed that the method of differential centrifugation carried out in this study could help to answer sufficiently well the current research question. Significant presence of microparticles could be detected with thromboelastometry and flow cytometry.

Discussion of thromboelastometry

Compared to the control measurement using PBS, the original cell-free supernatant S0.5 showed a CT-reduction. Presumably concentrated microparticles from the pellets P14 and P100 also demonstrated significant clotting time reduction. In contrast, the supposedly microparticle-free final supernatant S100 did not impact clotting time as compared to control.

As compared to medium control, a significant CT-reduction was already observed in S0.5, with an even more reduced CT in P14. Interestingly P100 reduced CT in the same range as did S0.5 (Figures 17 and Figures 19). From a qualitative point of view, considering the enhancement of the coagulation in thromboelastometry, it became

likely that the procoagulatory pattern of the original supernatant S0.5 is most likely mediated by microparticles. This was furthermore confirmed by the final assumed particle-free supernatant S100 did not impact blood coagulation in thromboelastometry.

Nevertheless it has to be kept in mind that the pellets P14 and P100 can' t be considered as consisting of microparticles only. P14 also bears at least cell debris and apoptotic bodies and P100 exosomes. These "by-products" of the centrifugation steps could also contribute to clotting time reduction.

Therefore further measurements were done with the same samples (supernatants and pellets) but additionally incubated with an anti-tissue-factor antibody for half an hour. There was a significant clotting time reversion for the original supernatant S0.5, as well for the microparticle-containing and diluted pellets P14 and P100. Surprisingly, although not enhancing coagulation more than the medium/PBS control, a significant clotting time prolongation was detectable for S100 when incubated with antibody against TF. We assume soluble tissue factor partly being responsible for this phenomenon.

To confirm that the used medium or diluents in this study presumably do not contain tissue factor, thromboelastometric control experiments with the same antibody were carried out showing the same clotting times regardless of the presence of an antibody or not. The fact the antibody incubation in PBS did not lead to any clotting time reversion, is not very surprising since PBS is a particle-free buffer, but it confirms the validity of the measurements. Also the medium, in which the cells were grown and which were shown to contain microparticles to some extent in flow cytometry, did not show any clotting time reversion upon anti-tissue factor antibody binding.

This fact confirms microparticles from EGM-2 contributing to the pellet P14 and P100 do not bear tissue factor activating coagulation in thromboelastometry.

It has to be kept in mind that anti-tissue-factor antibody addition did not lead to full clotting time reversion. Consequently a part of clotting time reduction is not due to tissue factor, but most likely to microparticles themselves, assumed to impact coagulation due their negative phosphatidyl-serine surface. [72]. It is well known that apoptotic bodies have phosphatidyl-serine on their surface too and might contribute to clotting time reduction. [79]

Because flow cytometry experiments revealed a relevant number of particles in EGM-2 in a gate < 0.9 µm, most pellets from differential centrifugation were diluted in PBS and not EGM-2.

However, to exclude that particles coming from EGM-2 contribute to clotting time reduction, EGM-2 itself was put under differential centrifugation. The samples were tested via ROTEM and no difference in clotting time reduction was observed.

Discussion of flow cytometry

To gain a deeper look inside the cell culture supernatant samples and their control media flow cytometry was used. In order to detect microparticles a gate < 0.9 µm was set with the help of the Biocytex Megamix. As expected nearly no events were detected within this gate for PBS or distilled water. In contrast, as already mentioned, a very high number of particles was detected in EGM-2. Their origin is most likely from the serum FCS and was the reason why most pellets were diluted in PBS instead of EGM-2. TNF-α stimulated HUVEC supernatants used for differential centrifugation but also unstimulated supernatants were analysed via flow cytometry and revealed a higher number of microparticles upon TNF-α stimulation, as was to be expected from

the thromboelastometric experiments. This effect could be confirmed in the cell-free supernatants S0.5 as well as in the pellets P14 and P100.

Flow cytometry also revealed a higher number of particles is concentrated in the diluted pellet as compared to the original supernatant S0.5. This nicely confirms that microparticles isolation was successfully carried out in this study.

The presumably particle-fee supernatant S100 gained from non-stimulated or unstimulated HUVECs resulted only in a quite low of events detected with flow cytometry. Furthermore, in contrast to the results obtained from S0.5 and both pellets P14 and P100 no increase of particle number was detected upon stimulation in S100. This suggests that all microparticles regardless of absolute numbers after stimulation were concentrated into the pellet P100. The smaller number of events detected in S100 are most likely not coming from microparticles, but could have various sources such as: protein aggregates or dust.

Another fact is also of interest: Even without TNF-α stimulation high numbers of particles for S0.5 and the pellets P14 and P100 could be detected. This means that also without TNF-α stimulation some sort of activation must happen, since literature says that microparticles from endothelial cells should be only released in response to cellular activation. [80] The source for that activation is mostly likely coming from the medium EGM-2. FCS seems very likely to be responsible, since many growth factors and other molecules with endothelial cell activation potential are present in serum.

A common marker for microparticles is Annexin V, which binds to phosphatidylserine (PS). [73] Because the pellet P14 showed the highest number of particles after TNF-α stimulation, it was used for PS but also tissue factor detection. FITC-labelled Annexin V was used for that purpose and anti-tissue factor antibody from American

Diagnostica in combination with Alexa 594 conjugated secondary antibody. Even though the majority of particles could be identified as Annexin V positive, no TF positive microparticles could be detected in first attempt.

When the experiments were repeated with a different secondary antibody on endothelial cells, but not endothelial cell culture supernatants, TF could be detected. The reason why HUVEC cells were tested for tissue factor was to test various anti-tissue factor antibodies but also to prove that microparticles found in the cell culture supernatants stem from these cells.

The following primary antibody was used for that purpose:

- Anti-tissue factor antibody-PE labelled from e-Bioscience
- Anti-tissue-factor antibody-PE labelled from BD
- Anti-tissue-factor-antibody from American Diagnostica in combination with Alexa 488 conjugated secondary antibody

In fact, compared to the isotype controls only anti-tissue-factor-antibody from American Diagnostica in combination with secondary antibody labelled with Alexa 488 showed an increase of fluorescence. When non-stimulated HUVEC were analysed this increase was rather low but quite high for TNF-α stimulated endothelial cells. The upregulation of the expression of TF on endothelial cells upon TNF-α stimulation makes perfect sense, since the connection between the two of them has been known for years.[81]

It appears surprisingly though that tissue factor is already expressed even without TNF-α stimulation. Tissue factor should only be expressed upon TNF-α stimulation. [82] This implies that some sort of activation must happen already when cells are

incubated with pure medium and serum. Indeed this is in line with the results from the previous study

[56] and goes along with the results we got of the number of microparticles being detected in non-stimulated HUVEC cell culture supernatants: Despite that in theory no microparticles should be released without stimulation a very high number was observed therefore some sort of activation happens also while 24 hours of medium/serum incubation. This is most likely to due to FCS, since as already discussed, containing many potentially activating molecules.

Discussion on antibodies used in this study

The reason why neither anti-tissue factor antibody purchased from BD nor e-Bioscience were binding to HUVEC is still unknown. All three antibodies used from the companies BD, e-Bioscience and American Diagnostica were monoclonal anti-human antibodies

from mouse as a host. That is why the secondary antibodies, conjugated to Alexa 488 and Alexa 594 were both against mouse directed. The main difference between the three of them is that BD and e-Bioscience antibodies are in contrast to antibody from American Diagnostica conjugated to a fluorophore and that they originate from different clones.

The fact that BD and e-Bioscience are labelled with a fluorophore could influence their binding capacity to a certain extent since some kind steric hindrance is possible. It is quite unlikely though since both e-Bioscience and BD antibodies are as the one from American Diagnostica all IgG antibodies. IgG antibodies have a structure which has been very successful in being conjugated to a fluorophore without interfering with its binding capacity. Much more likely though is the fact that they three

antibodies originate from different clones influence their binding capacity: The antibodies from e-Bioscience and BD originate from the clone HTF-1 whereas the antibody from American Diagnostica (AD) was produced by the clone TF9-10H10. Those clones, or hybridomas, are cells that are the product of cell fusion between immortal cancer cells and B-cells that produce the desired antibody. The B-cells had been isolated from an immunised mouse. [83]

Hybridomas are an endless source of antibodies, each clone producing monoclonal antibodies. If they were gained from the same donor, therefore were immunised with the same antigen, each clone of them produces antibodies which are directed against different epitopes of the same antigen. Consequently it could be easily happen antibodies against the same antigen but different clone bind equally efficiently to the antigen, since they are directed against different epitopes. [83]

Since it was clear now that the combination of anti-tissue factor antibody from AD and Alexa 488 conjugated secondary antibody worked well for the detection of tissue factor, the next step was the detection of tissue factor on microparticles. For that purpose the pellet with larger MPs and a very high number of particles upon TNF-α stimulation was chosen for the flow cytometry analysis.

First the flow cytometer FC 500 was calibrated with latex beads of a size of 500 nm, in order to set up a gate around 500 nm for microparticle detection. As expected no fluorescence was detected in distilled water. Compared to the isotype control there was a clear increase of fluorescent signal though when the pellet P14, collected from the supernatant of non-stimulated HUVEC cells, was used for flow cytometry. The signal could be detected with the help of anti-tissue factor antibody from AD and Alexa 488 conjugated secondary antibody. The fluorescent signal grew even stronger

when the pellet P14 from TNF-α stimulated HUVEC was analysed. These results are very consistent with former measurements: The HUVEC cells produce more tissue factor upon TNF-α stimulation and then release more TF-bearing microparticles, which then lead to an increased clotting time reduction in rotational thromboelastometry. These results most important prove that the particles detected bear tissue factor, which has to be found on the surface, otherwise it could have not been detectable with flow cytometry.

Alternatively, when secondary antibody labelled with Alexa 594 was used instead of Alexa 488 for the detection of tissue factor in pellet P14 in combination with anti-tissue factor antibody from American Diagnostica no fluorescent increase compared to the isotype controls could be observed. This was true for the measurements of the pellet P14 collected from non-stimulated HUVEC but also from TNF-α (10 ng/ml) stimulated endothelial cells. These results could actually explain quite well why no tissue factor positive particles could be detected in the first attempt when also Annexin V was also used for phosphatidylserine-detection. Alexa 594 conjugated secondary antibody is seemingly not working fine anymore. That could be because the fluorescence of the antibody had been fading or the polyclonal antibody - Alexa 488 conjugated secondary antibody is equally a polyclonal antibody- had been degraded and therefore is not binding to the primary anymore. In any case its usage was put on hold and secondary antibody labelled with Alexa 488 will be used for further experiments.

In this thesis I could prove that endothelial cell derived tissue-factor-bearing microparticles are involved in the process of facilitating blood coagulation, proven with ROTEM-measurements, differential centrifugation and flow cytometry. This is

very contrary to elder models of blood coagulation, which gives microparticles no part in blood coagulation. [84] To which extent exactly particles detected in pure EGM-2 contribute to flow cytometry measurements could not be fully answered yet, but they should not have a great impact on ROTEM, but needs further investigation.

5 Conclusion and Outlook

In conclusion the experiments could show that TNF-α stimulates endothelial cells to produce mediators that facilitate blood coagulation. These mediators have been further identified to be microparticles, which bear tissue factor on their surface. Differential centrifugation was shown to be a potent tool to isolate and characterise these microparticles further. The results from rotational thromboelastometry demonstrated a clear influence of isolated particles on reduction of clotting time. This effect could be partly reversed with the addition of anti-tissue factor antibody suggesting that a particle-bound tissue factor is involved in enhancing blood coagulation.

Flow cytometry experiments revealed a higher number of particles after cell stimulation as compared to non-stimulated cells. These particles carried phosphatidylserine on their outer surface, which is an important marker for microparticles, but also of apoptotic bodies. Furthermore, flow cytometry could detect tissue-factor bearing microparticles. The same antibody for microparticles was also used for endothelial cells and showed a higher tissue-factor expression upon TNF-α stimulation, which is coherent to the higher release of TF-bearing microparticles and also that their origin is indeed from these endothelial cells.

In order to make sure, that medium does not carry any tissue factor or Annexin V positive particles, which would be very unlikely since the ROTEM results proved that differently centrifuged samples did not show a different CT-times, EGM-2 will have to be also labelled with antibodies and Annexin V for flow cytometry. Also a tissue factor activity test is highly recommended in order to prove that really TF active particles are

involved in the process of blood coagulation. To exclude apoptotic bodies in the pellet

P14 from a 14 000 xg centrifugation a filter could be considered to use at 0.8 µm as suggested by Crescitell et al. [85]. Nonetheless a very small contribution to clotting time reduction can be assumed for apoptotic bodies, since none of them should be present in the pellet thanks to 100 000 xg centrifugation [85] and also a significant clotting time reduction without a complete reversion after tissue factor inhibition was observed.

This project could show that in contrast to common models, microparticles released by endothelial cells play a major role in the process of blood coagulation upon TNF-α stimulation and that part of these particles were bearing tissue factor on their surface, which was transmitting the signal to trigger the extrinsic pathway of blood coagulation.

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Abstract

Endothelial cells are well known for modulating haemostasis. Recent studies in the laboratory of the Ludwig Boltzmann Institute for Experimental and Clinical Traumatology could however show that endothelial cells as well as their culture supernatants accelerated blood coagulation. An incubation of whole blood with endothelial cell culture supernatants reduced viscoelastic clotting time. This effect could be reversed by an antibody against tissue factor. The aim of the current study was to isolate microparticles from endothelial cell culture supernatants and evaluate their effect on clotting time in rotational thromboelastometry (ROTEM). Microparticles are 0.1 – 1 µm small vesicles released upon cell stimulation. Therefore an up-regulation of microparticle release upon tumour-necrosis factor alpha (TNF-α) stimulation should be noticeable. Most important was to reveal if tissue factor can be found rather soluble or microparticle-bound in the endothelial cell culture supernatants.

For this purpose endothelial cells were stimulated with TNF-α up to 24 hours and subjected to ultracentrifugation. Microparticles concentrated into a pellet resulting from a centrifugation at 14 000 xg (P14) and 100 000 xg (P100) were mixed with recalcified citrated whole blood and analysed by thromboelastometry. Furthermore flow cytometry experiments were used to quantify of particle presence and to confirm tissue-factor presence on those particles.

The results showed that more procoagulatory mediators were released by endothelial cells upon TNF-α stimulation in comparison to non-stimulated cells. Following the differential centrifugation both pellets P14 and P100 harvesting microparticles (amongst others), showed a significant clotting time reduction, while the particle-free

supernatant S100 did not activate coagulation significantly. Clotting time reduction could be reversed in the particle-bearing pellets P14 and P100 after tissue factor inhibition with an antibody. The reversion for the pellets is most likely coming from microparticle-bound tissue factor. This could be confirmed by flow cytometry: In P14 tissue factor and phosphatidylserine (a common microparticle marker) positive particles were detected.

In conclusion, microparticles coming from endothelial cell culture supernatants could be separated via differential centrifugation and were assessed with flow cytometry and accelerated whole blood coagulation measured via thromboelastometry.

In this thesis it could be shown that procoagulatory mediators released by endothelial cells upon TNF-α stimulation are most likely tissue-factor bearing microparticles.

Zusammenfassung

Seit langem ist schon bekannt, dass Endothelzellen die Blutgerinnung modulieren. Erst kürzlich konnte das Ludwig Boltzmann Institut für Experimentelle und Klinische Traumatologie zeigen, dass Endothelzellen als auch Überstände von Endothelzellen aus der Zellkultur zur Blutgerinnung beitragen. Nach der Zugabe von Zellkulturüberständen zu frischem Blut konnte eine Reduktion der viskoelastisch gemessenen Gerinnungszeit beobachtet werden, die mit Hilfe von einem Antiköper gegen Gewebefaktor normalisiert werden konnte. Das Ziel der Arbeit war herauszufinden, ob Mikropartikel, die aus Endothelzellkulturüberständen isoliert werden, einen Einfluss auf die Blutgerinnungszeit, gemessen mittels Thromboelastometrie, haben. Mikropartikel sind kleine Vesikel mit einer Größe von 0.1 – 1 µm, die bei Zellstimulation ausgeschüttet werden. Somit sollte die Ausschüttung der Mikropartikel nach Stimulation der Endothelzellen mit Tumor-Nekrose Faktor (TNF-α) erhöht sein. Am wichtigsten war es herauszufinden, ob Gewebefaktor in den Zellkulturüberständen löslich oder an Mikropartikel-gebunden ist.

Zu diesem Zweck wurden Endothelzellen mit TNF-α bis zu 24 Stunden stimuliert und danach ultrazentrifugiert. Mikropartikel, die nach Zentrifugationen von 14 000 xg (P14) und 100 000 xg (P100), isoliert werden konnten, wurden mit Zitrat antikoaguliertem Blut vermischt und mittels Rotationsthromboelastometrie (ROTEM) analysiert. Zudem wurden Experimente mittels Durchflusszytometrie durchgeführt um die Mikropartikel zu quantifizieren bzw. um auf diesen Gewebefaktor auf diesen zu detektieren.

Die Ergebnisse zeigten, dass eine höhere Menge an prokoagulatorischen Faktoren durch TNF-α induzierte Endothelzellstimulation ausgeschüttet wurde in Vergleich zu

unstimulierten Endothelzellen. Nach differentieller Zentrifugation zeigten beide Pellets (P14 und P100), die Mikropartikel enthielten, eine signifikante Reduktion der Gerinnungszeit, während der partikelfreie Überstand (S100) die Blutgerinnung kaum aktivierte. Die Verringerung der Gerinnungszeit konnte durch die Inhibition von Gewebefaktor in den Pellets P14 und P100, in denen beiden Partikel gefunden wurden, reversiert werden. Die Reduktion der Gerinnungszeit ausgelöst von den Pellets stammt höchstwahrscheinlich von Mikropartikel-gebundenem Gewebefaktor. Die Vermutung konnte durch die Verwendung von Durchflusszytometrie bestätigt werden: Im Pellet P14 wurden sowohl Partikel mit Gewebefaktor als auch Phosphatidylserin detektiert (Phosphatidylserin ist ein typischer Marker für Mikropartikel).

Zusammenfassend konnte gezeigt werden, dass Mikropartikel aus den Zellkulturüberständen von Endothelzellen mittels einer differentiellen Zentrifugation isoliert werden können. Dies Mikropartikel können in Durchflusszytometrie entsprechend detektiert werden und führen zu einer Beschleunigung der Blutgerinnung im Vollblut, nachgewiesen durch die Thromboelastometrie.

Die Arbeit konnte zeigen, dass es sich bei prokoagulatorischen Faktoren, die von Endothelzellen unter TNF-α Stimulation ausgeschüttet werden, höchstwahrscheinlich um Gewebefaktor-tragende Mikropartikel handelt.

Curriculum Vitae

Personal Data

Name:	Cornelia Bonstingl
Staatsbürgerschaft:	Austrian

Work Experience

2013-2014	Employee of the Ludwig Boltzmann Institute for Experimental and Clinical Traumatology, Vienna
Specialised in	*Endothelial cell-derived microparticles*
Talk	*Speaker at the „Vascular Biology Meeting 2014" with the title*: "The influence of endothelial cell-derived microparticles on clotting time in thromboelastometry"
Poster-presentation	*Poster-presentation at the congress of the „Society of thrombosis and haemostasis research 2014" with the title:* "The influence of endothelial cell-derived microparticles on clotting time in thromboelastometry"
2014	Tutor at the University of Vienna (teaching genetics)
2013	Tutor at the University of Vienna (biochemistry course)
2012-2013	Teacher at the tutoring school „Lernquadrat" Döbling, Vienna
Specialised in	*Teaching Mathematics in groups or in one-to-one sessions*
2012	5-months laboratory work in the group of Greg Challis, University of Warwick, UK in the context of the Erasmus exchange programme
Specialised in	*Expession of recombinant proteins, which were participating in the synthesis of antibiotics*
2011	4-week internship in the hygienic laboratory of the MA39 of the city Vienna
2010	9-week internship in the group of Johann Mulzer, Department of Organic Chemistry, University of Vienna
Specialised in	*Synthesis of natural products*
2010	Tutor at the University of Vienan (chemistry course)

2009	4-week internship at the laboratory for construction building material at the Werk Peggau der Wietersdorfer & Peggauer Zementwerke GmbH, Styria
2006	4-week internship via GenAU (genome research in Austria) in the group of Wolfgang Schneider, which gave me the first time the chance to work in a laboratory whilst still being at high school

Educational Background

2011-2014	Master student of Biological Chemistry at the University of Vienna
Since 2013	Writing my master's thesis: "The influence of endothelial cell-derived microparticles on clotting time in thromboelastometry", which I am completing at the Ludwig Boltzmann Institute for Experimental and Clincial Traumatology
2011	Graduated with a bachelor degree in chemistry of the University of Vienna (average grade: 1.1)
2007	Passed A-level exams with honours at the Bundesoberstufenrealgymnasium Deutschlandsberg, Steiermark

Experience Abroad

2012	6-month studying though the Erasmus exchange program at the University of Warwick, UK

Language skills

Languages	German (native speaker)
	English (very good)

IT knowledge

Microsoft Office	Powerpoint, Excel, Microsoft Word
GraphPad Prism	

Scholarships

2010 and 2009 accepting the Performance Scholarship of the University of Vienna