Diploma thesis

Kinetic of clot formation and stress – strain behaviour of thrombi dependent on antiplatelet therapy

A non – therapeutic, biomedical study under the premises of fundamental research

for the attainment of the academic degree

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Abstract

Einleitung

Anfang des 21. Jahrhunderts sind Herz – Kreislauf Erkrankungen nach wie vor die häufigste Todesursache weltweit. (1) Manche Ausprägungen dieses Krankheitsbildes manifestieren sich als Insult, Embolie oder akuter Gefäßverschluss im Rahmen eines atherio-thrombotischen Ereignisses. Durch den progressiven Anstieg der Risikofaktoren (Hypertonie, Diabetes, Hypercholesterinämie, Fettleibigkeit, Alter, etc. (2)), vor allem in der modernen Welt, liegt die Notwendigkeit von prophylaktischen Maßnahmen auf der Hand. Eine der am etabliertesten Ansätze ist die Gabe eines Thrombozytenaggregationshemmer. (3) Der wichtigste Vertreter dieser Gruppe ist die Acetylsalicylsäure (ASS), welche unter dem Namen Aspirin eines der weitverbreitetsten Medikamente darstellt. Im klinischen Alltag wird die gerinnungshemmende Wirkung von ASS oft per Thrombelastometrie (ROTEM) monitiert. (4) Wir verwendeten ein Oszillationsrheometer um ein tieferes, komplexeres Verständnis für die Vorgänge während der Bildung eines Blutgerinnsels zu gewinnen.

Methoden

Wir untersuchten jeweils eine Vollblut – und eine Plasmaprobe der insgesamt 40 in die Studie eingeschlossenen Probanden. Die gemessenen Werte wie CT (Gerinnungszeit), G´init und G´plateau repräsentieren die Festigkeit eines Thrombus und sind vergleichbar mit Parametern des ROTEM. (5) Zusätzlich setzten wir das gebildeten Aggregat Scherkräften mit hohen Amplituden (LAOStress) aus, um dessen Eigenschaften unter extremen Bedingungen zu betrachten. Weiters wurden J – Module (Dynamische Übergang von elastischem Verhalten zu plastischer Deformierung), G´max (Maximale Festigkeit) und breakup stress (Maximale, externe Kraft) berücksichtigt und ausgewertet.

Ergebnisse & Schlussfolgerungen

Unterschiede zwischen Medikamenten – und Kontrollgruppe bezüglich Thrombusfestigkeit, maximal – externe Kraft sowie Neuanordnung der Fibrinfasern wurden sichtbar, waren jedoch nicht signifikant. Diese Erkenntnisse tragen ein Stück weit zum Sicherheitsgrad von ASS bei.

Durch Großteiles positive Korrelationen zwischen Thrombozyten / Fibrinogen Konzentration und unseren Messwerten wird die Schlüsselrolle der beiden Einflussgrößen einmal mehr untermauert. Obwohl Blutplättchen und Fibrinogen Konzentration nicht nur die Bildung sondern auch das Dehnungsverhalten von Thromben beeinflussen, scheint es als würden Thrombozyten maßgeblich in der kinetische Phase (Formation des Gerinnsels) wirken während die Fibrinogen Konzentration den Übergang von Elastizität zur plastischen Deformation diktiert.

Abstract

Introduction

In the 21st century, cardiovascular diseases are still leading cause of death, globally. (1) A part of this clinical picture, including insults, embolisms and acute vascular obliterations, can be triggered by atherothrombotic event. Because of progressively increasing risk factors (hypertension, diabetes, hypercholesterolaemia, obesity, age, etc. (2)) in the modern world, the importance of prophylactic countermeasures is obvious. One, well established approach, is the use of antiplatelet therapy. (3) The lead substance in this field is acetylsalicylic acid (ASA), commonly known as aspirin. The influence of ASA is often measured via rotational thrombelastometry (ROTEM) in clinical everyday life. (4) We used oscillatory shear rheometer to add deeper, more complex knowledge about the forming clot under antiplatelet therapy.

Methods

We analysed one whole blood and one plasma sample of all the 40 included patients. The measured values like CT (clotting time), G'_{init} and $G'_{plateau}$ correspond with stiffness and are comparable to merits of ROTEM. (5) In addition, we exposed the formed clot to high shear stress amplitudes (LAOStress) to examine its abilities under extreme circumstances. J – moduli (dynamic change from elastic behaviour to plastic deformation), G'_{max} (final stiffness) and breakup stress (maximal external force) were taken into consideration.

Results & Conclusion

Differences between treatment and control group regarding clot stiffness, clot breakup and the rearrangement of fibres were implied but not significant. These findings add a level of safety to the established use of ASA. Our data underlined the key role of platelet count and fibrinogen concentration, as were positive correlations for the better part of values.

Although platelets and plasma fibrinogen concentration regulate clot formation and distension, platelets appear to be more eminent in the kinetic phase while the fibrinogen concentration dominates clot deformation.

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1. Background

1.1. Blood

Blood is a suspension, which is composed by a liquid - called plasma - consisting mostly of water (90%), and solid parts like erythrocytes, thrombocytes and leukocytes. (6) A third important group of substances circulating in a solved or bound state are the electrolytes like calcium, sodium, potassium and magnesium, and small molecules and proteins. While circulating through an over 100.000 km long system of vessels, blood is reaching all cells in the human body. Every part of this "vital fuel" is responsible for essential tasks, containing the transport of oxygen and carbon dioxide, the protection of our organism from germs, the regulation of our temperature and many more. One process, which this work is committed to, is the ability of blood to change its state from fluid to solid during coagulation. (7) This mechanism requires certain basic parameters to be within physiological range, which are described in the following paragraphs.

1.2. Physiological conditions and blood components

Under normal circumstances certain parameters should be within predetermined ranges to qualify as physiological. A deviation can be caused by numerous events like a problem of ventilation (hyper- or hypoventilation), a lack in nutrition, intoxication, trauma just to name a few. Hereinafter are the most important blood components, including reasons of deviation, consequence of concentration levels and general facts, cited.

1.2.1. pH

The physiological range of the pH is 7.35 and 7.45, which is mostly influenced by the amount of CO_2, O_2 and HCO_3^- circulating in the bloodstream. If this parameter drops beyond 7.35, which can be caused by diarrhoea, hypoventilation or badly treated diabetes mellitus, it is called acidosis. Alkalosis on the contrary, results if the pH breaks above 7.45 because of bulimia nervosa with frequent loss of stomach acid or hyperventilation. (8)

1.2.2. Temperature

A healthy adult should have a body temperature between 36 °C and 37 °C. In situations of great physical effort, it can rise to 39°C but during normal circumstances, everything above 38°C is categorized as hyperthermia or fever. (9) Keeping this parameter within its range is vital because higher or lower temperature is strongly influencing processes like the transport of oxygen and the tendency of coagulation. (10)

1.2.3. Electrolytes

This group, also known as alkali metals, are highly important for numerous processes like keeping the electric potential in and around cells stable, functioning as co – transporter in absorption operations or playing a big part in the coagulation cascade. In the following table are the main electrolytes and their functions listed. (11)

Electrolyte	Function
Ca^{2+}	• 99% of all calcium in the body is bound as hydroxylapatit ($Ca_5[PO_4]_3OH$) in bones • intracellular, it is part of the signal cascade responsible for migration, secretion, metabolic changes, mitosis and muscle contraction • extracellular, it regulates the tendency of neuromuscular activity and controls the initiation of the coagulation cascade as well as the complementary system
Na^{+}	• the main task of sodium, with an intra – to extracellular ratio of 1 : 40, is to ensure the electric potential on the cellular membrane • sodium is also a key fragment in the osmotic regulation
K^{+}	• potassium is the most important intracellular electrolyte • it is involved in the intercellular communication, the regulation of heart contraction, the blood pressure and the acid – base – balance
Mg^{2+}	• half of all magnesium, like calcium, is bound in bones and teeth • the rest is part of the stimulus transmission and essential for the activity of many enzymes • furthermore, its metabolism is close – knit connected to the metabolism of calcium and potassium

Table 1.2.3.: Overview of electrolytes

1.2.4. Erythrocytes (red blood cells, RBC)

The main function of these components is transporting oxygen to the cells and removing carbon dioxide by free diffusion. Over the course of erythropoiesis in the bone marrow, the red blood cells are produced and released as bi – concave plates, in which cell organelles like ribosomes, mitochondria and a cell core are absent. This circumstance causes their inability for mitosis and necessity of anaerobic glycolysis for the purpose of generating energy. Haemoglobin is the most important molecule in erythrocytes, which empowers them to transport oxygen and carbon dioxide and causes the red colour of the blood. Furthermore the lifespan of these blood cells is limited to 120 days and the physiological ranges for RBC count should be between 4.8 – 5.9 Mio./µl for men and 4.3 – 5.2 Mio./µl for women. (12) Other important parameters, especially for basic labor diagnostics are:

-) MCH (Mean Corpuscular Haemoglobin – p.r. = 28 – 33 [pg 10^{-12} g])

→ serves as a main parameter for the diagnosis of anaemia

-) MCV (Mean Corpuscular Volume – p.r. = 83 – 97 fl [90 x 10^{-15} liter])

→ This is an important merit to determinate the origin of an occurring anaemia

-) MCHC (Mean Corpuscular Haemoglobin Concentration – p.r. = 30 – 36 g/dl)

→ MCHC – value is an additional factor to investigate the trigger of anaemia

1.2.5. Leukocytes

These, also called white bodies due to the absence of haemoglobin, including a huge diversity of cells and are mainly responsible for the defence of our organism against germs. The largest fraction of leukocytes is produced in the bone marrow and they differentiate, depending on certain growth factors, to their final type there. In contrast to red blood cells, white blood cells have a cell nucleus and are therefore able to pursue mitosis. In terms of diagnostics, two pathologies can be defined, which are leukopenia if the cell number is under 4000 cells/µL and leucocytosis in case the result of the flow cytometry is above 10000 cells/µL. This family of blood components can be divided into 3 general groups, which are structured in the table below. (Note that also the subgroups, marked with "▫", can be further categorized)

Group	Members	Function
Granulocytes	▪ Neutrophilic granulocyte ▫ Band neutrophile ▫ Segmented neutrophile ▪ Eosinophilic granulocyte ▪ Basophilic granulocyte	▪ They are capable of active migration to detect and neutralize germs. ▪ high levels of granulocytes can be found in case of infection, intoxication or allergic reactions.
Lymphocytes	▪ B – lymphocyte ▫ Plasma blast ▫ Plasma cell ▫ memory B cell ▪ T – lymphocyte ▫ cytotoxic T lymphocyte ▫ T helper cell ▫ suppressor T cells ▪ NK – cell (natural killer cell)	▪ Their main purpose is the defence against infections with the secretion of anti-bodies, initiation of apoptosis and presenting harmful substances to other parts of the immune system.
Monocytes	▪ Monocyte	▪ They circulate in the bloodstream for 12 – 48 hours, can migrate actively into tissue and differentiate there to their situational needed form

Table 1.2.5.: Three groups of leukocytes

1.2.6. Thrombocytes

Platelets, as their name indicates, are flat, round, core-less, 1 – 4 µm big, and 0.5 – 0.75 µm thick blood components who are produced by constriction from megakaryocytes in the bone marrow. Under the microscope the cytoplasm can be divided into two zones. The granulomere, which is centred and compounded by mitochondria, glycovesicles, lysosome, von – Willebrand – factor, fibronectin and many more. The peripheric hyalomer is rich in actin, myosin and tropomyosin, which form together the so-called microfilaments. These filaments govern the shape of thrombocytes and play an essential role, combined with the calcium-storing rough endoplasmic reticulum, during aggregation. (13)

Platelet activation is initiated by binding of von Willebrand factor (vWF) to glycoproteins located on the outside of their membrane, mainly by the collagen - receptor Ia/IIa and the fibrinogen - receptor IIb/IIIa,. (14) Platelets circulate in the bloodstream until they are removed after five to twelve days by the spleen, liver and lunge. If they are activated by an injury, they adhere to the wound so that the damaged vessel is closed. The physiological ratio is 150 000 – 350 000 thrombocytes per µL and is mostly screened preoperatively or in case of emergency

interventions. A value below 150 000 units/ µl is called thrombocytopenia, (15) which can be caused by Fanconi – anaemia, destruction of bone marrow, (16) huge blood loss, vitamin B12 deficiency (17) and folic acid and disseminated intravascular coagulation. (18) As a result of the reduced or even blocked coagulation, operations and procedures may lead to a lethal outcome. A thrombocytosis on the other hand is defined as a value above 450.000 units / µL and can be divided into two groups. The primary thrombocytosis, also called essential thrombocytosis, as a result of myeloproliferative neoplasm associated with a constant overactive distribution of platelets from the bone marrow, which increases the risk of thromboembolic events. The more common and less serious secondary thrombocytosis is caused by pregnancy, iron deficiency anaemia, infections or drugs like glucocorticoids and erythropoietin. (19)

1.3. Hemostasis

1.3.1. General

The term hemostasis comes from the Greek roots “heme” (blood) and “stasis” (stop, hold). Hemostasis is the combination of all underlying processes that have the objective of stopping or arresting unwanted blood flow. Therefore, also medical interventions like vessel stitches, compression bandages and hemostyptics (drugs that accelerate blood clotting) (20) apply in the broader sense as hemostasis. Nevertheless, the purpose of this work is discussing the internal part such as primary and secondary haemostasis including intrinsic and extrinsic systems with their collective pathways.

Blood under normal circumstances having a certain body temperature, a physiological ratio of pro– and anticoagulant factors, and normal levels of calcium, Vitamin K and thrombocytes is balanced in favour of anticoagulation. (7) A dysregulation in the coagulation system can lead to serious health issues no matter if it results in hypercoagulability syndromes or in hypocoagulability. (21) While the tendency for clot formation increases the risk for a deep venous thrombosis (22), pulmonary embolism (23) or arterial – obstructive events, a lack of pro- coagulant factors can lead to spontaneous, inner bleeding or life-threatening blood loss during surgery. The process of hemostasis gives the body the ability to reduce blood loss and combines the following steps. The process starts with vasoconstriction, caused by serotonin and thromboxane A2, leading to a deceleration of blood flow and an increase of wall shear stress[4] within the affected area. The following part is the adhesion and activation of thrombocytes, which is reinforced by the decreasing speed and volume of blood passing the injury. Finally, as a product of the plasmatic coagulation, an aggregate formed by fibrin polymers and platelets closes the damaged vessel.

1.3.2. Classification and pathways

Because of the complexity of hemostasis, two major phases can be described. Each of them differs in certain points of view, like “time of action”, dominating substances and final tasks. Its important to note, that while in theory, time intervals like the beginning and the ending of phases can be measured and pointed out separately, the majority of steps throughout coagulation take place simultaneously and not parted. The most general classification is the structure of primary hemostasis, including vasoconstriction, adhesion and aggregation, and secondary hemostasis, which can be further broken down into an intrinsic and extrinsic pathway.

1.3.2.1. Primary hemostasis

This initial part is responsible for the acute, most rapid prevention of blood loss within one to three minutes after an injury. Smooth muscles in arteries and arterioles, further proximal located than the vessel damage, are contracting because of increasing levels of angiotensin II, antidiuretic hormone (ADH), thromboxane, adrenalin or noradrenalin, diminishing blood flow. (24) If the vessels inner surface gets damaged, important anchor proteins from the extracellular matrix like collagen, fibrinogen and proteoglycan lay bare, starting phase two - adhesion. This allows the von – Willebrand – factor (vWF) to link with collagen and thrombocytes over their GPIb receptor, as well as fibronectin, a part of αMβ2 integrin, connecting thrombocytes to proteoglycan and collagen. (25)

These two major engagements create a thin but first layer of protection over the affected locus. For platelet aggregation, the last step of primary haemostasis, GPIIb / IIIa receptors are expressed on the extracellular membrane of activated thrombocytes empowering them, in combination with vWF, to crosslink in absence of fibrinogen. This mentioned activation of platelets takes place after an exuberant stimulus of a foreign surface or agonists like collagen, thrombin, adenosine diphosphate (ADP), or adrenalin. Furthermore, the appearance of the cells switches from their passive plate shape to a spherical V-shape with several pseudopodia differing in length. (26)

In the following chain reaction, more thrombocytes attach to the forming clot and activate coagulation factor X, which is a main substance of the secondary hemostasis. This blood clot is instable and reversible, to the point of certain mediator levels and missing fibrin connections.

1.3.2.2. Secondary hemostasis

The secondary hemostasis needs six to ten minutes and is the actual part of blood coagulation, establishing a solid fibrin network with embedded thrombocytes and erythrocytes. According to the classic model an intrinsic and an extrinsic system can be differentiated as part of the cascade. For better illustration and due the variety of factors involved, please find a scheme below of what will be discussed in the next paragraphs.

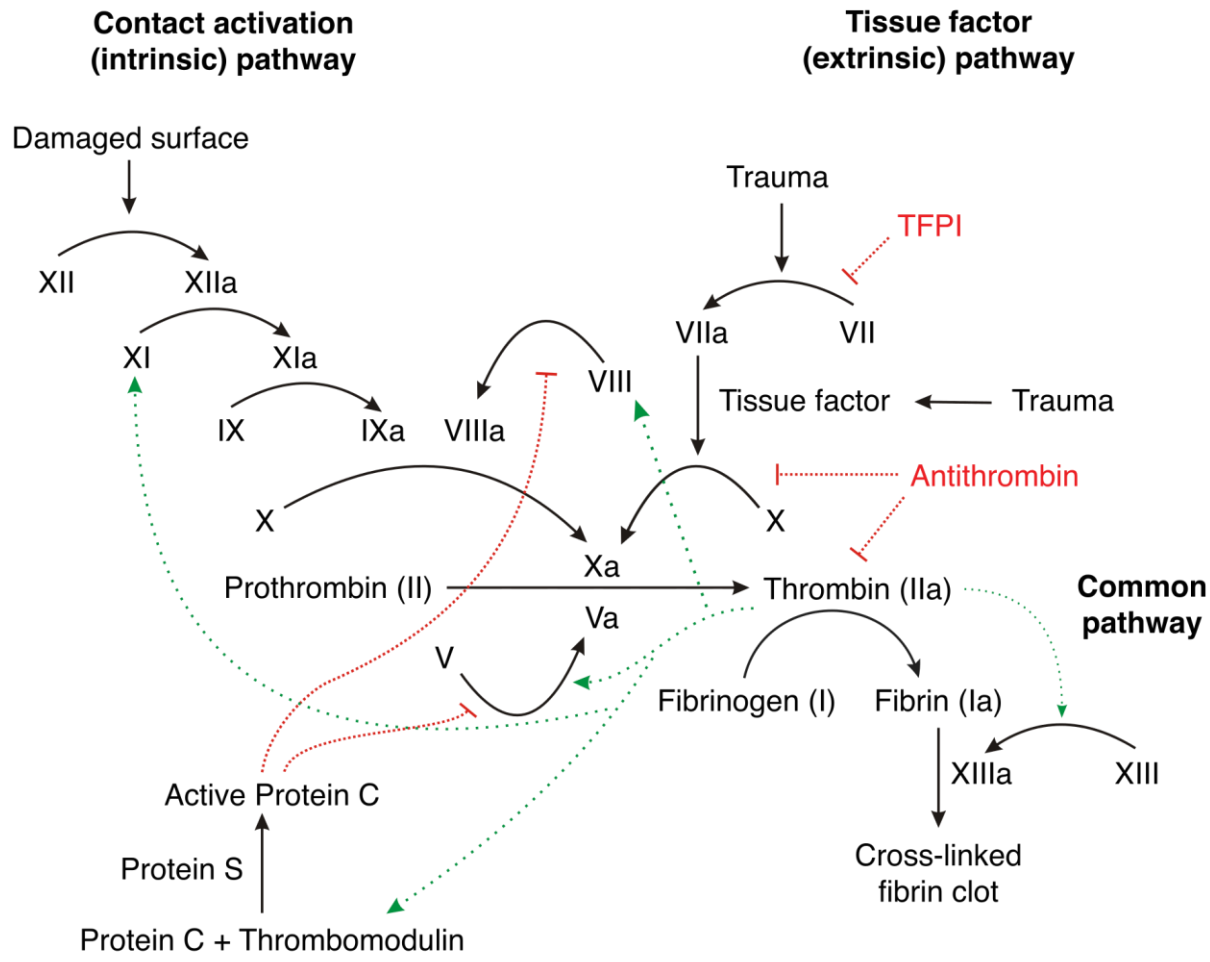

Figure 1.3.2.: Hemostasis (Coagulation cascade)

1.3.2.2.1. Intrinsic system

The main operational area of the intrinsic system is the thrombocytes extracellular membrane where its purpose is the transformation of fibrinogen to its active metabolite fibrin (detailed described in 1.3.4.). This reaction is not only triggered by exposure of subendothelial tissue but also other foreign surfaces like a central venous catheter (CVC) or an artificial heart valve, justifying the importance in clinical everyday life. The initiation takes place when the Hageman – Factor, f.(XII), meets a negatively – charged surface self – reinforced by kallikrein, causing

a change of state from f.(XII) to the active f.(XIIa).
f.(XIIa) and over positive feedback of thrombin f.(XI) is converted to f.(XIa), which consequently activates f.(IX). Simultaneously thrombin forces f.(VIII), a glycoprotein produced by endothelial cells and megakaryocytes, out of his bond with vWF and transforms it to f.(VIIIa), a factor three to the power of ten catalyst for the next step.
f.(IXa), with his co – factor f.(VIIIa), connect to a complex that activates f.(X) to f.(Xa) leading to the collective, final pathway of intrinsic and extrinsic system. (26)

1.3.2.2.2. Extrinsic system

For the most cases the triggering event is a damaged endothelia associated with contact of f.(III), which is technically a receptor, and f.(VII) resulting in the creation of f.(VIIa) which afterwards is able to activate, in addition with calcium, the so called Stuart – Prower - Factor or f.(X) to f.(Xa). From here on it flows into the same shared pathway as the intrinsic system. (26)

1.3.2.2.3. Collective pathway

From the activation of f.(X) into f.(Xa) till the end, both system have this pathway in common. f.(Va) and f.(Xa) combined, in presents of calcium ions, form together the prothrombinase – complex. This molecule is able to catalyse the transformation from the inactive prothrombin (f.(II)) to its active form, thrombin (f.(IIa)). Now, thrombin can dissociate from the surface of the platelets and cleave fibrinogen (f.(I)) into the smaller fibrin monomers (f.(Ia)). Furthermore f.(IIa) is stimulating the formation of f.(XIIIa.), which is responsible for the cross – linking of f.(Ia), and ensures a positive feedback of f.(VII) to f.(VIIa), leading to more prothrombinase – complexes.
Finally, a few minutes after a solid clot has formed, serum is pressed out of it by the retraction of the network. The result is an even stronger clasp of the underlying defect. (27) (28)

1.3.3. Thrombin

Thrombin, also known as f.(IIa), is synthesised in the liver and circulates as prothrombin, which consists of 579 amino acids after translatory modifications, in our blood. The prothrombinase – complex cleaves prothrombin to thrombin, transforming it into a protease and enabling the two major abilities:

- *Plasma protein – effect*

  As the name indicates, the first main assignment of thrombin is the interaction with other plasma proteins, which are involved in the coagulation cascade. It catalyses the cleavage of fibrinogen to fibrin and promotes the activation of the factors V, VIII and XI resulting in a positive feedback loop. (29)

- *Vascular cell – effect*

  This pathway is mediated by thrombin receptors or protease – activated receptors (PARs) which are dominantly found on the surface of thrombocytes and smooth muscle cells. There they enable platelets and are in involved in proliferation, migration and atherosclerotic processes within smooth vascular muscle cells. (30)

The so – called thrombin inhibitors comprise a group of substances, which decrease the prothrombotic effect of thrombin. The most important representatives are Dabigatran, Argatroban, Hirudin, Lepirudin, Desirudin and Bivalirudin, which are all direct anticoagulants. An example for an indirect inhibitor would be heparin because it increases the affinity of antithrombin, a physiological negative feedback protein, to thrombin by a factor 1000. (31)

1.3.4. Fibrinogen and Fibrin

These two molecules are highly important because fibrin is the driving force especially for the elastic behaviour of the clot while under shear stress and the fibrin network´s final structure. Fibrinogen is encoded in the distal cluster of chromosome 4 (32), produced in the liver and consists of three polypeptide chain pairs (33), designated Aα, Bβ, and γ. These fibrinopeptides only represent 2 % of the total molecule mass, their absence changes the fibrin solubility radically. (34) (35)

Fibrin network

- Thrombin – depended cleavage of fibrinogen

  Thrombin cleaves A fibrinopeptide and B fibrinopeptide simultaneously. The latter is released in relevant quantity after A fibrinopeptide cleavage. (36) B fibrinopeptides dissolving rate grows by a factor 7 while polymerization, suggesting that it is cleaved from fibrin polymers predominantly. (37) Furthermore, thrombin has a strong bond with fibrin polymers within the forming clot, leading to a slower dissociation in comparison to thrombin adsorption. (38)

- Polymerization

  After fibrinopeptides A are cleaved, the underlaying binding sites (A or “knobs”) located in the central domain are exposed. Their complementary binding pockets (α or “holes”) are located in the C – terminal gamma chain. The same principle and nomenclature applies to B and β. (39) (40) These regional circumstances cause the monomers connecting to each other in staggered mode showing a 22.5 nm repeat in transmission electron microscopy to generate two – stranded protofibrils in a half – shifted manner. (41) After protofibrils reach a certain length (usually between 600 – 800 nm), they aggregate around their edges and become fibres. (42) α - and β - pockets are always exposed, allowing not only fibrin – fibrin connections, but also fibrin – fibrinogen interactions. (35)

- Crosslinking

  The main player within this process is f.(XIIIa), a plasma transglutaminase, which is fifty - fifty released as platelet factor XIII, a 2-A chain dimer, or circulating as plasma f.(XIII), a 2-A and 2-B tetra chained molecule, inside the bloodstream. (43) In presence of fibrin and calcium, thrombin can cleave the A_2B_2 zymogen form of f.(XIII), leading to a dissociation of B – chains and ultimately exposes the active site cysteine. (44)

  Since plasma f.(XIII) is missing B – chains, it is activated faster than its plasma circulating relative. Despite this temporal difference, both precursors catalyse in their active form the α - α and γ - γ crosslinking in fibrin monomers, oligomers and protofibrils as well as fibrinogen molecules. The resulting covalent bonds increase the resistance regarding mechanic forces and proteolytic processes while clot formation. (45)

- Platelet integration

  As established above, platelets circulate in inactive form though the vessels to prevent spontaneous, unwanted thrombosis upon activation. In case of an injury, fibrinogen and fibrin can connect for instance to endothelial cells and thrombocytes, playing the role of an “anchor piece” vital to adhesion of platelet to the fibrinogen network. Another important function, which takes place after fibrin binds to a platelet, is a reorganisation of the platelet’s cytoskeleton and a higher affinity of integrin receptors for their ligands. Consequently, the bigger molecule, fibrinogen, can bridge two platelets over their activated αIIbβ3, resulting in a crosslinking between thrombocytes. (46)

1.3.5. Plasminogen and plasmin

Because clot formation has a temporary purpose like closing acute bleedings, the human body has ways of dissolving fibrin complexes. As the headline indicates, the most relevant kind is controlled by plasminogen and plasmin.

Circulating plasminogen binds to fibrin, where the already present tissue – type plasminogen activator (tPA) catalyses the production of its active form. The created molecule, plasmin, cleaves fibrin and fibrinogen leading not only to a partial disintegration of the clots network but also a creation and exposition of new binding sites for plasminogen and tPA. The latter represents a positive feedback loop explaining the acceleration of lysis within the process. (47)

1.4. Coagulation disorders

Easy to recognize, that this group of disease is summing up disorders, which have impacts on the above-mentioned process of clot formation, regardless of whether the effect is an acceleration or a slowdown. This hypernym can be further subcategorized by a higher or a lower tendency of haemorrhage and if the condition is inherent or acquired. In terms of diagnostics, the most important steps are a very detailed anamnesis and of course a coagulation diagnostic via laboratory measuring prothrombin time, partial thromboplastin time (PTT) and platelet count. These basic values can be followed by advanced diagnosis like fibrinogen and antithrombin concentration as well as D – dimer and Anti – factor Xa – activity. Due to the fact of enormous variety of coagulation disorders there will be only the most important noted in the paragraphs below.

1.4.1. Haemorrhagic diathesis (Minus - coagulopathy)

This subgroup combines all diseases that affects bleeding tendency in an accelerated way. It can be broken down into 4 categorize including:

1.4.1.1. Thrombocytopathies

- Hereditary/genetic (48)
    - Bernard – Soulier – syndrome *(autosomal recessive inheritance; 1 : 1 000 000)*
    - Storage – pool – disease *(secretion – disorder; autosomal recessive inheritance)*

- Drug – induced *(caused by acetylsalicylic acid, Clopidogrel or chemotherapeutic agents)*

1.4.1.2. Thrombocytopenia

This subgroup is defined with platelets score smaller than 150 000 units/µl. (15)

- Production disorder
    - Wiskott – Aldrich – syndrome *(X – linked recessive inheritance; symptoms: eczema, thrombocytopenia and recurrent infections)*
    - Fanconi and aplastic anemia
    - Bone marrow disease like leukaemia / bone marrow damage caused by drugs or toxic substances *(incl. Myelodysplastic syndrome, thrombocytopenic purpura, heparin induced thrombopenia)*
    - Lack of substrate and factor *(vitamin – B12, folic acid)*

- Shorten lifespan disorder
    - Transfusion accident *(unexpected reaction after a blood transfusion)*
    - Idiopathic thrombocytopenic purpura *(destruction of platelets in the spleen)*
    - Disseminated intravascular coagulation *(intravascular activation of coagulation, resulting in a lack of coagulation factors)*
    - Heparin induced thrombopenia *(a drop of platelet number below 50% of the baseline value after a heparin therapy)*

- Distribution disorder
    - Splenomegaly *(gain in volume of the spleen)*

1.4.1.3. Coagulopathies

Coagulopathies can be genetic or acquired and are disorders regarding plasmatic coagulation or fibrinolysis. (49)

- Inherent *(mostly caused by the lack of one or more coagulation factors. Haemophilia and Von – Willebrand – Jürgens – Syndrome represent more than 95% of coagulopathies)*
  - Haemophilia A *(f.(VIII) is missing or inactive → disorder of the secondary haemostasis)*
  - Haemophilia B *(f.(IX) – deficiency; X – linked recessive inheritance)*
  - Von – Willebrand – Jürgens – syndrome *(quantitative and qualitative deviations regarding the von – Willebrand - factor)*
  - Rosenthal – syndrome *(missing f.(XI) consequential bleeding after minor traumas around joints)*
  - Hagemann – syndrome *(lack of f.(XII))*
  - Factor – XIII – deficiency *(concentration of f.(XIII) < 1 % of p.r.; tissue repair disorder, increased bleeding)*
- Acquired
  - Vitamin – K – deficiency *(hepatic failure, malabsorption, cholestasis, therapy vitamin – K – antagonists)*
  - Immuncoagulopathy *(anti – bodies against coagulation factors)*
  - Systemic hyperfibrinolysis *(st.p. lunge and prostate operations or malignoma)*

1.4.1.4. Vascular haemorrhagic diathesis

This last subgroup with a higher tendency of bleeding is caused by an exorbitant permeability of the small blood vessels. The optical and most impressive sign of this diseases is subcutaneous - petechial bleeding.

- Inherent
- Morbus Osler *(autosomal – dominant inheritance; pathological dilation of vessels telangiectasis)*
- Acquired
  - Henoch – Schönlein purpura *(IgA – vasculitis, auto - immune)* (50)
  - Senile purpura *(age – related fragility of capillary)*
  - Skorubt *(caused by poor or missing collagen production within the vessels)*

1.4.2. Thrombophilia (Plus - coagulopathic)

In contrast to the above mentioned, this group of disease is characterized by a tendency of blood clotting which can be triggered by a lot of factors. Like the haemorrhagic diathesis, this cluster can be divided in regard of an inherent or acquired ethology. Furthermore, the definition or criteria of a thrombophilic illness can be determent by the so-called Virchow – Trias including,

1.) Existence of a laboratory diagnostic attested hyper - coagulability
2.) Verification of a biochemically, defined defect
3.) Differentiation between primary (inherent) and secondary (acquired) kind of thrombophilia

1.4.2.1. Primary (inherent) risk factors

- Activated protein C resistance *(weak anticoagulant response to activated protein C, leading to an increased risk for venous thrombosis)*
- G20210A – mutation *(this gen is responsible for the expression of f.(II) or prothrombin; the mutation creates an overexpression up to a factor three)*
- Protein C deficiency *(diminished inactivation of the coagulation cascade, caused by a mutation of the protein C gen or a liver damage)*
- Protein S deficiency *(Co – factor for protein C; vitamin K depended synthesized in the liver)* (51)

1.4.2.2. Secondary (acquired) risk factors

- Age (most important factor)
- Adiposities
- Smoking
- Estrogenic contraceptives
- Pregnancy
- Congestive heart failure

While the secondary forms can be observed during different basic disease and conditions, the genetic or primary risk factors are responsible for round about half of all cases reported. This fact consequently indicates that the acquired risk factors, which are mainly caused by an individual lifestyle, can be avoided up to a certain percentage. (52)

2. Anticoagulation

This process is defined by the prophylactic or therapeutic inhibition of hemostasis, caused by an application of anticoagulant drugs (anticoagulants) and distanced from the physiological anticoagulation driven by antithrombin, protein C, α_1-antitrypsin and more. Furthermore, it can be structured into temporary anticoagulation, including pre -, peri – and postinterventional, and long – term treatments. The benefits of such an interference regarding the physiological balance of the haemostatic system must be measured out constantly against the associated increased risk of provoking a lethal bleeding. Addressing this issue and trying to objectify decisions grading scores like the HAS – BLED – Score has been established. These types of systems are not meant to be strictly followed by but can be helpful tools to determinate the best fitting therapy or prophylactic approach for patients individually.

2.1. HAS – BLED - Score

For every attested subcategory there is one point added to the final score. A total of three or higher indicates a higher risk of bleeding. As the score can potentially reach up to nine, this is the value with the most fragile circumstances. (53)

H	Hypertension	1 point
A	Abnormal kidney or liver function	1 point each
S	Stroke in the anamnesis	1 point
B	Bleeding in the anamnesis	1 point
L	Labile INR (international normalized ratio)	1 point
E	Elderly (age > 65)	1 point
D	Drugs or/and alcohol	1 point each

Figure 2.1.: HAS – BLED – Score

2.2. Drugs

2.2.1. Low molecular weight heparin (LMWH)

As their name suggests, this group of substances is similar to conventional heparin but differs concerning shorter carbohydrate – chains, which fluctuates between 4 to 36 single saccharides. Nevertheless, the most LMWH molecules have an average carbohydrate – chain length of 10 – 20 single sugars and middle molecular weight of 3500 to 8000 Dalton.

Effect mechanism

LMWH forms a complex with antithrombin, which is able to inhibit the activity of f.(Xa). The previously discussed shortened carbohydrate chains are responsible for the lower affinity, in comparison to conventional heparin, to prothrombin (f.(II)), resulting in more precise and safer control during usage.

Pharmacokinetics

The bio – availability of LMWH is after subcutaneous application round about 90 %, compared to unfractionated heparin with 20 %, and a biological half – life up to 18 hours, which makes a single dose per day possible. After an intravenous administration, the biological half – life of LMWH moves between 2 and 3 hours, for what reason its the preferred way during operations. Due its renal elimination a restricted or lowered kidney function can lead to accumulation.

Substances

Enoxaparin (Clexane®); Certoparin; Dalteparin; Nadroparin; Reviparin; Tinzaparin

Side effects

local reaction; risk of bleeding; anaphylaxis; thrombopenia (HIT = heparin induced thrombopenia); effluvium; osteoporosis (54)

2.2.2. Penta saccharide

This chemical compound contains five with each other linked monosaccharide – units, which imitate the same effect as heparin.

Effect mechanism & side effects

After binding to antithrombin, the created complex inhibits f.(Xa) and as a consequence the ongoing process of coagulation. Furthermore its higher selectivity for the antithrombin – binding locus and the lack of affinity for thrombin, f.(IXa), platelet factor 4 and thrombocytes accounts for its balmier side effects, especially HIT.

Substances

Fondaparinux (Arixtra®), Idraparinux and Idrabiotaparinux (55)

2.2.3. Coumarin derivatives

Coumarins are synthetic compounds with structural similarity to fragments of vitamin K, therefore they interfere as competitive antagonist in the vitamin – K – metabolism.

Effect mechanism & side effects

More precisely they inhibit the vitamin – K – depended on γ – carboxylation of coagulation f.(II), f.(VII), f.(IX), f.(X) as well as the anticoagulant protein C and S in the liver. The influence on the anticoagulant proteins is the reason for the most feared side effect, the coumarin / warfarin necrosis.

This epiphenomenon is caused by the shorter biological half – life of protein C and S in comparison to the procoagulant factors and justifies the parallel application of heparin. Coumarin derivatives are also embryopathic and can responsible for big haematomas after minor traumas. Note that all coumarine-associated effects occur 2 to 3 days after intake and keep up depending on their half-life, estimated as an average of 29 hours (range 18 to 52 hours)

Substances

- Phenprocoumon (Marcumar®, Falithrom®)

The application happens oral with a nearly full and fast resorption rate. It gets metabolized in the liver and half of the active concentration eliminated by the kidney within 10 to 14 days.

- Warfarin (Coumadin®)

This derivate has very similar properties regarding absorption and metabolism but differs with a middle elimination half – life of 45 hours. (56)

2.2.4. Direct oral anticoagulants (DOACs)

This relatively new cluster enables another therapeutic approach regarding effect mechanism. While the above-mentioned substances are operating as a cofactor to antithrombin causing an affinity increase up to the factor 1 000, DOACs inhibit curtain coagulation factors directly and can be broken down into two groups. The major advantages of them are the easy form of application and the absent necessity of periodic coagulation surveillance.

2.2.4.1. Anti f.(IIa) – type – DOACs

The first group, with its lead substance dabigatran (Pradaxan®), decelerates the activity of thrombin (f.(IIa)) and is mostly used as a postoperative anticoagulants or prophylactic in cases of atrial fibrillation.

Effect mechanism & pharmacokinets

This thrombin – inhibition leads to its key elements of function, which are preventing the conversion of fibrinogen to fibrin and obstructing the thrombin – induced activation of platelets. After oral application of Dabigatran-etexilat (prodrug) and its activation by the liver and blood plasma due hydrolysis the plasma concentration peeks between half an hour and two hours, which can be prolonged post interventional to six hours.

Side effects & contraindications

Similar to other anticoagulants the most important side effect is bleeding, especially during double treatments like heparin or warfarin. Severe renal failure (creatinine clearance < 30 ml/min), liver dysfunctions, artificial cardiac valve and a temporal – over lapsing application of ketoconazole, itraconazole or cyclosporin are red flags for the therapy with dabigatran.

2.2.4.2. Anti f.(Xa) – type – DOACs

The so called factor Xa inhibitors interfere, as their name indicates, with the Stuart – Prower – factor, which consequently is detained from activating prothrombin (f.(II)) to thrombin (f.(IIa)). This group can be sub categorized into direct (NOACs – new oral anticoagulants) and indirect factor Xa inhibitors. The most important members of this group are rivaroxaban (Xarelto®), apixaban (Eliquis®) and edoxaban (Lixiana®). (57)

2.2.5. Non – vitamin K antagonist oral anticoagulants (NOACs)

While the bio – availability of direct anticoagulants like rivaroxaban, apixaban and edoxaban moves between 50 % and 100 %, its biological half – life differs from 9 to 14 hours. The range of indications is very similar to those of anti – f.(IIa) – type inhibitors with an addition of deep venous thrombosis, pulmonary embolism and insult.

2.2.5.1. Indirect f.(Xa) – inhibitors

The most important members of this cluster are danaparoid sodium, low molecular weight heparin and fondaparinux, binds selective to antithrombin III resulting in a factor 300 empowerment of f.(Xa) inhibition. The indications in comparison to NOACs are tendentially, not least because of their non-oral application, used in acute settings like coronary syndromes, myocardial infarction and major interventions. (58)

2.3. Occurring drugs

The following two pharmaceuticals are highlighted because of the recurring importance in this work. This chapter will also cover two essential pathways for better understanding why and how these two drugs are influencing the coagulation cascade.

2.3.1. Acetylsalicylic acid (ASA)

This, at the end of the 19th century by Felix Hoffmann discovered, substance can be sorted in the category of NSAIDs (nonsteroidal anti – inflammatory drugs) and is an irreversible inhibitor of the cyclooxygenase. The empirical formula of ASA is $C_9H_8O_4$ and its molar mass is 180,16 g/mol. Depending on dosage three effects can be seen: (59)

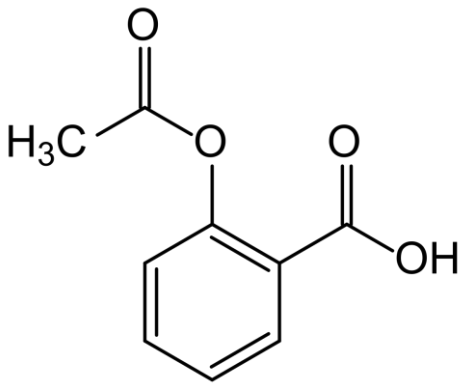

Figure 2.3.1.: Structure formula of acetylsalicylic acid

- 30 – 50 mg → the aggregation of platelets is inhibited
- 0,5 – 2 g → the decentral perception of pain is lowered
- 2 – 5 g → antiphlogistic and antipyretic effects occur (obsolete)

2.3.1.1. Cyclooxygenase

Cyclooxygenases (COX) are enzymes within the arachidonic acid cascade where they catalyse its transformation to prostaglandins and thromboxane. Prostaglandin, a substance belonging to the group of eicosanoids, is involved in the process of pain mediation and inflammation. (60) The creation of thromboxane by fusion of fatty acids largely takes place in thrombocytes, including the most important member, thromboxane A_2. This particular associate, in combination with protein G, plays a great role inducing vasoconstriction, promoting the aggregation of thrombocytes and draining thrombocyte granules.

Because of the irreversible inhibition of cyclooxygenase and the fact that thrombocytes are unable to reproduce COX, the effects of ASA last for the life span of platelets. As it has been established above those levels for antiphlogistic and antipyretic purpose are no longer administrated, because of the risk for side effects in higher dosage, acetylsalicylic acid is indicated within a wide field of medical conditions such as: (61)

- *Minor to medium pain*, like headache, back -, joint -, muscle – and periodic pain
- *Inhibition of thrombocytic aggregation*
  - unstable angina pectoris
  - acute myocardial infarction
  - reinfarction prophylaxis
  - postinterventional after PTCA (percutaneous coronary intervention) or stent implantation
- *prophylactic treatment* for TIA (transient ischemic attack) or ischemic stroke

Toxic adverse effects start occurring with a daily intake of six grams, which is the reason why the anti-inflammatory and fewer lowering impacts are obsolete nowadays. The unwanted events reach from gastrointestinal discomfort to renal dysfunction up to neurological symptoms like tinnitus and vertigo. Two phenomena are to be emphasized:

- *Ion trapping*

  This term defines the accumulation of ions in a cell or a cell compartment based on different pH – depending lipophilicity. While 50% of ASA (pKa = 3,5) is ionized within the gastric mucosa (pKa = 2) it nearly dissociates completely after entering the cell, causing an increase of intracellular protons (H^+). Because of this damaging potential and the risk of ulcer progression, ASA is normally taken in combination with a proton pump inhibitor.

- *Reye´s syndrome (white liver disease)*

  This acute encephalopathy mostly occurs three to five days after an infection of the upper respiratory system and the administration of salicylic drugs. The symptoms range from emesis, somnolence and lethargy to severe complications like brain oedema, spasm, dispone and coma. Reason for this potentially lethal condition is a malfunction of mitochondria within the liver tissue, leading to high levels of neurotoxic ammonia and lactate. (62)

Acetylsalicylic acid is a compound of well-known pharmaceutics like Aspirin® and Thomapyrin® but we will focus on the treatment with Thrombo ASS® (100mg)

2.3.2. Clopidogrel

This substance, a derivate of thienopyridine (Iscover® and Plavix®), using a different approach to inhibit the aggregation of thrombocytes. Its empiric formula is $C_{16}H_{16}ClNO_2S$ with a molar mass of 321.82 g/mol, and its main target is the $P2Y_{12}$ - receptor on platelets. (63)

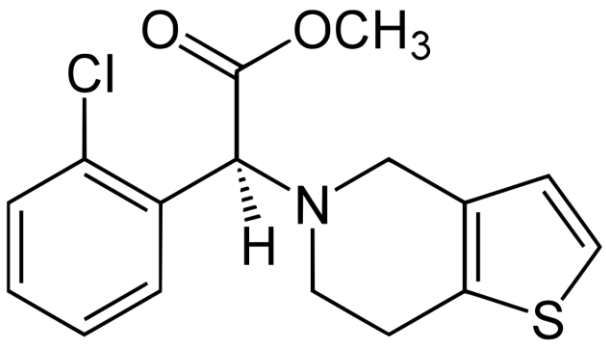

Figure 2.3.2.: Structure formula of clopidogrel

2.3.2.1. P2Y12 - receptor

The purpose of this G – protein linked receptor, which is located on the surface of thrombocytes, is regulating the aggregation. ADP (adenosine diphosphate), the physiological ligand, triggers the active form after binding and initiates this pathway of the coagulation cascade.

There are four main members of the so-called ADP – receptor – blockers who are all working according to their name but mention that there are differences regarding time of impact. While Clopidogrel, Ticlopidin and Prasugrel form an irreversible inhibition lasting for the whole life span of platelets, Ticagrelor´s effect is shortened by its reversible binding behavior.

As Clopidogrel is only a prodrug, it passes through oxidation then hydrolysis by the cytochrome P450 – system (more specific CYP2C19 enzyme) within the liver before it is transformed to his active metabolite. This substance binds very selective to the $P2Y_{12}$ – receptor and consequently inhibits the ADP – depending, clot forming GPIIb / IIIa – pathway.

Because of the cytochrome P450 empowerment close attention must be payed regarding individual fluctuations of blood levels and their accompanied differences concerning effect peak and biological half-life. Furthermore, interactions while simultaneous administration with antidepressants, PPI and tranquilizers, who share the same liver metabolism, can possibly occur. (64)

Indications and side effects

The monotherapy with Clopidogrel is approved for secondary prophylaxis after myocardial infraction, ischemic stroke, haemodialysis and PAD (peripheral arterial disease). The dual therapy, combined with acetylsalicylic acid, is established in cases of acute coronary syndrome or as off – label – usage after interventions like coronary stenting and PTA. (65)

Possible side effects are bleedings, hematoma, haematuria, diarrhoea and rarely leukopenia.

3. Rheology

The name originates from the Greek "ῥέος"– rheos, meaning „stream" and it describes the behavior of materials exposed to a shear deformation, in this case blood or more specific the forming clot. As rheological properties are relationships understood between stresses and strains, they can be measured by setting these factors in proportion.

3.1. Terminology and Definitions

3.1.1. Viscoelasticity

Viscoelasticity is the attribute of a material that displays not only viscous but also elastic behaviour subsequently to external force, and a change of viscoelasticity occurs within the process of clot formation. This perception is the reason why the following definitions, physical quantities and formulas are essential whenever working with suspensions like blood. (66)

Viscosity

Liquids are composed of molecules, which can differ in size and weight. If a liquid is forced to move, these molecules slide along each other. Due to internal friction a flow resistance emerges, which fluctuates with temperature, surrounding surface (adhesion), applied pressure and deformation. In general, if low viscosity fluids are driven by the same pressure difference as a high viscosity fluid, they flow faster, which can be mathematically depicted by Hagen – Poiseuille´s law.

This law states that the blood flow (volume per time) is directly proportional to the difference of pressure and to the fourth potency of the tube radius. (67)

$$V = \frac{r^4 * \prod * \Delta P * t}{8 * \eta * l}$$

Elasticity

This term describes the ability of a material to return to its original state after an external force is released. It describes the solid-like property of materials. The more elastic a subject is, the more it will remain its shape when exposed to an external force, or alternatively, it will break when the deformation becomes too high. (68) This phenomenon is based on the atomic structure of solids and explained by Hooke´s law. Solids, especially metals, maintain their lattice texture via strong ionic linkage or metal bonding. Suspensions and gels, however, have also viscous

(fluid-like) properties, and keep their integrity by non-covalent bonding, resulting in a weaker molecular assembly that allows yielding or strain stiffening. (69)

3.1.2. Plasticity

Plastic deformation describes an irreversible change of an object´s structure, not only from a visual standpoint but also on the molecular level, responding to a certain amount of stress. Most materials yield when they transition from elasticity to plasticity (70), fibrous materials can undergo a period of stiffening prior to yielding.

3.1.3. Shear

When energy is applied to a unidirectional motion system, the components respond, depending on their physical properties, for instants molecular composition, temperature and pressure, with some form of loading due. The five fundamental types of loading are compression, tension, torsion, bending and shear. (71) The last one is used in rheology and will be discussed below.

Shear stress (τ)

Shear stress (τ) is defined as the shear force (F) [N] per cross – sectional area (A) [m²]. Its unit is N/m² which is equivalent to 1 Pascal (Pa) and therefore can be calculated as follows: (72)

$$\tau = \frac{F}{A}$$

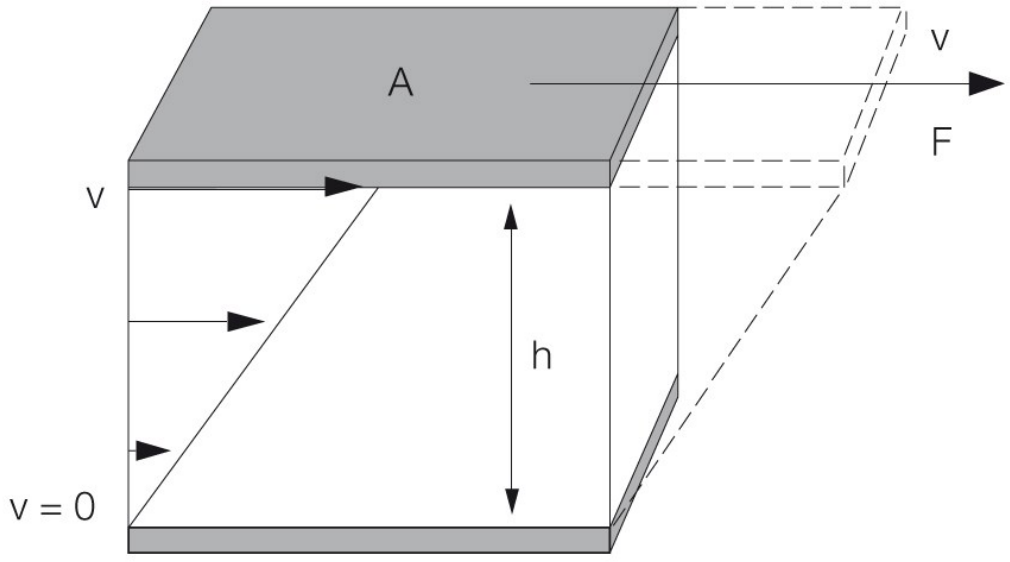

Figure 3.1.3.1: Calculation of shear stress using the two – plates model with shear area (A), gap width (h), shear force (F) and velocity (v).

Shear strain (γ)

Shear strain, also called shear deformation, can be understood as the result of shear stress. In consequence of applied shear stress, the substance between the two plates tries to compensate the kinetic absorption by shifting towards the edges. Since both involved magnitudes, not only the deflection path (s) but also the shear gap (h), have meter as their unit, the shear strain has no dimension, is normally stated in percentage and calculated as followed: (72)

$$\gamma = \frac{s}{h}$$

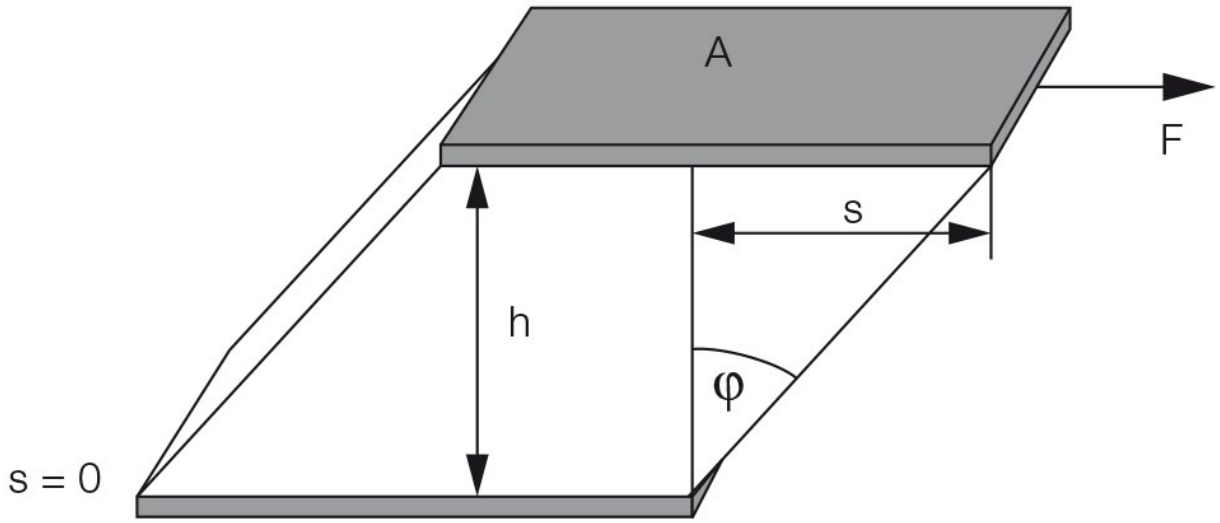

Figure 3.1.3.2: Model for better understanding of a shear test, based on a two-plate run. The illustration includes shear area (A), gap width (h), shear force (F), deflection path (s) and the deflection angel (φ).

3.1.4 Phase shift (δ)

Phase shift is measured when stress or strain are applied in oscillatory mode. Over the course of every cycle the deflection angel (φ) is recorded, showing the shift of the output phase (e.g. shear deformation) in relation to the input phase (e.g. shear stress). If a material is purely elastic, the input and output signals are overlapping. In fluids, the phase shift is 90°. Because blood has predominantly viscous properties, the output signal is retarded (phase shifted, close to 90°). When a clot starts to form, this phase shift progressively diminishes, but never reaches zero due to the several fluidic properties of fully formed clots stemming from the water content or the gliding of components that results in energetic loss.

3.1.5. Shear moduli

Shear moduli track elastic (solid-like: G′) and viscous (fluid-like, G′′) properties of materials. When a clot forms there is a switch from mainly viscous to mainly elastic. This dynamic change of property can be measured by G′ and G′′.

- Storage modulus G′

  This parameter, also referred to as G prime, is a benchmark of a substance′s tendency to bounce back in its original shape after being deformed by an external force. For a material being able to react in this specific way it must contain an inner structure, which is predominant the case in solids.

- Loss modulus G′′

  As the name indicates, the loss modulus G′′ or G double prime reflects the amount of energy, which is lost during deformation. This energy loss is a result of a phenomenon called internal friction. In a substance with viscoelastic behaviour, the different components interact with each other on a molecular level, leading to the so – called frictional heat. External force that is converted to this specific energy form is no longer available to the system and therefore lost.

- Complex shear modulus G*

  The entirety of viscoelastic behaviour of a sample, under the basic conditions of an oscillatory shear test, can be described by the complex shear modulus G*. For better understanding of the correlations between the three main moduli you will find an illustration on the next page. (73) (72)

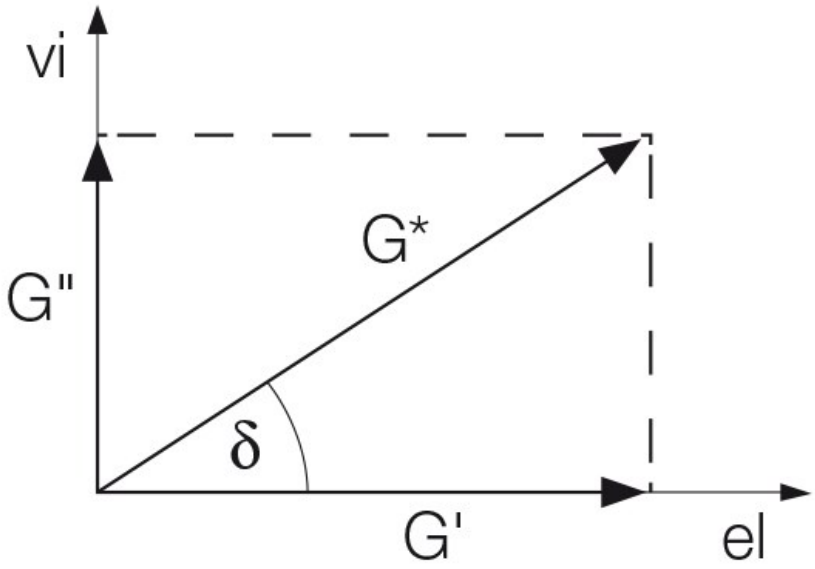

Figure 3.1.5.: This Illustration should help understand the relationship between elastic behaviour (G') on the x – axis, the viscous portion (G'') on the y – axis and their influence on the complex shear modulus G. Furthermore, you can find the phase – shift angel (δ) – between G' and G*.*

Since the point of interest within this work is mainly the elastic behaviour of blood, we will focus on G' and its change over the course of every run. G' is smaller than G'' in the beginning of coagulation because of the mostly liquid properties of blood.. With the progression of procoagulant reactions, the samples composition changes radically in favour of elastic characteristics resulting in an increase of the storage modulus (G') over the loss modulus (G´´) and a subsequent decrease of the phase – shift angle (δ).

3.2. Rheometer (MCR 301, Anton Paar®)

For all sample analyses the rheometer Physica MCR 301 (Anton Paar, Graz, Austria) was used, which is based on a permanent magnet synchronous drive principle including a torque range between 0.1 µNm and 200 mNm with a torque resolution of 0.001 µNm, and an accuracy of up to 0.2 µNm. Its frequency ranges from 10-5 to 100 Hz, and the maximum rise of torque is 1500 Nm/s.

Furthermore, the rheometer is equipped with an optical encoder enabling an internal, digital resolution of 0.012 µrad. (real resolution < 1 µrad).

Figure 3.2.: Exemplary setup for the test runs. The most important components are numbered and explained on the following page. Please find more detailed information regarding manufacturing, operative parameters and technical specifications in the product manual "MCR 301 series E" provided by Anton Paar®.

3.2.1. Part details

1. H – PTD 200

   This Peltier – controlled hood is designed for keeping the sample on a constant temperature level. With its cap – like form, the differences within the forming clot is minimalized while the measurement system stays unaffected.

Figure 3.2.1.1: Thermo - chamber

2. Moving cone: CP50 – 1/S

   The cone (stainless steel) is the upper part of the test system and has the surface roughened to facilitate adhesion of the clot.

Figure 3.2.1.2: Upper plate

3. Caps (hood and evaporation blocker)

   The plastic caps as well as the ring enhance the level of consistency regarding temperature inside the chamber. Please note that the remaining gaps are filled with silicone liquid for the same reason.

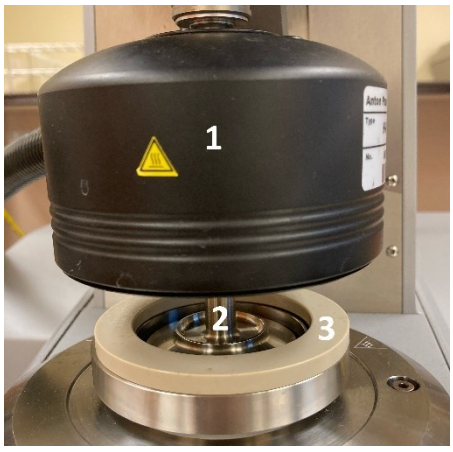

Figure 3.2.1.3: Sealing caps

4. Stationary plate

   The plate is the bottom part of the test system It has the same surface properties like the upper cone.

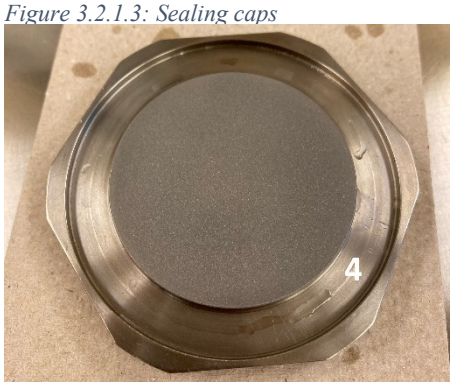

Figure 3.2.1.4: Carrier plate

4. Aims of the study

Since cardiovascular disease and their prophylaxes, acute treatment and postinterventional care, within an aging society, will keep gaining importance, it was our main goal to acquire a deeper understanding of two frequently used drugs regarding molecular effects while clot formation as well as endpoints like yielding and maximal stress amplitudes.

While using rheological analysis, we were looking for similarities as well as differences regarding the kinetic of clot formation, and the deformability of fully formed clots.

4.1. Test parameters: Kinetic

4.1.1. CT (clotting time) ~ *[in minutes]*

We set this parameter to the point within the timely development of clot formation where G´ remains above 1 Pa for the first time. As we established above (q.v. 3.1.3. Shear) that the lead physical properties of blood change over the course of the run from viscous to elastic, this is the first point of interest, corresponding to the gel point or in other words: coagulation time.

4.1.2. $G'_{plateau}$ and $G'\text{-}t_{plateau}$ ~ *[in Pa and minutes]*

These two landmarks represent the finalisation of clot formation (the fully formed clot) and complete the process of the first sample run.

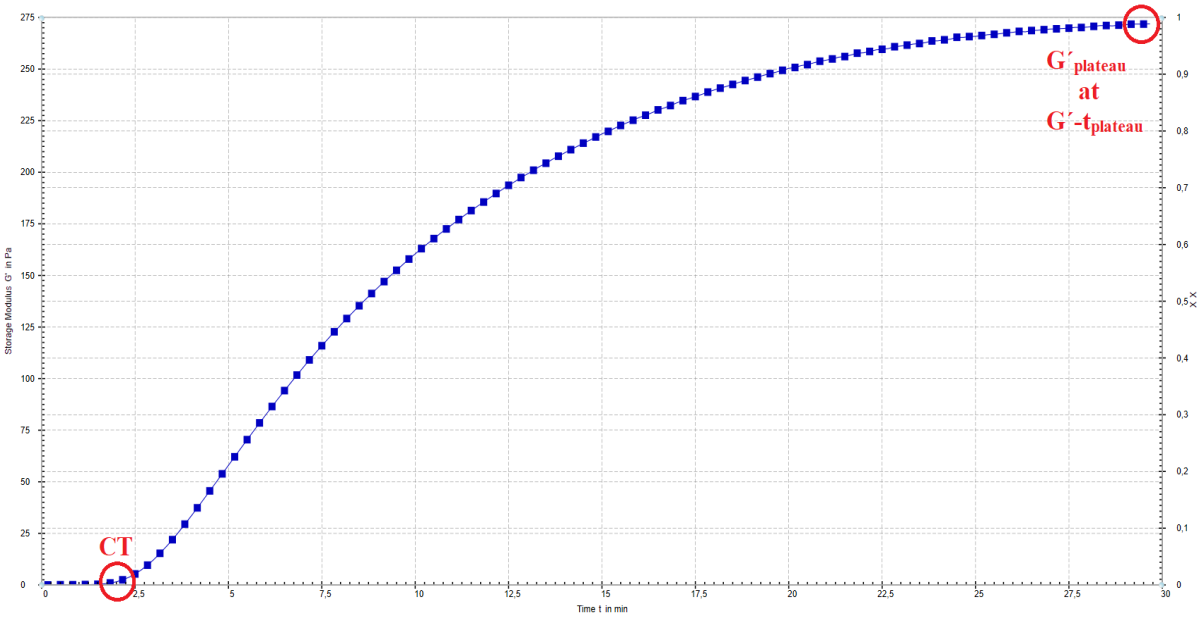

*Figure 4.1.2.: Illustrating an exemplary kinetic curve of a whole blood sample highlighting three important measuring points including CT (clotting time) , **$G'_{plateau}$** (plateau maximum) and **$G'\text{-}t_{plateau}$** (time to **$G'_{plateau}$**).*

4.1.3. Initial clot firmness ~ *[in Pa and minutes]*

This parameter corresponds with the A10-parameter in thrombelastometry. It marks the point of inflection of the kinetic curve and was determined by the use of the maximum of its first derivative, which was determined by the Rheocompass software (compare with Figure 4.1.3). From the x- and y-values of this point, the initial clot firmness (G'_{init}) and the corresponding time ($G'\text{-}t_{init}$) were used as parameters. (5)

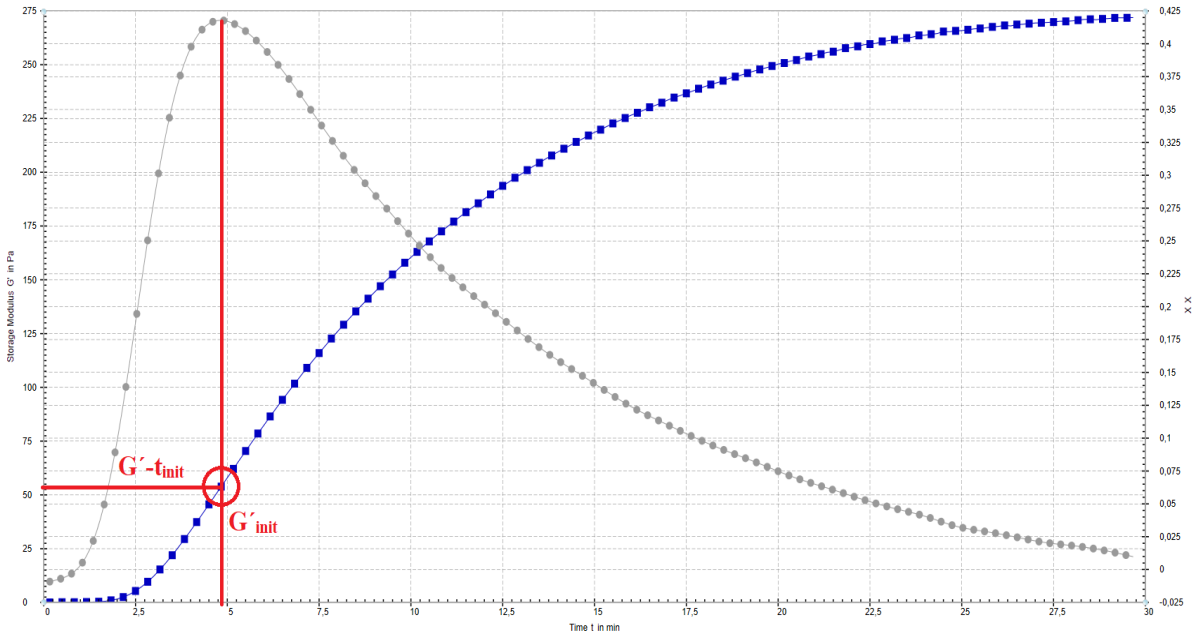

Figure 4.1.3.: This graph displays the same kinetic curve as figure 4.1.2. but in addition of the grey line, which represents the first derivation of G´. This calculation provides the above-mentioned turning point, enabling the determination of G'_{init} and $G'\text{-}t_{init}$.

4.1.4. G´23 and G´20 ~ *[in Pa]*

Because of differing G´ - t plateau merits, we established these two checkpoints to have a clear, objective cut-off for measuring G´ values towards the end of clot formation. For whole blood we set this point to 23 minutes after test begin (G´23). Since plasma tends an increased clotting time, we shortened the equivalent time to 20 minutes.

4.2. Test parameters: Deformability, measured by LAOStress

After the kinetic run reached the G´ plateau values, indicating the fully formed clot (q.v. 4.1.2.), the testing program is switched to LAOStress while the clot remains in the entire test chamber.(5) Now the thrombus is exposed to increasing sinusoidal shear stress amplitudes at constant 1 Hz frequency in a manner described in 3.1.3. As a result of this dynamic force input to the clot, the fibrin network progressively changes its geometric arrangement in a unilateral manner also increasing the clots total stability (making clots stiffer) until it breaks apart when the deformation becomes too high. Before their breaking point, the clot shows a gradual reduction of its integrity (yielding), collapse and destruction. During this metamorphosis, shear stress thresholds and their corresponding J´-moduli (reflecting clot compliances) are determined, representative to several stages throughout this process. (5) The parameters obtained in the LAOStress test are described in the following.

4.2.1. Divergence of J´M and J´L moduli (divergence point) ~ *[in Pa - logarithmic]*

This parameter shows the shear stress needed to drive the clot out of its mechanical equilibrium by cyclic loading. From now on plastic deformation takes place. This parameter was read by the aid of the stress softening ratio: the first value of $\triangle_{soft}$ above 0.005 in the data table was used to determine this shear stress threshold ($\boldsymbol{\tau}_{DP}$). In addition, also the time needed to reach this threshold was taken (t_{DP}).

From this divergence point on, the fibrin fibres start to re-order themself in a unidirectional manner. Clot compliance (both J´-moduli) first increases, reflecting softening (compare with Figure 4.2.2) until J´L reaches its maximum.

4.2.2. J´M_{max} and J´L_{max} with their shear stress thresholds (τM and τL) ~ *[in 1/Pa and Pa]*

At the J´L-maximum, softening switches to partial stiffening. At the J´M-maximum the whole clot starts to stiffen with shear (macroscopic shear-stiffening). The J´M-maximum is an important value since it reflects clot compliance when all fibers are unidirectionally oriented. After this point affine deformation takes place. This means also that the rearrangement of the internal fibrin network - softening (bending of some fibers) together with stiffening (stretching of other fibers) - is finalized. From now on only fiber stretching occurs resulting in a decrease of both compliances until the clot starts to yield (both compliances increase because the deformation is too large). The shear stress thresholds ($\boldsymbol{\tau}$L, $\boldsymbol{\tau}$M) indicate the necessary external force to result in these the metamorphotic changes at J´L_{max} and J´M_{max}.

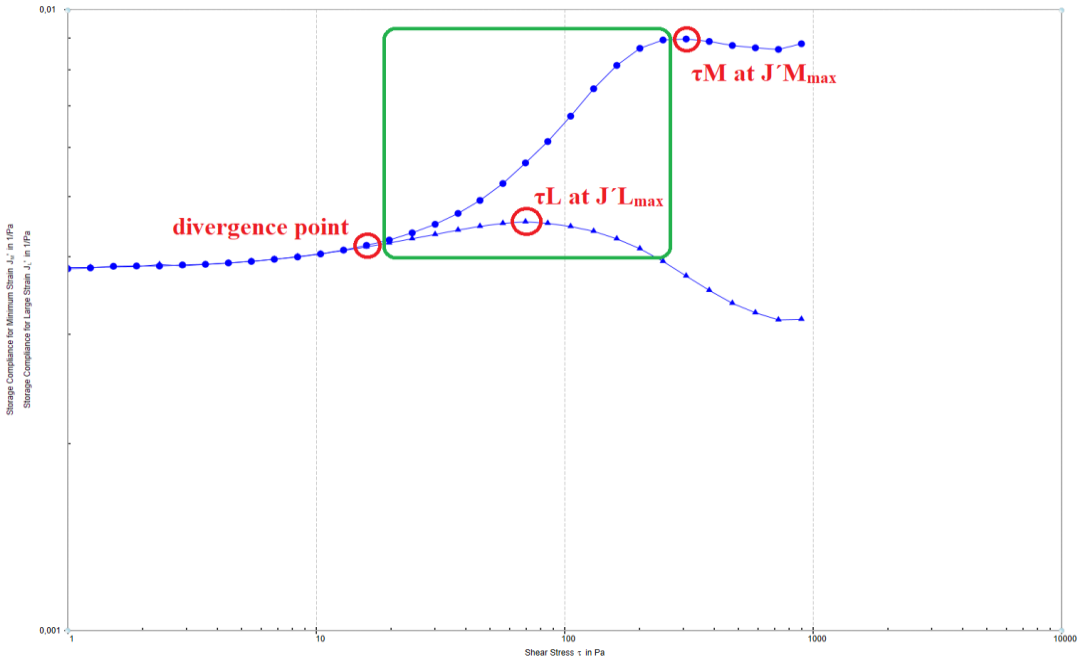

Figure 4.2.2.: J – modules illustrating the divergence point followed by a differing J´Mmax from J´Lmax. Also note the green box between divergence point and J´Mmax, which represents the in paragraph 1.3.4. "Fibrinogen and Fibrin", mentioned rearrangement of the fibrin network.

4.2.3. breakup shear stress ~ *[in Pa - logarithmic]*

This shear stress threshold represents the endpoint of the test and the highest possible shear stress the clot can sustain. It is the last measured value of the sample run. The corresponding G´-value reflects the highest stiffness the clot can attain through stiffening of its fibers before it breaks apart (Figure 4.2.3).

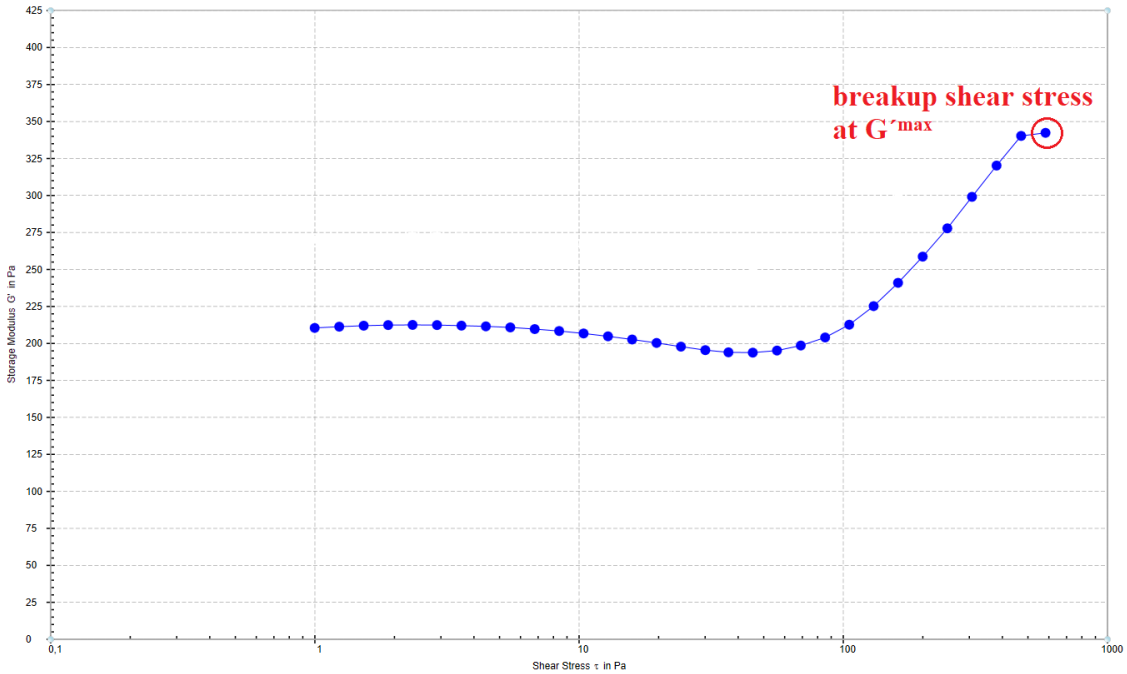

Figure 4.2.3.: This diagram sets shear stress τ and storage modulus G´ in context from the last point of the kinetic run (G´plateau) till break up shear stress with the corresponding G´max, representing the final merit of the rheometric measurement.

5. Study design and sample recovery

5.1. Initial Phase

We started working on the structure of this medical study in November 2018. After we applied the necessary changes regarding patients insurance, samples sizes and criteria of patient suitability, the ethics committee of "Barmherzige Schwestern Linz" which is responsible for clinical trials within the "Vinzenz Group" approved our request in May 2019.

The design can be defined as a non – therapeutical, biomedical study under the premisses of fundamental research with a strong focus regarding angiology, visceral and general surgery.

5.2. Criteria of patient suitability

In the following table one can find inclusion criteria concerning past medical history as well as current state of health and medication.

Criteria of patient suitability	
Signed letter of agreement	YES
Sex	Male or female
Age	40 – 75*
Body mass index	≤ 35 kg/m^2
ASA - Score	≤ III
Admission of heparin containing drugs such as Lovenox, Enoxaprin, Inhixa and others	NO
Admission of contraceptive	NO
Admission of complex, herbal or mineral substances with the purpose of performance enhancement or weight loss	NO
Known coagulative disorder	NO

Table 5.2.: Criteria of patient suitability

**Please note, that there have been made exceptions regarding age for the reason of bigger sample sizing. Despite the increasing interval, all patients were full aged adults as well as politically and medically mature.*

5.3. Standardized process of sample recovery

The main number of patients were recruited in the "Krankenhaus Göttlicher Heiland". In this scenario, the first step was to determinate whether there is a suitable patient fulfilling the criteria, mentioned in paragraph 4.2. If there was a suitable patient the responsible physician was consulted for any circumstances within the ongoing treatment that would prohibit the patient in participating our research. As soon as it was clear that the further process regarding an optimal outcome for the patient is in no way harmed or prolonged, physical and laboratory parameters were added to the sample protocol (q.v. 5.4.). After giving informed consent, three blood samples were taken. One 3.5 mL EDTA tube, was drawn first remove tissue factor from the Vacuette system, followed by two 3.5 mL sodium citrate tubes. The latter were put into insulated bags for transport to the Centre for Biomedical Research at AKH-Leitstelle 1Q. The time from withdrawal to measurement was noted.

5.4. Sample protocol

As mentioned, after the patient signed the letter of agreement, important basic data were noted (q.v. figure 5.4.). Information regarding lifestyle like smoking, last meal and taking the pill, has been obtained by interview before blood draw. The protocol is structured into the following subsections for better survey as well as error prevention.

- Personal data

  General information like sex, age and BMI but also sample number, ASA – Score and group affiliation are taken into registry. Despite the following subsection "Medication", the two drugs acetylsalicylic acid and clopidogrel are separately mentioned in the first paragraph.

- Status post

  A table for gathering interventions, which took place in the last four weeks, was insert in this part of the protocol, to rule out any acute medications with possible coagulation effects.

- Present checkpoints

  This list registers data right before the blood draw as well as shortly after. The most important ones are the nutrition status (has the patient eaten today?), are all boxes checked concerning transportation and the exact time of sample recovery.

- Laboratory values

  The most recent and important blood levels analysed from in vivo whole blood draw are copied from the digital patient file.

- Medication

  All current drugs including dosage and daily intervals are transferred from the patients' chart to this table.

- Time recording

  This paragraph is for timeline documentation throughout the entire sample run. The two main sizes are staring time (time of the kinetic test) and duration (kinetic and LAOStress).

- Run control

  This last section is a final personal reminder for doublechecking the essential settings prior to the test runs. Furthermore, the time of centrifugation is deliberately located at the very end because of the strict chronological steps of procedure, described in "4.5. Process of sample analysis".

Please find the whole illustration beyond but note that the original sample protocol – always connected to the letter of consent – is in German language and has been translated into English.

Sample protocol

Number: _____ ASA – Score: _____
Sex: M / W BMI: _____
Age: _____ Heparins: O YES O NO
Group: A / B / 0 Pill: O YES O NO

Taking: O ASA (_____mg/day) O Clopidogrel (_____mg/day)

Status post

	Localization	Days
PTA		
By Pass		
Patch		
Thrombektomie		
CT - Angio		

Eaten today? YES / NO
Blood draw position? sitting / laying
Transportation: O Transport box O Transport Cylinder O at room temperature
Time of blood draw: _____
Begin of analysis: _____

	Whole Blood	Plasma
Thrombocyte		
Fibrinogen		
aPTT		
PTZ		
GOT		
GPT		
GGT		

Medication	Morning	Midday	Evening	Night

	Whole blood		Plasma	
	Kinetic	*LAOStress*	*Kinetic*	*LAOStress*
Starting Time				
Duration (min:sec)				

Run Control: O Zero gap set / controlled
O Temperature set to 37 °C
O Centrifugation of the whole blood sample at 1500 turns / min. for 8 min
(Time of centrifugation: _____)

Figure 5.4.: Translated sample protocol sheet, containing personal data, interventional history, parameters at the time of blood draw, current medication and sample run table.

5.5. Process of sample analysis

After the sample transfer to the AKH was completed, the following steps were taken, always in the same order as described in the list below. Whole blood samples, due to decreased storage stability, were prioritized over plasma samples in terms of analytical order.

- Boot the rheometer and synchronize with the RheoCompass - Software
- Turn on the water – cooling system
- Insert the measuring system (sand-blasted stainless steel, cone-plate, diameter 5 cm)
- Set zero – gap (0.1 mm) and temperature chamber to 37 °C
- Insert the evaporation blocker
- Seal the evaporation blocker with silicone (no contact of the clot with silicone)
- Turn the sample tube three times upside down to resuspend RBCs
- Lay 40 µL of $CaCl_2$ into a 1.5 mL "Eppendorf" – tube
- Pipette 600 µL of sample in this tube, mix once by pressing to the first pressure point
- Superimpose the reactivated sample blister - free in the middle of the bottom plate
- Descend the cone to the measuring gap (0.1 mm) and trim if necessary
- Start the first analytic run (kinetic of clot formation) and note the time
- Centrifugate the second tube (20 °C, 1500 U/min, 8 min) and note the time
- Pipette at least 650 µL of plasma into a new "Eppendorf" – tube
- After the whole blood sample run is finished, clean the test system by brushing under water and rinsing
- Dry the test system and insert it again to the rheometer
- Finally, repeat the whole process with the plasma sample as described in point three → "Set the zero – gap (0.1mm)"

6. Statistics & Results

This chapter covers basic data, laboratory results and rheometry results. This section is ordered into subsections. Every subsection contains a table followed by a description highlighting the most relevant differences and similarities. Descriptive statistics including mean values, standard deviation, minimum, median, maximum and diagrams was performed by Microsoft Excel Version 2201. IBM SPSS Statistics 27.0 was used to calculate correlation coefficients and p – values for the descriptive statistics. Multivariate regression analysis was performed by Stata Corp Stata 17.

6.1. Basic data and laboratory results

23 individuals of the 40 included patients (21 female and 19 male) belonged to the treatment groups, which were further subcategorized into "Mono" (acetylsalicylic acid as single therapy) and "Dual" (Combined anti – platelet – therapy with acetylsalicylic acid and clopidogrel). Since the quantity of the dual group was only five, observations regarding treatment will refer to the mono - group only, if not stated otherwise.

		Mean value	Standard deviation	Minimum	Median	Maximum
18 Mono (6♀/ 12♂)	Age [years]	*59,67*	*11,97*	*27*	*61,5*	*78*
	BMI [nondimensional]	*26,78*	*3,86*	*19,9*	*26,6*	*34,5*
	ASA - Score [nondimensional]	*2,11*	*0,56*	*1*	*2*	*3*
	$t^{(analyse)}$ [minutes]	*61,94*	*25,88*	*10*	*56,5*	*119*
	PLT [G/L]– Whole blood	*131,56*	*60,62*	*56*	*123,5*	*284*
	PLT [G/L] - Plasma	*194,28*	*193,07*	*68*	*147,5*	*904*
	FIB [mg/dl]	*375,56*	*46,47*	*261*	*384*	*444*
17 Controls (11♀/ 6♂)	Age [years]	*52,29*	*16,6*	*25*	*52*	*81*
	BMI [nondimensional]	*26,86*	*5,23*	*18,5*	*26*	*39,4*
	ASA - Score [nondimensional]	*1,32*	*0,44*	*1*	*1*	*2*
	$t^{(analyse)}$ [minutes]	*70,82*	*36,07*	*12*	*58*	*138*
	PLT [G/L] – Whole blood	*122,76*	*48,2*	*41*	*112*	*227*
	PLT [G/L] - Plasma	*119,47*	*46,37*	*46*	*116*	*248*
	FIB [mg/dl]	*373*	*88,18*	*203*	*376*	*544*

Table 6.1.: Basic data distribution and laboratory results sorted by treatment group (upper table) and control group (bottom table)

The mean age in the treatment group with 59.6 years was approximately 7 years above average age in the control group. BMI, platelet count and fibrinogen were nearly identical, displaying a good baseline in terms of comparability. The difference concerning thrombocyte count in plasma samples originates from an outlier increasing the mean value from 148,7 to 190,67 and the maximum from 403 to 904 G/L. The ASA – score difference is worth mentioning and the result of patient selection is considered as control vector X in the multivariate regression analysis.

Furthermore, the number of smokers was higher in the treatment group (12/18) compared to the proband group (7/17). Due to the fact that most of the patients who received acetylsalicylic acid as a prevention therapy were recruited in the hospital, this group exhibited 3 interventions in the last 4 weeks before participating in this study. These three patients have been included because an influence from intraoperative medication on physiological haemostasis has been suspended.

6.2. Test results

The first two tables display the descriptive statistic (mean value, standard deviation, minimum, median and maximum) of rheometric parameters. The first table (6.2.1.4) covers values from whole blood samples while the second table (6.2.2.6) displays values from plasma samples. If diagrams legend includes a correlation curve, the number in brackets states the corresponding r – value.

6.2.1. Whole blood samples

Differences between the study groups are minimal (both in kinetic and LAOStress tests) and only occurred at the beginning of the kinetic test. CT was shorter in the treatment group compared to the control group (q.v. figure 6.2.1.1.) There was a weaker relationship of clot stiffness (G'_{23}) with platelet count in the patient group compared to the control group. (figure 6.2.1.2.)

The generally high standard deviations arise from the limited number of samples in both study groups (n=17). Nevertheless, tendencies and orientations get more distinct in plasma runs while running multivariate regression analysis (will be described later; q.v. 6.3.).

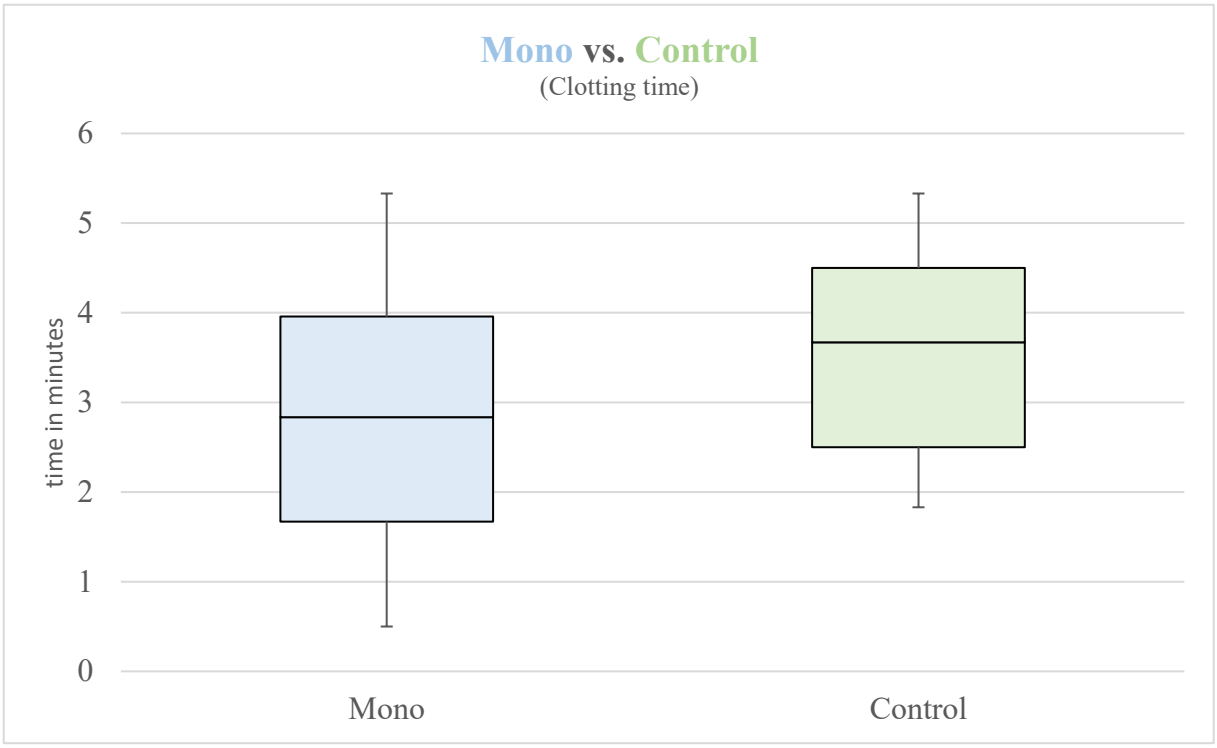

Figure 6.2.1.1.: Whole blood samples comparing the clotting time of coagulation between patients taking acetylsalicylic acid and the control group.

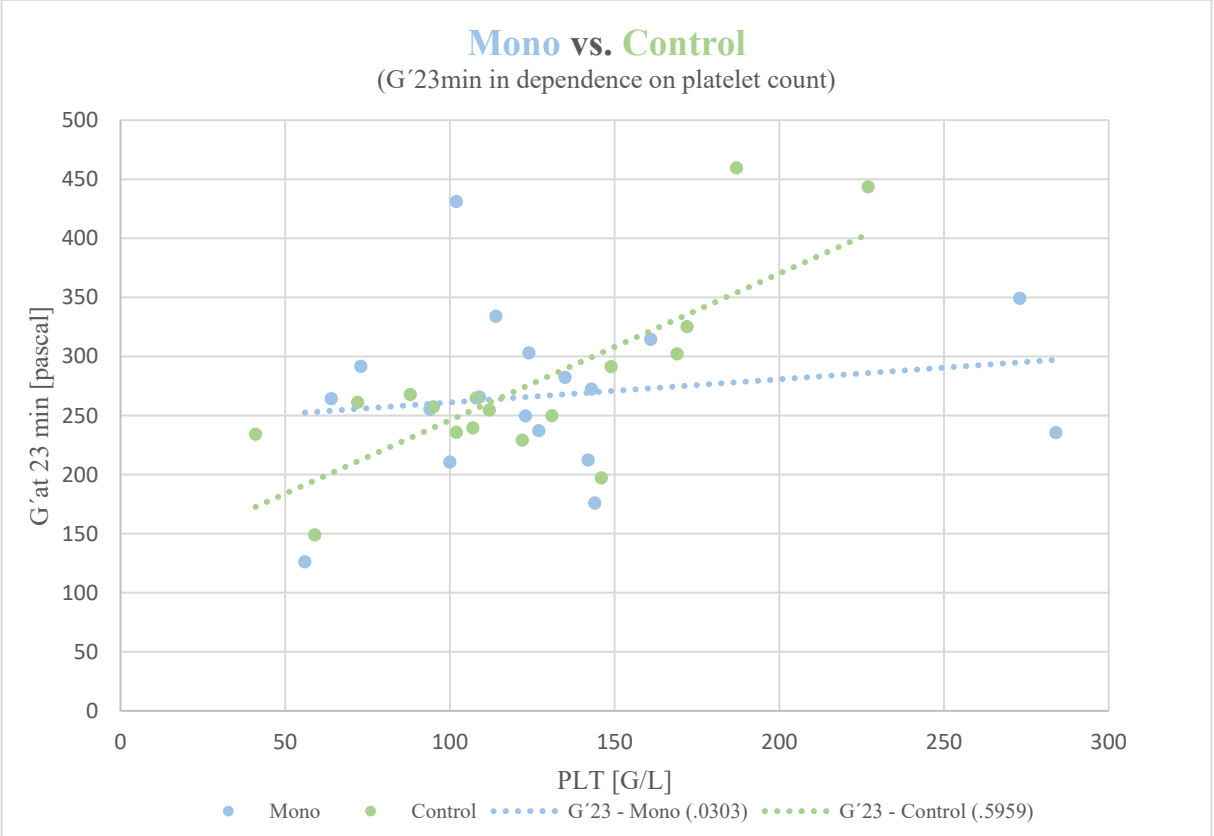

Figure 6.2.1.2.: Scatter plots displaying the correlation between platelet count and the corresponding G´ value at 23 minutes during kinetic whole blood runs. Furthermore, gradient differences are visible regarding the linear regression line indicating a positive interrelation.

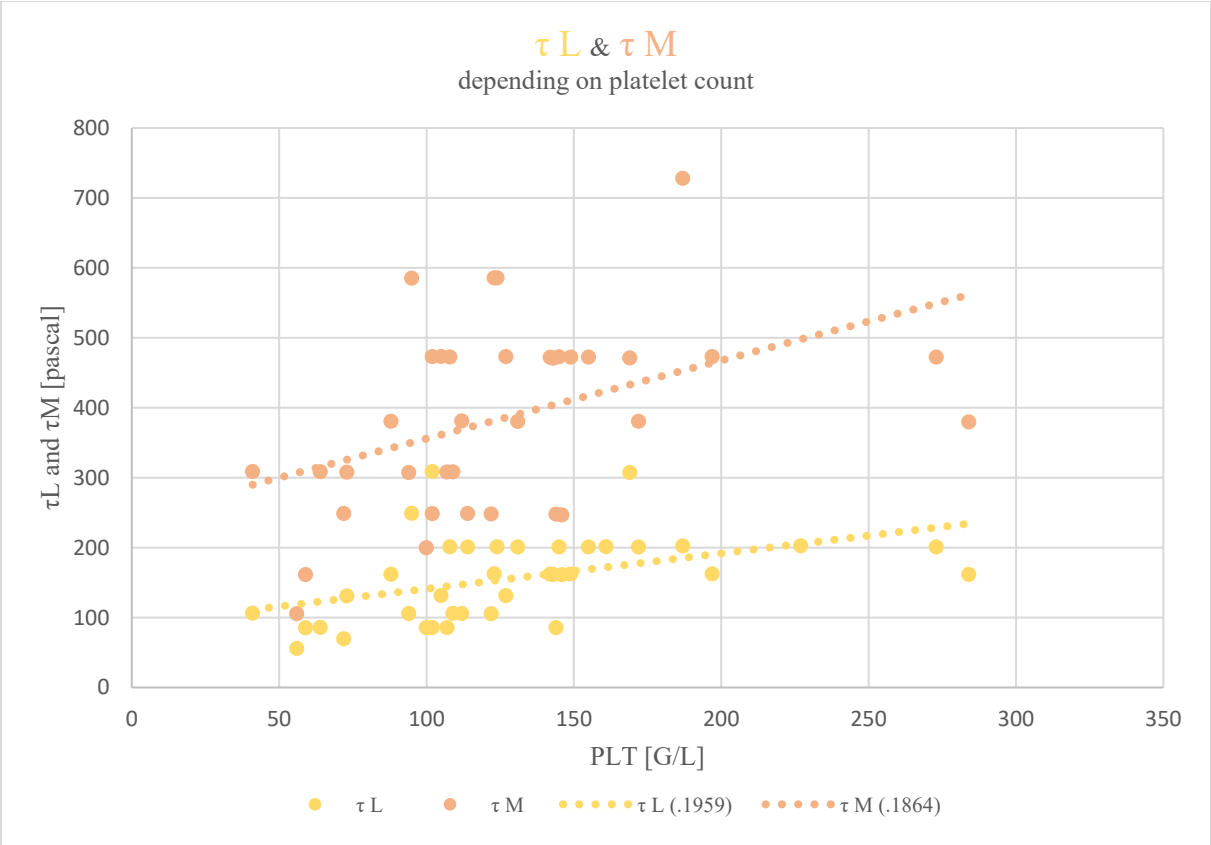

Figure 6.2.1.3.: Relationship between platelet count and τ L (p=0.009) respectively τM (p=0.047). This significance levels where later calculated in chapter 6.3. multivariate regression analysis and indicate a strong positive correlation.

		Whole blood results	Mean value	Standard deviation	Minimum	Median	Maximum
Treatment	*Kinetic*	CT [minutes]	*2,853*	*1,487*	*0,5*	*2,835*	*5,33*
		G'-t_{init}[minutes]	*7,363*	*2,191*	*3,71*	*7,34*	*11,8*
		G'_{init} [pascal]	*64,24*	*17,93*	*31,48*	*60,6*	*97,9*
		G'_{23} [pascal]	*267,26*	*68,26*	*126,19*	*264,94*	*431,14*
		G'- $t_{plateau}$ [minutes]	*28,933*	*3,92*	*23*	*28,25*	*34,7*
		$G'_{plateau}$ [pascal]	*294,95*	*69,66*	*127,55*	*287,49*	*445,77*
	LAOStress	t_{DP} [minutes]	*7,650*	*0,699*	*6,107*	*7,619*	*8,615*
		τ DP [pascal]	*18,26*	*6,59*	*8,40*	*15,92*	*30,11*
		τL [pascal]	*149,67*	*62,31*	*55,73*	*161,28*	*308,63*
		τ M [pascal]	*371,45*	*138,88*	*105,37*	*343,95*	*585,43*
		$J'M_{max}$ [pascal]	*346,19*	*79,19*	*144,54*	*353,81*	*442,24*
		breakup shear stress [pascal]	*783,34*	*265,36*	*376,91*	*723,29*	*1374,9*
		G'_{max} [pascal]	*403,91*	*84,03*	*198,97*	*404,05*	*560,92*
Control	*Kinetic*	CT [minutes]	*3,598*	*1,199*	*1,83*	*3,67*	*5,33*
		G'-t_{init}[minutes]	*8,122*	*2,297*	*4,8*	*7,67*	*12,8*
		G'_{init} [pascal]	*65,33*	*16,83*	*37,69*	*60,51*	*94,52*
		G'_{23} [pascal]	*274,23*	*77,55*	*148,88*	*257,39*	*459,62*
		G'- $t_{plateau}$ [minutes]	*31,629*	*2,718*	*23,8*	*32,2*	*34*
		$G'_{plateau}$ [pascal]	*315,03*	*90,62*	*179,39*	*290,21*	*546,38*
	LAOStress	t_{DP} [minutes]	*8,025*	*0,748*	*6,107*	*8,082*	*9,273*
		τ DP [pascal]	*20,20*	*6,53*	*10,37*	*19,58*	*30,12*
		τL [pascal]	*158,24*	*67,00*	*69,49*	*161,64*	*307,09*
		τM [pascal]	*376,22*	*145,17*	*161,19*	*380,35*	*727,76*
		$J'M_{max}$ [pascal]	*355,26*	*93,53*	*215,39*	*340,93*	*639,78*
		breakup shear stress [pascal]	*805,85*	*335,97*	*380,42*	*723,3*	*1706,5*
		G'_{max} [pascal]	*424,05*	*119,05*	*254,43*	*416,21*	*720,23*

Table 6.2.1.4: Description of the most important whole blood landmarks during both test runs.

6.2.2. Plasma samples

Differences between the study groups became more obvious in plasma samples because the influence of RBCs was removed. (5)

CT was lower in the patient group. Also, G'_{init} and $G'\text{-}t_{init}$ were slightly lower (q.v. figure 6.2.2.1.). There is a positive correlation between platelet count / fibrinogen concentration and G'_{20} (q.v. 6.2.2.2.) in all samples (combining both study groups). This analogy carries on for platelet count / fibrinogen concentration regarding τL and τM (q.v. 6.2.2.3 and 6.2.2.4). Whilst increasing platelet counts led to stiffer clots in the control collective, we found a negative correlation between platelet count and maximal stiffness from patients receiving acetylsalicylic acid with a r – value of 0.0999 (q.v. 6.2.2.6.). The same chart also demonstrates the positive impact of fibrinogen on G'_{max}. Finally, a positive correlation between FIB and G'_{max} ($p < 0.001$) including both groups underlined the eminent character of fibrinogen for this last parameter. This is displayed in figure 6.2.2.7.

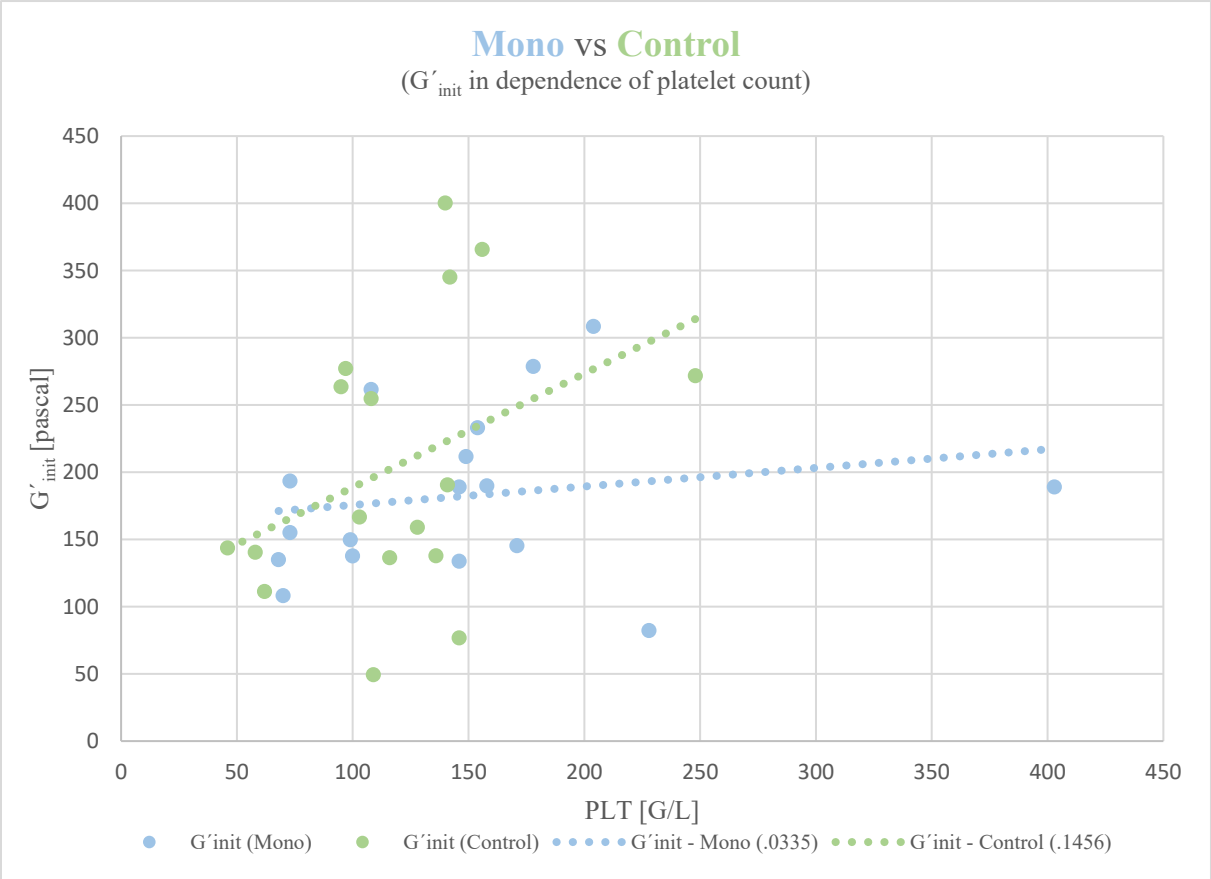

Figure 6.2.2.1.: On this chart you find a confrontation of G'_{init} merits in dependence on platelet count divided into a treatment and a control group. Please not that one outlier belonging to the mono collective was excluded due to an exorbitant PLT value of over 900. This exception had only minor influence of the displayed curve.

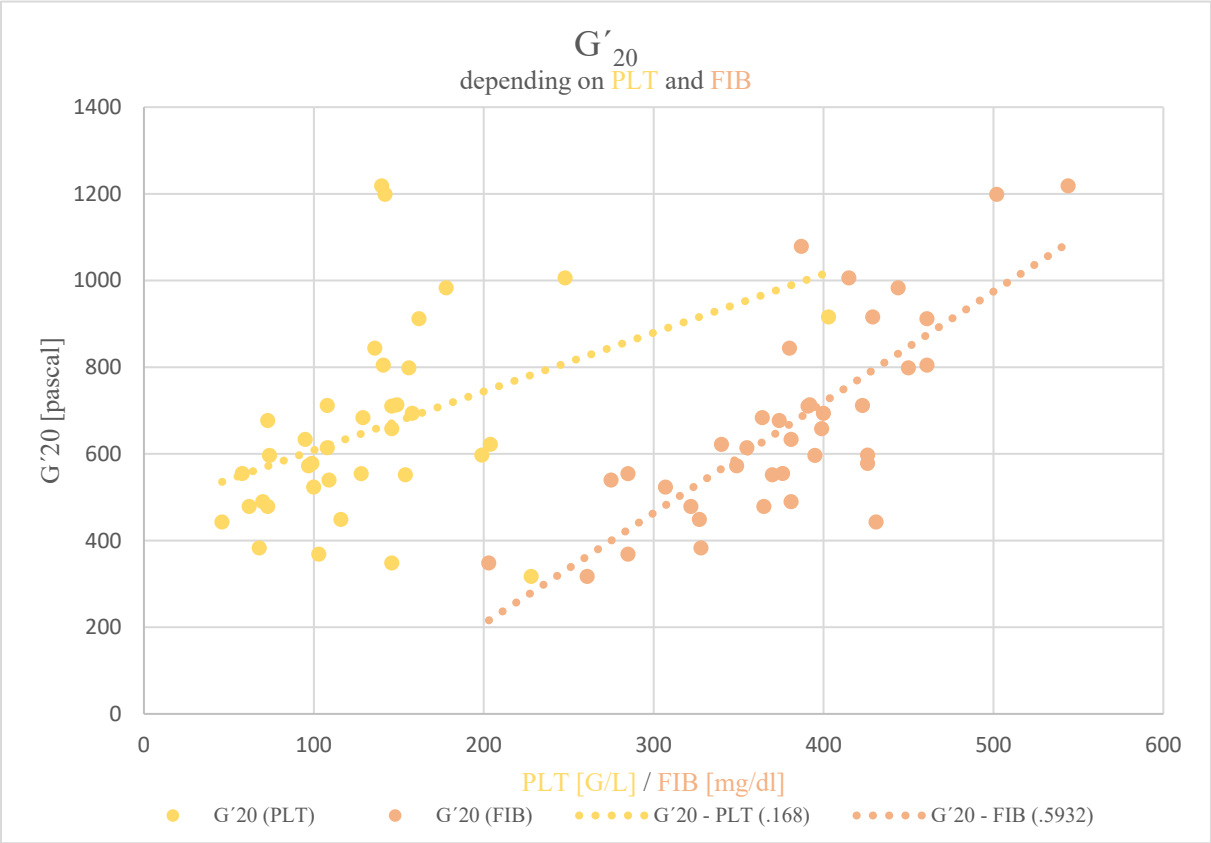

Figure 6.2.2.2.: Scatterplot including correlation curve displaying the positive link between platelet count / quantity of fibrinogen and the corresponding clot stiffness at t = 20 min. We excluded the same outlier from figure 6.2.1.1..

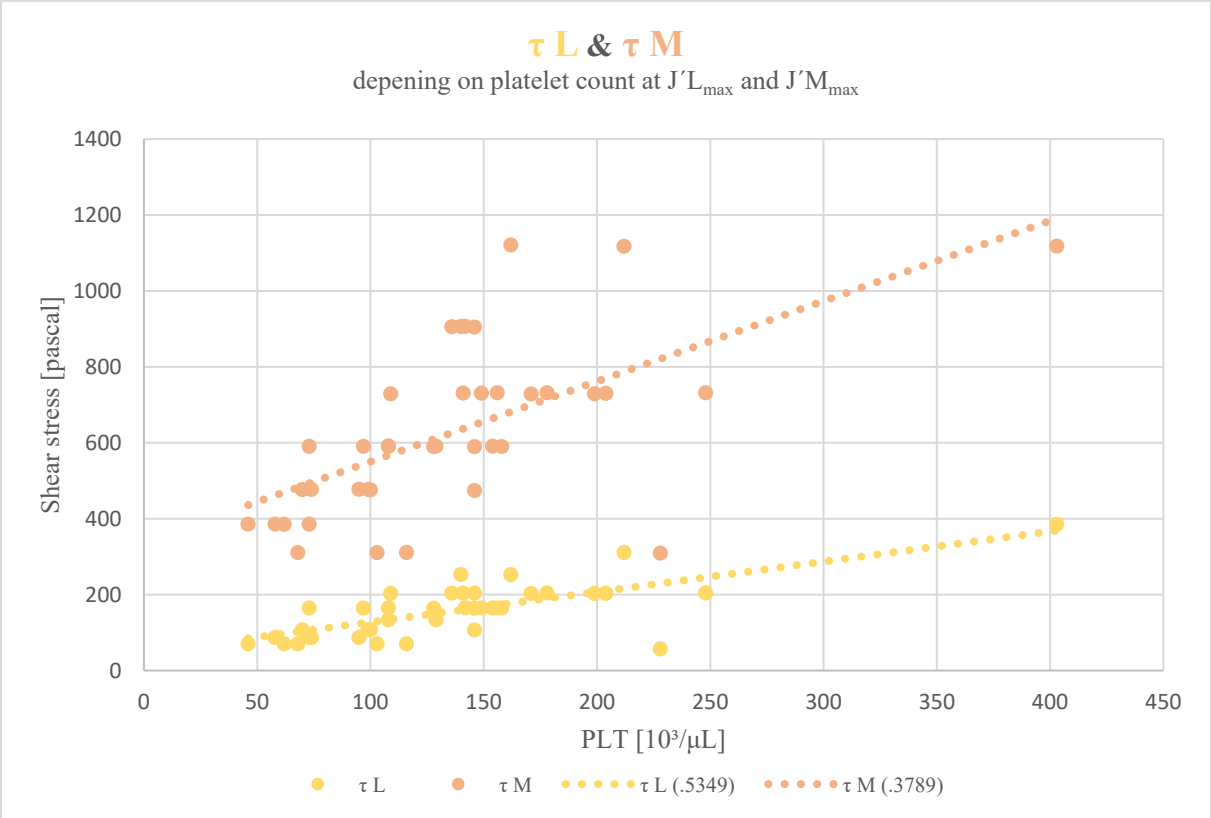

Figure 6.2.2.3.: On display is τ L and τ M in dependence on the platelet count at J´L$_{max}$ and L´M$_{max}$. The positive relationship between increasing PLT merits and corresponding needed shear stress is clearly visible.

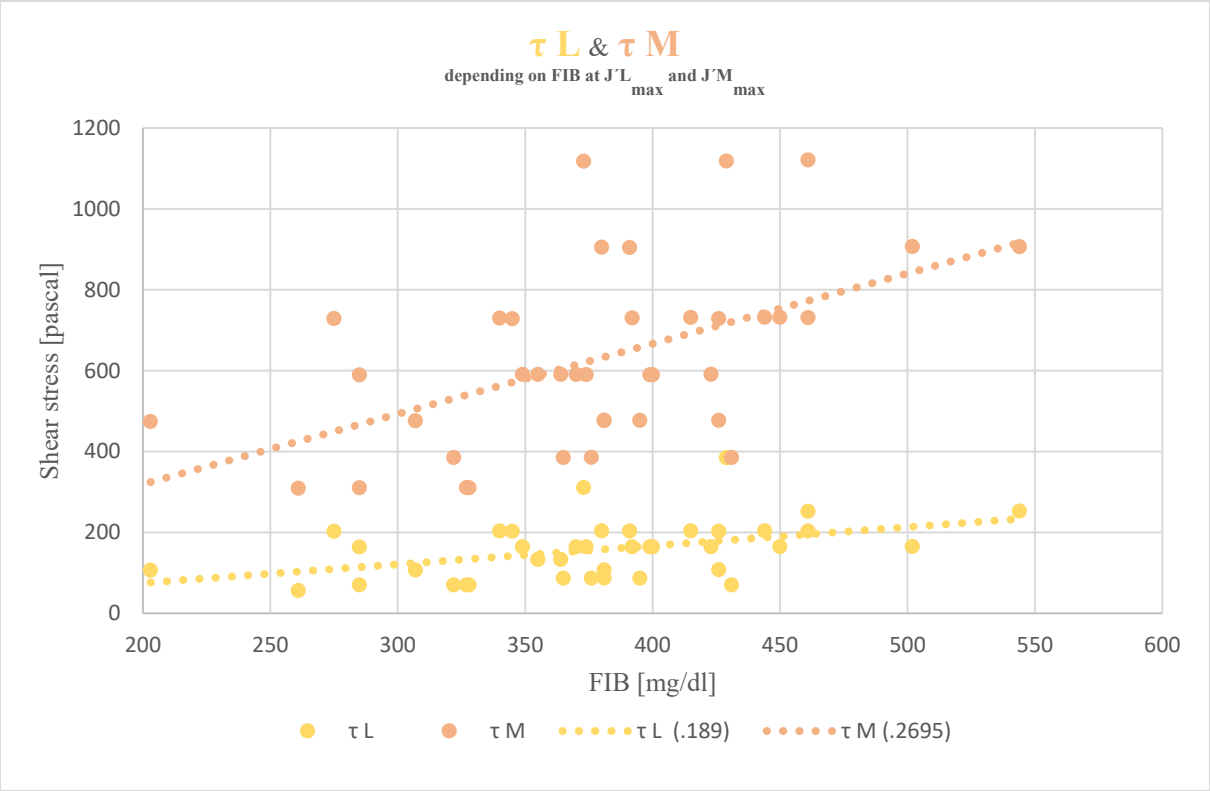

Figure 6.2.2.4.: This diagram has the same y – axis as the figure 6.2.2.3. but the baseline is switched to fibrinogen. In contrast to the chart before fibrinogen and shear stress including their linear correlations seem to differ drastically apart in terms of positive influence. Especially the link between fibrinogen and τ M constitutes to spiral more rapidly upwards.

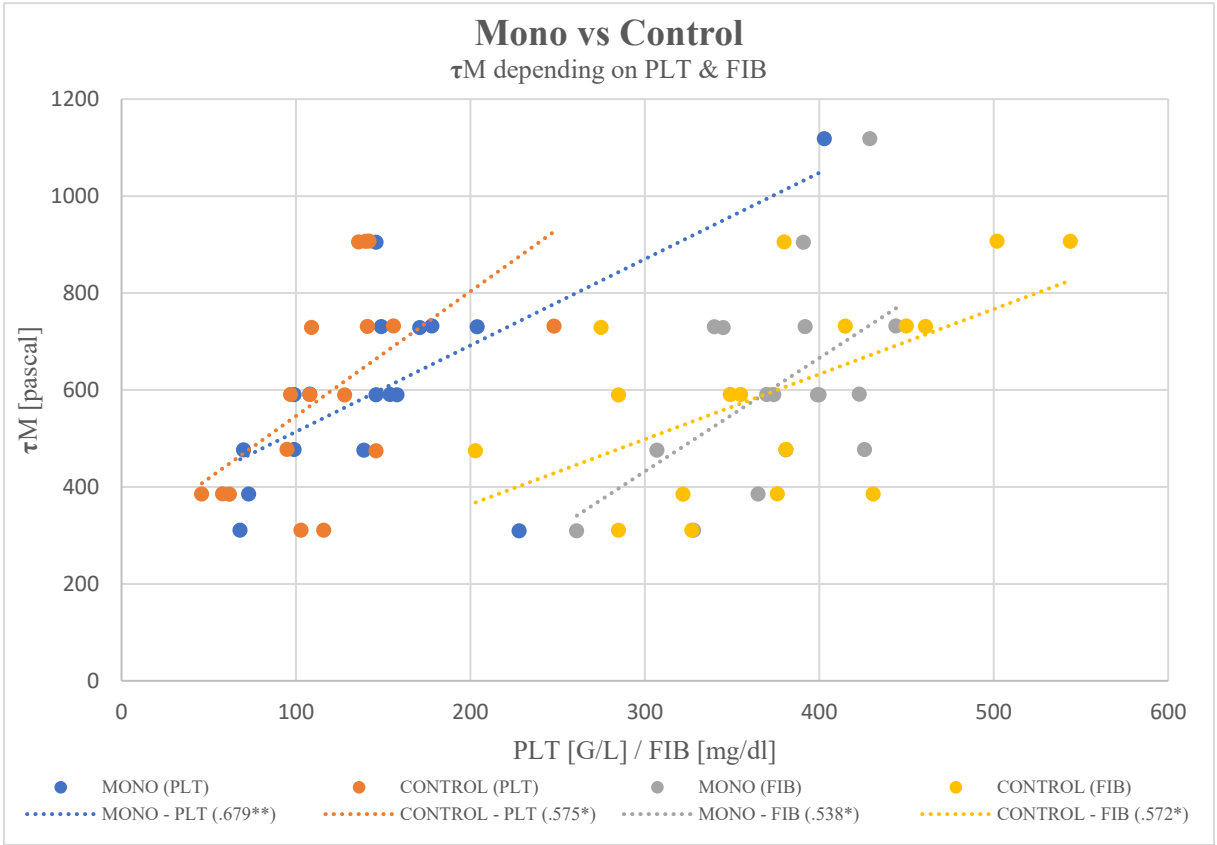

Figure 6.2.2.5.: Positive correlation (coefficient in brackets) between platelet count / fibrinogen concentration and τM in the mono group and the control collective. The mono - platelets curve is shallower, compared to the patients with acetylsalicylic treatment. The opposite seems to be the fact concerning fibrinogen concentration and τM.

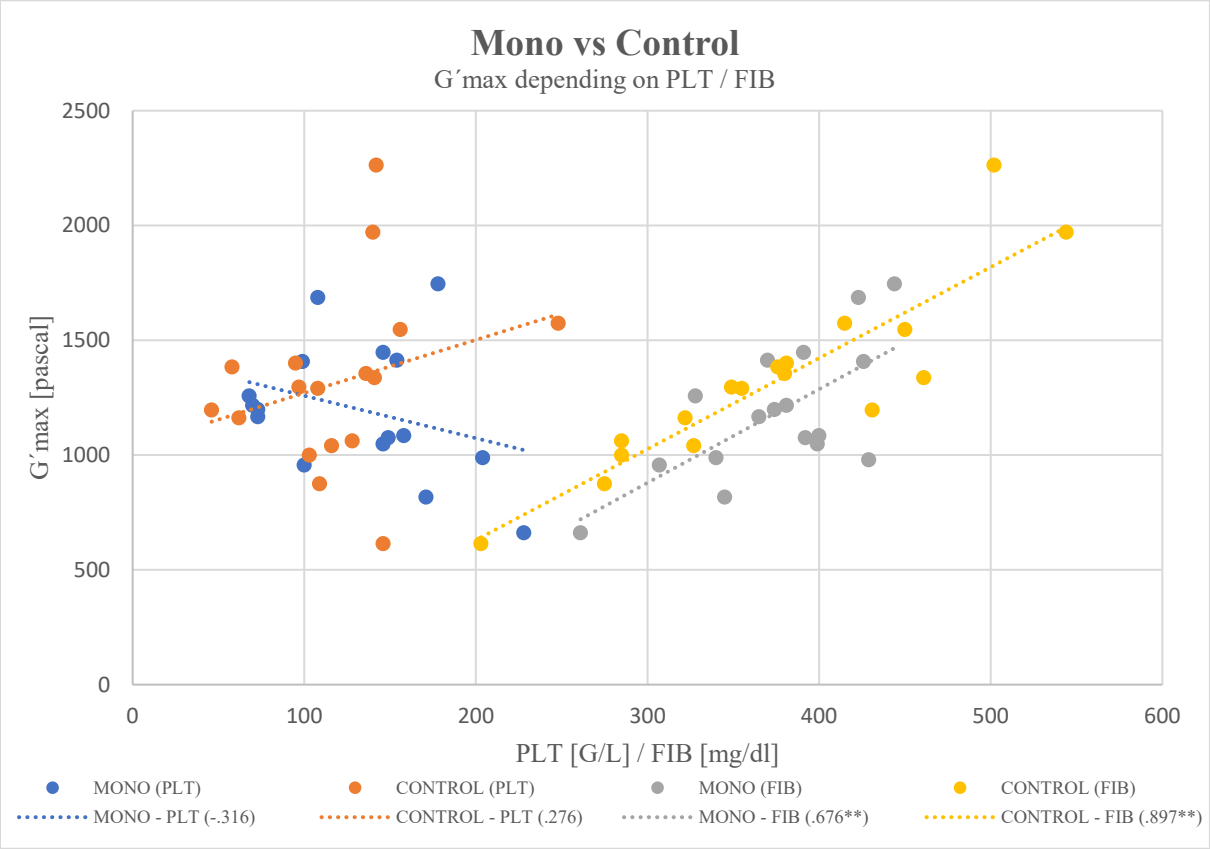

Figure 6.2.2.6.: This chart displays the impact of platelet count / fibrinogen concentration on G´$_{max}$ (final stiffness). There is a strong positive, statistically significant correlation between fibrinogen levels and G´$_{max}$, in both groups. The brackets state the correlation coefficient.

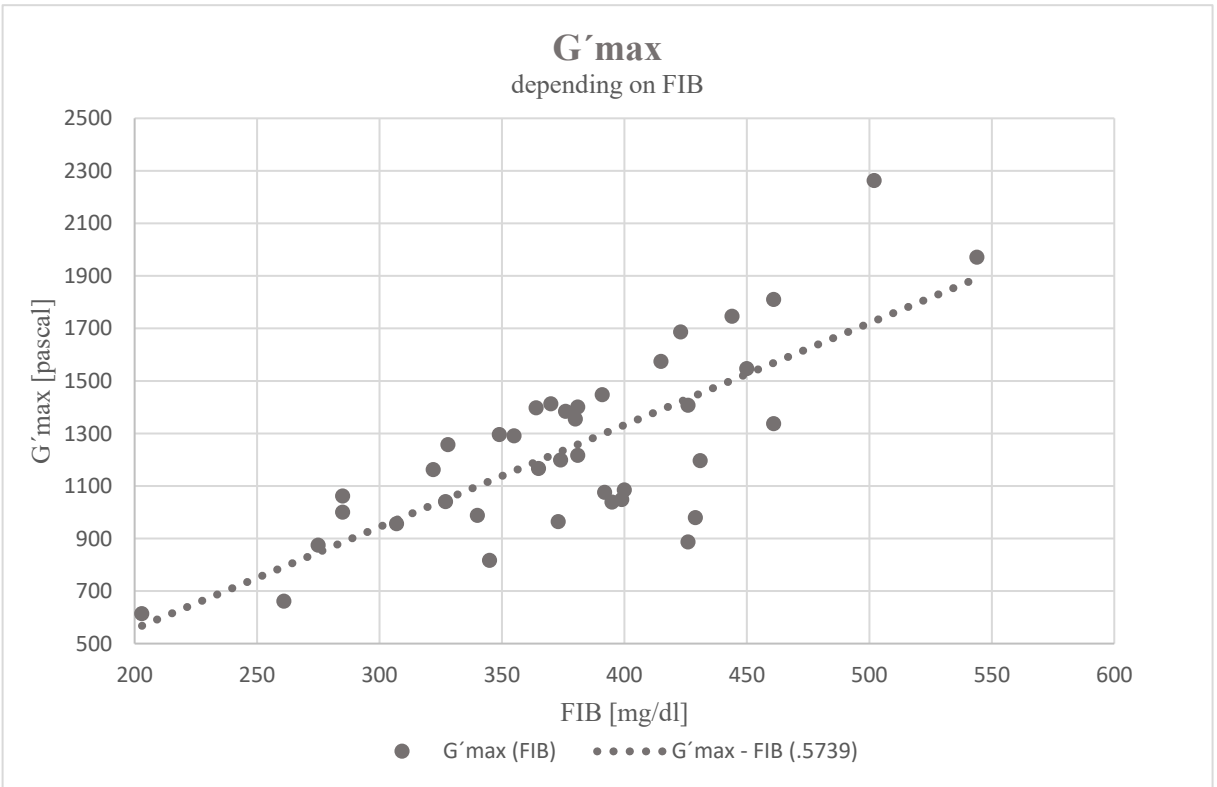

Figure 6.2.2.7.: Correlation between G´ max and the quantity of fibrinogen. The p - value < 0.001 indicates a very strong positive relationship between increasing concentrations of fibrinogen and G. This supports the essential role of fibrinogen for clot formation.

		Plasma results	Mean value	Standard deviation	Minimum	Median	Maximum
Treatment	*Kinetic*	CT [minutes]	*2,084*	*1,737*	*0,167*	*1,415*	*5,5*
		G´-t_{init}[minutes]	*7,658*	*3,497*	*3,5*	*6,83*	*13,5*
		G´$_{init}$ [pascal]	*192,81*	*74,44*	*82,04*	*188,93*	*371,38*
		G´$_{20}$ [pascal]	*651,82*	*201,29*	*316,89*	*657,98*	*1078,4*
		G´- t plateau [minutes]	*24,978*	*3,009*	*17,5*	*25*	*30,5*
		G´$_{plateau}$ [pascal]	*709,45*	*218,24*	*315,22*	*711,46*	*1230,7*
	LAOStress	t_{DP} [minutes]	*8,303*	*1,431*	*5,119*	*8,905*	*9,871*
		τ DP [pascal]	*32,81*	*14,83*	*6,78*	*37,28*	*57,01*
		τL [pascal]	*159,83*	*75,31*	*56,17*	*164,22*	*384,68*
		τM [pascal]	*607,40*	*207,49*	*308,96*	*590,15*	*1117,9*
		J´M_{max} [pascal]	*880,21*	*206,58*	*437,68*	*889,44*	*1291,9*
		breakup shear stress [pascal]	*2547,18*	*949,10*	*1383*	*2122,6*	*4971,5*
		G´$_{max}$ [pascal]	*1184,84*	*288,00*	*660,67*	*1166,4*	*1745,4*
Control	*Kinetic*	CT [minutes]	*2,785*	*0,750*	*1,33*	*2,83*	*3,83*
		G´-t_{init}[minutes]	*8,554*	*2,895*	*4*	*7,6*	*13,8*
		G´$_{init}$ [pascal]	*205,17*	*102,93*	*49,22*	*166,49*	*400,06*
		G´$_{20}$ [pascal]	*671,82*	*267,82*	*347,96*	*572,12*	*1218,2*
		G´- t plateau [minutes]	*26,935*	*3,762*	*20,7*	*27*	*34,8*
		G´$_{plateau}$ [pascal]	*749,53*	*286,18*	*381,05*	*704,08*	*1343,3*
	LAOStress	t_{DP} [minutes]	*8,353*	*1,031*	*6,931*	*8,411*	*9,893*
		τ DP [pascal]	*32,19*	*14,08*	*15,90*	*30,13*	*57,10*
		τL [pascal]	*142,14*	*59,91*	*69,84*	*163,86*	*252,29*
		τM [pascal]	*596,28*	*207,12*	*310,31*	*590,24*	*906,5*
		J´M_{max} [pascal]	*993,12*	*337,75*	*505,88*	*971,81*	*1742,6*
		breakup shear stress [pascal]	*2675,19*	*731,78*	*1704,4*	*2621,8*	*4025,7*
		G´$_{max}$ [pascal]	*1315,27*	*389,42*	*613,42*	*1295,6*	*2262,3*

Table 6.2.2.8.: Descriptive statistic of the most important plasma blood landmarks during both test runs.

6.3. Multivariate regression analysis

The advantage for this type of calculation is that possible disruptive factors or bias (control vector X) caused by natural differences become partially bowdlerised, allowing a more accurate assessment of potential correlations.

6.3.1. Multivariate regression model

Beyond is the formula, which was applied to the data set. It provides the list of numbers, for instant the regression coefficient, standard error, p – value, and used sample size.

$$y_i = a_i + \beta_1 T_i + \beta_2 PLT_i + \beta_3 FIB_i + \boldsymbol{X}_i + \varepsilon_i$$

- y_i variables ..... *{CT, $G'_{init}{}^{x}$, G'-t_{init}, G'-$t_{plateau}$, G'_{20}, G'_{23}, $G'_{plateau}$, t_{DP}, τ_{DP}, τL, τM, $J'M_{max}$, breakup shear stress, G'_{max}}*
- α_i ..... intercept
- $\beta_1 T_i$ ..... treatment dummy *[1 = mono]*
- $\beta_2 PLT_i$ ..... platelet dummy *[1 = 10^3 units/µl]*
- $\beta_3 FIB_i$ ..... fibrinogen dummy *[1 = 1 mg/dl]*
- X_i ..... control vector *{sex, age, BMI, ASA – score, smoker, abstinent, intervention, contraceptive, $t^{(analyse)}$}*
- ε_i ..... error term

6.3.2. Calculated values

The following tables are arranged in a “dual – numeric” manner. The greater number represents the regression coefficient while the smaller number below is the corresponding standard error. The former is to be constructed as a tendency of change. If the value on the very left column increases by one in its own unit, the associated variable shifts accordingly. Please note that the algebraic sign is more eminent than the actual factor, meaning that the assumption of a positive or a negative correlation has a higher validity compared to the effective change in units.

6.3.2.1. Kinetic results (whole blood)

The table below shows the statistical values of whole blood samples during the kinetic run. Also here, CT was not significantly different between study groups (q.v. figure 6.2.2.1). Nevertheless, G'_{23} and platelet count correlated positively. The same relationship appears to be the case for $G'_{plateau}$ and platelet count, but it should be taken into consideration that the $G'_{plateau}$ and G'-$t_{plateau}$ have been taken before the exact plateau was reached.

		CT [minutes]	G'-t_{init} [minutes]	G'_{init} [pascal]	G'_{23} [pascal]	G'-$t_{plateau}$ [minutes]	$G'_{plateau}$ [pascal]
Variables	Treatment [yes/no]	-.087 *(.635)*	.305 *(1.003)*	2.019 *(9.380)*	-14.420 *(28.407)*	-4.150 *(1.835)*	-28.554 *(32.598)*
	PLT [G/L]	.003 *(.004)*	.002 *(.007)*	.106 *(.062)*	.521* *(.188)*	.0174 *(.012)*	.637* *(.216)*
	FIB [mg/dl]	.007 *(.004)*	.008 *(.007)*	.002 *(.063)*	-.094 *(.190)*	.003 *(.012)*	-.049 *(.218)*
Control vector	Sex [male/female]	.616 *(.567)*	.854 *(.895)*	14.581 *(8.375)*	38.940 *(25.366)*	.780 *(1.639)*	42.808 *(29.108)*
	Age [years]	-.026 *(.025)*	-.019 *(.039)*	.265 *(.365)*	.958 *(1.106)*	.110 *(.071)*	1.385 *(1.269)*
	BMI [numeric]	.079 *(.057)*	.157 *(.089)*	.652 *(.835)*	-.509 *(2.528)*	-.073 *(.163)*	-.413 *(2.901)*
	ASA [numeric]	-.387 *(.499)*	-.8547 *(.787)*	-2.323 *(7.365)*	5.399 *(22.307)*	-1.512 *(.163)*	-4.449 *(25.598)*
	Smoker [yes/no]	.476 *(.578)*	.421 *(.912)*	-.772 *(8.532)*	-8.058 *(25.839)*	3.251 *(1.670)*	-1.343 *(29.650)*
	Abstinent [yes/no]	.038 *(.849)*	.387 *(1.341)*	2.334 *(12.546)*	9.754 *(37.996)*	-1.254 *(2.455)*	9.603 *(43.601)*
	Intervention [numeric]	-1.087 *(.801)*	-2.241 *(1.265)*	6.805 *(11.836)*	102.183* *(35.848)*	.848 *(2.316)*	111.627* *(41.136)*
	Contraceptive [yes/no]	-1.300 *(1.728)*	2.091 *(2.729)*	3.678 *(25.523)*	-31.939 *(77.299)*	7.924 *(4.995)*	-2.498 *(88.702)*
	$t^{(analyse)}$ [minutes]	-.015 *(.007)*	-.024 *(.0118)*	.033 *(.110)*	.861* *(.334)*	-.033 *(.022)*	.774 *(.383)*

Table 6.3.2.1.: Results from the multivariate regression analysis regarding the kinetic run of whole blood samples.

** $p < 0.05$ ** $p < 0.005$ *** $p < 0.0005$*

6.3.2.2. LAOStress results (whole blood)

The subsequent LAOStress measurement displayed a similar picture. We found no statistically robust differences between a patient receiving acetylsalicylic acid and the probands in the control group. What we saw is a positive correlation between platelet count and the shear stress thresholds τL and τM as well as τDP.

		t_{DP} [minutes]	τ DP [pascal]	τ L [pascal]	τ M [pascal]	$J'M_{max}$ [pascal]	breakup shear stress [pascal]	G'_{max} [pascal]
Variables	Treatment [yes/no]	-.441 *(.450)*	-.906 *(2.905)*	2.600 *(28.783)*	86.810 *(69.120)*	39.151 *(44.301)*	95.566 *(138.678)*	18.171 *(42.366)*
	PLT [G/L]	.003 *(.003)*	.046* *(.019)*	.551* *(.190)*	.951* *(.447)*	.305 *(.286)*	.358 *(.915)*	.009 *(.2795)*
	FIB [mg/dl]	-.000 *(.003)*	-.020 *(.021)*	-.225 *(.207)*	-.764 *(.483)*	-.360 *(.309)*	-1.611 *(.996)*	-.2771 *(.3042)*
Control vector	Sex [male/female]	.010 *(.419)*	5.288 *(2.703)*	.960 *(26.780)*	111.091 *(62.670)*	93.678 *(40.121)*	131.119 *(129.029)*	89.144 *(39.418)*
	Age [years]	-.021 *(.018)*	-.115 *(.113)*	-1.324 *(1.120)*	-.261 *(2.640)*	1.500 *(1.690)*	12.262 *(5.394)*	4.446* *(1.648)*
	BMI [numeric]	.020 *(.045)*	.310 *(.287)*	2.329 *(2.846)*	13.916 *(6.901)*	7.104 *(4.418)*	3.833 *(13.714)*	2.347 *(4.190)*
	ASA [numeric]	.490 *(.355)*	4.694 *(2.292)*	-12.734 *(22.709)*	-45.064 *(51.961)*	-23.725 *(33.265)*	-128.302 *(109.412)*	-42.567 *(33.425)*
	Smoker [yes/no]	-.396 *(.413)*	-5.239 *(2.662)*	-22.602 *(26.381)*	-64.314 *(64.274)*	-38.248 *(41.148)*	-91.984 *(127.104)*	-10.687 *(38.830)*
	Abstinent [yes/no]	.177 *(.618)*	3.862 *(3.984)*	-14.966 *(39.482)*	2.906 *(88.579)*	59.629 *(56.707)*	189.815 *(190.228)*	84.613 *(58.115)*
	Intervention [numeric]	-.173 *(.569)*	3.066 *(3.668)*	96.786* *(36.346)*	30.745 *(109.286)*	48.242 *(69.963)*	-2.267 *(175.116)*	47.680 *(53.498)*
	Contraceptive [yes/no]	-.646 *(1.219)*	-13.452 *(7.863)*	-110.979 *(77.915)*	-211.016 *(183.292)*	-18.487 *(117.341)*	414.008 *(375.402)*	161.150 *(114.685)*
	$t^{(analyse)}$ [minutes]	-.000 *(.005)*	.072 *(.035)*	.635 *(.342)*	2.099* *(.762)*	1.291 *(.488)*	5.179* *(1.650)*	1.510* *(.504)*

Table 6.3.2.2.: Results from the multivariate regression analysis regarding the LAOStress run of whole blood samples.

** $p < 0.05$ ** $p < 0.005$ *** $p < 0.0005$*

6.3.2.3. Kinetic results (plasma)

After eliminating the influence of erythrocytes, the importance of platelets and fibrinogen became more distinct. We found strong positive relations between platelets and stability parameters like G'_{init}, G'_{23min} and $G'_{plateau}$. Also here, $G'_{plateau}$ was taken before exactly the plateau was reached, but nevertheless a very similar correlation between these three values from the kinetic runs and fibrinogen was found. Clot formation was slightly (but insignificantly) different in the patient group.

		CT [minutes]	G'-t_{init} [minutes]	G'_{init} [pascal]	G'_{20} [pascal]	G'-$t_{plateau}$ [minutes]	$G'_{plateau}$ [pascal]
Variables	Treatment [yes/no]	-.182 *(.726)*	-.1563 *(1.635)*	-34.774 *(31.232)*	-51.868 *(59.551)*	-2.894 *(1.606)*	-71.414 *(60.102)*
	PLT [G/L]	.003 *(.002)*	.005 *(.004)*	.278** *(.078)*	.576** *(.147)*	.003 *(.004)*	.697*** *(.149)*
	FIB [mg/dl]	-.002 *(.005)*	-.003 *(.011)*	.746** *(.216)*	2.652*** *(.409)*	.008 *(.011)*	2.815*** *(.415)*
Control vector	Sex [male/female]	.251 *(.667)*	1.203 *(1.502)*	38.087 *(28.689)*	40.690 *(55.766)*	.946 *(1.475)*	68.433 *(55.207)*
	Age [years]	-.005 *(.0285)*	-.033 *(.064)*	-.735 *(1.227)*	-2.648 *(2.325)*	.0625 *(.063)*	-2.747 *(2.361)*
	BMI [numeric]	.086 *(.066)*	.307 *(.148)*	4.724 *(2.833)*	-7.181 *(5.401)*	.141 *(.146)*	-2.613 *(5.452)*
	ASA [numeric]	-.350 *(.596)*	-.302 *(1.341)*	24.275 *(25.625)*	66.069 *(48.520)*	-.9949 *(1.318)*	67.753 *(49.310)*
	Smoker [yes/no]	.086 *(.670)*	.537 *(1.510)*	12.737 *(28.838)*	-75.453 *(54.911)*	3.873* *(1.483)*	-47.330 *(55.494)*
	Abstinent [yes/no]	.543 *(1.076)*	2.499 *(2.424)*	34.747 *(46.298)*	-.414 *(88.810)*	.658 *(2.381)*	42.838 *(89.094)*
	Intervention [numeric]	-.378 *(.952)*	-.717 *(2.144)*	-20.635 *(40.953)*	-72.332 *(82.328)*	-.530 *(2.106)*	-94.386 *(78.808)*
	Contraceptive [yes/no]	-1.346 *(2.089)*	6.941 *(4.705)*	130.669 *(89.869)*	-141.3 *(173.298)*	11.316 *(4.621)*	11.757 *(172.939)*
	$t^{(analyse)}$ [minutes]	.017* *(.008)*	.033 *(.018)*	.247 *(.350)*	-.739 *(.662)*	.019 *(.018)*	-.450 *(.673)*

Table 6.3.2.3.: Results from the multivariate regression analysis regarding the kinetic run of plasma samples.

** $p < 0.05$ ** $p < 0.005$ *** $p < 0.0005$*

6.3.2.4. LAOStress results (plasma)

Table 6.3.2.4. displays the strong influence of fibrinogen on clot deformation, supporting its essential role on elasticity and shear-stiffening. Platelets seem to exhibit a similar importance restricted on medium shear stresses (τL to τM), where the inner rearrangement of fiber geometry takes place. A difference between treatment and control group concerning τL (r was present.

		t_{DP} [minutes]	τ_{DP} [pascal]	τL [pascal]	τM [pascal]	$J'M_{max}$ [pascal]	breakup shear stress [pascal]	G'_{max} [pascal]
Variables	Treatment [yes/no]	.837 *(.538)*	9.811 *(6.132)*	44.361* *(16.518)*	70.559 *(62.643)*	-116.38 *(84.988)*	169.498 *(389.013)*	-108.917 *(113.291)*
	PLT [G/L]	.002 *(.003)*	.0377 *(.035)*	.553*** *(.094)*	1.297** *(.358)*	.071 *(.486)*	-4.001 *(2.224)*	-.878 *(.648)*
	FIB [mg/dl]	.012* *(.004)*	.128* *(.045)*	.438** *(.120)*	1.746** *(.456)*	3.403*** *(.618)*	-.344 *(2.830)*	3.478*** *(.824)*
Control vector	Sex [male/female]	.436 *(.494)*	6.140 *(5.637)*	24.543 (15.183)	81.209 *(57.582)*	77.722 *(78.123)*	488.773 *(357.587)*	96.765 *(104.139)*
	Age [years]	-.049 *(.023)*	-.538 *(.260)*	-1.524* *(.699)*	-4.474 *(2.652)*	-2.291 *(3.599)*	38.757* *(16.471)*	4.116 *(4.797)*
	BMI [numeric]	.014 *(.049)*	.163 *(.553)*	-.489 *(1.490)*	-2.584 *(5.651)*	-1.897 *(7.667)*	-40.020 *(35.092)*	-2.720 *(10.220)*
	ASA [numeric]	-.140 *(.437)*	-1.404 *(4.979)*	.922 *(13.410)*	23.735 *(50.859)*	94.188 *(69.000)*	-504.222 *(315.833)*	4.387 *(91.979)*
	Smoker [yes/no]	-.556 *(.491)*	-8.762 *(5.596)*	-46.887* *(15.074)*	-115.264 *(57.166)*	-44.664 *(77.558)*	306.927 *(355.004)*	55.004 *(103.387)*
	Abstinent [yes/no]	-.438 *(.808)*	-5.972 *(9.209)*	20.275 *(24.807)*	-37.682 *(94.079)*	17.661 *(127.637)*	15.537 *(584.230)*	52.160 *(170.143)*
	Intervention [numeric]	-.983 *(.698)*	-6.685 *(7.958)*	-22.777 *(21.436)*	-100.01 *(81.294)*	-103.047 *(110.292)*	94.687 *(504.833)*	-80.974 *(147.021)*
	Contraceptive [yes/no]	-1.71 *(1.543)*	-19.759 *(17.595)*	-97.030 *(47.393)*	-325.241 *(179.737)*	-47.812 *(243.851)*	188.612 *(1116.169)*	163.521 *(325.058)*
	$t^{(analyse)}$ [minutes]	-.003 *(.006)*	-.008 *(.068)*	-.603** *(.183)*	-1.777* *(.693)*	-.362 *(.941)*	-1.185 *(4.306)*	.828 *(1.254)*

Table 6.3.2.4.: Results from the multivariate regression analysis regarding the LAOStress run of plasma samples.

** $p < 0.05$ ** $p < 0.005$ *** $p < 0.0005$*

7. Discussion

7.1. Whole blood

The shorter CT (clotting time) of the treatment group compared to control group (q.v. figure 6.2.1.1.), was surprising because someone would expect this parameter to be prolonged under ASA therapy. This could indicate that clopidogrel (65) should be added to the therapeutic regime to compensate the underlaying tendency of spontaneous platelet aggregation. An increased dosage of ASA will not correspond with an enhanced prophylactic protection, but will rather result in accumulative gastrointestinal side effects. (74) The positive correlation between platelet count and clot stiffness emphasizes the significance of platelets (5), and the necessity to keep this parameter within a physiological range. In addition to this context, the relationship between platelets and clot stiffness is weaker in the treatment group (patients receiving acetylsalicylic acid) than in untreated volunteers. Since thrombocytes are main players during clot formation, a clot stiffness that is less coupled with the platelet count, would line up with the therapeutic effect of ASA. (25) (59) The reduced stiffness of clots from the patient group appeared again while comparing correlations between platelet count and G'_{init} (q.v. figure 6.2.2.1.)

Thrombi under antiplatelet therapy tend to be looser having wider pores, and the fibrin network is composed of thicker fibers. (75) This could be an explanation for the lowered final stiffness of clots from our patients receiving acetylsalicylic acid q.v. 6.2.2.6.). These findings could help with clinical decision-making. Looser clots tend to shiver during TEA (thrombendarteriectomy) combined with a catheter. In some cases, this can lead to restenosis in a further peripheric located arterial branch. But a clot with wider pores will at same time better respond to remodeling or lysis. (76)

7.2. Conclusion (plasma)

The results from plasma samples are more distinct. This is because in whole blood clots, RBCs might form a layer of aggregates at the bottom of the test plate. If so, the tested material is inhomogeneous, and its response will be attenuated or even incorrect. Other reason can be the blockage of the fiber response by entrapped or incorporated RBCs. (77) This could be one reason why LAOStress values from plasma samples exhibit higher stiffening ability and breakup stresses. (78) At least, these tests display better the properties of the fiber meshwork. Since platelets, fibrin, and their crosslinking are key elements for a clot to form, their effect on clot formation and the behavior at plastic deformation is eminent.

Entering the LAOStress – phase, we saw strong impacts of increasing platelet count on shear stress thresholds (τL and τM) (compare figure 6.2.2.3.). These findings support data from whole blood samples but are more pronounced in plasma samples. Increasing the platelet count had a weaker influence on the shear stress thresholds that are indicative for clot softening and stiffening (τL,τM) in the patient group. This means that platelets are less potent in the patient group to modify clot rearrangement, since they are partially blocked by anti-platelet therapy (q.v. figure 6.2.2.5. and table 6.3.2.4.).

As the meshwork of fibrin fibers determines the outcomes in the LAOStress run, we expected certain interrelations between fibrinogen concentration and rheological variables. While the number of platelets seemed to affect softening and stiffening likewise, the positive correlation between fibrinogen concentration and τM appeared be stronger than its effect on τL (q.v. figure 6.2.2.4.). This means that, within our volunteers, fibrinogen levels affected stiffening more than softening. These observations got backed by highly significant correlation coefficients, displayed in table 6.3.2.3. Not only the rearrangement of fibrin fibres (35) but also clot strength at maximum stretch of fibers was strongly dependent on the samples fibrinogen concentration. This is illustrated in figure 6.2.2.7.

7.3. General Conclusion

To sum up, the differences between treatment group and control group, regarding clot stiffness, clot breakup at high deformation, or the re-arrangement of fibers before clot breakup, were implied but not significant. Only clotting time was reduced in patients. These findings add a certain level of safety to the existing, well documented and widely used acetylsalicylic acid therapy with its prophylactic and post-interventional benefits. (79) (3)

But the data presented here underline the role of platelet count and fibrinogen concentration as two main influences of clot characteristics. Most of the correlations we explored were positive. This implies that an increased value of thrombocytes and/or fibrin(ogen) will enhance magnitudes like clot stiffness, clot stiffening during plastic deformation, and the shear stresses needed for a transformation of clot architecture. Although platelets and plasma fibrinogen concentration regulate clot formation and distension, platelets appear to be more eminent in the kinetic phase while the fibrinogen concentration dominates clot deformation.

8.1. Limitations

Due to the comparably small number of included individuals, it is not possible to make general assumptions. All interpretations regarding differences between patients receiving acetylsalicylic acid and probands without antiplatelet therapy, relate to those two specific collectives. Furthermore, to make statements of universal validity, we propose a lager patients number. Lastly, we want to flag that the physiological ASA nonresponse rate (approx. 24% globally) was not taken into consideration. (80)

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