EVALUATION OF NOVEL LABORATORY METHODS USING WHOLE BLOOD TESTING IN DIFFERENT DISTURBANCES OF HAEMOSTASIS

Ph.D. thesis

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2009
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<tr>
<td>ACS</td>
<td>acute coronary syndrome</td>
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<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
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<tr>
<td>AMP</td>
<td>adenosine monophosphate</td>
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<tr>
<td>APC</td>
<td>activated protein C</td>
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<td>APTT</td>
<td>activated partial thromboplastin time</td>
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<tr>
<td>APY</td>
<td>apyrase</td>
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<tr>
<td>ARC</td>
<td>AR-C69931MX</td>
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<tr>
<td>ASA</td>
<td>acetylic salicylic acid, aspirin</td>
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<tr>
<td>AT</td>
<td>antithrombin</td>
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<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
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<tr>
<td>AU</td>
<td>aggregation unit</td>
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<tr>
<td>AUC</td>
<td>area under the aggregation curve</td>
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<tr>
<td>C4BP</td>
<td>complement 4 binding protein</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>calcium-chloride</td>
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<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
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<tr>
<td>CFT</td>
<td>clot formation time</td>
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<tr>
<td>CI</td>
<td>confidence interval</td>
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<tr>
<td>COX1</td>
<td>cycloooxygenase-1</td>
</tr>
<tr>
<td>CT</td>
<td>clotting time</td>
</tr>
<tr>
<td>DRVV</td>
<td>diluted Russell's viper venom</td>
</tr>
<tr>
<td>DVT</td>
<td>deep vein thrombosis</td>
</tr>
<tr>
<td>EC₅₀</td>
<td>half maximal effective concentrations</td>
</tr>
<tr>
<td>ETP</td>
<td>endogenous thrombin potential</td>
</tr>
<tr>
<td>FII</td>
<td>coagulation factor II, prothrombin</td>
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<tr>
<td>FV</td>
<td>coagulation factor V</td>
</tr>
<tr>
<td>FVIII</td>
<td>coagulation factor VIII</td>
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<tr>
<td>FVQ506</td>
<td>factor V Leiden</td>
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<td>FX</td>
<td>coagulation factor X</td>
</tr>
<tr>
<td>FXa</td>
<td>activated factor X</td>
</tr>
<tr>
<td>FXIIa</td>
<td>activated factor XII</td>
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<tr>
<td>G</td>
<td>gauge</td>
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<tr>
<td>G/l</td>
<td>giga per litre</td>
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<tr>
<td>GP</td>
<td>glycoprotein</td>
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<td>Abbreviation</td>
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<tr>
<td>IC$_{50}$</td>
<td>half maximal inhibitory concentrations</td>
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<td>IP$_3$</td>
<td>inositol triphosphate</td>
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<tr>
<td>LA</td>
<td>lupus anticoagulant</td>
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<tr>
<td>LMWH</td>
<td>low molecular weight heparin</td>
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<td>LTA</td>
<td>light transmission aggregometry</td>
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<tr>
<td>MA</td>
<td>maximal amplitude</td>
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<tr>
<td>MCF</td>
<td>maximum clot firmness</td>
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<tr>
<td>MEA</td>
<td>multiple electrode aggregometry</td>
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<tr>
<td>min</td>
<td>minute</td>
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<tr>
<td>MRS</td>
<td>MRS2179</td>
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<tr>
<td>n</td>
<td>number</td>
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<tr>
<td>OR</td>
<td>odds ratio</td>
</tr>
<tr>
<td>p</td>
<td>probability, statistical significance</td>
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<tr>
<td>PA</td>
<td>platelet aggregation</td>
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<tr>
<td>PAR-1</td>
<td>proteinase activated receptor 1</td>
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<td>PC</td>
<td>protein C</td>
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<td>PGE$_1$</td>
<td>prostaglandin E$_1$</td>
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<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PLC</td>
<td>phospholipase-C</td>
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<tr>
<td>PRP</td>
<td>platelet-rich plasma</td>
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<tr>
<td>PS</td>
<td>protein S</td>
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<tr>
<td>PT</td>
<td>prothrombin time</td>
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<tr>
<td>ROTEG</td>
<td>rotation thrombelastography</td>
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<tr>
<td>SD</td>
<td>standard deviation</td>
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<tr>
<td>SPC</td>
<td>single platelet counting</td>
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<tr>
<td>TEG</td>
<td>thrombelastography</td>
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<tr>
<td>ThP</td>
<td>thrombophilia</td>
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<tr>
<td>TRAP-6</td>
<td>thrombin receptor-activating peptide-6</td>
</tr>
<tr>
<td>TT</td>
<td>thrombin time</td>
</tr>
<tr>
<td>TxA$_2$</td>
<td>thromboxan A$_2$</td>
</tr>
<tr>
<td>U/ml</td>
<td>unit per millilitre</td>
</tr>
<tr>
<td>VASP</td>
<td>vasodilator-stimulated phosphoprotein</td>
</tr>
<tr>
<td>VTE</td>
<td>venous thromboembolism</td>
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<tr>
<td>vWF</td>
<td>von Willebrand factor</td>
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1. Introduction

Haemostasis is a very complex and well regulated process, which is designed to maintain blood in a fluid state under physiologic conditions, but it is primed to react to vascular injury in an explosive manner to stem blood loss by sealing the defect in the vessel wall. The three “traditional” components of haemostasis are the endothelium, the platelets and the coagulation system. The participation of platelets in haemostasis is a fundamental component of this physiologic process. The reactions involved include adhesion to the cut end of a blood vessel, spreading of adherent platelets on the exposed subendothelial surface, secretion of stored platelet constituents, and formation of large platelet aggregates. In addition, platelet membrane sites become available for adsorption and concentration of clotting factors, and plasma coagulation is accelerated, resulting in the formation of a fibrin network that reinforces the otherwise friable platelet plug. The firm platelet-fibrin clot subsequently retracts into a smaller volume, a process that is also platelet-dependent [1]. These two main components of haemostasis – platelets and plasmatic proteins - are interdependent. The importance of leukocytes in coagulation and fibrinolysis has also been established [2,3]. The platelet adhesion process is different under different shear conditions, for example at high shear rates plasma von Willebrand factor (vWF) and glycoprotein (GP) Ib/IX are required, while at low shear rates other proteins are sufficient for the adhesion of platelets.

Several laboratory assays have been designed to measure haemostasis. First, simple haemostatic tests, like bleeding time and coagulation time were used, which were mainly made for the detection of hemorrhagic diatheses. Then plasma-based global haemostatic assays were developed, like activated partial thromboplastin time (APTT) and prothrombin time (PT), which could be automated and are now widely used in routine laboratories. However, these tests are robust, unable to detect mild defects and to show the location of the disorder. With the development of single factor determinations this problem was thought to be have been solved, but clinical experience showed that certain haemostasis abnormalities could not be diagnosed by them. Additionally, single factor determinations without good screening assays are very costly. As we get more and more information about
physiological haemostasis, there is a tendency to develop methods, which can better model the physiological conditions, such as flow conditions and shear, and the use of whole blood is preferred. The methods using whole blood testing are good candidates for point of care testing, so the test can be used even in acute situations.

1.1. Epidemiology and importance of haemostatic abnormalities

Haemostasis is the consequence of balanced interactions of cellular and molecular components responsible for the maintenance of an intact circulation [4]. Dysfunction of cells or proteins participating in the haemostasis system may result in thrombophilia or a haemorrhagic diathesis.

1.1.1. Bleeding disorders

Haemostatic imbalance may be inherited or acquired. A wild range of inherited bleeding disorders have been described, originating from an inadequate function of the platelets as seen in Glanzmann’s disease and the Bernard Soulier syndrome, or caused by deficiency in procoagulant proteins such as fibrinogen or coagulation factor II, V, VII, VIII, IX, X, XI and XIII. Some bleeding disorders such as inherited FV and FX deficiency are rare, occurring only in one per million inhabitants. Other bleeding disorders, such as haemophilia A are more common, occurring in 1:10000 [1].

1.1.2. Thromboembolic disease

Hypercoagulable states can present themselves as arterial or venous thromboembolic disease.

The major cause of death and disability in the developed world is arterial vascular disease. Its main etiological factor is atherosclerosis, along with embolisation mainly of cardiac origin. Depending on the affected part of the arterial branch, we can talk about cardiovascular, cerebrovascular, aortic, peripheral and
renal vascular disease. Symptoms develop when growth or rupture of the plaque reduces or obstructs blood flow; symptoms vary with the affected arteries.

In recent years, age-related mortality attributable to atherosclerosis has been decreasing, but in 2005, cardiovascular diseases, primarily coronary and cerebrovascular atherosclerosis still caused almost 870,000 deaths in the US (more than cancer and almost 9 times more than injuries) [5]. Atherosclerosis is rapidly becoming more prevalent in developing countries, and as people in developed countries live longer, incidence will further increase. By 2020, atherosclerosis among cardiovascular diseases is expected to be the leading cause of death worldwide. Eastern European countries such as the Ukraine, the Russian Federation, Hungary, and the Czech Republic have among the highest and increasing CVD rates in the world, which is in marked contrast to most economically stable European countries where declines in CVD mortality rates have been experienced over the past 30 years [6].

Deep vein thrombosis (DVT) and pulmonary embolism represent different manifestations of the same clinical entity, which is referred to as venous thromboembolism (VTE). The incidence rate of a first venous thrombosis is 1 to 2 events per 1000 patient-years [7]. According to population studies, the average annual incidence rates of VTE was 117 per 100,000 person-years [8]. Venous thromboembolism is predominantly a disease of older age. Incidence rates increase markedly with age for both men and women and for both deep vein thrombosis and pulmonary embolism [8,9]. The overall age-adjusted incidence rate is higher for men than women (male:female sex ratio is 1.2:1) [8]. Pulmonary embolism accounts for an increasing proportion of VTE with increasing age for both genders [8]. Venous thromboembolism recurs frequently; about 30% of patients develop recurrence within the next 10 years [10]. The hazard of recurrence varies with the time since the incident event and is highest within the first 6–12 months.

At least 1 out of 5 of the venous thromboembolism cases is fatal, although available autopsy data suggest that this figure is probably a significant underestimation of actual mortality. In Hungary, the mortality rates of venous
thromboembolism were 10.8, 19.8 and 10.7 per 100000 inhabitants in the years 1970, 1990 and 1999, respectively [11,12]. Moreover, pulmonary embolism accounts for 5-10% of deaths in hospitalized patients, making VTE the most common preventable cause of in-hospital death [13].

Morbidity is also associated with two long-term complications: chronic thromboembolic pulmonary hypertension and post-thrombotic syndrome. Chronic pulmonary thromboembolism with pulmonary hypertension is seen in up to 5% of patients as a result of the incomplete resolution of a thrombus [14]. These patients are functionally limited because of progressive exertional dyspnoea, chest pain, syncope, and lower extremity oedema. Post-thrombotic syndrome is characterized by leg pain, oedema, other signs of venous insufficiency, and eventually leg ulceration as a result of prolonged venous hypertension. At least 30% of patients with venous thromboembolism develop this chronic debilitating disease [15].

In some cases, risk factors for both venous and arterial disease may be present, making the clinical picture more complex [16].

1.2. The role of haemostatic investigations in arterial thrombotic disease

Thrombosis plays a central role in the pathogenesis of acute coronary syndrome (ACS); this is supported by the presence of thrombi at the site of a ruptured coronary plaque at autopsy, in atherectomy specimens from patients with unstable angina, and on angioscopy and angiography of patients with unstable angina. Indirect evidence of ongoing thrombosis in ACS is provided by the elevation in levels of markers of platelet activation and fibrin formation. Additionally, a marked improvement in the clinical outcome of patients with ACS is achieved with specific antithrombotic therapy using acetylsalicylic acid (aspirin), heparin, platelet GP IIb/IIIa inhibitors and clopidogrel. Because of the central role of platelets in the pathophysiology of ACS, antiplatelet therapy is one of the cornerstones of therapy and is directed at decreasing the formation of TxA₂ (aspirin), inhibiting the adenosine diphosphate (ADP) receptor pathway of platelet activation (ticlopidine and clopidogrel), and directly inhibiting platelet aggregation (GP IIb/IIIa inhibitors) [1]. The efficacy of various antiplatelet agents in preventing
cardiovascular and thrombotic complications has been established in large-scale clinical trials. Composite data from such studies tend to mask individual responsiveness to the drugs being investigated. In fact, antiplatelet drugs that are effective and safe in one individual may be ineffective or harmful in another [17].

According to laboratory measures of platelet activation and aggregation a large proportion of people fail to respond in the anticipated manner when treated with aspirin, they are thus termed “aspirin resistant” [18,19]. The clinical relevance of aspirin resistance was recently demonstrated in a study of stable patients with cardiovascular disease who were found to have a greater than threefold increase in the risk of major adverse events during long-term follow-up compared with those on aspirin who exhibited normal inhibition of platelet aggregation [20].

The thienopyridines ticlopidine and clopidogrel inhibit platelet function by irreversibly blocking the binding of ADP to its P2Y$_{12}$ platelet receptor. Both of these drugs are inactive and require conversion to their active platelet-inhibitor metabolites by the hepatic cytochrome P450 system in vivo. Clopidogrel has a more favourable side effect profile and a more rapid onset of action than does ticlopidine [21,22]. Interindividual variability in platelet inhibition by clopidogrel and the occurrence of "clopidogrel resistance" has been recently documented by several groups [23-26]. Although not conclusively demonstrated, one study suggested that clopidogrel resistance increases the risk of coronary stent thrombosis [26]. It is also becoming increasingly clear that there is considerable intrinsic variability in the responsiveness of individuals to antiplatelet agents, which is mostly genetically determined. There is growing evidence that differences in the effect of not only aspirin, but also clopidogrel is of clinical importance [26-31].

This widespread use of antiplatelet therapy in cardiovascular medicine and evidence suggesting a clinically relevant drug resistance have created a demand for simple assays to determine the effectivity of antiplatelet treatment. Point-of-care platelet function testing in acute settings and rational pharmacogenomic approaches would permit more individualized treatment, in some cases requiring dosing changes or the use of alternate drugs to optimize antiplatelet therapy. The clinical value of the so far known point-of-care technologies, such as the platelet function analyzers PFA-100 (Dade-Behring) and VerifyNow (Accumetrics), remains to be
proved convincingly [32]. Optical platelet aggregometry is still accepted as gold standard, but this is not a standardized method [33]. Preanalytical interferences related to blood sampling and complicated sample processing add substantial technical imprecision to the already large biological variability of platelet aggregation. Optical aggregometry requires expert personnel and time-consuming centrifugation steps to obtain platelet-rich and platelet-poor plasma. Aggregation correlates with the increase of light transmittance in platelet-rich plasma (PRP) after addition of a platelet agonist. However, PRP is an artificial milieu deficient in giant platelet subspecies as well as erythrocytes and leukocytes, which are regarded as critical modulators of platelet function in vivo [34]. Thus, whole blood platelet aggregometry has several advantages. We were the first to describe a new, promising whole blood aggregometry, called multiple electrode aggregometry (MEA) and thus prepare it for further studies.

1.3. The role of haemostatic investigations in venous thromboembolism

Venous thromboembolism is now recognized as a complex, multifactorial disease, involving both environmental exposures (e.g. clinical risk factors) and both genetic and environmental interactions [35]. Environmental risk factors include immobilization, surgery, malignancies, pregnancy, puerperium, and exogenous female hormones [36]. Genetic abnormalities increasing the risk of a thrombotic event have been known for several decades and include deficiencies of the natural anticoagulants antithrombin (AT), protein C (PC), and protein S (PS). Additional biochemical risk factors for a thrombotic event are factor V Leiden (FVQ506), prothrombin (FII) G20210A, high levels of factors VIII, IX, or XI, homocysteine, and fibrinogen [37-43]. A certain combination of risk factors is necessary, each adding to the thrombotic event potential, to exceed the thrombosis threshold and put an individual at risk.

In order to improve survival, avoid recurrence, prevent complications, and reduce health care costs, the occurrence of VTE must be reduced. To reduce VTE incidence, persons at risk for VTE must first be identified. Thrombophilia (the tendency to develop thrombosis) can be inherited, acquired, or both. Recent family
based studies indicate that VTE is highly inheritable and follows a complex mode of inheritance involving environmental interaction [44-46]. Still, many cases of venous thromboembolism remain “idiopathic”.

Studies hold the potential for further stratifying individual patients into high- and low-risk for incident and recurrent VTE, targeting prophylaxis to those who would benefit most, and ultimately, reducing the occurrence of VTE [35]. However, extensive testing for the presence of a thrombophilic state is quite costly. Screening should be reserved for patients who sustain their first event prior to 50 years of age, have a history of recurrent events, or who have a first-degree relative with a venous thromboembolic event that also occurred prior to the age of 50 [47].

According to the “Guidelines of the Ministry of Health: The prevention and treatment of thromboembolism”, thrombophilic state can be divided into two types:

A. Mild thrombophilia: negative family history, the first VTE occurs after 45 years of age. In this case the recommended examinations are: activated protein C (APC)-resistance, FVQ506 and FIIG20210A mutation, lupus anticoagulant (LA), fasting homocysteine level. The occurrence of multiple genetic defects is 1-2%.

B. Severe thrombophilia: the first VTE event occurs prior to 45 years of age, recurrent VTE, have a first-degree relative with a venous thromboembolic event that also occurred prior to the age of 45. In this case the recommended examinations are: AT, PC, PS activity, APC-resistance, FVQ506 and FIIG20210A mutation, LA, fasting homocysteine level. The occurrence of multiple genetic defects is ca. 10%.

In a group of patients with a history of VTE only 40-70% has an underlying thrombophilia (depending on the inclusion criteria). This can be explained from the existence of not yet identified prothrombotic abnormalities, which hold the same thrombotic event potential as the known prothrombotic abnormalities or the presence of acquired risk factors.

Additionally, the diagnosis of the various haemostatic disorders using routine laboratory tests and single factor determinations is time consuming and/or expensive. There is no single haemostasis laboratory test that has the capacity to accurately illustrate the clinical effects of procoagulant or anticoagulant interventions up to now, and is capable of diagnosing overall hypocoagulation or hypercoagulation states of blood. Thrombelastography, which is a global
haemostatic method, is potentially capable of diagnosing thrombophilic states in the most natural environment: whole blood. We will discuss the applicability of rotation thrombelastography in detection of thrombophilia in VTE patients.
2. Multiple electrode aggregometry

2.1. Introduction

The study of the role of platelets in the pathogenesis of ischemic vascular diseases and the monitoring of anti-platelet drug effects in patients with cardio- and cerebrovascular diseases require reliable platelet function tests. Several techniques are in use to measure platelet aggregation.

The most commonly used method is light transmission aggregometry (LTA, “Born” aggregometry) employing citrated or heparinised PRP [48]. Disadvantages of this technique include the need of centrifugation to separate other blood cells from platelets, which are also known to influence platelet function [49,50]. Besides, PRP does not contain every blood platelet. The platelet recovery rate is only 61 to 90% of total, depending on the separation methods used [51,52]. This usually leads to the loss of giant platelets which may be both hypo- and hyperactive [53]. All these factors may artificially alter the measured platelet aggregation response. For these reasons, novel methods have been introduced to measure platelet aggregation in whole blood.

Single platelet counting (SPC) is one of these methods, which is characterized by the measurement of the loss of platelets after stimulation of anticoagulated blood by using conventional haematology analyzers, which has been described in detail in [54], and is widely used [55-59]. Single platelet counting, however, does not differentiate between micro- and macroaggregates. Measurements of aggregation kinetics are laborious, since it requires the measurement of platelet count for each time point.

Another method to measure platelet aggregation in whole blood is impedance aggregometry, which was introduced by Cardinal and Flower [60]. It is based on the attachment of platelets on two platinum electrodes, which results in an increase of electrical resistance between the electrodes. The change of resistance (called “impedance” as an alternating current is applied in order to prevent electrolysis) is continuously recorded. This is proportional to the amount of platelets sticking to the electrodes. Riess et al. compared whole blood impedance
aggregometry with LTA of PRP [61]. They found that there was a good correlation between the two techniques when aggregation was induced by ADP or collagen. As compared with PRP, impedance aggregometry in whole blood was more sensitive to the aggregating effect of thrombin, ristocetin, and arachidonic acid, or to the inhibitory effect of prostacyclin or aspirin [61]. Platelet function in lipemic blood can also be evaluated by impedance aggregometry [62]. However, impedance aggregometry, as realized in commercial instruments (e.g. Chrono-log®) has some problems. The re-usable electrodes have to be cleaned between analyses, which is impractical and a possible source of error.

For these reasons, a novel instrument to measure platelet aggregation in diluted whole blood by impedance aggregometry has been developed. This instrument uses disposable test cells with duplicate impedance sensors. The method is called multiple electrode aggregometry (MEA) because it uses 4 electrodes per test cell.

2.2. Aims of the study with multiple electrode aggregometry

1. Validating this novel impedance aggregometry method by using platelet agonists ADP and collagen, considered as the most important agonists in atherosclerotic conditions and furthermore thrombin receptor-activating peptide (TRAP)-6, which powerfully mimics the effects of thrombin exerted on proteinase activated receptor 1 (PAR-1).

2. Examining the in vitro effect of two anticoagulants: the commonly used sodium citrate (12.9 mmol/l) and the direct thrombin inhibitor hirudin.

3. Comparing MEA to another sensitive method for platelet aggregation: single platelet counting, using the same agonists.

4. Evaluating the in vitro platelet aggregation inhibitory effects of aspirin and the ADP scavenger enzyme apyrase as measured by MEA and SPC.
5. Investigating the contribution of the two platelet ADP receptors (P2Y₁ and P2Y₁₂) in platelet aggregation by using the P2Y₁ and P2Y₁₂ antagonists, i.e. MRS2179 and AR-C69931MX.

6. Testing MEA’s ability to detect the aggregation inhibitory effect of per os clopidogrel treatment ex vivo, exhausting the synergistic effect of prostaglandin E₁ (PGE₁).

### 2.3. Materials and methods

#### 2.3.1. Materials

ADP, epinephrine, apyrase (grade VII), the ADP receptor P2Y₁ antagonist MRS2179 and PGE₁ came from Sigma (Taufkirchen, Germany), acetylsalicylic acid from Fluka. Collagen (Horm) was obtained from Nycomed Pharma (Munich, Germany), tri-natriumcitrate dihydrate was from Merck (Darmstadt, Germany). The ADP receptor P2Y₁₂ antagonist AR-C69931MX (2-trifluoropropylthio-N-(2-(methylthio)ethyl)-β,8dichloromethylene ATP) was a gift from AstraZeneca R & D Charnwood (Loughborough, United Kingdom). As hirudin, recombinant lepirudin (Refludan, Schering AG, Germany) was used. TRAP-6 (SFLLRN-OH, thrombin receptor activating peptide-6) was obtained from Dynabyte Medical (Munich, Germany). Clopidogrel was Iscover from Bristol-Myers Squibb Pharma EEIG (Uxbridge, UK).

#### 2.3.2. Preparation of blood

Blood was collected from healthy volunteers using a 19 G needle and plastic syringe containing either 1/10 volume recombinant hirudin (final concentration in blood ~200 U/ml; 13 µg/ml) or 1/10 volume sodium citrate (final concentration in blood 12.9 mmol/l) or using 5 ml Vacutainer tubes with buffered citrate with the same final concentration of sodium citrate after informed consent was obtained. All
volunteers denied taking any medication which might affect platelet function in the two weeks preceding the experiments.

For some experiments, acetylic salicylic acid (ASA, final concentration in blood 1 mmol/l) was dissolved in the sodium citrate solution or saline solution before addition of hirudin, to achieve total dissolution. In case of the ex vivo experiments, blood was taken from six healthy volunteers before and 3, 6 and 78 hours after administration of the first dose of clopidogrel (300 mg loading dose on day 1 and 75 mg on day 2, 3 and 4) and at least 2 weeks after the last drug ingestion. Measurements were performed between 0.5 and maximal 4 hours after venipuncture. The blood was kept in closed polyethylene tubes at room temperature during the experiment.

2.3.3. Measuring platelet aggregation by multiple electrode aggregometry

Whole blood aggregation was determined using a new generation impedance aggregometer (Multiplate® analyser, Dynabyte Medical, Munich). The device has 5 channels for parallel tests, and a single use test cell with duplicate impedance sensors, each consisting of 2 straight electrode wires (blood contacting area: 3 mm length, 0.3 mm diameter for each sensor wire). The impedance change determined by each sensor is recorded independently. Due to the use of 2 duplicate electrodes per test cell the technique is called “multiple electrode aggregometry” (MEA). The instrument is called “Multiplate®”, short for “multiple platelet function analyzer”, indicating the multiplicity of channels and sensors per channel of the device. During the analysis the sample-reagent mixture is stirred using a discardable PTFE (poly-tetra-fluoro-ethylene) -coated magnetic stirrer (800 U/min) (Figure 2.1.).
In order to prepare a 1:1 dilution of whole blood, first preheated (37° C) saline (288 µl) was placed into the test cells. Into the saline we added 12 µl of the appropriate dilutions of inhibitors or other test substances; the ADP scavenger apyrase (final concentration 10 U/ml), the P1Y₁ receptor inhibitor MRS2179 (final concentrations from 0.1 µmol/l to 200 µmol/l), the P1Y₁₂ receptor inhibitor AR-C69931MX (final concentrations from 0.1 nmol/l to 2000 nmol/l), the prostaglandin PGE₁ (final concentrations 6 or 10 nmol/l, depending on the anticoagulation used), CaCl₂ for recalcification of citrated blood (final concentration 2 mmol/l) or 12 µl saline for control tests to reach 300 µl volume. Afterwards we added 300 µl of anticoagulated whole blood - with or without aspirin, as described above (Figure 2.2.).
In one experimental series, a special version Multiplate® apparatus with a different stirring device was tested in order to analyze the effect of turbulence on platelet aggregation. In this apparatus, the stirrer was turned alternately to the right and left by 180°. In order to attain sufficient flow in the cup a larger (6x3 mm vs. 4x2 mm) stirrer was applied.

After 3 minutes of incubation and stirring at 37°C, the measurements were started by adding 12 µl of the appropriate agonist solution or saline to the 1:1 diluted blood. The impedance change caused by the adhesion and aggregation of platelets on the electrode wires was continuously detected. The results registered by the two sensors provided two aggregation curves. The two curves typically did not differ much; if they did, the measurement was considered to be faulty and was repeated. This difference was only roughly estimated but only occurred in less than 5% of the tests. In this way the duplicate sensors serve as an internal control. The mean values of the two determinations are expressed in arbitrary “aggregation units” (AU). The test time was typically 5 minutes. The area under the aggregation curve (AUC) was used to express the aggregation response over the measured time (AU*min) (Figure 2.3.).
Aggregation was triggered using ADP, collagen or TRAP-6. Platelet aggregation did not alter from 30 to 240 minutes after venipuncture (data not shown) when measured by MEA. The method’s reproducibility (intra-assay variability) was found to be 6±3% (mean coefficient of variations [%CV] ±SD, n=8).

The maximal aggregation (AU) and the area under the aggregation curve (AUC) showed a linear correlation in case of ADP and TRAP-6 -induced platelet aggregation. The correlation was exponential in the case of collagen-induced platelet aggregation.
2.3.4. Single platelet counting

Single platelet counting (SPC) was performed as previously described by Fox et al., and Haseruck et al. [54,59], with the exception that diluted blood (1:1 dilution with saline) was used in order to make the results comparable with those of the MEA measurements. Aliquots of diluted blood (15 µl) were removed from the Multiplate® test cells just before adding the agonist or saline, or at different times after addition of the agonist, and transferred into a fixation buffer (30 µl). The fixation buffer consisted of NaCl (150 mmol/l), Na$_2$EDTA (4.6 mmol/l), Na$_2$HPO$_4$ (4.5 mmol/l), KH$_2$PO$_4$ (1.6 mmol/l) and formaldehyde (0.16 w/v%) (pH 7.4). Fixed samples were counted using the Sysmex Platelet Counter PL-100 (TOA Medical Electronics, Kobe, Japan). In the counter, the blood particles (diluted according to the instructions) pass through the small aperture of the transducer where an electric signal is produced. The size of this signal is directly proportional to the size of the particle. After counting and a subtraction process, the number of the particles of the size of a single platelet is arrived at. Percentage aggregation was calculated as percentage loss of single platelets compared to baseline count. All platelet counts were done in duplicate.

2.3.5. Manual platelet counting

To measure platelet concentration in blood from individual donors, anticoagulated blood (20 µl) was diluted with 2 ml Thrombo Plus (Sarstedt, Germany) haemolysis buffer and platelets were counted in a Neubauer chamber according to the manufacturer’s instructions.

Platelet concentrations of blood from different donors ranged between 164 and 395 G/l. Platelet aggregation induced by ADP (5 µmol/l) (Figure 2.4.) or collagen (2.5 µg/ml) (not shown) seemed to be independent of the platelet concentration in the normal range, regardless of whether blood was anticoagulated with citrate or hirudin.
Figure 2.4. Independence of platelet aggregation from thrombocyte concentration. Maximal platelet aggregation induced by 5 µmol/l ADP was measured by multiple electrode aggregometry in citrated (A, n=22) or in hirudin-anticoagulated (B, n=16) blood. Statistical analysis was performed by linear regression analysis.

2.3.6. Phase contrast microscopy

In some cases, after an aggregation reaction was completed, the Multiplate® electrodes with the adherent thrombocytes were carefully washed 3-4 times with PBS buffer and then fixed with 3.7 % formaldehyde for 10 minutes at room temperature. After a PBS wash, the electrodes were carefully cut off, placed on a glass slide and covered with Moviol and a glass cover slip. Phase contrast microscopy was performed using an inverse Zeiss microscope (Axiovert 200 M) and the corresponding Axiovision software.

2.3.7. Statistical analysis

Results are reported as mean ± SD from 4-8 experiments conducted on different blood samples. Intra-assay, intra- and interindividual variabilities were expressed as %CV (coefficient of variations, calculated as SD/mean x 100). Intra-assay variability was determined in two independent donors by assaying the same blood 4 times simultaneously. Half maximal effective concentrations (EC50) were generated manually from every single dose-response curve, based on the following equation: Y50=(y_max−y_min)/2+y_min, the fitting X50 was read from the graph (where
Y_{50}: half of maximal aggregation, \( y_{\text{max}} \): maximal aggregation (AUC), \( y_{\text{min}} \): control, without platelet stimulus AUC). Subsequently, the EC_{50}s were statistically evaluated (mean ± SD, t-tests). Half maximal inhibitory concentrations (IC_{50}) were determined correspondingly. Statistical comparisons of samples were performed by paired and unpaired Student’s t-test or linear regression analysis using Microsoft Excel 2000. Differences were considered significant when p was < 0.05.

2.4. Results

2.4.1. Spontaneous platelet aggregation and ADP-, collagen- and TRAP-6-induced platelet aggregation as measured by multiple platelet aggregometry

To evaluate the method, we measured spontaneous platelet aggregation, platelet aggregation induced by a „weak“ platelet agonist ADP, and two „strong“ platelet agonists, collagen and the PAR1 receptor agonist TRAP-6. In multiple platelet aggregometry a small, but considerable amount of spontaneous platelet aggregation was found, which ranged from 0-123 AU*min during the measurements. However, when turbulent stirring was applied (alternate movement of the stirrer to right and left by 180°, as described above) spontaneous aggregation was much higher (280±108 AU*min, mean±SD, n=6 in hirudin-anticoagulated blood) as compared to normal, circular stirring (37±23 AU*min, mean±SD, n=8, p<0.05) (Figure 2.5.).
ADP induced a moderate platelet aggregation in anticoagulated whole blood in a time- and dose-dependent manner (Figure 2.6. A). Maximal platelet aggregation induced by ADP was highly variable between different donors (Figure 2.7. A, B), it ranged from 27 AU*min to 900 AU*min. When ADP-induced platelet aggregation was assessed for the same two donors on different days over a time period of 4 months, platelet aggregation was fairly constant. For donor “A” it ranged from 276 to 435 AU*min (365±68, mean±SD, n=5, CV: 18%), and for donor “B” it ranged from 413 to 483 AU*min (448±49, mean±SD, n=3, CV: 11%).
Collagen induced platelet aggregation obtained with impedance aggregometry started after a timelag, which changed in a dose- and donor-dependent manner (Figure 2.6. B). The maximal aggregation induced by collagen (2.5 µg/ml) in citrate- and hirudin-anticoagulated blood varied between different donors (Figure 2.7. C, D). It ranged from 225 to 899 AU*min. Maximal aggregation after collagen-stimulation varied less than after ADP stimulation of blood (compare with Figure 2.7. A, B).
Figure 2.7. Distribution of ADP- and collagen-induced platelet aggregation measured by MEA. Platelet aggregation induced by 5 µmol/l ADP in citrated (A) or in hirudin-anticoagulated blood (B), and 2.5 µg/ml collagen in citrated (C) or in hirudin-anticoagulated blood (D) of 29, 21, 20 and 18 different healthy donors, respectively, assessed by multiple electrode aggregometry.

With phase contrast microscopy we observed that while collagen resulted in a higher platelet aggregation signal than ADP when measured by MEA, it induced more platelet layers to adhere to the electrodes than ADP (Figure 2.8.). Platelet aggregates could be seen only on one side of the electrode. In the case of spontaneous platelet aggregation we saw no apparent platelet aggregation using phase contrast microscopy, whereas using fluorescence microscopy some adhered platelets and red blood cells could be recognised on the surface of the electrode (not shown).
TRAP-6-induced platelet aggregation was characterized by a rapid initial response without a lag phase and a rapidly reached maximal aggregation (Figure 2.6. C). The maximal aggregation induced by TRAP-6 (20 µmol/l) in citrate- and hirudin anticoagulated blood varied between different donors and ranged from 230 to 1028 AU*min. The interindividual variation of aggregation was less after TRAP stimulation than after stimulation with ADP (data not shown).

When measured by MEA, spontaneous platelet aggregation did not correlate with stimulus-induced platelet aggregation of citrate- or hirudin- anticoagulated blood. ADP-, collagen- and TRAP-6-induced maximal platelet aggregation showed a linear correlation with each other in blood anticoagulated with citrate or hirudin (Figure 2.9. and data not shown). The half maximal effective concentrations for ADP, collagen and TRAP-6 are represented in Table 2.1. (page 26.).
Figure 2.9. Correlation between collagen- and ADP-induced maximal platelet aggregation (PA). Platelet aggregation induced by 5 µmol/l ADP or 2.5 µg/ml collagen was measured by multiple electrode aggregometry in citrated blood (n=17). Statistical analysis was performed by linear regression analysis.

2.4.2. Effect of different anticoagulation techniques

Citrate, the most commonly used anticoagulant in haemostasis measurements, complexes divalent cations, lowering the millimolar concentration of ionized calcium into the micromolar range (less than 5 µmol/l) [64]. Calcium is an important participant in coagulation, and it may also affect platelet aggregation. Hirudin, which prevents clotting by inhibiting the action of thrombin, has the advantage of preserving the physiological concentration of ionized calcium and magnesium [65,66]. Thus to investigate the effect of anticoagulation, we measured platelet aggregation parallely in both citrate- and hirudin-anticoagulated whole blood. We found differences in spontaneous aggregation, agonist-induced platelet aggregation, and also the effect of different inhibitors.

Spontaneous platelet aggregation was higher in citrated blood than in hirudin-anticoagulated blood. In hirudin-anticoagulated blood, but not in citrated blood, pre-treatment with apyrase significantly reduced spontaneous platelet aggregation. Aspirin had no effect on spontaneous platelet aggregation when either anticoagulants were used (Figure 2.10).
Figure 2.10. Effects of anticoagulation and inhibition by apyrase and aspirin on spontaneous platelet aggregation. Spontaneous platelet aggregation measured by multiple electrode aggregometry in citrated (open bars) or hirudin-anticoagulated blood (closed bars) after 5 minutes of stirring. Data are mean±SD (n=8). Asterisk indicates statistical significance of the effects of apyrase (10 U/ml, APY) or aspirin (1 mmol/l, ASA) as compared to control (*p<0.05). Cross indicates statistical significance of the difference between citrated and hirudin-anticoagulated blood (p<0.05).

In the case of agonist-induced platelet aggregation we observed that all agonists induced higher aggregation in hirudin-anticoagulated blood as compared to citrated blood. Maximal platelet aggregation induced by ADP was lower in citrated blood (304±136 AU*min) than in hirudin-anticoagulated blood (483±224 AU*min, mean±SD; n=20; p<0.00001) (Figure 2.7. A, B). Figure 2.11. shows that this difference could be observed for all ADP concentrations used, but was not apparent when measured by single platelet counting.
Figure 2.11. ADP-induced platelet aggregation in citrate and hirudin-anticoagulated blood. Dose–response curves of ADP assessed by multiple electrode aggregometry (A) and single platelet counting (B). Data are mean±SD (n=5). Asterisks indicate statistical significance of the difference between hirudin- and citrate-anticoagulated blood (*p<0.05).

The maximal aggregation induced by collagen was again lower in citrate- than in hirudin-anticoagulated blood (Figure 2.7. C, D). The values (mean±SD) were 421±128 AU*min and 674±110 AU*min, respectively (p<0.0001, n=19, see also Figure 2.15. C, D). The EC\textsubscript{50} of collagen was lower in citrated than in hirudin-anticoagulated blood, indicating that the platelets were more, rather than less, sensitive to collagen-stimulation in citrate-blood as compared with hirudin-blood (Table 2.1., p<0.01). TRAP-6-induced platelet aggregation was higher in hirudin-anticoagulated blood than in citrated blood measured by MEA. The mean±SD was 691±184 AU*min and 531±194 AU*min in hirudin- and citrate-anticoagulated blood, respectively (p<0.0001, n=15).

2.4.3. Comparison of multiple electrode aggregometry and single platelet counting

Spontaneous platelet aggregation during stirring of diluted whole blood was observed by both single platelet counting (Figure 2.12.) and MEA (Figure 2.10.).
Figure 2.12. Spontaneous platelet aggregation in whole blood assessed by single platelet counting was not significantly affected by apyrase (10 U/ml, APY), aspirin (1mmol/l, ASA) or the type of anticoagulation used (citrated blood: open bars, hirudin-anticoagulated blood: closed bars). Data are mean±SD (n=8).

By comparing the time-course of ADP-induced platelet aggregation measured by single platelet counting or MEA, it was observed that maximal platelet aggregation was reached more rapidly according to single platelet counting. Also, platelet aggregation induced by 1 µmol/l ADP was partly reversible according to single platelet counting (Figure 2.13), which was not observed with MEA (Figure 2.6. B).

Figure 2.13. Time course of ADP induced platelet aggregation measured by single platelet counting. Data are representative of 5 experiments.
The dose-response curves for ADP were similar in both methods (Figure 2.11). However the EC$_{50}$ of ADP measured by single platelet counting tended to be lower than when measured by MEA (Table 2.1).

<table>
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<tr>
<th></th>
<th>ADP (µmol/l)</th>
<th>Collagen (µg/ml)</th>
<th>TRAP (µmol/l)</th>
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<tr>
<td></td>
<td>EC$_{50}$</td>
<td>p</td>
<td>EC$_{50}$</td>
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<tr>
<td><strong>Citrate</strong></td>
<td></td>
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<tr>
<td>SPC</td>
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<td>0.33±0.08 (4)</td>
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<td><strong>Hirudin</strong></td>
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<tr>
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<td>MEA</td>
<td>1.00±0.56 (4)</td>
<td></td>
<td>0.63±0.17 (4)</td>
</tr>
</tbody>
</table>

Note: Data are mean±SD (n in parenthesis). The statistical significance (p-values) was assessed between the data of single platelet counting and MEA.

The collagen dose-response curves were different in both methods of aggregation measurement (see also Figure 2.15, page 34.). The half maximal effective concentration (EC$_{50}$) of collagen to induce platelet aggregation was significantly lower when aggregation was measured by single platelet counting as compared to MEA, both in citrate- and hirudin-anticoagulated blood (Table 2.1.). TRAP-6, as a strong platelet aggregation-inducing agent, caused irreversible aggregation at all examined concentrations as measured by MEA. The dose-response curves of TRAP-6 were similar in both methods of aggregation measurement (see also Figure 2.16.) shown by comparison of the EC$_{50}$ values (Table 2.1.). No significant effect of the type of anticoagulation was found when ADP-, collagen-, or TRAP-6-induced maximal platelet aggregation was assessed by single platelet counting.
2.4.4. Evaluation of the in vitro platelet aggregation inhibitory effects of aspirin and apyrase

In clinical settings, the two most commonly used platelet aggregation inhibitors target the cyclooxygenase-1 (COX1) enzyme and the ADP receptor \( \text{P}2\text{Y}_{12} \). We used the COX inhibitor aspirin and the ADP scavenger enzyme apyrase in vitro to test MEA’s ability to detect their action in comparison with single platelet counting.

As already mentioned above, spontaneous platelet aggregation was significantly reduced by pre-treatment with apyrase, but not with aspirin in hirudin-anticoagulated blood. No effect of apyrase or aspirin on spontaneous platelet aggregation measured by MEA in citrated blood or by single platelet counting in citrated or hirudin-anticoagulated blood was found (Figure 2.10. and Figure 2.12.).

On the other hand, when turbulent stirring was applied (alternate movement of the stirrer to right and left by 180°) the high spontaneous platelet aggregation could be reduced by aspirin and by apyrase which indicates a role of TxA\(_2\) released from activated platelets and ADP released from activated platelets and/or damaged red blood cells (Figure 2.14.).
Figure 2.14. Effects of apyrase (10 U/ml, APY) and aspirin (1 mmol/l, ASA) on spontaneous platelet aggregation induced by turbulent stirring. Spontaneous platelet aggregation was measured in hirudin-anticoagulated blood while being stirred turbulent (the stirrer was turned alternately to the right and left by 180°) for 5 minutes in a Multiplate® test cell. Data are mean±SD (n=6). Asterisks indicate statistical significance of the effects of apyrase (10 U/ml, APY) or aspirin (1 mmol/l, ASA) as compared to control (*p<0.05).

The collagen-induced platelet aggregation measured by single platelet counting or MEA was partially inhibited by the ADP scavenger enzyme apyrase (10 U/ml) and aspirin (1 mmol/l). The inhibitory effect of aspirin was more pronounced when aggregation was measured by MEA in comparison to single platelet counting. Inhibition by apyrase was most pronounced in hirudin-anticoagulated blood when aggregation was measured by MEA (Figure 2.15.).
Aspirin did not inhibit TRAP-6-induced platelet aggregation according to single platelet counting or MEA in citrate- or in hirudin-anticoagulated blood. Apyrase slightly inhibited platelet aggregation at various TRAP-6 concentrations in both single platelet counting and MEA (Figure 2.16.).
2.4.5. Investigating the contribution of the two platelet ADP receptors (P2Y₁ and P2Y₁₂) in platelet aggregation

2.4.5.1. Evaluation of the effective inhibitory concentrations of the ADP receptor antagonists MRS2179 and AR-C69931MX on ADP-induced platelet aggregation

ADP-induced platelet aggregation measured by MEA was concentration dependently inhibited by the ADP receptor P2Y₁ inhibitor MRS2179 and the P2Y₁₂ inhibitor AR-C69931MX. The aggregation was completely inhibited by certain concentrations of the antagonists (>100 µmol/l MRS2179 and >100 nmol/l AR-C69931MX) (Figure 2.17.).
We determined the half maximal effective inhibitory concentrations (IC$_{50}$) of platelet aggregation induced by 5 µmol/l ADP of the antagonists using different anticoagulation techniques. The IC$_{50}$ values for AR-C69931MX were 3 nmol/l in hirudin-anticoagulated blood and 4 nmol/l in citrated blood, while for MRS2179 they were 1.5 µmol/l in hirudin-anticoagulated blood and 6 µmol/l in citrated blood (Figure 2.18).

Very low concentrations of MRS2179 (0.1 µmol/l) seemed to enhance platelet aggregation to some extent (by 20%). Based on these findings 1 µmol/l or (in case of the ex vivo experiments) 100 nmol/l concentration of AR-C69931MX and 100 µmol/l of MRS2179 were chosen in order to evaluate the role of the ADP receptors in spontaneous platelet aggregation or platelet aggregation induced by different agonists.
Figure 2.18. Inhibition of platelet aggregation induced by 5 µmol/l ADP by AR-C69931MX (empty squares) and MRS2179 (diamonds) in citrate- (A) and hirudin- anticoagulated (B) blood. Data are mean±SD (n=5).
2.4.5.2. Inhibition of spontaneous platelet aggregation by MRS2179 and AR-C69931MX

The spontaneous platelet aggregation as measured by MEA was significantly inhibited by both ADP receptor antagonists in hirudin-anticoagulated blood; there was no detectable additive effect between the two antagonists when added together. Spontaneous platelet aggregation was 44.9 AU*min, 17.0 AU*min, 15.2 AU*min and 13.9 AU*min for control, with the addition of 1 µmol/l AR-C69931MX, 100 µmol/l MRS2921, or both, respectively (results are mean ± SD). These results indicate that spontaneous platelet aggregation by trace amounts of ADP in stirred hirudin-blood is mediated by activation of both the P2Y\textsubscript{1} and P2Y\textsubscript{12} receptor.

In citrated blood, however, only the P2Y\textsubscript{1} antagonist MRS2179 had statistically significant inhibitory effect (it reduced spontaneous platelet aggregation by 40%) (Figure 2.19), indicating that only P2Y\textsubscript{1} activation is required. Spontaneous platelet aggregation was 36.2 AU*min for control, 32.4 AU*min, 21.7 AU*min and 18.9 AU*min, with the addition of 1 µmol/l AR-C69931MX, 100 µmol/l MRS2921, or both, respectively (results are mean ± SD).

![Figure 2.19. Effects of AR-C69931MX (1 µmol/l, ARC) and MRS2179 (100 µmol/l, MRS) on spontaneous platelet aggregation in citrate- (open bars) or hirudin-anticoagulated blood (closed bars). Data are mean±SD (n=9). Asterisks indicate statistical significance of the effects of AR-C69931MX or MRS2179 (*p<0.05) as compared to control.](image-url)
2.4.5.3. Inhibition of collagen–induced platelet aggregation by MRS2179 and AR-C69931MX

Both ADP receptor antagonists showed approximately the same effect if hirudin anticoagulation was used. MRS2179 (100 µmol/l) inhibited platelet aggregation induced by collagen (0.5-1-2.5 µg/ml) by 71.6-49.1-39.8 (8.1-24.9-17.3) %, respectively and AR-C69931MX (1 µmol/l) by 62.0-55-30 (20.8-10.7-9.9) %, respectively (data are mean (SD)). MRS2179 had less effect in citrated blood, which difference was statistically significant, when aggregation was induced by 1 or 2.5 µg/ml collagen. The combination of both antagonists showed synergism in inhibition of collagen induced platelet aggregation (Figure 2.20.).

If we relate all aggregation data to the collagen-induced platelet aggregation achieved in hirudin blood without adding an antagonist (100%), it is apparent that the relative amount of the residual platelet aggregation was about the same in citrated and hirudin-anticoagulated blood after adding an ADP receptor antagonist, and this reduction was enhanced when they were added together (Figure 2.21.)
Figure 2.20. Effects of MRS2179 (100 µmol/l, MRS) and AR-C69931MX (1 µmol/l, ARC) on platelet aggregation induced by collagen (0.1-2.5 µg/ml) in citrate- (a) or hirudin-anticoagulated blood (b). Data are mean±SD (n=5). Symbols indicate statistical significance of the effects of AR-C69931MX or MRS2179 (*p<0.05) as compared to control, or interaction between AR-C69931MX or MRS2179 (‡p<0.05).
2.4.5.4. Inhibition of TRAP-6-induced platelet aggregation by MRS2179 and AR-C69931MX

There was a clear inhibition of AR-C69931MX in both anticoagulants used on TRAP-6-induced platelet aggregation, to about 25-80% of control aggregation. The higher the TRAP-6 concentrations, the less inhibition of aggregation could be achieved with AR-C69931MX. MRS2179, however did not inhibit the TRAP-6-induced platelet aggregation in citrated blood, on the other hand, it significantly enhanced the aggregation induced by 10 µmol/l TRAP-6 (Figure 2.22. A). In hirudin-anticoagulated blood however, MRS2179 was able to inhibit 3.75 µmol/l TRAP-6-induced platelet aggregation to the same extent as AR-C69931MX, but this inhibitory potential declined with the increase in the TRAP-6 concentration. Despite of the loss of the inhibitory effect of MRS2179, a synergism between the antagonists could be observed even at higher TRAP-6 concentrations (Figure 2.22. B).
Figure 2.22. Effects of MRS2179 (100 µmol/l, MRS) and AR-C69931MX (1 µmol/l, ARC) on platelet aggregation induced by TRAP-6 (2.5-20 µmol/l) in citrate- (A) or hirudin-anticoagulated (B) blood. Data are mean±SD (n=4). Symbols indicate statistical significance of the effects of AR-C69931MX or MRS2179 (*p<0.05) as compared to control, or interaction between AR-C69931MX and MRS2179 (‡p<0.05).
2.4.6. Clopidogrel responsiveness measured by MEA in blood (ex vivo studies)

Control ("spontaneous") platelet aggregation seemed not to be affected by clopidogrel intake. Inhibition of ADP (5 µmol/l)-induced platelet aggregation 3h to 3 days after clopidogrel intake was dependent on the volunteer tested (Figure 2.23.). In hirudin-anticoagulated blood, 2 persons (P1 and P2) showed more than 90% inhibition of aggregation (clopidogrel responsive), whereas 2 persons (P3, P5) were clopidogrel-resistant (<10% inhibition). Two persons (P4, P6) were partially responsive to clopidogrel (49 and 57% inhibition). The maximal effect could be seen already 3 hours after the administration of the loading dose (300 mg) of clopidogrel, and the effect was fairly constant throughout the experiment. Addition of AR-C69931MX (100 nmol/l) to the test cells in vitro maximally inhibited ADP-induced platelet aggregation before and after clopidogrel-intake in all volunteers, thereby indicating the functional P2Y\textsubscript{12} receptor reserve after clopidogrel-intake (i.e. clopidogrel could not inhibit platelet aggregation completely). Similar observations were made, when citrate was used as anticoagulant. However, ADP-induced aggregation was again lower in citrate- than in hirudin-anticoagulated blood, and the inhibition by clopidogrel was therefore less detectable (data not shown).

Addition of PGE\textsubscript{1} (10 nmol/l in case of hirudin anticoagulated blood, 6 nmol/l in case of citrated blood) itself inhibited platelet aggregation by 30-50%, and enhanced the clopidogrel induced platelet inhibition from ~40% to ~60-85% in hirudin blood. Except of P1 and P2, whose ADP-induced platelet aggregation was completely inhibited by clopidogrel, the relative inhibition was higher when using additional PGE\textsubscript{1} than ADP alone (Figure 2.24. and Figure 2.23).

As expected from the results of incomplete inhibition of TRAP- and collagen-induced platelet aggregation by AR-C69931MX, TRAP-6 and collagen-induced aggregation was only slightly affected after clopidogrel intake. Collagen-induced platelet aggregation remained practically unchanged after clopidogrel intake (data not shown). Two weeks after the last clopidogrel intake, aggregation values returned to the previous levels (Figure 2.24.).
Figure 2.23. Effects of per os clopidogrel treatment, in vitro added PGE$_1$ and AR-C69931MX (100 nmol/l, ARC) on platelet aggregation induced by ADP (5 μmol/l) in hirudin-anticoagulated blood on 6 healthy individuals. Lines and symbols indicate the inhibitory rate (%) of clopidogrel with (□) or without (▲) the addition of PGE$_1$ related to the platelet aggregation at time point 0 (100%) with (□) or without (▲) the addition of PGE$_1$, respectively.
Figure 2.24. Effects of per os clopidogrel treatment and in vitro added PGE$_1$ and AR-C69931MX (100 nmol/l, ARC) on platelet aggregation induced by ADP (5 µmol/l) in citrate (A) and hirudin-(B) anticoagulated blood of 6 healthy individuals. Data are mean±SD (n=6). Symbols indicate statistical significance of the effects of clopidogrel (*p<0.05) as compared to the aggregation before clopidogrel intake, or the effect of PGE$_1$ or AR-C69931MX (‡p<0.05).
2.5. Discussion

We were the first to introduce a new technique for whole blood aggregometry based on the impedance method. The new method applies duplicate impedance sensors each consisting of two straight electrode wires. Based on the application of a total of 4 electrodes for the analysis, the method is called “multiple electrode aggregometry” (MEA). By the application of an electrical signal, the measurement is independent of optical variables in the sample (e.g. lipemia) [62]. Each analysis provides a kinetic signal which is characterized by the area under the curve (AUC, expressed as AU*min). We found that the instrument was easy to handle and the results were highly reproducible.

In whole blood aggregometry, unlike light transmission aggregometry, there is no possibility to adjust the platelet concentration. Therefore we investigated the correlation between whole blood platelet concentration and the maximal aggregation responses to different agonists. Previously, Sharp et al. showed a strong correlation between whole blood platelet count and ADP-induced platelet impedance changes [67]. Our data, however, did not show a dependency of the aggregation induced by collagen, ADP and TRAP on the platelet concentrations within the normal range of platelet counts in healthy volunteers (Figure 2.4.). According to our observations with phase contrast microscopy, the greater amount of platelets aggregated on the surface of the electrode, the higher signal was detected by the instrument.

We observed a great interindividual variability in both spontaneous and agonist-induced platelet aggregation when measured by MEA, and the variability of platelet aggregation was most pronounced after stimulation with ADP. A linear correlation was shown between the aggregations induced by different agonists. No correlation was observed, however, between spontaneous platelet aggregation and stimulated platelet aggregation.

All of the investigated agonists elicited significantly higher platelet aggregation responses measured by MEA in hirudin-anticoagulated blood than in citrated blood, confirming previous findings of Wallén et al. [64]. Interestingly, spontaneous platelet aggregation according to MEA was less in hirudin-blood than
in citrated blood (7% vs. 16% of maximal aggregation, respectively), which excludes the possibility of an artefact caused by hirudin. However, since spontaneous aggregation was not inhibited in citrated blood by apyrase or by aspirin, consequently neither ADP nor TXA₂ contributed to the more pronounced spontaneous aggregation. The higher spontaneous aggregation in citrate-anticoagulated vs. hirudin-anticoagulated blood could be explained by small traces of active thrombin possibly present in blood anticoagulated with citrate.

MEA was compared to single platelet counting, one of the standard techniques of platelet aggregation analysis in whole blood. We found that the results obtained by MEA compared, in general, well with the single platelet counting method in diluted blood.

Some spontaneous platelet aggregation by stirring of diluted whole blood was observed using both single platelet counting and MEA. Armstrong et al. reported spontaneous aggregation determined by single platelet counting which was inhibited by the membrane stabilizer chlorpromazine [68]. In our experiments, spontaneous aggregation determined by single platelet counting was not significantly inhibited by apyrase or aspirin. In hirudin-blood spontaneous platelet aggregation was significantly inhibited by apyrase when measured by MEA, which suggests a role of ADP that could be released either from red blood cells or platelets. We found that the use of a different MEA instrument causing more turbulence due to uneven stirring resulted in significantly higher spontaneous platelet aggregation. This can be explained by both the release of ADP and the formation of Txₐ₂ from activated platelets, since under these conditions spontaneous aggregation could be inhibited by both apyrase and aspirin.

Besides the good agreement of platelet aggregation responses determined by SPC and MEA, differences between the two methods were also observed. Single platelet counting was more sensitive in the detection of platelet aggregation induced by low concentrations of ADP (non-significant) and collagen (significant), while there was no difference in sensitivity towards TRAP-6-induced platelet aggregation. These differences are probably due to the fact that single platelet counting method measures recruitment of platelets into platelet aggregates which can be as small as two platelets, while impedance aggregometry is dependent on firm attachment of
platelets onto the metal sensors. Accordingly, platelet aggregation measured by the single platelet counting method was more rapid than when measured by MEA. The reversible phase of aggregation induced by ADP was not detectable by impedance aggregometry. However, the normal blood cation concentration seems to be important for platelet spreading and platelet aggregation as measured by impedance aggregometry, since the ADP-, collagen- and TRAP-induced platelet aggregation was lower in citrate-anticoagulated blood when measured by MEA, but it did not affect platelet aggregation measured by SPC.

The sensitivity of impedance aggregometry towards low dosages of aspirin has previously been shown by Sathiropas et al. [69] in ex vivo experiments. Riess et al. showed that the anti-platelet effect of a single oral dose of aspirin on collagen-induced platelet aggregation could be detected for a longer period by impedance aggregometry than by the turbidimetric method [61]. In the MEA device, aspirin had a significant inhibitory effect even at the highest concentration of collagen used (2.5 µg/ml) in contrast to inhibition measured by single platelet counting. Using impedance aggregometry Wallén et al. also found that inhibition of platelet aggregation by aspirin was more pronounced in citrated blood compared to hirudin treated blood [64]. However, this could not be confirmed by our study.

When comparing platelet inhibition of the ADP scavenger apyrase in citrate- and hirudin-anticoagulated blood, we found that inhibition - as measured by both methods - was more pronounced in hirudin-anticoagulated blood. These findings are in accordance with the observations of Storey et al. on the effects of ADP receptor antagonists in hirudin-anticoagulated blood measured by single platelet counting [70].

Adenosine diphosphate is generally known as a week agonist of platelet aggregation. However, it plays an important role in the amplification of platelet aggregation induced by several “strong” agonists. ADP binds to two G-protein-coupled purinoceptors on platelet surface, the ADP receptor P2Y1 and P2Y12. The P2Y1 is linked to a Gq protein, and its activation leads to the activation of phospholipase-C (PLC), and this accounts for most of the elevation in cytosolic Ca\(^{2+}\) induced by ADP, via formation of inositol triphosphate (IP\(_3\)) and release of Ca\(^{2+}\) from intracellular stores [71,72]. This receptor was formerly known to initiate
the platelet aggregation induced by ADP [73,74]. Jin and Kunapuli demonstrated that also the G_i protein P2Y_{12} is needed for platelet aggregation, reducing cyclic AMP levels via inhibition of adenylate cyclase [75].

The fact that the ADP scavenger enzyme apyrase inhibited the spontaneous, collagen-induced, as well as TRAP-6-induced platelet aggregation proves the role of ADP released during platelet activation. Hence we investigated the role of the two platelet ADP receptors, P2Y_1 and P2Y_{12} in the contribution of the effect of released ADP.

Aleil et al. showed, that inhibition of P2Y_{12} receptor in citrate-PRP results in a decreased, reversible platelet aggregation when measured by turbidimetric method, which cannot be diminished by increasing the antagonist concentration [76]. Storey at al. however reported that the potent P2Y_{12} receptor inhibitor AR-C69931MX (now in phase III drug developing studies known as cangrelol) in the same concentration completely blocked platelet aggregation, when measured by turbidimetry, while in single platelet counting a transient, reversible aggregation could be observed [77]. However, they used hirudin anticoagulation, which may be an explanation for the difference, as it is known, that low divalent cation concentration enhances the release reaction in citrated blood [78]. MRS2179 abolished platelet aggregation using either of the methods, when hirudin anticoagulation was used.

When determining the effective concentrations of the ADP receptor antagonists on ADP induced platelet aggregation by MEA, regardless of the anticoagulation used, we did not observe this reversible phase of aggregation and at a certain AR-C69931MX concentration the aggregation was completely inhibited. The same was observed when using MRS2179. These findings suggest that activation of both receptors is essential to the firm attachment of platelets to the MEA electrodes, and perhaps therefore to other non-physiological surfaces such as atherosclerotic plaques.

The inhibitory effect of the used antagonists was concentration dependent, in accordance with findings observed by turbidimetry or VASP phosphorilation [76], single platelet counting [77], PAC-1 expression assessed by whole blood flow cytometry [79]. IC_{50} values of AR-C69931MX were similar in hirudin- and citrate-
anticoagulated blood, whereas MRS2179 was more effective in hirudin-anticoagulated blood. This finding suggests that in citrated blood, the P2Y₁ receptor or its transduction pathways are already impaired probably due to the low cation concentration.

We have shown that spontaneous platelet aggregation was inhibited by apyrase only in hirudin-anticoagulated blood. Additionally we observed that both ADP receptor antagonists inhibited spontaneous platelet aggregation in hirudin anticoagulated blood. Surprisingly, spontaneous platelet aggregation was inhibited by MRS2179 also in citrated blood, despite MRS2179 being more effective in hirudin blood.

Both ADP receptor antagonists inhibited collagen induced platelet aggregation, suggesting that the secondarily released ADP is important in collagen induced platelet aggregation. Penz et al. demonstrated that collagen structures in human atheromatous plaques play a central role in thrombus formation by activating the platelet glycoprotein VI (GP VI) [80]. According to the current models, the binding of collagen to GP VI results in the activation of phospholipase Cγ2 isoform (PLCγ2) and a raise in the free cytosolic Ca²⁺ concentration and the activation of protein kinase C (PKC) [81]. The activation does not directly involve Gαq or Gαi activation, but results in release reaction, and activation of other cell-surface receptors, including integrin αIIbβ₃ and α2β1 due to inside-out signalling. It is also known, that only a small portion of platelets are directly activated by collagen, even at high collagen concentration, probably because of its bulky nature. Thus the secondarily released agonists play an important role in collagen-induced platelet aggregation.

The effect of the two antagonists was inversely proportional to the collagen concentration, suggesting that the released ADP has more importance at low collagen concentrations, but at higher collagen concentrations other mechanisms come to the front, although even at 2.5 μg/ml collagen both antagonists still had a significant inhibitory effect on platelet aggregation measured by MEA. Compared to single platelet counting, multiple electrode aggregometry appears to be more sensitive to detect the ADP receptor inhibition, since Storey et al. used the same type of collagen (Horm) to demonstrate the effect of AR-C69931MX and MRS2179...
on collagen-induced platelet aggregation as measured by single platelet counting, and in hirudin anticoagulated blood there was no inhibitory effect of the ADP receptor antagonists at 2 µg/ml collagen [70].

We observed that MRS2179 had a markedly lesser effect in citrated blood than in hirudin blood, but the overall residual platelet aggregation was about the same in both anticoagulants. On the other hand, the platelet aggregation obtained in citrated blood was decreased related to the aggregation obtained in hirudin-anticoagulated blood. These findings suggest that this decrease in aggregation is due to the impairment of a P2Y₁ dependent pathway because of the not physiological divalent cation concentrations in citrated blood. As this effect could not be detected in citrated blood before by single platelet counting (measuring microaggregation), we suppose, that the P2Y₁ receptor dependent pathway is responsible for macroaggregate formation, thus the formation of large, stable haemostatic aggregates in vivo, which are essential for haemostasis by strong platelet plugs, paving the way for clot retraction [82].

TRAP-6 is a synthetic peptide ligand of the thrombin-receptor PAR-1, a member of the G-protein coupled receptors found on platelets. We found, that TRAP-6 induced platelet aggregation can be partially inhibited by P2Y₁₂ receptor blockade; however, the P2Y₁ inhibitor MRS2179 has little effect on platelet aggregation. These findings are consistent with the fact, that activated PAR-1 directly activates Goₐ protein, and secondarily enhances the release reaction. The released ADP promotes the platelet aggregation mainly due to the P2Y₁₂ receptor, since the P2Y₁ receptor-linked Goₐ is already activated. The effect of the antagonists, just like at collagen-induced aggregation, was inversely proportional to the TRAP-6 concentration, suggesting that the released ADP is of greater importance at low TRAP-6 concentrations. This is in accordance with the findings of Nylander et al., who investigated platelet inhibition by single platelet counting and by the platelet activation marker PAC-1 expression (antibody recognizing the active conformation of αIIbβ₃) with flow cytometry [79].

In hirudin anticoagulated blood, however, when inducing platelet aggregation with low TRAP-6 concentrations we observed marked inhibition by MRS2179. One possible explanation for this is that so low TRAP-6 concentrations
do not completely activate $\alpha_q$, thus released ADP can activate the other part. However, this effect could not be observed in citrated blood.

After we had demonstrated, that MEA had the ability to detect the inhibition factor of ADP receptors, we started to investigate the ex vivo platelet aggregation inhibitory effect of clopidogrel, which, along with aspirin, is one of the most widely used ADP receptor antagonists in the secondary prevention of ACS. Through its active metabolites formed in the liver, it irreversibly inhibits the platelet ADP receptor $\text{P2Y}_{12}$, resulting in decreased platelet aggregation. In an experiment on a small population of healthy individuals we demonstrated, that when measured by MEA, the currently clinically recommended clopidogrel dose (saturation dose followed by a sustaining dose) significantly decreased the ADP induced platelet aggregation as early as three hours after the drug intake. A large variation in the extent of decrease could be observed among the volunteers. Amongst our test group 2/6 persons showed clopidogrel resistance and 4/6 persons had clopidogrel responsiveness when we compared inhibition to the platelet aggregation measured before clopidogrel intake. This indicates that two of the volunteers were regarded as clopidogrel resistant, in two volunteers we detected complete, in other two partial inhibitory effects. As we concluded, clopidogrel does not develop a full inhibition of the platelet ADP receptor $\text{P2Y}_{12}$, since in vitro added AR-C69931MX could increase the inhibitory effect. It has been previously shown, that the effects of $\text{P2Y}_{12}$ antagonists are enhanced by natural prostaglandins such as $\text{PGE}_1$, as prostaglandins increase the intracellular cAMP concentrations and PKA activation, thus interfering with $\text{P2Y}_{12}$ receptor activation lowering the cAMP concentration in activated platelets [83]. It has also been shown, that VASP phosphorilation was more effective in detecting the clopidogrel effect, if ADP was combined with $\text{PGE}_1$ or iloprost [76,84]. In our study, $\text{PGE}_1$ generally enhanced the inhibitory effect of clopidogrel, as seen in the increased inhibitory rate on Figure 2.23., with the exception that the already low platelet aggregation after the addition of $\text{PGE}_1$ in P1 and P2 could not be inhibited in the same proportion, as the other volunteer’s platelet aggregation. In short, ADP induced platelet aggregation of hirudin-anticoagulated blood together with $\text{PGE}_1$ proved to be the best combination when using multiple electrode aggregometry to detect the clopidogrel effect in healthy
volunteers. TRAP-6 or collagen induced platelet aggregation was not suitable for recording the clopidogrel effect. To define the limit between responders and non-responders, larger studies would be required both in healthy populations and among ACS patients. In our study, the maximal platelet inhibitory effect of clopidogrel was already detectable after 3 hours of drug ingestion, which is in accordance with the result of previous studies [85].

There are some remaining questions. The very high variability among individuals raises difficulties in establishing a reference interval for the different tests and inhibitions by different drugs. Thus, the extent of inhibition may only be calculated if we have values before the inhibiting agent was used. Otherwise, this high variability raises the question, whether or not high platelet aggregation also observed in a young, healthy group of volunteers reflects high platelet reactivity and so acts as a risk factor for ACS or stroke, which may present themselves later in their life. Manoharan et al. in a prospective study demonstrated that impedance aggregometry is able to distinguish between platelet hypo- and hyperactivity in myeloproliferative disorders, which was also beneficial in considering therapeutic options [86]. Optical aggregometry has been shown previously to be inadequate in detecting platelet hyperactivity [87,88]. These areas also need further investigation.

Based on our study, several, practically important investigations have been made. For example, Sibbing et al. analyzed the platelet reactivity of 149 patients scheduled for coronary angiography after clopidogrel treatment, and demonstrated that ADP-induced platelet aggregation measured with MEA decreased significantly after clopidogrel treatment. Platelet aggregation measured with LTA and simultaneously in whole blood with MEA correlated significantly [63]. Afterwards, this group demonstrated that patients, who were defined as clopidogrel low responders (n = 323) according to their MEA results after a clopidogrel loading dose of 600 mg, compared with normal responders (n = 1,285), had a significantly higher risk of definite early drug-eluting stent thrombosis within 30 days (2.2% vs. 0.2%; odds ratio (OR): 9.4; 95% confidence interval (CI): 3.1 to 28.4; p < 0.0001). Mortality rates were 1.2% in low versus 0.4% in normal responders (OR: 3.2; 95% CI: 0.9 to 11.1; p < 0.07) [89].
In conclusion, by using different agonists we demonstrated that MEA is a fast, convenient platelet function testing method that enables the measurement of platelet aggregation in diluted whole blood even in point-of-care form. Using hirudin for blood anticoagulation proved to be better than citrate. Aggregation measurements were convergent to those obtained by single platelet counting. MEA was able to detect the inhibitory effect of aspirin, apyrase and ADP receptor inhibitors in vitro and also ex vivo at different concentration of agonists, and thus is a good candidate for testing even the early effects of antiplatelet drugs in various clinical settings.
3. The use of rotation thrombelastography in the diagnosis of thrombophilia

3.1. Introduction

Citrated plasma has been used predominantly in routine coagulation analysis, for several decades. The initial formation of fibrin can easily be detected photometrically, and employing activated partial thromboplastin time (APTT) and prothrombin time (PT) methodologies, plasma has been widely utilized in the diagnosis and monitoring of patients with major coagulation abnormalities. New understanding of the biochemistry of physiological haemostasis, in particular the tissue factor-dependent generation of thrombin has altered our view of the clotting process [90,91]. The importance of thrombin generation patterns in various coagulation abnormalities has been established [92,93]. Moreover, a significant role of the platelets in promoting thrombin generation has been widely appreciated [94,95]. While plasma contains the majority of the coagulation factors implicated in the coagulation process, whole blood includes phospholipid bearing cells and platelets with an important ability to support coagulation. Recent studies have pointed to the importance of the functions of platelets and leukocytes during coagulation and fibrinolysis [2,3]. Thus, choosing whole blood for coagulation analysis appears more favourable compared to study of plasma coagulation by PT- and APTT – based methods that include excessive activation. Furthermore, often unphysiological phospholipids are included in the assays to substitute for natural cell surface phospholipids. Moreover, since 95% of the coagulation takes place after the initiation of clot formation, continuous profiles of coagulation may provide additional information on the entire haemostatic process [4,96,97].

In 1948, Hartert introduced the trombelastographic principle (TEG) that records viscoelastical changes during coagulation [98]. Thrombeleastographic analysis of coagulation can be carried out on plasma as well as on whole blood. Initially, thrombelastography attracted most interest in research laboratories. Although the time course of thrombin generation in plasma and the endogenous thrombin potential (ETP) may be useful coagulation parameters, clotting involves
components other than thrombin (e.g. platelets, fibrinogen). The continuous coagulation profiles of thrombelastography may provide a more accurate reflection of in vivo biology, covering initiation, development and final clot strength during whole blood clot formation [99].

Today, thrombelastography is utilized in various clinical settings, e.g. monitoring liver transplantation and cardiac surgery [100], it has also been used to investigate hypocoagulation (in haemophilia A [99,101], rare coagulation disorders [102], anticoagulant therapy and dilutional coagulopathy [103-105], hypercoagulation [106-112] and the ex vivo testing of haemostatic interventions [113]. Thrombelastography has been shown to reflect the clinical efficacy of activated prothrombin complex concentrate (aPCC) and recombinant activated factor VII (rFVIIa) in patients with haemophilia A with inhibitors and in patients with acquired haemophilia [101,114]. Although the TEGs ability to detect hypercoagulability of whole blood has been proved early and extensively in various situations, there are only sporadic reports about the detectability of thrombophilia by the method [107,112].

Recently a newly developed portable TEG instrument (ROTEG® Coagulation Analyzer, Pentapharm Co., Munich, Germany) has been marketed [115]. In this TEG, data are continuous, digital, and retrievable for further calculations. It proved to be a convenient method for overall patient-near haemostatic profiling [116,117].

In our study we investigated 55 patients with clinically opposed thrombophilia and compared their ROTEG® parameters to those of 66 healthy volunteers. Our aims were to investigate the applicability of rotation thrombelastography in differentiating between thrombophilia patients and healthy subjects, and to differentiate between hereditary and acquired thrombophilias. We additionally investigated the effect of LMWH-s and gender on ROTEG® parameters.
3.2. Aims of the examination of ROTEG® method in various thrombophilia conditions

1. Examining the applicability of rotation thrombelastography to detect hypercoagulation state in patients with venous thromboembolism, using different assays.

2. Evaluating the value of rotation thrombelastography in the diagnosis of thrombophilia.

3. Comparing the coagulability of the two genders if measured by rotation thrombelastography.

4. Testing the sensitivity of two assays, INTEG and nNATEG to sodium-heparin and low molecular weight heparin.

3.3. Materials and methods

3.3.1. Subjects and preparation of blood and plasma samples

Blood (10 ml) was collected from 66 healthy volunteers (age: 34.9±10.7 years; male:female=32:34) and 55 consecutive patients (age: 37.6±12.6 years; male:female=12:43) with personal history of venous thromboembolic disease referred to our Haemostasis Outpatient Clinic using a 19 G needle and 10 ml native, silicone coated glass Vacutainer tubes and 5 ml Vacutainer tubes with buffered citrate (final concentration of sodium citrate in blood 12.9 mmol/l) after informed consent was obtained. All healthy subjects denied taking any medication that might affect platelet function or coagulation, in the two weeks preceding the experiments.
Patients with thromboembolic disease on oral anticoagulant therapy were converted to a prophylactic dose LMWH (typically 0.01 ml nadroparinux/kg or 1 mg enoxaparine/kg once daily) at least 14 days before venipuncture. Blood was only taken at least 3 months after the last VTE event, after 12 h fasting, in morning hours, 24 hours after the last dose of LMWH. ROTEG® measurements, routine haemostatic tests (PT, aPTT, fibrinogen, TT) were performed typically between 0.5-4 hours after venipuncture, except for the native blood samples which were tested immediately after venipuncture. The blood was kept in closed Vacutainer tubes at room temperature during the experiment. Plasma samples for specific factor determinations were centrifuged at 2000 g for 20 minutes, aliquoted and kept frozen at -70°C until the tests were performed.

3.3.2. Haemostatic assays and thrombophilia factor determinations

Haemostatic assays

Prothrombin time, fibrinogen, activated partial thromboplastin time, thrombin time, antithrombin, protein C, protein S activity and APC ratio were measured using an ACL 200 coagulation instrument and IL (Instrumentation Laboratory, Milano, Italy) reagents or kits according to the manufacturer’s instructions.

The prothrombin time and the fibrinogen level was determined using the IL PT-Fibrinogen kit, which contains a recombinant rabbit tissue factor relipidated in a synthetic phospholipid blend and combined with calcium chloride, buffer and preservatives. In the PT-test, the addition of the reagent to the patient plasma initiates the activation of the extrinsic pathway. This results ultimately in the conversion of fibrinogen to fibrin, with formation of a solid gel. Fibrinogen is quantitated by relating the absorbance of light-scatter during clotting to a calibrator [118-120].

The APTT was determined using the IL Test™ APTT Lyophilized silica kit containing bovine cephalin and micronized silica as a contact activator, which is used to stimulate the production of factor XIIa by providing a surface for the
function of high molecular weight kininogen, kallikrein and FXIIa. This contact activation is allowed to proceed at 37°C for a specific period of time. Calcium is then added to trigger further reactions and the time required for clot formation is measured. Phospholipids are required to form complexes which activate factor X and prothrombin [121,122].

The thrombin time was measured using IL Test™ Thrombin Time Kit. The addition of thrombin to the plasma samples results in the conversion of fibrinogen to fibrin, with the formation of solid gel. The time between addition of thrombin to the plasma sample and clot formation is thrombin time [123].

Antithrombin activity was measured by using the IL Antithrombin kit, which is an assay based on a synthetic chromogenic substrate and on FXa inactivation. The measurement contains two stages: 1. Incubation of the plasma with the FXa reagent in presence of an excess of heparin. 2. Quantification of the residual FXa activity with a synthetic chromogenic substrate. The paranitroanilin released is monitored kinetically at 405 nm and is inversely proportional to the antithrombin level in the test sample [124].

Protein C functional activity was determined with IL Test™ ProClot kit based on the prolongation of an APTT assay in the presence of activated protein C. The anticoagulant effect of activated protein C is due to its sensitivity to FV and FVIII levels. APC in plasma samples is generated by Protac®, a rapid in vitro PC activator derived from the venom of the copperhead snake Agkistrodon contortrix contortrix [125].

Protein S functional activity was determined with IL Test™ Protein S assay by measuring the degree of prolongation of a prothrombin time in the presence of bovine thromboplastin, calcium ions and activated protein C. The protein S activity is proportional to the prolongation of the PT of a protein S deficient plasma to which diluted sample has been added. Activated protein C is generated by activation of PS deficient plasma with Protac® [126].

APC resistance was determined using the IL Test™ APC™ Resistance V kit. Sample plasma is prediluted with Factor V Reagent Plasma and incubated with the APTT reagent for a standard period of time. Coagulation is triggered by the addition of CaCl₂ in the absence and presence of APC and the time of clot
formation is recorded. The APC ratio is calculated with the following formula: APC ratio = APTT with APC / APTT without APC [127].

For the determination of lupus anticoagulants (LA) we used HemosIL™ LAC Screen and LAC Confirm tests. In LAC Screen test a simplified diluted Russell’s Viper Venom Test (DRVV) reagent is used to screen for the presence of lupus anticoagulants. In LAC Confirm test a phospholipid-rich DRVV reagent is applied to confirm the presence of lupus anticoagulants. Russell’s viper venom, in the presence of calcium, directly activates factor X (in the test sample). LAC Screen and LAC Confirm are therefore unaffected by contact factor abnormalities, factor VII, VIII and IX deficiencies or inhibitors [128].

Immunologic assays

For the measurement of the antithrombin and protein C antigen levels anti-human antithrombin polyclonal antibody (DAKO) and anti-human protein C polyclonal antibody were used on rocket electrophoresis system according to Laurell, respectively [129,16].

The free protein S antigen level in the samples was determined using the Coaliza® Protein S Free kit (Chromogenix), which is based on a procedure described by Dahlback and colleagues. The microplate wells are precoated with C4BP, which has a very high affinity for binding free protein S antigen in plasma. After the sample and conjugate incubation, unbound material is washed away and bound protein S, in complex with C4BP is detected with the addition of a substrate-chromogen. The amount of color in the wells, when expressed in a double logarithmic scale, is directly proportional to the amount of free protein S antigen in the plasma sample [130].

Plasma homocysteine level determinations were performed by IMx homocysteine immunoassay on IMx Analyzer (Abbott) in the Department of Laboratory Medicine, Faculty of Medicine, University of Pécs, Hungary.
Molecular genetics

FV:Q506 mutation analysis was performed according to Zöller and Dahlbaeck, FII G20210A mutation analysis according to Poort and co-workers publication [131,132].

3.3.3. Rotation thrombelastography

We analyzed the blood samples by using modified thrombelastograph® coagulation analysis (ROTEG®; Pentapharm Co., Munich, Germany), which is based on the thrombelastograph® system (TEG®) after Hartert [133]. Technical details of the ROTEG® coagulation analyzer are described in the literature [134-136]. TEG measures shear elastic modulus during clot formation and subsequent fibrinolysis (Figure 3.1.)

Figure 3.1. Basic elements of rotation thrombelastography.
ROTEG® shows good correlation with conventional TEG® determination [137], and an excellent reproducibility and precision [138]. In the literature, inconsistent references exist on the stability and reproducibility of TEG measurements after sample storage at room temperature. Depending on the references [139-141], TEG measurements produced accurate and reproducible results within 30 min up to 4 hours. The automatic pipetting system makes the ROTEG® coagulation analyzer easy to handle in daily routine. ROTEG® uses a ball-bearing system for power transduction, which makes it less susceptible to mechanical stress, movement, and vibration. Furthermore, the activation of the test samples accelerates the measurement process and seems to enhance reproducibility when compared with conventional TEG analysis [142]. The main variables of ROTEG® analysis are "clotting time" (CT, time from start of measurement until the start of clot formation) corresponding to the reaction time (r time) in a conventional TEG, "clot formation time" (CFT, time from the beginning of clot formation until an amplitude of 20 mm is reached) in accordance with the coagulation time (k time), "maximum clot firmness" (MCF, which represents the maximum firmness which the clot has reached in this measurement, it is the maximal vertical expansion of the
thrombelastograph), which is equivalent to the maximum amplitude, and the alpha-angle (this parameter is given by the angle between the centre line and a tangent to the curve through the 2 mm amplitude point, which represents the kinetics of clot formation), and the maximum lysis (ML, which represents the maximum fibrinolysis detected during the measurement. It is defined as the difference between the MCF and the lowest amplitude after reaching MCF during the measurement and is given as <\% of MCF>) (Figure 3.2.).

The following measurements were performed:

1. Native TEG (nNATEG): native whole blood was placed into the test cell immediately after venipuncture and the test was started without delay.

2. Non-activated TEG (NATEG): Just before running the assay, citrated blood samples were recalcified with 20 µL of CaCl₂ 0.2 M (Start-TEG; Pentapharm Co., Munich, Germany) and the test was started without an added activator.

3. Intrinsic TEG (INTEG): NATEG + activation using surface activator (partial thromboplastin from rabbit brain, 20 µL of In-TEG reagent, Pentapharm Co., Munich, Germany) for monitoring the intrinsic system (factors XII, XI, IX, VIII, X, II, I and platelets).


In some cases, 16 µL of appropriate dilutions of nadroparine were added to the test cells before starting the measurement.

3.3.4. Statistical analysis

Results are reported as mean ± SD from the measurements conducted on different subgroup of patients or healthy volunteers. Statistical comparisons of samples were performed by paired and unpaired Student’s t-test or linear regression analysis using Microsoft Excel 2000. Differences were considered significant when p was < 0.05.
3.4. Results

3.4.1. Comparison of patients with a history of thromboembolic disease and control subjects

55 consecutive patients with personal history of venous thromboembolic disease referred to our Haemostasis Outpatient Clinic were tested. In the group of patients with thromboembolic anamnesis the CT in INTEG assay, the CFT in INTEG and EXTEG assays were significantly shorter, the MCF in INTEG and EXTEG assays and the alpha angle in INTEG and EXTEG assays were significantly higher when compared to the control group. (Figures 3.3. and 3.4.) There were no significant differences between the two groups in NATEG assay (Figure 3.5.). Surprisingly, when using nNATEG assay, CFT proved to be significantly longer and MCF and alpha angle significantly lower compared to the healthy volunteers (Figure 3.6.).

![Figure 3.3. Comparison of ROTEГ® parameters of healthy volunteers (filled bars) and VTE patients (open bars) in INTEG test (recalcinated anticoagulated whole blood with contact activator) (data are mean±SD).](image)
Figure 3.4. Comparison of ROTEG® parameters of healthy volunteers (filled bars) and VTE patients (open bars) in EXTEG test (recalcinated anticoagulated whole blood with tissue factor) (data are mean±SD).

Figure 3.5. Comparison of ROTEG® parameters of healthy volunteers (filled bars) and VTE patients (open bars) in NATEG test (recalcinated anticoagulated whole blood) (data are mean±SD).

Figure 3.6. Comparison of ROTEG® parameters of healthy volunteers and VTE patients in nNATEG test (native whole blood) (data are mean±SD).
3.4.2. Comparison of patients with positive and negative thrombophilia results

Out of the examined 55 patients 34 (61%) proved to be thrombophilia (ThP) negative, 21 (39%) were ThP positive (13 FV Leiden heterozygotes, 2 FV Leiden homozygotes, 2 FII20210 heterozygotes, 1 lupus anticoagulant positive, 1 protein C deficient, 2 hyperhomocysteinaemia patients). When we compared the results of ThP negative and positive patients, we found no significant differences; although the CT and CFT values were still significantly lower if compared to the controls in case of the INTEG assay (Figure 3.7.).

Figure 3.7. Comparison of ROTEG® parameters of healthy volunteers, thrombophilia positive and negative VTE patients in INTEG test (recalcinated anticoagulated whole blood with contact activator) (data are mean±SD).

3.4.3. Effect of low molecular weight heparin on blood coagulation measured by ROTEG®

Regarding the confusing results in nNATEG test, we carried out some additional experiments. As most of the VTE patients received LMWH prophylaxis during this period, we hypothesized a disturbing effect of LMW-heparin to the results. We tested the sensitivity of two ROTEG® assays (INTEG and nNATEG) to nadroparine, a commonly used low molecular weight heparin in vitro. We found, that nadroparine impairs whole blood coagulation in a dose dependent manner. The assay nNATEG was much more sensitive to low doses of nadroparine than INTEG (IC50s for clotting time were 0.65 and 15 U/ml, respectively) (Figure 3.8.).
3.4.4. Effect of gender on blood coagulation measured by ROTEG®

Observing that the male to female ratio was significantly different between patients and controls, we investigated, if there was a difference between the genders in the control group. The results showed that there were no significant differences in nNATEG, NATEG and INTEG parameters between male and female controls. In EXTEG, however, CT and CFT were significantly shorter; MCF and alpha-angle were higher in women compared to men (Figure 3.9.).

Figure 3.8. Effect of nadroparine on blood coagulation in nNATEG (left) and INTEG (right) assays. Symbols represent the measured parameters. CT (diamond) and CFT (rectangle) is shown on the left y axis, whereas MCF (triangle) and alpha angle (x) is demonstrated on the right y axis.

Figure 3.9. Effect of gender on blood coagulation in EXTEG assay. Male data are represented in open bars, female data in closed bars. Data are mean±SD (n=58). Asterisk indicates statistical significance (*p<0.05).
3.5. Discussion

The knowledge of the risk of a thrombotic event recurrence and its determinants is relevant to clinical policy regarding screening for thrombophilia, duration of anticoagulant treatment, and prophylactic strategies in circumstances of increased risk.

The profile of whole blood coagulation by thrombelastography can be considered as an indirect measure of thrombin generation. One of the main features of classical TEG without added activators or anticoagulants was to maintain the physiologic conditions as much as possible. The new generation rotation thrombelastograph allies the naturality of classical TEG with the advantages of the modern medicine. Activated ROTEG® assays provide results much faster than conventional assays, and due to the mechanical modifications, described earlier, it can be used as point of care coagulation testing.

In our study, we compared 66 healthy volunteers with 55 consecutive patients with personal history of venous thromboembolic disease referred to our Haemostasis Outpatient Clinic using the standard assays of the recently developed rotation thrombelastography. We demonstrated, that patients with previous thromboembolic disease were more hypercoagulable when compared to controls, using either by tissue factor or by contact activator activated assays. Previously TEG has shown a promising ability to detect known or established hypercoagulable states during pregnancy and postpartum [143-145], during the presence of cancer [146,147] and in association with surgery [148].

Although the high sensitivity of TEG to hypercoagulability has been described in numerous studies, clear association between TEG parameters and thrombophilia has not been established. O’Donnel et al. examined thrombophilia patients, and concluded, that TEG cannot be used as a sole initial screening test in patients referred for thrombophilia testing, as it fails to identify 43% of underlying thrombophilic traits. TEG maybe a useful adjunctive test, particularly in patients in whom the regular thrombophilia screen proves unremarkable [112].

A study by Traverso et al. [149] included 100 patients undergoing elective abdominal surgery, and in the patients randomized to receive no postoperative
heparin thromboprophylaxis, TEG maximal amplitude (MA) value showed the ability to predict the occurrence of DVT with a sensitivity of 72.2% and specificity of 69%. Finally, Wilson et al. performed TEG every other day in 250 patients who had undergone proximal femoral fracture repair and showed that patients suffering from postoperative DVT had a significantly higher level of hypercoagulability as measured by TEG® than did those who did not suffer DVT [150]. Postoperative TEG® MA values in the hypercoagulable range are associated with a higher risk of postoperative thrombotic complications including pulmonary embolism, DVT, myocardial infarction, and ischemic stroke [108].

Miall et al. demonstrated that in a group of pregnant women there was a significant correlation between TEG parameters and PT, APTT and AT level, and mid trimester loss, but not with other thrombophilic defects [109]. Schreiber et al showed correlation between hypercoagulability determined by TEG after serious injury and female gender [110].

Since the clinical parameters of patients and controls differed significantly in the male to female ratio, and previously a significant difference between ROTEG® profiles of genders was observed [140], we compared data between males and females. Females proved to be more hypercoagulable than males in case of tissue factor activated ROTEG®. Interestingly, Sorensen et al. described that in TEGs activated by minute amounts of tissue factor, females expressed a higher clot formation velocity as compared to males, which is consistent with our findings. On the other hand, the other assays (i.e. NATEG, INTEG, nNATEG) were not affected by genders. So despite the imbalance between the genders in our study, we can conclude that the results of the contact activator activated thrombelastography seem to be reasonable. After all, our findings of tissue factor activated thrombelastography are in accordance with those of Hvitfeldt et al., who investigated 34 venous and 42 arterial TE patients with activation by minute amounts of tissue factor, and found that patients with a history of venous or arterial thromboembolism had a significantly greater hypercoagulant whole blood coagulation as defined by a shortened clotting time together with an accelerated maximum velocity of clot propagation [107].
In the nNATEG test, oddly, the VTE patients proved to be hypocoagulable when compared to the control group. As most of the patients received LMWH as VTE prophylaxis during the investigation, we suspected that the heparin had a role in this effect. Although we did not have the opportunity of measuring heparin concentration (anti-Xa activity) in these samples, the high sensitivity of nNATEG (it was almost 100 times more sensitive than INTEG) of both low molecular weight and unfractionated heparin could explain the apparent discrepancy of the results. However, using activated assays can eliminate the disturbing effect of heparin traces.

Surprisingly, we did not find significant differences between the parameters of patients with thrombophilia proven by laboratory tests and those with no thrombophilia trait, although most of the found defects are not considered as severe thrombophilia. This and the relatively small case number may mask the differences detectable by ROTEG®. However, since the tested patients were clinically considered to have thrombophilia, the lack of difference between the two groups can also be explained by the existence of not yet identified prothrombotic abnormalities, which hold the same thrombotic event potential as the known ones.

Although the differences between the thrombosis and the control group are small, and thus the assay is per se not capable of identifying patients with thrombotic tendency, there is a possibility of developing more sensitive assays based on rotation thrombelastography.

In conclusion, our study indicated that rotation thrombelastography, first of all INTEG assay was able to detect hypercoagulability in patients with venous thromboembolism, which was independent of the presence of laboratorially detected thrombophilic traits.
4. Summary

In this study, we have investigated two whole blood haemostasis methods to promote the research and treatment of cardiovascular disease and thrombophilia.

As described in the first part of the work, we were the first to introduce a newly developed whole blood platelet function analyzer technique, the multiple platelet aggregometry.

Our main results are:

1. We indicated that MEA is a reliable, reproducible platelet aggregation method. Different platelet agonists induced platelet aggregation in donor-, time-, and dose dependent manner, which was not affected by platelet count within the normal platelet count range.

2. We showed that when measured by MEA, as citrate seems to inhibit platelet aggregation, the use of the direct thrombin inhibitor lepirudin as an anticoagulant is preferable to the use of trinatrium citrate.

3. Comparing MEA to SPC we found, that results are highly comparable, while SPC was much more time consuming than MEA.

4. We concluded that MEA is able to detect the expected inhibitory effect of aspirin and apyrase to a greater extent than SPC.

5. MEA could detect the inhibitory effect of selective ADP receptor antagonists in vitro.

6. MEA was also capable of the ex vivo testing of clopidogrel effectiveness. MEA may be a good candidate for further studies in large populations of cardiovascular patients.
In the second part, we investigated rotation thrombelastography in the detection of thrombophilia.

**Our main results are:**

1. Rotation thrombelastography was proved to detect thrombotic tendency in patient with former thromboembolism.

2. We were the first to investigate the applicability of the most commonly used ROTEG® tests (nNATEG, NATEG, INTEG, EXTEG) in thrombophilia, and found that INTEG especially has the ability to detect hypercoagulable state.

3. There was no difference between the ROTEG® parameters of TE patients with or without thrombophilic trait. This suggests that clinically both groups of patients had the same tendency to thrombosis, probably due to an underlying, so far unknown factor.

4. In the group of healthy volunteers the women seemed to have a higher tendency to thromboembolism, when compared to the men, when we used the tissue factor activated test.

5. Sodium-heparin and LMWH both inhibited the coagulation when measured by native or contact factor activated assay, the former being much more sensitive to both kinds of heparins.
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6. Publications of the author

Book chapter:


Papers:


Total impact factor: 8.816

Published abstracts:


7. Acknowledgements

These studies were carried out at the 1st Department of Internal Medicine, Medical School of the University of Pécs and at the Institute for Prevention of Cardiovascular Diseases of the University of Munich between 2001 and 2005.

I want to express my thanks to my teacher and project leader, Professor Hajna Losonczy, who mentored my studies and gave support and useful advice during my work.

I want to express my thanks to Professor Wolfgang Siess, who gave me the opportunity to join his team, and directed my work on the field of platelet aggregation studies. I am also very grateful for all the team members, who accepted me and helped me a lot. Special thanks to Sandra Penz for the very useful discussions.

I am really thankful to Marianna David, Agnes Nagy and all other members of the Heamatology Department, who lead my way to haematology, and tirelessly taught me on haematology and haemostasis, the importance of the relationship between patients and doctors, and laboratory work.

I convey my thanks to Marianna Kecskés, Mrs. László Temesi, Mrs. Ádám Vető, Éva Somos, and Beáta Meng for their excellent help in learning and performing haemostasis laboratory methods.

I express my gratitude and thanks to my friends and family for their encouragement and support during my studies and work.