

**PLATELET AGGREGATION AND VISCOSITY  
MEASUREMENTS IN THE CLINICAL PRACTICE AND  
IN BASIC RESEARCH**

**Ph.D. thesis**

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## LIST OF ABBREVIATIONS

ACS	acute coronary syndrome
ADP	adenosine diphosphate
AGE	advanced glycation endproducts
ASA	acetyl salicylic acid
ATP	adenosine triphosphate
AZT	3'-azido-3'-deoxythymidine (zidovudine)
CAD	coronary artery disease
cAMP	cyclic-3'5'-adenosine monophosphate
CAPRIE	Clopidogrel versus Aspirin in Patients at Risk for Ischaemic Events
CORE	Collaborative Organization of RheothRx Evaluation
COX	cyclooxygenase enzyme
DMEM	Dulbecco's modified Eagle's medium
eNOS	endothelial nitrogen monoxide synthase
ESPS	European Stroke Prevention Study
ET-1	endothelin-1
GP IIb/IIIa receptor	platelet glycoprotein IIb/IIIa receptor
Hct	hematocrit
ICAM	intercellular adhesion molecule
IL-1	interleukin-1
iNOS	inducible nitrogen monoxide synthase
IS	ischemic stroke
LoRRca	laser-assisted optical rotational cell analyzer
LDL	low density lipoprotein
MCP-1	monocyte chemotactic protein-1
MCSF	macrophage colony stimulating factor
NAD <sup>+</sup>	nicotinamide adenine dinucleotide
NO	nitrogen monoxide
PAF	platelet-activating factor
PARP	poly(ADP-ribose) polymerase

PBS	phosphate buffered saline
PCI	percutan coronary intervention
PFA-100	platelet function analyzer
PGI <sub>2</sub>	prostacyclin
PPP	platelet-poor plasma
PRP	platelet-rich plasma
RBC	red blood cell
ROS	reactive oxygen species
SEM	standard error of mean
TIA	transient ischemic attack
TNF	tumor necrosis factor
TXA <sub>2</sub>	thromboxan A <sub>2</sub>
VCAM	vascular cell adhesion molecule
vWF	von Willebrand factor

## 1. INTRODUCTION

Atherothrombosis, defined as an atherosclerotic plaque disruption with superimposed thrombus formation represents the principal cause of morbidity and mortality in the industrialized countries with gaining importance in the developing nations as well. More than a century ago, Rudolph Virchow described first the principal factors involved in atherosclerosis and thrombus formation known as Virchow's triad. It includes arterial wall damage with endothelial injury and dysfunction; the presence of various pro-thrombotic factors in the circulating blood and altered local hemorheological parameters. Despite the extended research on this field though, the exact pathomechanism is still debated and not fully elucidated yet.

It is generally recognized, that atherosclerosis is a diffuse, ongoing, sterile inflammation initiated asymptotically in early childhood as a "response to injury" [129]. The process begins with the functional impairment of the vascular endothelium lining the entire blood vessel system. This imperative autocrine/paracrine organ is composed of unique, multifunctional cells that provide an anticoagulant, but selectively permeable barrier essential in maintaining vascular homeostasis and vessel tone. Endothelial cells are also responsible for the regulated synthesis and secretion of potent vasodilator substances including prostacyclin ( $\text{PGI}_2$ ), bradykinin, adenosine and most importantly, nitric oxide (NO). NO has an inevitable physiological role in the regulation of blood pressure and local blood flow. Reduced synthesis of the molecule in atherosclerotic vessels contributes to an impaired vasomotor function, enhanced platelet aggregation and smooth muscle cell proliferation. On the contrary, vasoconstrictor agents such as endothelin-1 (ET-1), angiotensin II and thromboxane  $\text{A}_2$  ( $\text{TXA}_2$ ) offset the efficacy of the vasodilator molecules mentioned above contributing to the minute-by-minute control of the vascular tone and local blood flow.

The endothelial regulation of inflammatory and immune responses is also of importance affecting the progression of atherosclerosis. While resting endothelial cells do not consecutively express molecules that can attract leukocytes from flowing blood, upon activation by thrombin, inflammatory cytokines (i.e., IL-1, TNF) or bacterial endotoxins these cells express a series of adhesion molecules on their luminal surface (i.e., VCAM, ICAM-1, E-selectin) initiating a multi-step process. Leukocytes first

transiently tether and roll on the endothelial surface, then adhere to it and migrate through the cell layer. Finally, they emigrate from the vascular lumen and move towards the site of tissue damage or infection. Following the early emigration of neutrophils, monocytes also leave the vascular bed promoted by the vascular cell adhesion molecules (VCAM). It is important to note that pro-oxidant molecules, present in hyperlipidemia, hyperglycemia and oxidative stress, upregulate the membrane expression of VCAM providing a plausible link to the early accumulation of monocytes within the vessel wall.

Endothelial cells are considered to be the main source of the circulating von Willebrand factor. This highly multimeric glycoprotein has two distinct biological functions: it acts as the carrier of coagulation factor VIII and is also a cofactor required for platelets to bind to exposed subendothelial collagen upon vessel wall damage. Furthermore, plasma concentrations of von Willebrand factor might have potential as a marker for the assessment of endothelial dysfunction in vivo [12]. Elevated levels of the molecule have been demonstrated in patients with chronic cardio- and cerebrovascular diseases and were also associated with the prediction of adverse clinical events [66]; possibly by the promotion of thrombus formation. Further endothelium derived molecules, such as the pro-coagulant factor XIII and the anticoagulant thrombomodulin are pivotal in maintaining the critical balance of vascular homeostasis.

During the early phases of the atherosclerotic process, no gross morphologic changes can be detected within the vessel wall, except for the slight impairment of endothelium-dependent vasodilatation. Isolated macrophages containing negligible amounts of oxidized lipid droplets might appear. Subsequently, in response to the accumulating lipid particles, especially oxidized low density lipoprotein (LDL), endothelial and smooth muscle cells tend to secrete monocyte chemoattractant protein-1 (MCP-1) and macrophage colony stimulating factor (M-CSF) that both promote monocyte chemotaxis, adhesion and their differentiation to macrophages. These cells further accumulate oxidized LDL leading to fatty streak formation. As lipid accretion overwhelms the uptake by macrophage-derived foam cells, small extracellular lipid pools appear within the vessel wall that - in conjunction with certain cytokines and platelet-derived growth factors - stimulate the proliferation and migration of adjacent smooth muscle cells forming the intimal coating of the expanding atherosclerotic plaque. Beneath this proteoglycan-rich layer, an organized lipid core develops and the lesion is classified as atheroma at this stage. Since fibrosis is not a predominant feature

of atheroma, it is structurally weak and might be susceptible of fissuring caused by mechanical forces. Otherwise, smooth muscle cells will form a fibrous cap consisting of collagen and connective tissue that might cause the substantial diminution of the vascular lumen. If the fibrous cap is destabilized by altered hemorheological parameters, increased mechanical forces or enzymatic degradation by metalloproteases (i.e., collagenase, gelatinase, elastase), the plaque may rupture exposing highly thrombogenic structures. Thus, platelets may contact and adhere to the subendothelial von Willebrand factor (vWf), collagen and fibronectin initiating their local activation [54]. This is followed by the synthesis and release of TXA<sub>2</sub> and adenosine diphosphate (ADP) from  $\alpha$  and dense granules, which proaggregatory molecules recruit additional thrombocytes to the site of vascular injury [51,54,71]. The common impact of these agonists is to activate specific signaling pathways and to generate a number of second messengers leading to the functional expression of glycoprotein (GP) IIb/IIIa receptors further promoting platelet aggregation. The resultant platelet-rich thrombi may induce partial or complete occlusion of the atherosclerotic vessel leading to the clinical manifestations of acute coronary syndrome (ACS), transient ischemic attack (TIA) or ischemic stroke (IS) [71,121].

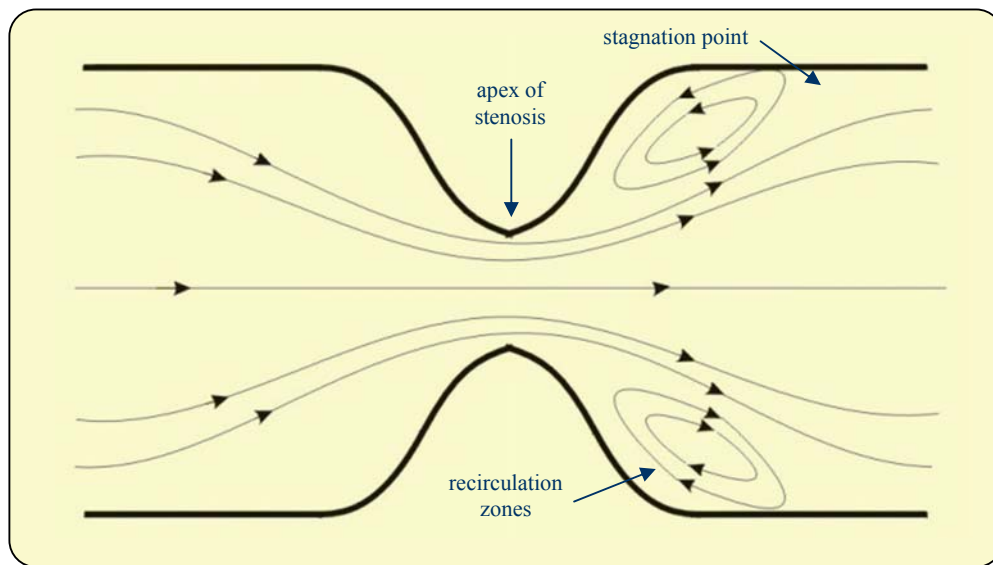
The role of “classic” risk factors in the initiation and progression of atherosclerosis has been revealed a long time ago (i.e., age, male gender, hypertension, hyperlipidemia, genetic background, smoking, obesity and the lack of physical exercise). Further epidemiological studies have demonstrated that patients with diabetes mellitus or glucose intolerance are also at a higher risk for coronary heart disease [74]. Underlying mechanisms include the enhanced generation of cytokines, free radicals and advanced glycation endproducts (AGE). Glycosylation as well as the oxidative damage of proteins and lipoproteins lead to the structural and functional impairment of the affected molecules [38]. Impaired hemorheological parameters, particularly enhanced red blood cell aggregation are also of great importance [25]. Lipoprotein(a) is a well-described risk factor for coronary artery disease (CAD) that has a molecular structure similar to that of plasminogen. As a consequence, it may reduce plasmin formation and thus impair thrombolysis [87]. LDL, especially oxidized LDL diminishes endothelium-derived NO activity by the down-regulation of the endothelial nitrogen monoxide synthase (eNOS), decreased receptor-mediated NO release and NO inactivation via enhanced superoxide anion production [86]. Impaired homocysteine

metabolism has also been implicated as a risk factor for atherosclerosis [48]. While the exact pathomechanism is not completely elucidated yet, direct toxic effects on endothelial cells as well as more indirect mechanisms have been postulated. Enhanced cyclooxygenase (COX) activity has been documented in patients with essential hypertension reducing the availability of NO via free radical production. As a result, there is an increase regarding the biological activity of ET-1 and the expression of various adhesion molecules on the endothelial cell surface [148]. Several trials have consistently demonstrated the important role of oxygen free radicals in physiological processes, such as metabolism and cellular defense. On the contrary, they have been implicated in the development and progression of a wide range of diseases including atherosclerosis, ischemia-reperfusion injury, diabetes mellitus, inflammation and immunological disorders. When generated in excessive amounts, free radicals are considered to be highly toxic as they can oxidatively modify proteins, nucleic acids, carbohydrates and cause lipid peroxidation. Erythrocytes are one of the most susceptible cells as they are continuously exposed to high oxygen tensions. Under normal conditions, well-developed antioxidant defense mechanisms can render protection against the deleterious effects of constantly generated free radicals. In contrast, enhanced oxidative stress or spoiled protective mechanisms might lead to the functional impairment of the red blood cells (RBC). Structural damages are also initiated; alterations of the intracellular milieu and membrane characteristics might have a serious impact on hemorheological parameters (i.e., erythrocyte deformability and aggregation) leading to disturbed microcirculatory blood flow [8]. Employing drugs with the potential of scavenging these destructive radicals might be favorable throughout the treatment and prevention of cardiovascular diseases [58].

To further emphasize the importance of hemorheological parameters and shear stress, we should note that early atherosclerotic lesions are site-specific, plaques do not occur randomly throughout the vascular system as would be expected by the systemic nature of risk factors [76]. Instead, they preferentially develop in areas where blood flow changes direction and where longitudinal forces exerted on the arteries are increased [50]. Also, in highly atherosclerotic vessels normal laminar flow conditions might not be maintained since the stenotic narrowings induce flow disturbances modifying cell-cell and cell-vessel interactions [5]. In these areas secondary streams might develop in directions away from the primary flow leading to the formation of a



vortex or a recirculation zone between the mainstream and the vascular wall (Figure 1.1.1). As a consequence, a prominent increase of shear stress occurs at the apex of the stenosis causing further endothelial damage, enhanced platelet activation and deposition. Analyses of the axial distribution of these depositions indicated that the greatest thrombocyte accumulation occurs at the apex of the atherosclerotic plaque leading to the progression of the vascular lesion. These findings highlight the inevitable preventive role of antiplatelet drugs diminishing shear-induced platelet aggregation and also the importance of blood viscosity contributing to the endothelial cell damage.



**Figure 1.1.1:** Effect of stenotic lesions on laminar blood flow.

Blood is a non-Newtonian suspension of erythrocytes, leukocytes, platelets, chylomicrons, carbohydrates, proteins and electrolytes. Its viscosity can be defined as an intrinsic resistance to flow caused by the internal friction developing between adjacent fluid layers [156]. At a given temperature, blood viscosity is primarily the function of the cell concentration, shear rate, red blood cell aggregation, deformability and plasma viscosity. As erythrocytes occupy the largest volume fraction of blood cells, hematocrit value (Hct) is considered to be one of the most dominant factors. Unlike for Newtonian fluids, blood viscosity is strongly dependent on the shear rate, it is significantly elevated under low-flow conditions when compared to that measured at higher shear rates. This shear-thinning behavior can be explained by the flow-dependent variations in erythrocyte aggregation and deformation [7]. Red blood cells tend to aggregate to form

rouleaux under low flow conditions when the shear stress is insufficient to disperse the cells. However, increasing flow rates lead to their progressive disaggregation and thus a noteworthy decrease in blood viscosity [9]. Normal human erythrocytes are remarkably deformable; they easily get elongated in response to higher shear rates with their long axis aligned with the flow. Red blood cell deformability is of crucial pathophysiological importance in the coronary microcirculation where the average capillary diameter might be as low as 3-5 $\mu$ m, well below the dimensions of an erythrocyte [157]. Plasma behaves as a Newtonian viscous fluid and also has a remarkable impact on regional blood flow. Plasma viscosity is a function of the concentration of suspended proteins, especially those with high molecular mass and structural asymmetry (i.e., fibrinogen, some globulins) and also lipid fractions. The importance of fibrinogen molecule is further emphasized by the fact that it is considered to be a significant determinant of red blood cell and platelet aggregation *in vivo*.

Several clinical and epidemiological studies have provided compelling evidence that hemorheological parameters are primary risk factors for the development and progression of cardio- and cerebrovascular diseases [78,6,37,18,139]. The coronary vessel system represents a special part of the circulation as there is a continuous fluctuation in blood flow, perfusion pressure and shear rate due to the cardiac cycle and the narrowest capillaries of the body can be found in the myocardium. Therefore the role of rheological alterations can be of superior importance than in any other parts of the circulatory system [78,6,157]. The Framingham Study initiated over half a century ago, the Puerto Rico Heart Health Program and the Honolulu Heart Program established the importance of hematocrit in the development and progression of cardiovascular diseases [73,140,18]. The Framingham and Northwick Park Studies demonstrated that elevated plasma fibrinogen concentration is a major cardiovascular risk factor [99]. The Monica Project revealed the great impact of whole blood and plasma viscosity for the first time. The Edinburgh Artery Study and the Caerphilly and Speedwell Collaborative Heart Disease Study provided further compelling evidence regarding the role of hemorheological variables in the development of cardiovascular diseases [144,174,84,89]. In the Physicians' Health Study elevated baseline plasma fibrinogen concentration was associated with an increased risk of future myocardial infarction during a five-year follow-up period and was independent from any other risk factors [93]. Lowe et al. suggested blood viscosity to be associated with the extent of coronary

heart disease, but they could not demonstrate such a correlation regarding plasma viscosity [88]. Other authors revealed impaired hemorheological variables in patients with angiographically proven coronary artery disease, but they were unrelated to the clinical severity [123]. Previous data of our group also found hemorheological parameters (hematocrit, plasma fibrinogen, plasma and whole blood viscosity) to be significantly impaired in patients with ACS and chronic ischemic heart disease when compared to that of normal, healthy subjects [78,155]. Administering a nonionic block copolymer surfactant (RheothRx) to patients with acute myocardial infarction significantly reduced erythrocyte aggregation and also improved several hemorheological parameters highlighting its potential usefulness in a variety of diseases [154]. Kesmarky and his co-workers revealed major changes in some rheological and free radical associated parameters following percutaneous transluminal coronary angioplasty that might have a remarkable impact on the final outcome of the intervention [79]. Other trials confirmed the inevitable role of disturbed hemorheological variables in the development and progression of hypertensive retinopathy and other hypertension associated organ damages [101,158,161]. Habon et al. showed that the evaluation of various hemorheological parameters along with other diagnostic procedures could be of assistance in the clinical decision-making in patients with suspected ischemic heart disease [59].

In addition to the aforementioned trials, numerous studies suggested the importance of altered rheological parameters in the development of cerebrovascular diseases as well [46]. The Edinburgh Artery Study revealed that hematocrit, whole blood and plasma viscosity and plasma fibrinogen concentration represent a major risk factor for stroke [91]. Furthermore, they have been implicated in the pathomechanism of lacunar brain infarcts and TIA [98]. Szapary et al. demonstrated that hematocrit, blood viscosity, plasma fibrinogen concentration, erythrocyte aggregation and deformability are significantly impaired in patients with chronic ischemic cerebrovascular disorders. In addition, the observed rheological alterations were in great agreement with the severity of the carotid artery stenosis [147].

We should note that - in spite of the abundant, large case clinical trials cited above -, most hemorheological measurements are still lacking a strict standardization. Numerous devices exist aiming to characterize red blood cell aggregation; however, a widely accepted, “gold standard” method is not available to date. The Myrenne MA-1

was the first, easy to operate aggregometer and is still the most commonly utilized apparatus characterizing erythrocyte aggregation photometrically. Besides its great advantages (i.e., minimal amount of blood required for a test, short measurement time), the Myrenne has several limitations including the lack of temperature control and the limited amount of data provided by the basic instrument. A number of methods and devices have been developed to assess red blood cell deformability (St. Georges' filtrometer, ektacytometer, LoRRca), however, most of the tests are time consuming and the results are hardly comparable. Although numerous whole blood viscometers exist on the market providing accurate viscosity measurements, unfortunately, most of the tests are time consuming, have a potential biohazard risk and thus they are inappropriate for rapid, bedside testing. Thus, the development of a fast, reliable and accurate whole blood viscometer providing rapidly available, comparable and consistent data is emerging. The employment of standardized hemorheological methods might promote further epidemiological studies evaluating associations between blood rheology and various diseases.

As detailed previously, numerous risk factors have been identified to date damaging the cell layer providing the internal lining of the vascular system and thus promoting endothelial dysfunction and the expansion of atherosclerosis. Following its initiation in early childhood this sterile inflammation progresses over time and culminates in plaque formation. Upon the rupture of these lesions occlusive thrombi might develop leading to the clinical manifestations of ischemic vascular diseases. We should also note the great importance of blood rheology in atherosclerotic vessels as impaired hemorheological parameters might further reduce microcirculatory blood flow thus enhancing clinical symptoms caused by the pre-existing stenotic lesions.

Individually controlled and adjusted antiplatelet therapy has an exceptional importance in the therapy of ischemic vascular disorders and also throughout the primary and secondary prevention. Detecting abnormal hemorheological parameters, most importantly elevated whole blood viscosity profiles might assist physicians to modify them favorably in the near future and thus further reduce the morbidity and mortality of cardio- and cerebrovascular diseases.

## 2. AIMS OF THE INVESTIGATIONS

1. To assess the efficacy of conventional antiplatelet medications (acetyl salicylic acid and thienopyridine derivatives) via platelet aggregometry and to investigate the prevalence of non-responder subjects in a large patient population with chronic cardio- and cerebrovascular diseases.
2. To monitor the platelet function of previously enrolled cooperative individuals with chronic vascular diseases up to two years. Meanwhile, we evaluate the hypothesis that the sensitivity to a fixed-dose antiplatelet medication progressively decreases during long-term therapy.
3. To correlate the long-term prevalence of adverse clinical events (recurrent acute coronary syndrome, stroke or TIA) with the efficacy of the antiplatelet medication estimated by optical platelet aggregometry in a subset of our patients.
4. To evaluate the hypothesis that poly(ADP-ribose) polymerase inhibitors with adenine-like molecular structure - acting as competitive antagonists - can effectively interfere with ADP-induced platelet aggregation. Furthermore, we intend to explore whether the experimental PARP inhibitors can antagonize heparin-induced platelet hyperreactivity.
5. To test the reliability and reproducibility of a newly developed scanning capillary viscometer. To emphasize the importance of standardization, we intend to provide direct comparisons between the results obtained by the Rheolog™ and data collected using cone-plate and Couette viscometer systems over a wide range of shear rates. Our further aim is to assess the effects of storage time and storage temperature on whole blood viscosity profiles.

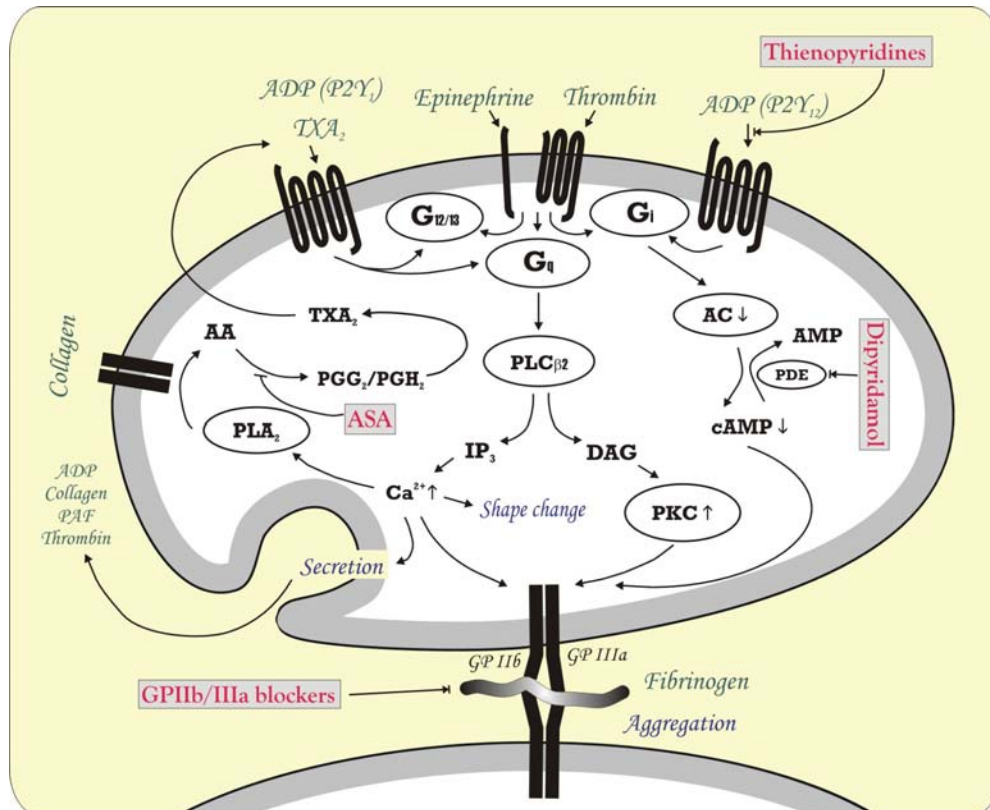
### **3. RESISTANCE TO ROUTINE ANTIPLATELET MEDICATION AND THE EFFICACY OF LONG-TERM ASPIRIN AND THIENOPYRIDINE THERAPIES**

#### **3.1 Introduction**

Cardio- and cerebrovascular diseases are the most frequent causes of morbidity and mortality in the developed nations with growing importance in the developing countries as well. Resting platelets are biconvex, discoid bodies that circulate as individual cells without adhering to the normal vascular endothelium [54]. Although at sites of vessel injury thrombocytes play a central role in the physiology of primary hemostasis, their possible role in atherogenesis has only been recently revealed [55,122]. Angiographic and angioscopic studies have further demonstrated that pathological platelet activation and aggregation are pivotal events promoting intravascular thrombus formation [4,47,151].

Considering platelets' crucial role in the development and progression of occlusive vascular diseases, numerous drugs with improved effectiveness have been developed to influence discrete mechanisms of clot formation (Figure 3.1.1). The cyclooxygenase inhibitor acetyl salicylic acid (ASA) was introduced into medical practice in 1899 and was mainly used for its analgesic, anti-inflammatory and antipyretic effects for a long time. Its ability to inhibit platelet aggregation was first revealed decades later and soon became the standard antithrombotic agent used in the clinical practice. Biochemically, ASA acetylates the hydroxyl group of the serin-530 amino acid of cyclooxygenase, irreversibly inactivating both isoforms of the enzyme [114]. However, it is important to note that the serum concentration required to inhibit COX-2 activity is considerably higher than needed to inhibit COX-1. Numerous trials have verified its beneficial effect in the treatment of cardiovascular diseases [3,127], and the regular administration of aspirin has been the mainstay of both primary and secondary preventions. Multi-center studies involving large patient populations have provided gripping evidence that aspirin therapy provides a 25% relative risk reduction of acute coronary syndrome, ischemic stroke and vascular death over the placebo group [3,53,150]. In subjects with unstable angina, aspirin reduced the incidence of

myocardial infarction and coronary death by 68% within the first 30 days of follow-up, and by 62% after twelve month when compared to the control group [127]. In a placebo controlled trial performed among individuals with previous myocardial infarction, it diminished the incidence of recurrent ischemic periods by 50% and also cardiovascular mortality by 23% within the initial five weeks following the acute event [67].



**Figure 3.1.1:** Possible mechanisms of platelet activation and the inhibitory effect of various antiplatelet drugs (G: G protein, PLC: phospholipase C, IP<sub>3</sub>: inositol triphosphate, DAG: diacylglycerol, PKC: protein kinase C, AC: adenylyl cyclase, PDE: phosphodiesterase, AMP: adenosine monophosphate, PLA<sub>2</sub>: phospholipase A<sub>2</sub>, AA: arachidonic acid, PGG<sub>2</sub>: prostaglandin G<sub>2</sub>, PGH<sub>2</sub>: prostaglandin H<sub>2</sub>, TXA<sub>2</sub>: thromboxane A<sub>2</sub>).

The greater attention directed to the understanding of platelet pathophysiology led to the development of novel drugs with improved antiplatelet efficacy [132]. The metabolites of thienopyridine derivatives (activated by the cytochrome P450 CYP3A4 enzyme) selectively and irreversibly block the binding of adenosine diphosphate to the

P2Y<sub>12</sub> receptor on the platelet surface [19,137]. These agents effectively interfere with platelet activation, degranulation and also reduce the expression of the active form of GP IIb/IIIa receptor on the platelet surface thus preventing the binding of fibrinogen to this complex. On a molar basis clopidogrel has greater antiplatelet efficacy than ticlopidine and serious side effects are also rare [3]. Numerous trials have verified the preventive value of thienopyridine derivatives throughout the secondary prevention of ischemic vascular diseases. For instance, in a population of 3069 patients with previous transient ischemic attack or amaurosis fugax ticlopidine provided an additional 21% relative-risk reduction over aspirin monotherapy [63]. Also, the CAPRIE study indicated that clopidogrel has superior efficacy than ASA in terms of prevention of ischemic stroke, myocardial infarction and vascular death [17]. In patients with unstable angina or myocardial infarction without ST segment elevation the combination therapy of ASA and clopidogrel proved to have superior efficacy than aspirin alone [100].

Dipyridamole is a well-known antiplatelet agent that has been available for many years. The drug reduces the uptake of adenosine into endothelial cells and erythrocytes in a dose dependent manner, so its local concentration available for platelet cyclic-3`5`-adenosine monophosphate (cAMP) synthesis increases. Also, dipyridamole inhibits phosphodiesterase enzyme activity thus raising the intracellular levels of cAMP and cGMP considerably [94]. Via these mechanisms, platelet aggregation is reduced significantly in response to various agonists, such as platelet-activating factor (PAF), collagen and ADP. Two large case clinical trials, the European Stroke Prevention Studies (ESPS) have examined in detail the benefits of aspirin and/or dipyridamole for the secondary prevention of ischemic stroke and TIA. Over the 2-year period of ESPS-1, it revealed that the combination therapy of ASA and dipyridamole decreased the relative risk of stroke by 16% over aspirin monotherapy [43]. The ESPS-2 trial demonstrated that dipyridamole monotherapy applied as part of the secondary prevention reduced the risk of recurrent ischemic stroke considerably; however, it did not diminish the rate of mortality [35].

The final common pathway for platelet aggregation is the binding of fibrinogen to the GP IIb/IIIa complex [104,153]; blocking this receptor with a highly effective antagonist (e.g., eptifibatide) completely inhibits platelet aggregation induced by any of the agonists or even their combination [105,116].



Heparin is an anticoagulant agent used in the prevention and management of venous and arterial thrombosis as well as during invasive procedures. Although unfractionated heparin inhibits thrombin-induced platelet aggregation [40], the molecule conversely potentiates platelet TXA<sub>2</sub> formation and thus enhances their aggregation provoked by a range of agonists, including ADP, PAF and epinephrine [2,133,172]. Most importantly, this effect is detectable in therapeutic serum concentrations applied to prevent clot formation [172]. Molecules binding to the ADP receptors on the platelet surface might have potential therapeutic benefits in counteracting this pro-thrombotic effect of unfractionated heparin.

Despite the obvious beneficial effects of ASA, it fails to prevent the recurrence of serious atherothrombotic events throughout the secondary prevention in some patients and persistent platelet aggregation has also been suggested in a significant number of high-risk subjects (5 to 40%) [77,15,56]. These phenomena are often referred as “aspirin resistance”; however, the term remains poorly defined to date. It can imply the clinical inability of aspirin to protect individuals from ischemic vascular events despite the regular administration of the drug (clinical description) [11,170,20]. The laboratory definition describes the inability of aspirin to produce an anticipated effect on one or more laboratory tests of platelet function, such as inhibiting aggregation, thromboxane biosynthesis and causing the prolongation of bleeding time (biochemical aspirin resistance) [57,112,41,62]. The underlying mechanism of the drug resistance has not been completely elucidated yet, but may involve the activation of platelets by COX-independent pathways, ASA-insensitive biosynthesis of TXA<sub>2</sub> caused by COX-1 gene polymorphism, collagen-induced platelet hyperaggregability and some classic risk factors, such as smoking [16,41,52]. Also, homozygote individuals to the P1<sup>A2</sup> allele might show reduced response to ASA therapy (Papp E. et al., unpublished data). While the existence of aspirin non-responder patients has been accepted for years, thienopyridine resistance has only been recently revealed [168]. It is also important to note that some recent trials indicated that patients might become progressively less sensitive to the applied antiplatelet medication over long-term follow-up [120,64].

As mentioned previously, the occurrence of serious ischemic vascular events might be significantly reduced by the regular administration of aspirin or thienopyridine derivatives. However, the laboratory monitoring of platelet function is not conducted routinely these days leading to the strategy of “one size fits all” for dosing ASA,

ticlopidine and clopidogrel as well [70]. Also, the pharmaceutical inactivation of distinct thrombocyte functions might increase the risk of serious bleeding complications. Considering the individual differences regarding the sensitivity to platelet-active agents, monitoring platelet function to predict the risk of treatment failures and to apply the lowest, but still efficient daily drug doses might be favorable throughout the secondary prevention of vascular disorders. Numerous laboratory techniques have been developed with the aim to assess the efficacy of routine antiplatelet therapy. Although the optimal test is still debated, the most widespread method is the optical aggregometry described by Born [13]. Measurements are performed in platelet-rich plasma in the presence of various agonists (ADP, collagen, epinephrine and arachidonic acid are employed most frequently); the rate and extent of increase in light transmission is recorded by a photometer for 10 or 20 minutes. Impedance aggregometry is an alternative assay performed in diluted, anticoagulated whole blood [126]. Upon the addition of a pro-aggregatory molecule (inductor) to the sample, platelet aggregates form around the surface of the electrodes. As a consequence, electric impedance increases that is monitored throughout the measurement. A recently developed platelet aggregometer is the platelet function analyzer (PFA-100), where the closure time of a 150 $\mu$ M diameter aperture of a coated nitrocellulose membrane is recorded by a computer program [85]. Collagen-ADP and collagen-epinephrine are applied generally to the surface of these filters. Additional methods include ACT (ability of platelets to decrease clotting time) and the determination of the serum extruded from a clot upon retraction [107]. Eikelboom and colleagues raised the possibility that measuring the urinary levels of 11-dehydro thromboxane B<sub>2</sub> can prospectively identify patients relatively resistant to the conventional aspirin therapy [41]. These subjects might benefit from higher daily ASA doses or the administration of thienopyridine derivatives. Further studies are required to validate and standardize the results of laboratory tests mentioned above and to correlate them independently with the clinical outcome.

To investigate the effectiveness of routine antiplatelet drugs and to assess the prevalence of non-responder subjects, optical platelet aggregometry was performed in a large patient population. As an initial measurement we evaluated the efficacy of conventional antiplatelet medication in five distinct patient populations. Subsequently, the platelet function of cooperative participants was monitored regularly up to 24 months ( $\pm$ 1 month). Their antiplatelet medication was repeatedly adjusted or

modified according to the laboratory results. As little prospective data are available for follow-up in the literature to date, in a pilot study we correlated the long-term prevalence of adverse clinical events (recurrent acute coronary syndrome, stroke or TIA) with the efficacy of the antiplatelet medication in a subset of our patients.

## **3.2 Patients and methods**

### *3.2.1 Initial visit*

The efficacy of routine antiplatelet medication was initially evaluated in 2819 patients (1664 males, 1155 females, mean age: 62±12 years) on aspirin, thienopyridine or combination therapy. Five subgroups were formed based on the indication of the antiplatelet drugs and the reliability of follow-up:

- **Subgroup I** (*patients with previous acute coronary syndromes followed-up at the First Department of Medicine, Division of Cardiology, University of Pecs, Medical School*)

149 subjects with previous acute coronary syndrome (91 males, 58 females, mean age: 65±12 years) were enrolled in this subgroup. These high-risk patients were strictly followed-up by a specialist in cardiology and were regularly controlled in our laboratory as well. The effectiveness of the antiplatelet medication was evaluated six months following the acute event.

- **Subgroup II** (*chronic cardiovascular inpatients at the Department of Cardiac Rehabilitation, State Hospital of Balatonfured*)

We elaborated the aggregometry results of 461 subjects with previous ACS (322 males, 139 females, mean age: 62±10 years) being under continuous medical attendance at the Department of Cardiac Rehabilitation, State Hospital, Balatonfured. During the entire cardiac rehabilitation program specialists in cardiology regularly and strictly controlled both the drug therapy and the compliance of these patients. Platelet aggregometry was performed at least six weeks following the acute event and on the second week of the rehabilitation program.

- **Subgroup III** (*patients with chronic cardiovascular disease visiting the Outpatient Department of Cardiology, First Department of Medicine, University of Pecs, Medical School*)

The effectiveness of routine antiplatelet therapy was evaluated in 645 individuals with chronic cardiovascular disease (410 males, 235 females, mean age: 61±12 years). Ischemic heart disease was the most common indications for the applied medication. Merely some of these patients were regularly seen by a cardiologist or followed-up by a physician between the acute event and the performed laboratory test.

- **Subgroup IV** (*patients with chronic cerebrovascular disease seen at the Department of Neurology, University of Pecs, Medical School*)

1206 subjects (662 males, 544 females, mean age: 60±11 years) with chronic cerebrovascular disease (at least 3 months following an acute event) were enrolled in this group. 881 and 325 patients experienced ischemic stroke and TIA, respectively. The individual diagnosis was confirmed using CT or MRI in each case. Neurologists did not control regularly the efficacy of the antiplatelet therapy and compliance of these subjects prior to the laboratory test. Besides the regular aspirin or thienopyridine medications 88% of these patients were on a neuroprotective agent (pentoxiphyllin, piracetam or vinpocetin).

- **Subgroup V** (*patients from various family practices in Baranya County*)

The efficacy of antiplatelet medication was estimated in 358 patients (181 males, 177 females, mean age: 66±10 years) seen from six family practices in Baranya County. The most common indications for the antiplatelet therapy included ischemic heart disease, chronic phase cerebrovascular disease, or peripheral arterial disease in this subgroup. Merely some of these patients were regularly seen by a specialist or even by the general practitioner subsequent to the initiation of the platelet inhibitory therapy.

The efficacy of the antiplatelet therapy was evaluated in the entire patient population and in each subgroup separately. As we can note, the most widespread indication for the treatment was either ischemic heart disease or chronic cerebrovascular disease and a specialist in cardiology, neurology, or a family practitioner initiated the laboratory test in each case. Subjects with previous acute coronary syndrome or stroke

were enrolled at least 3 months subsequent to the acute event. According to our initial findings the antiplatelet efficacy of 300 and 325mg ASA/day was not different significantly, both of them are referred as a daily dose of 325mg in the further data analysis. Informed consent along with a detailed questionnaire assessing compliance were obtained from each patient at the laboratory visits. The institutional committee on human research approved the study.

### *3.2.2 Patient follow-up*

Following the initial laboratory test we encouraged our patients from subgroups III, IV and V to join our follow-up study. 71% of the addressed subjects responded and were enrolled; the efficacy of their antiplatelet medication was monitored every 6 month ( $\pm 1$  month) for an average of 2 years ( $\pm 1$  month). If the applied low-dose (100 or 200mg) ASA medication was proved to be ineffective on any of the visits, the daily dose of the drug was increased up to 325mg and an extra laboratory test was performed within four weeks to verify the efficacy of the modified treatment. If a patient on a higher daily aspirin dose (300 or 325mg) was found to be non-responder, the administration of a thienopyridine derivate (either 500mg ticlopidine or 75mg clopidogrel) was proposed and the test was repeated in a month. Further visits were completed according to the initial schedule.

As part of the follow-up study, we also evaluated the hypothesis that the sensitivity to a fixed-dose antiplatelet medication progressively decreases during long-term therapy. The individual responsiveness to low and high daily doses of aspirin along with the platelet inhibitory effect of thienopyridine derivates were continuously monitored throughout the follow-up period.

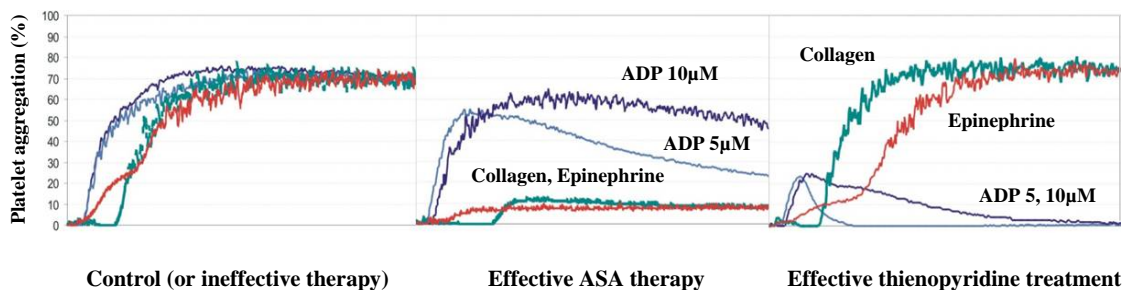
### *3.2.3 Prospective study*

In a prospective, pilot study the possible association between the ex vivo efficacy of the antiplatelet medication and the frequency of cardio- and cerebrovascular morbidity and mortality was evaluated. Results from 330 patients (186 males, 144 females, mean age:  $62 \pm 12$  years) with baseline and repeated platelet aggregometry have been analyzed to date; the average follow-up period was 24 months ( $\pm 2$  months). Two subgroups were formed based on the prevalence of adverse clinical events within the study period: 1) 35 patients presenting an acute coronary syndrome or a

cerebrovascular event; 2) 295 subjects without any acute ischemic events. The efficacy of the applied antiplatelet medication was evaluated in the subgroups separately.

### 3.2.4 Platelet aggregation measurements

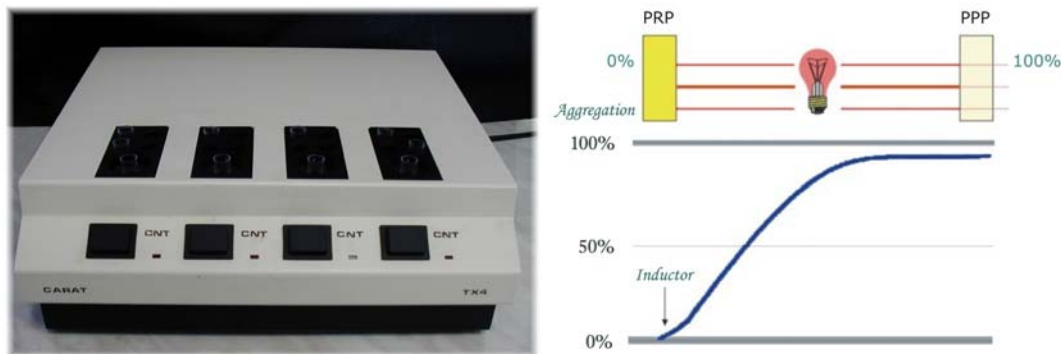
Fasting blood samples were collected into Vacutainer<sup>®</sup> tubes via antecubital venipuncture and were immediately mixed with 1:10 volume of trisodium citrate (3.8%). Platelet-rich plasma (PRP) was prepared by spinning the tubes at 150g for 10 minutes. After carefully removing PRP, the remaining specimen was further centrifuged at 2500g for 10 minutes to obtain platelet-poor plasma (PPP). Aliquots of 450 $\mu$ l PRP or PPP were pipetted into glass cuvettes and 50 $\mu$ l of ADP (final concentrations: 5 and 10 $\mu$ M), collagen (2 $\mu$ g/ml), or epinephrine (10 $\mu$ M) was added to the PRP so as to induce platelet aggregation. Epinephrine and collagen are widely accepted to estimate the antiplatelet efficacy of acetyl salicylic acid, while ADP is used to verify the effectiveness of thienopyridine derivates (Figure 3.2.1). Samples were incubated at 37°C and continuously stirred at 1000rpm throughout the measurements. The aggregation curve was recorded for 10 minutes using a Carat TX-4 platelet aggregometer (Carat Ltd., Budapest, Hungary) utilizing the turbidimetric method described by Born [13] (Figure 3.2.2). Maximal platelet aggregation was expressed as a percentage of 100% light transmission, calibrated for each specimen. All measurements were completed within 2 hours following venipuncture.



**Figure 3.2.1:** Characteristic platelet aggregation curves representing a control measurement, an effective ASA and an appropriate thienopyridine therapy.

Initially we re-validated the normal range (mean $\pm$ 2SD) of maximal platelet aggregation for each agonist (Table 3.2.1) by performing the aggregometry test for 250 drug-free, control subjects (140 males, 110 females, mean age: 35 $\pm$ 12 years). The

antiplatelet therapy of a patient on aspirin was considered to be ineffective if the maximal aggregation indices achieved by collagen and epinephrine were not significantly different from that of controls [29]. In case of thienopyridine derivatives the applied medication was considered to be inappropriate if the ADP ( $5\mu\text{M}$ ) induced platelet aggregation was within the range established for untreated, healthy subjects [29]. Results acquired with  $5$  and  $10\mu\text{M}$  ADP were in great agreement in all cases; the higher inductor concentration was employed to verify the data obtained with  $5\mu\text{M}$  ADP.



**Figure 3.2.2:** Carat TX-4 platelet aggregometer utilizing the light transmission method described by Born.

**Table 3.2.1:** Reference ranges (average $\pm$ 2SD) for maximum platelet aggregation indices achieved by different agonists in the control population (ineffective antiplatelet therapy). ADP is accepted to assess the antiplatelet efficacy of thienopyridine derivatives, while collagen and epinephrine are used to estimate the sensitivity to aspirin.

<b>Reference ranges for maximum platelet aggregation (average<math>\pm</math>2SD)</b>			
<i>ADP <math>5\mu\text{M}</math></i>	<i>ADP <math>10\mu\text{M}</math></i>	<i>Collagen <math>2\mu\text{g/ml}</math></i>	<i>Epinephrine <math>10\mu\text{M}</math></i>
62-91%	62-91%	64-92%	60-88%

All aggregation curves were automatically analyzed by the computer program of the platelet aggregometer and by two independent medical doctors who were blinded to the drug therapy of the subjects.

### 3.3 Results

#### 3.3.1 Initial visit

94% of the recruited patients were on either aspirin or thienopyridine monotherapy; a drug combination (daily dose of 100 or 325mg ASA in combination with either 500mg ticlopidine or 75mg clopidogrel) was administered in merely 6% of the population (Table 3.3.1). We can note that most patients were on a low-dose ASA therapy, only 12% of the subjects took 325mg on a regular basis. Clopidogrel was administered slightly more frequently than the other thienopyridine derivate (Table 3.3.1).

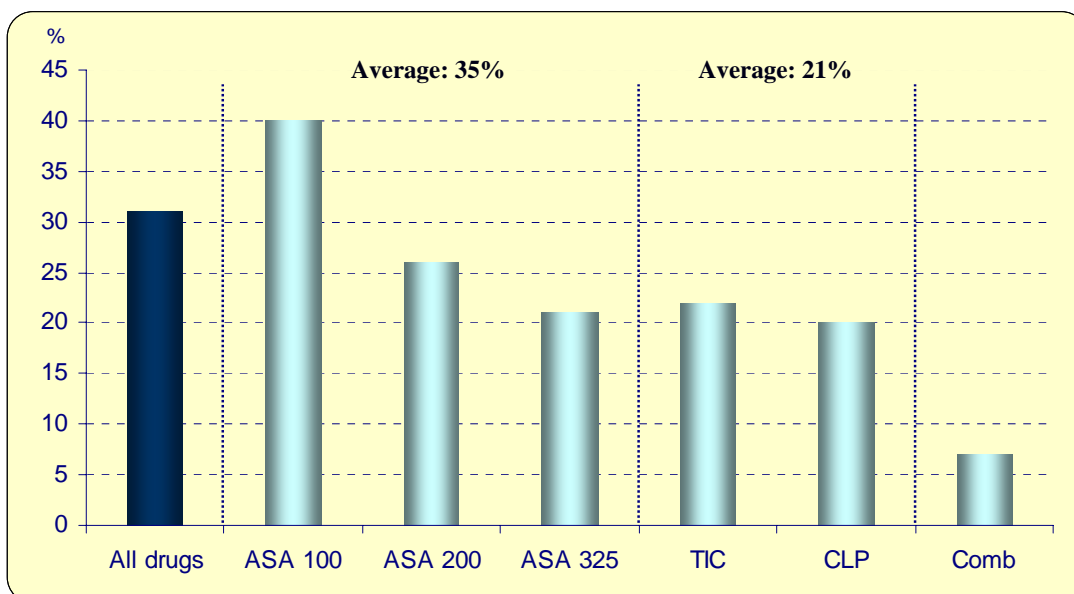
**Table 3.3.1:** Detailed antiplatelet medication of the study population recorded at the initial visit.

Subgroups	Antiplatelet drugs (mg)					
	<i>ASA 100</i>	<i>ASA 200</i>	<i>ASA 325</i>	<i>TIC 500</i>	<i>CLP 75</i>	<i>COMB</i>
<i>Subgroup I (%)</i>	37	8	25	2	7	21
<i>Subgroup II (%)</i>	65	10	3	7	6	9
<i>Subgroup III (%)</i>	51	2	18	4	15	10
<i>Subgroup IV (%)</i>	55	6	9	12	16	2
<i>Subgroup V (%)</i>	58	10	17	4	9	2
<b>Total (%)</b>	<b>55</b>	<b>6</b>	<b>12</b>	<b>8</b>	<b>13</b>	<b>6</b>

Considering the total examined population of 2819 subjects, the applied antiplatelet medication was found to be ineffective in 31% according to our laboratory tests (Figure 3.3.1). Inadequate ASA monotherapy was recorded in 35% of our patients; however, we can note an obvious inverse correlation between the aspirin dosage and the ratio of non-responsive individuals: 40% vs. 21% observed at low and high daily ASA doses, respectively (Figure 3.3.1). The frequency of ineffective medication was found to be 21% among subjects on a thienopyridine derivate with no significant differences



between the antiplatelet efficacy of ticlopidine and clopidogrel. As anticipated, combination therapy was found to be effective in most cases (Figure 3.3.1).



**Figure 3.3.1:** The ratio of non-responder patients considering all antiplatelet agents (all drugs). The prevalence of drug-insensitive platelet aggregation is also detailed in case of three different doses of acetyl salicylic acid (ASA), ticlopidine (TIC), clopidogrel (CLP) and combination therapy (Comb). Values recorded at the initial visit are shown.

45% of patients in subgroup I were on a low-dose aspirin monotherapy, ticlopidine and clopidogrel administration was recorded in 2% and 7%, respectively. A drug combination was employed in 21% representing the highest value among the subgroups. 30% of the population on 100mg ASA showed reduced sensitivity to the medication. When applying higher daily doses of the drug, the ratio of ineffective therapy progressively diminished and reached as low as 24% in case of 325mg aspirin a day. Thienopyridine derivatives and combination therapy were found to be inappropriate in merely 18% and 9% of this subgroup, respectively (Table 3.3.2).

78% of subjects in subgroup II received ASA on a regular basis at the Department of Cardiac Rehabilitation. 12% of these individuals were found to be insensitive to the therapy despite the tight control provided by physicians and the medical staff. This value represents the lowest ratio of aspirin non-responders observed in any of the subgroups. While the proportion of adequate ticlopidine and clopidogrel therapies reached as high as 90% and 93%, respectively, the combination of aspirin and

a thienopyridine derivate proved to be appropriate in all patients in this subgroup (Table 3.3.2).

According to the aggregometry tests, 38% of the 465 patients on a low-dose ASA in subgroup III were insensitive to the medication (Table 3.3.2). As anticipated, when increasing the daily dose of the drug to 325mg the efficacy of the therapy improved significantly in this subgroup as well. 5 $\mu$ M and 10 $\mu$ M ADP promoted platelet aggregation equivalent to that observed in the control population in about one quarter of the thienopyridine treated subjects. We can note that the ratio of ineffective medication was as low as 9% in case of a combination therapy (Table 3.3.2).

**Table 3.3.2:** The ratio of drug insensitive platelet aggregation revealed in case of ASA, ticlopidine (TIC), clopidogrel (CLP) and combination (Comb) therapy in the total population and in each of the subgroups.

Subgroups	Resistance to antiplatelet drugs					
	<i>ASA 100</i>	<i>ASA 200</i>	<i>ASA 325</i>	<i>TIC 500</i>	<i>CLP 75</i>	<i>COMB</i>
<i>Subgroup I (%)</i>	30	33	24	0	18	9
<i>Subgroup II (%)</i>	23	10	0	10	7	0
<i>Subgroup III (%)</i>	46	39	18	26	24	9
<i>Subgroup IV (%)</i>	43	28	23	23	21	13
<i>Subgroup V (%)</i>	48	39	31	36	25	13
<b>Total population</b>	<b>40</b>	<b>26</b>	<b>21</b>	<b>22</b>	<b>20</b>	<b>7</b>

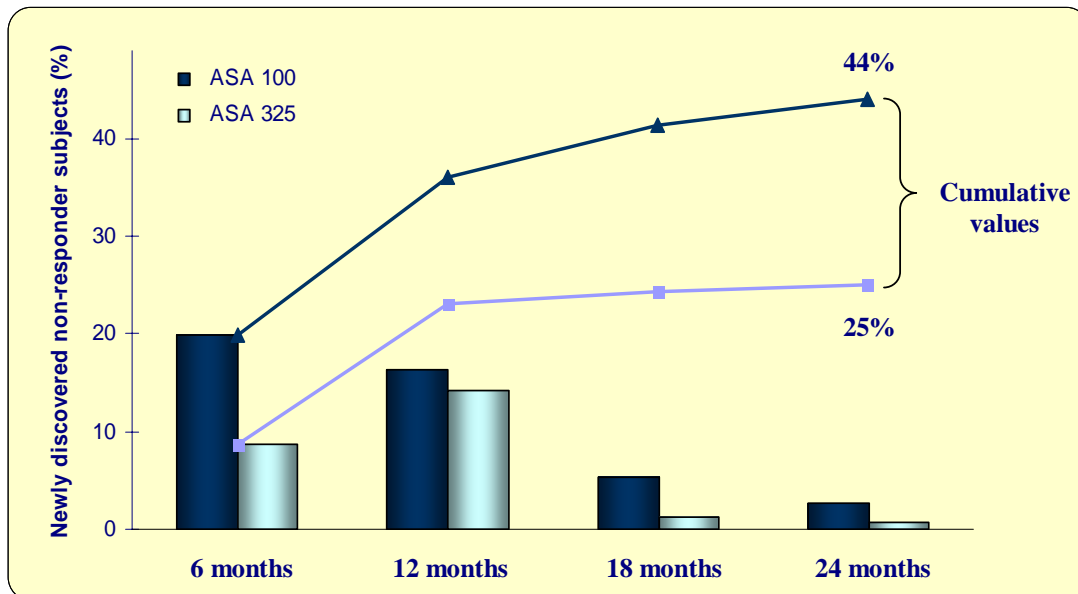
847 subjects in subgroup IV were on ASA medication with the low daily dose of the drug representing the most frequently administered medication in this subgroup as well. Aspirin was found to be ineffective in 39% of this population on average with an obvious association between the applied daily drug dose and the efficacy of the treatment. Despite the regular administration of 500mg ticlopidine or 75mg clopidogrel, persistent platelet activation was demonstrated in 22% of subjects. Combination therapy was rare in this subgroup, and as expected, its efficacy was superior to that of any antiplatelet drugs applied in monotherapy (Table 3.3.2).

A great majority (85%) of patients seen from family practices (subgroup V) received ASA monotherapy. The ratio of ineffective antiplatelet medication was the highest in this subgroup reaching as high as 43%. An ADP receptor blocker medication was rarely administered, the prevalence of inappropriate drug effect reached as high as 29%. Merely some of the enrolled subjects were on a combination therapy, although the occurrence of individuals with drug insensitive platelet aggregation was the highest within this subgroup (Table 3.3.2).

### *3.3.2 Patient follow-up*

Following the initial laboratory visit, we monitored the antiplatelet therapy of our cooperative patients for up to 2 years. Furthermore, we investigated whether the prolonged administration of a fixed daily dose of ASA or thienopyridine derivate provokes reduced sensitivity to these agents. The aggregometric responses to collagen and epinephrine decreased in 44% of patients on a daily dose of 100mg ASA throughout the follow-up period with the maximum aggregation indices reaching similar values observed in the control population. Of these subjects, 45% and 37% converted to non-responder within the initial six or twelve months of the study, respectively (Figure 3.3.2). Following an improper aggregometry result with 100mg ASA, the daily drug dose was increased up to 325mg. The test performed within 30 days subsequent to the therapy adjustment revealed appropriate laboratory effect in 79% of previously ASA insensitive subjects. Similar, although less prominent phenomena could be noted regarding the aspirin sensitivity of patients on a high daily dose of the drug; 25% of them converted to non-responder over the entire follow-up period with the 12 months visit revealing therapy insensitive platelet aggregation in most cases (Figure 3.3.2).

If 325mg/day aspirin did not diminish collagen and epinephrine induced platelet aggregation, the administration of a thienopyridine derivate was proposed. The regular daily doses of ticlopidine and clopidogrel provided efficient platelet inhibition in 89% of previously ASA non-responder patients when tested one month following the therapy modification. It is important to note that our assay did not reveal any remarkable change regarding the antiplatelet efficacy of either ticlopidine or clopidogrel throughout the entire follow-up period.



**Figure 3.3.2:** The ratio of newly discovered aspirin non-responder patients at each visit (bar graphs). The solid lines represent the cumulative ratio of subjects with ASA insensitive platelet aggregation revealed throughout the follow-up.

### 3.3.3 Prospective study

As a pilot, prospective study we addressed the possible association between the antiplatelet efficacy of ASA (including all aspirin doses) and thienopyridine derivatives estimated by platelet aggregometry and the long-term clinical outcome (24 months). Despite the regular administration of antiplatelet medications, the ratio of inappropriate laboratory tests reached as high as 50% among individuals developing an ischemic event (acute coronary syndrome, stroke or TIA) within the study period. In contrast, therapy resistant platelet aggregation could be revealed in merely 30% of the population without a negative clinical outcome. Despite the low number of enrolled individuals, our results demonstrate a significant relative risk reduction in patients with an appropriate aggregometry test ( $p < 0.001$ ). We should note that no remarkable discrepancy could be detected between the two populations regarding the indication of the therapy and the prescribed antiplatelet medications. This observation might reveal a considerable association between the ex vivo aggregometry results and the long-term clinical effectiveness of secondary prevention.

### 3.4 Discussion

Platelets have a well-identified, critical role in the development of cardiovascular diseases including coronary artery thrombosis, stroke and peripheral vascular diseases [130]. In case of an atherosclerotic plaque disruption blood might be exposed to subendothelial structures within the damaged vessel wall. As a consequence, aberrant and excessive platelet aggregation occurs leading to occlusive thrombi formation [71].

Antiplatelet agents were proved to reduce the incidence of serious vascular events throughout the secondary prevention [3]. Several drugs with improved effectiveness have been developed to influence discrete mechanisms of clot formation. The cyclooxygenase inhibitor ASA has been the standard antiplatelet agent for decades; however, several patients develop recurrent atherothrombotic events despite the regular administration of aspirin during the long-term follow-up. Possible mechanisms involved in ASA resistance have been summarized previously [16]. Increasing the daily drug dose to 325mg might be beneficial; however, these subjects will certainly not be resistant to the side effects of the high aspirin dose [3]. Thienopyridine derivatives are alternative antiplatelet agents employed in the secondary prevention of cardio- and cerebrovascular diseases; severe side effects are also less frequent. In contrast with primary expectations though, recent studies confirm the occurrence of thienopyridine non-responder patients in the general population [168]. Considering the number of agonists leading to platelet activation, the combination of aspirin and a thienopyridine derivative, or pharmaceutical interventions blocking the final common pathway of clot formation - the GP IIb/IIIa receptors - represent the most effective antiplatelet medications available today [116,153].

In the first part of the present study we evaluated the efficacy of routine antiplatelet medication in the general population. Inappropriate platelet inhibition was revealed in approximately one third of the aspirin-treated subjects with the highest ratio among patients on a low daily drug dose. We can note an obvious association between the applied ASA dose and the inhibition of platelet function *ex vivo*. This finding was also verified in an earlier trial, where numerous hemorheological parameters and platelet aggregation were monitored for a year in subjects with previous ACS [95]. 21% of the study population was on a thienopyridine derivative with the indication of ASA

non-response or drug intolerance. Despite the improper drug effect revealed in some subjects - confirming the existence of thienopyridine non-response -, ticlopidine and clopidogrel provided appropriate platelet inhibition for 79% of these high-risk patients. The observed resistance to thienopyridine derivatives might be explained by the great variability in body mass indices, inappropriate compliance or by the individual differences in the metabolic activity of the liver enzymes also affected by certain medications employed throughout the secondary prevention. The combination of ASA and a thienopyridine derivative proved to be effective in a great majority of the population. Although this therapy is obligatory following percutaneous coronary interventions (PCI) and - as anticipated - the most effective way to inhibit platelet function, serious and undesirable side effects may occur [149]. Applying the lowest, but still effective daily drug doses might help to diminish the risk of major bleeding disorders in these subjects. Further studies are required to elucidate possible drug interactions interfering with an effective and safe antiplatelet therapy.

Following the initial data analysis we assessed the efficacy of routine antiplatelet medication in each subgroup separately. The lowest ratio of ASA non-responder patients was discovered in subgroup II followed by subgroup I. These subjects with previous ACS were regularly followed-up by a specialist in cardiology; their compliance is thought to be appropriate. Our assumption was also supported by the software of the platelet aggregometer estimating a compliance value over 92%. A higher ratio of ineffective ASA therapy was revealed in subgroup IV, subgroup III and subgroup V that might be explained - at least in part - by the irregular or even absent medical visits resulting in their slightly lower compliance. Detecting these subjects with better reporting, by measuring the urinary 11-dehydrothromboxane B<sub>2</sub> level or by the routine use of arachidonic acid in platelet aggregometry tests might help to reduce the number of non-compliant patients and thus ASA non-response. Our laboratory results revealed several individuals showing reduced sensitivity to ticlopidine and clopidogrel in each subgroup. In accordance with our findings with aspirin, the ratio of ineffective thienopyridine therapy was slightly higher in subgroups III, IV and V than among subjects participating in a strict follow-up program. These results further confirm the importance of platelet function testing and the value of regular medical controls throughout the secondary prevention.

In the following part of our study the effect of long-term ASA and thienopyridine administration was assessed. The inhibition of platelet function achieved by a fixed daily dose of aspirin progressively decreased over the follow-up period in a remarkable number of subjects. The observed decline of platelet sensitivity was dependent on the daily drug dose; the number of patients converted to non-responder was significantly higher among subjects on 100mg/day ASA than in case of higher drug doses. In contrast with these findings, the antiplatelet efficacy of ticlopidine and clopidogrel was found to be persistent in 99% of the population for the entire period of 24 months. Our findings further suggest that - based on the laboratory results - increasing the daily aspirin dose within the recommended range of 75-325mg might be beneficial for a large patient population. However, given the increasing rate of morbidity related to side effects, currently no clinical indication exists for the routine use of higher daily ASA doses throughout the secondary prevention of cardiovascular diseases [3]. It is also important to note that platelets from the great majority of previously aspirin non-responder subjects showed proper sensitivity when administering a thienopyridine derivate.

In the second part of the study we attempted to substantiate a linkage between the laboratory effectiveness of routine antiplatelet therapy and the long-term prevalence of atherothrombotic events. Our preliminary data reveal a strong positive correlation between the individual responsiveness to ASA or thienopyridine derivates and the occurrence of undesirable clinical events. Improving and monitoring the efficacy of antiplatelet medication might help to reduce the prevalence of recurrent ischemic episodes by 40% among high-risk subjects. These results suggest the importance of platelet function tests and the absolute value of regular medical control throughout the secondary prevention. However, further prospective studies are required to confirm our initial findings in a large patient population.

### **3.5 Study limitations**

A large number of patients participated in the present, non-randomized study. Two independent medical doctors evaluated the platelet aggregometry results in each case. They were blinded to the antiplatelet medication as well as the diagnosis and the

possible adverse clinical events. Patient compliance was assessed by detailed questionnaires and the aggregometer software at each visit; although direct laboratory measurements were not employed to control the validity of these data.

### **3.6 Conclusions**

In accordance with previous reports, our results indicate that the standard daily doses of aspirin do not develop appropriate antiplatelet efficacy in a significant number of patients. This study also confirms the occurrence of thienopyridine non-responders examining a large patient population for the first time. Detecting subjects with reduced platelet sensitivity and adjusting their medication individually might help to reduce the occurrence of further ischemic events as well as the prevalence of serious side effects. Appreciating platelets' role in the development and progression of ischemic vascular diseases, individually adjusted antiplatelet therapy might improve the value of secondary prevention.



## 4. INHIBITION OF ADP-EVOKED PLATELET AGGREGATION BY SELECTED POLY(ADP-RIBOSE) POLYMERASE INHIBITORS

### 4.1 Introduction

Poly(ADP-ribose) polymerase (PARP) is a 116kDa Zn<sup>2+</sup>-finger nuclear protein present exclusively in eukaryote cells. Thirteen subtypes of the enzyme have been identified to date with PARP-1 discovered initially and examined the most extensively. Its biological activity is dramatically and transiently induced by DNA damage, especially caused by reactive oxygen species (ROS). In fact, a direct correlation between the activity of the enzyme and the extent and number of single-strand DNA breaks has been documented previously [117]. Upon binding to the damaged DNA, PARP forms homodimers and catalyzes the cleavage of cellular NAD<sup>+</sup> to ADP-ribose and nicotinamide units. The enzyme utilizes multiple, occasionally over 200 of these ADP-ribose units to synthesize and covalently attach poly(ADP-ribose) chains to various nuclear acceptor proteins [22,163]. As poly(ADP-ribosylation) confers a highly negative charge, it exerts a dramatic effect on the function of target proteins.

Although the exact range of the physiological roles of PARP has not been completely elucidated yet, it certainly involves nine main functions:

- It has been implicated in DNA repair and the maintenance of genomic integrity [21,33,34]. This function is indicated by delayed DNA base-excision repair and by the high frequency of sister chromatid exchange in PARP-1 deficient cells exposed to ionizing radiation or treated with alkylating agents [34].
- PARP-1 regulates the production of various inflammatory mediators, such as the inducible nitric oxide synthase (iNOS) or intercellular adhesion molecule-1 [83,176]. NFκB is the key transcription factor controlling the synthesis of these proteins, and PARP has been shown to act as a co-activator in the NFκB-mediated transcriptions [108].
- The enzyme has a major role regulating replication and differentiation. This function is supported by the observation that poly(ADP-ribose) metabolism is accelerated in the nuclei of proliferating cells [72]. Furthermore, several

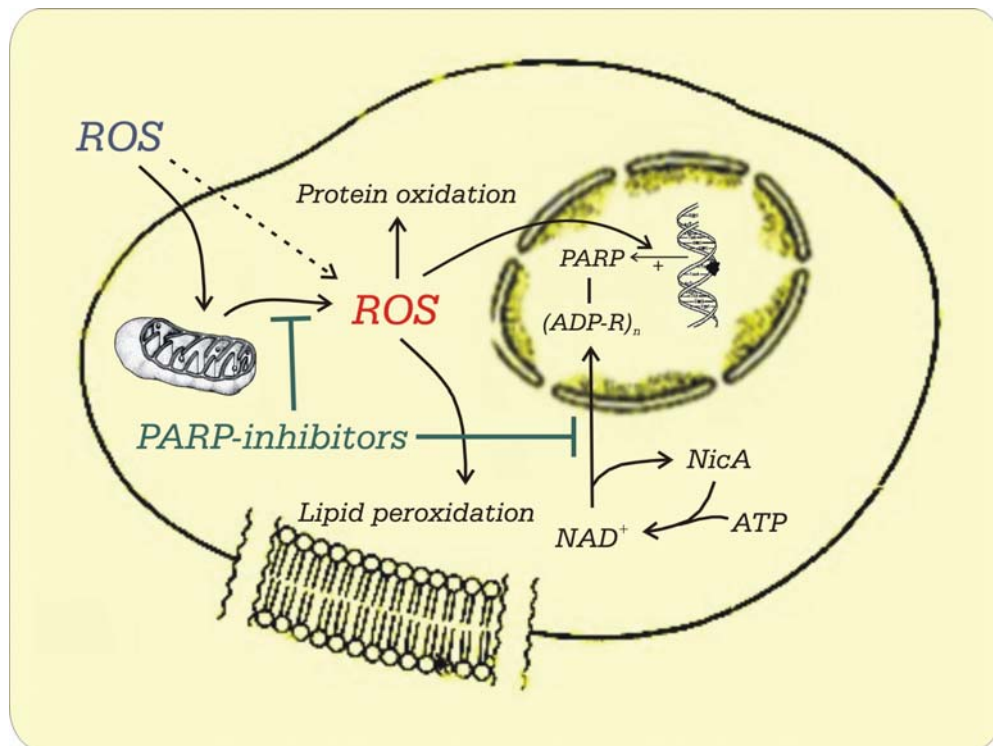
replication factors as well as the centromere have been shown to serve as substrates for PARP [135].

- Poly(ADP-ribosylation) has been implicated in the regulation of telomerase activity. Although results from previous studies are controversial, the enzyme might have an important role in maintaining telomere length [134,138].
- The activation of PARP has been proposed to represent a cell-elimination pathway allowing the removal of critically damaged cells. Severe genotoxic noxa might lead to the overactivation of the enzyme thus the depletion of  $\text{NAD}^+$  and ATP leading to necrotic cell death [10,162].
- Poly(ADP-ribose) polymers might be used as an emergency source of energy to synthesize ATP [96].
- Poly(ADP-ribose) might serve as a signal for protein degradation in oxidatively damaged cells [26,160].
- Poly(ADP-ribose) polymers can establish non-covalent bonds with specific sites of various proteins (e.g., histones, p53) and thus modify their function [119]. These polymers are formed as a result of the poly(ADP-ribose) glycohydrolase activity [32].
- Poly(ADP-ribosylation) might be involved in the regulation of cytoskeletal organization. The overexpression of PARP-1 has been proved to disrupt the structure of the cytoskeletal F-actin molecules thus leading to aberrant cell and tissue morphology [159].

As detailed above, PARP plays a pivotal role in maintaining genomic integrity through the modulation of cellular responses upon DNA injury. In contrast, permanently high ROS concentrations present under various clinical and experimental conditions (i.e., myocardial and brain ischemia-reperfusion, diabetes or anticancer therapy) might cause an extensive DNA damage leading to excessive PARP activation (Figure 4.1.1). The ensuing intracellular  $\text{NAD}^+$  depletion compromises mitochondrial substrate oxidation as well as ATP production culminating in necrotic cell death due to energy depletion [69,152]. Recognizing the biological function of PARP led to the hypotheses that inhibiting the enzyme can partially prevent ROS induced cell damages [102,131].

Intensive research has been devoted in the last decade to synthesize small molecules that can effectively bind to the PARP enzyme thus blocking its activity.

Numerous experimental PARP inhibitor agents have been proved to be successful in limiting ischemia-reperfusion injury of the cardiac muscle and brain tissue. They were effective in delaying the progressive functional impairment in chronic heart failure and the development of streptozotocin-induced diabetes. Also, these molecules reduced the occurrence of acute photodamage, endothelial dysfunction and the extensive tissue damage observed in shock [163,36,45,61,109,110,111,118,146,145,173]. Knockout animal models have further demonstrated that disrupting the PARP gene increases cell tolerance against oxidative damages [42,65].



**Figure 4.1.1:** Deleterious effects of reactive oxygen species (ROS) and the mechanisms of cell protection provided by PARP inhibitor molecules.

As discussed previously, hemorheological parameters might be significantly altered by reactive oxygen species. Also, oxidative stress triggered by ischemia-reperfusion in patients with ACS or in subjects following percutaneous coronary interventions may result in a remarkable thrombocyte activation leading to thrombosis and subsequent re-occlusion [49,68,79]. The role of ADP is inevitable and widely accepted in provoking and amplifying platelet aggregation both under physiological circumstances and the pathological conditions mentioned above. Furthermore, it is a

commonly applied agonist in laboratory tests so as to induce platelet aggregation. It is important to note that the structure of the most potent PARP inhibitors resembles to that of the adenine moiety of ADP-ribose and, thus, can interact with the substrate-binding site of the enzyme [22,163]. From this perspective it is tempting to speculate that ADP-, or adenine-mimicking agents might also bind to the ADP receptors present on platelets' surface - acting as competitive antagonists - and thus inhibit their aggregation. This novel feature of PARP inhibitors would further extend the variety of recently available antiplatelet drugs employed throughout the secondary prevention of occlusive vascular diseases. Especially, since agents blocking thrombogenic platelet functions have been proved to reduce significantly the risk of recurrent ACS and stroke over long term follow-up [3].

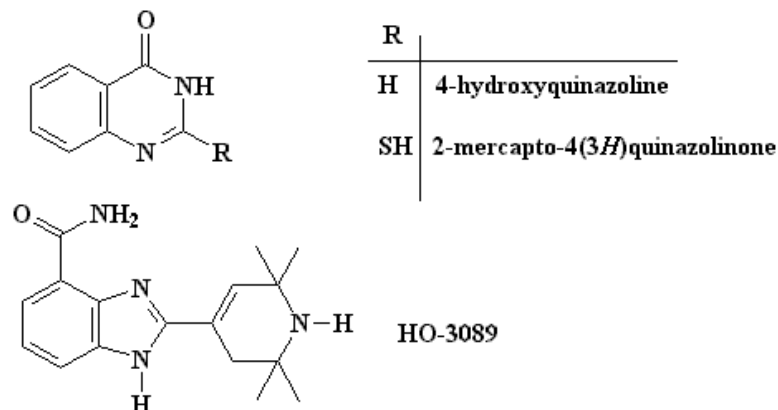
Based upon the aforementioned context, we tested the hypothesis that poly(ADP-ribose) polymerase inhibitors with adenine-like molecular structure can impede platelet aggregation. Since localized thrombocyte activation, adhesion and aggregation contribute to the development and progression of myocardial ischemia, efforts to optimize the treatment of cardiac ischemia-reperfusion injuries would welcome novel agents that render protection for cardiomyocytes, microcirculation as well as blood cells at the same time. Our results demonstrate for the first time that selected PARP inhibitors may fulfill this role by delivering myocardial cell protection as well as by exerting beneficial effects on tissue perfusion - via blocking platelet aggregation induced by ADP - under conditions of reduced coronary flow [163,61].

## **4.2 Materials and Methods**

### *4.2.1 Chemicals and reagents*

Acetyl salicylic acid was a gift from Sanofi-Synthelabo Ltd. (Le Plessis-Robinson, France), eptifibatide was purchased from Schering-Plough Ltd. (New York, USA). 4-hydroxyquinazoline and 2-mercapto-4(3*H*)-quinazolinone were obtained from Sigma-Aldrich Chemie Ltd. (Steinheim, Germany). HO-3089 was synthesized at the Institute of Organic and Medicinal Chemistry, University of Pecs, Medical School. The molecular structures of PARP inhibitors utilized in the present study are demonstrated on Figure 4.2.1. ADP, collagen, and epinephrine were purchased from Carat Ltd.

(Budapest, Hungary). Sodium-heparin was obtained from Biochemie Ltd. (Vienna, Austria). All other reagents were used at the highest purity commercially available.



**Figure 4.2.1:** Chemical structure of the examined poly(ADP-ribose) polymerase inhibitors: 4-hydroxyquinazolinone, 2-mercapto-4(3H)-quinazolinone and HO-3089.

#### 4.2.2 Oxidative challenge of cultured cardiomyocytes

H9c2 cardiomyocytes, a clonal line derived from embryonic rat heart, were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum and 2mM pyruvate in a humidified atmosphere of 95% air and 5% CO<sub>2</sub> at 37°C. Before reaching confluence, the cells were split, plated at low density in culture dishes (approx.  $2 \times 10^4$  cell/well) and cultured for 24 hours. Cardiomyocytes were then incubated without (negative control) and with 1mM hydrogen peroxide for three hours either untreated (positive control) or treated with 4-hydroxyquinazolinone, 2-mercapto-4(3H)-quinazolinone (both in 5, 10, 20, 50, 100, 500, 1000, and 1500 $\mu$ M), or HO-3089 (in 0.1, 0.5, 1, 2, 10, 20, 50, 100, and 500 $\mu$ M). At the end of the incubation period cell survival was determined by the MTT assay as the percentage of survival in negative control samples.

#### 4.2.3 Test validation and the antiplatelet effect of PARP inhibitors

Venous blood samples were drawn from healthy adult donors into Vacutainer<sup>®</sup> tubes containing trisodium citrate (3.8%). An experimental PARP inhibitor or a well-known antiplatelet drug (ASA or eptifibatide) - all dissolved and diluted in distilled water - was added to the tubes at various final concentrations: 4-hydroxyquinazolinone,

2-mercapto-4(3*H*)-quinazolinone (both in 100, 500, 1000, and 1500 $\mu$ M) and HO-3089 (in 10, 20, 50, 100, and 500 $\mu$ M). ASA (in 0.25, 0.5, 1, 2, 7, 20, and 70 $\mu$ M) and eptifibatide (in 100, 200, 300, 400, 500, and 1000ng/ml) served as control drugs. Equal volume of distilled water was added to the control tubes. Samples were incubated and continuously mixed at 37°C for 20 minutes to ensure optimal drug effect.

#### *4.2.4 The effect of incremental ADP concentrations*

Following the incubation period, PPP and PRP were prepared according to the method detailed previously in chapter 3.2.4. ADP (final concentrations: 5 and 10 $\mu$ M), collagen (2 $\mu$ g/ml) and epinephrine (10 $\mu$ M) were utilized as inductors throughout the experiment. Aggregation curves were monitored up to 10 minutes using the Carat TX-4 optical platelet aggregometer (Figure 3.2.2). We compared the maximal aggregation indices of the samples to that of control in each PARP-inhibitor concentration. All measurements were carried out within 2 hours following venipuncture and were repeated 15 times using blood samples from different individuals for every applied compound concentration. It is well documented that optical aggregometry is not suitable to assess platelet aggregation in case of any hemolysis. Therefore, the desensitization of platelet receptors by ADP released from damaged erythrocytes could be excluded.

#### *4.2.5 Heparin induced platelet hypersensitivity*

In a subsequent experiment we evaluated the antiaggregatory effect of PARP inhibitors on thrombocytes stimulated with increasing ADP concentrations. Initially, platelet aggregation was almost completely blocked by adding high concentrations of 4-hydroxyquinazoline, 2-mercapto-4(3*H*)-quinazolinone or HO-3089 to the samples (2000, 2000, and 1000 $\mu$ M, respectively). Following the twenty-minute incubation period, platelet aggregation was induced with ADP applied in various final concentrations (2, 5, 10, 15, 20, and 40 $\mu$ M). We compared the extent of maximum aggregation to the control measurement in each case.

It is well documented that heparin enhances platelet aggregation *in vivo*, and also when stimulated by any of the inductors *in vitro* [97]. In the following part of the study we evaluated the hypotheses that PARP inhibitors can effectively antagonize heparin-induced platelet hyperreactivity. Besides the experimental molecule, sodium-heparin (final concentration: 5U/ml) was also added to the samples prior to the

twenty-minute incubation period. Sample preparation and testing were performed according to the method described previously with the exception that platelets were stimulated by incremental ADP concentrations: 0.5, 1, 2.5, 5 and 10 $\mu$ M. The extent of maximum aggregation was compared to that of control (sample incubated with the same amount of heparin but without any PARP inhibitor) in each case.

#### *4.2.6 Statistical analysis*

Data are presented as means $\pm$ SEM. Samples were compared to the control values using the Students' *t* test.

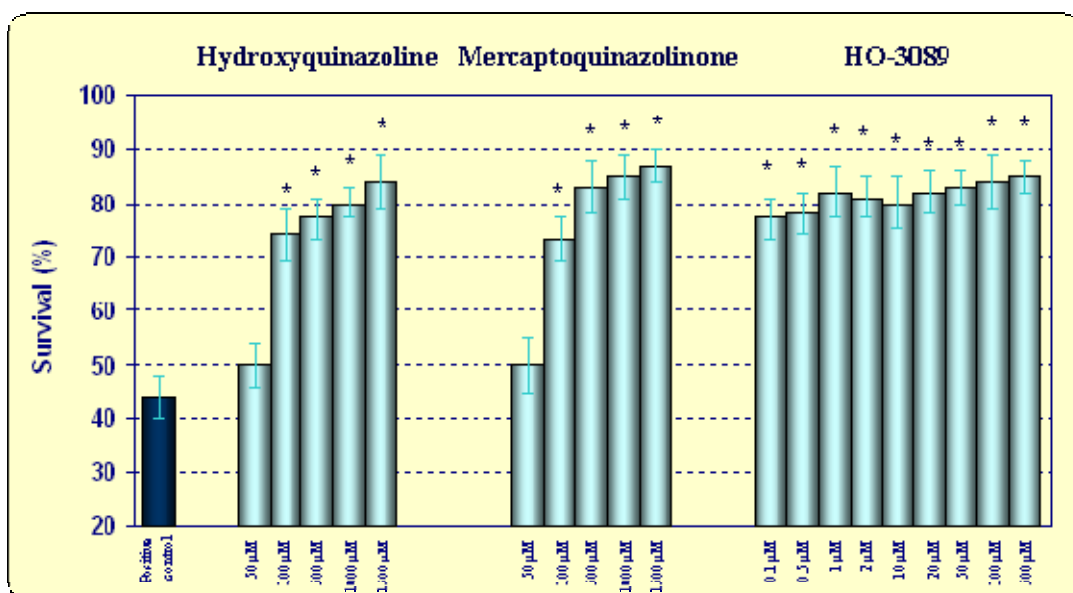
### **4.3 Results**

#### *4.3.1 Effect of PARP inhibitors on the survival of cardiomyocytes during oxidative insult*

Following the three-hour incubation period of H9c2 cardiomyocytes in the presence of 1mM hydrogen peroxide, merely 44 $\pm$ 6% of the cells survived. 4-hydroxyquinazoline and 2-mercapto-4(3*H*)-quinazolinone could not significantly improve the survival of the cells in the concentration range of 5-50 $\mu$ M. Nevertheless, in 100 $\mu$ M and above, 4-hydroxyquinazoline and 2-mercapto-4(3*H*)-quinazolinone protected the cells significantly when compared to the untreated samples ( $p < 0.01$ ). In the meantime, all the examined concentrations of HO-3089 (0.1-500 $\mu$ M) promoted the survival of H9c2 cells remarkably ( $p < 0.01$ ) (Table 4.3.1, Figure 4.3.1).

#### *4.3.2 In vitro test validation*

Two clinically used antiplatelet agents were utilized at the first stage of our study to validate the in vitro measurements. As anticipated, acetyl salicylic acid decreased collagen- and epinephrine-induced platelet aggregation in a concentration-dependent manner. Significant inhibitory effect could be detected as of 1 $\mu$ g/ml up to the entire examined concentration range (Figure 4.3.2). An in vitro concentration of 2 $\mu$ g/ml and 7 $\mu$ g/ml correspond to the eventual serum concentrations observed upon the oral administration of 100mg and 325mg ASA/day, respectively [115,113]. At the same time, acetyl salicylic acid had merely minimal impact on ADP-induced platelet aggregation.

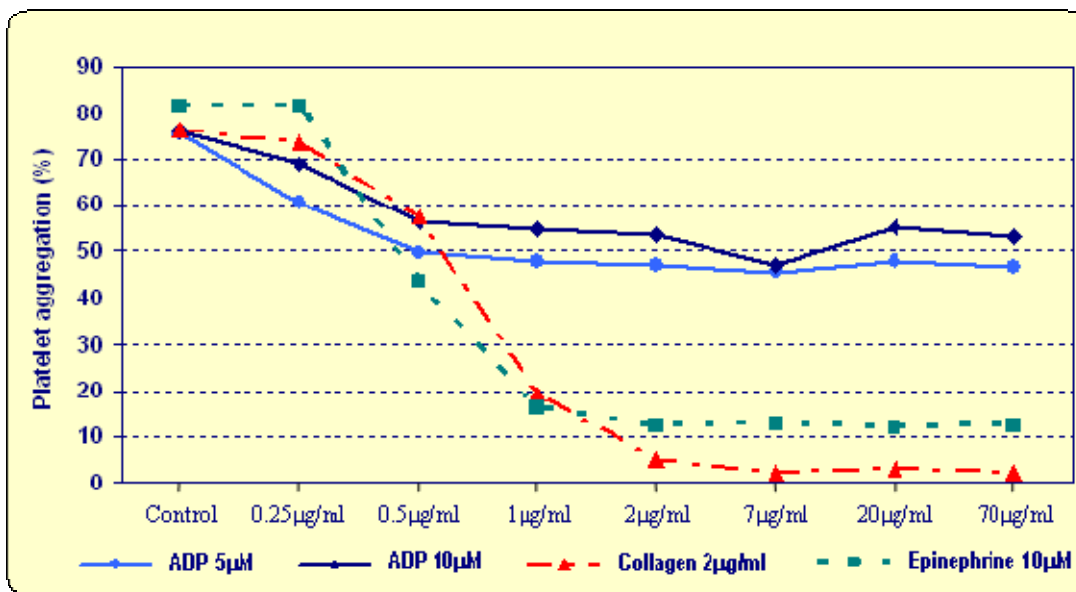


**Figure 4.3.1:** Effect of PARP inhibitors on the survival of H9c2 cardiomyocytes during three-hour hydrogen peroxide-induced (1mM) oxidative stress. Survival rates are expressed as the percentage of the cell count in negative (untreated) controls (mean $\pm$ SEM) (\*p<0.01). Positive control: survival in the presence of 1mM H<sub>2</sub>O<sub>2</sub> without any PARP inhibitor. All conditions were carried out in quadruplicate.

**Table 4.3.1:** IC<sub>50</sub> values of the experimental PARP inhibitors for H9c2 cardiomyocyte survival and for remarkable antiplatelet activity in the presence of 5 $\mu$ M or 10 $\mu$ M ADP.

Hydroxyquinazoline	Mercaptoquinazolinone	HO-3089
<b>IC<sub>50</sub> values considering the survival of cardiomyocytes</b>		
92 $\mu$ M	96 $\mu$ M	35nM
<b>IC<sub>50</sub> values of antiplatelet activity in the presence of 5<math>\mu</math>M ADP</b>		
1720 $\mu$ M	1310 $\mu$ M	185 $\mu$ M
<b>IC<sub>50</sub> values of antiplatelet activity in the presence of 10<math>\mu</math>M ADP</b>		
3065 $\mu$ M	2380 $\mu$ M	432 $\mu$ M





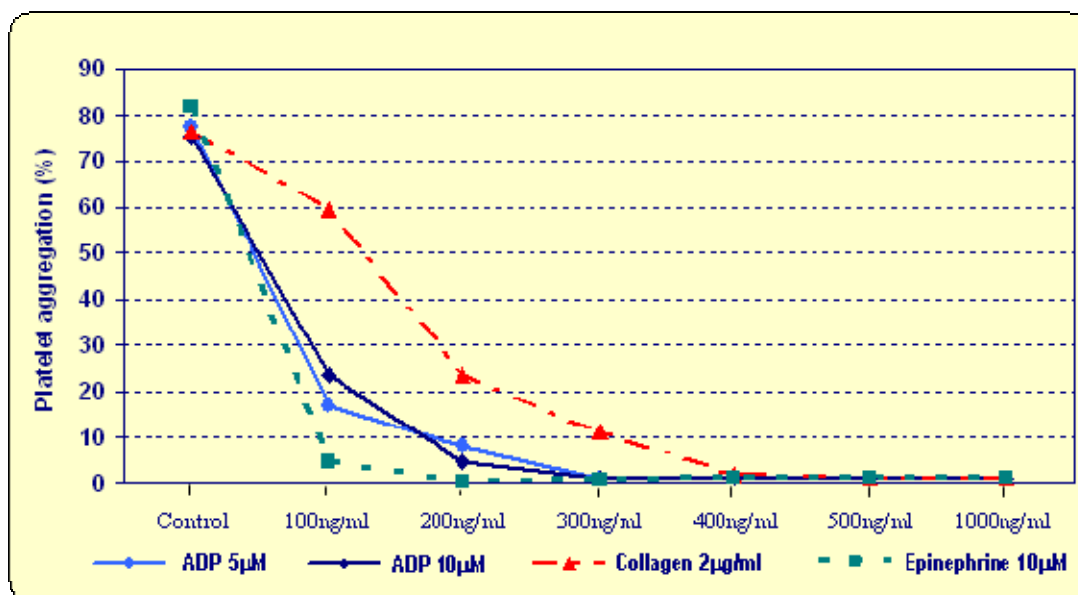
**Figure 4.3.2:** Dose-response curves of human platelets obtained when using ADP (5µM and 10µM), collagen (2µg/ml) and epinephrine (10µM) as agonists in the presence of increasing acetyl salicylic acid concentrations.

The inhibitory effect of eptifibatide was proportional to the applied drug concentration and could totally block platelet aggregation stimulated by any of the agonists in a concentration as low as 400ng/ml, well below its normal *in vivo* serum level (approximately 1000ng/ml during continuous intravenous administration) [31]. By blocking the GP IIb/IIIa platelet fibrinogen receptors, eptifibatide completely abolishes platelet aggregation independent of the type of activation used (Figure 4.3.3).

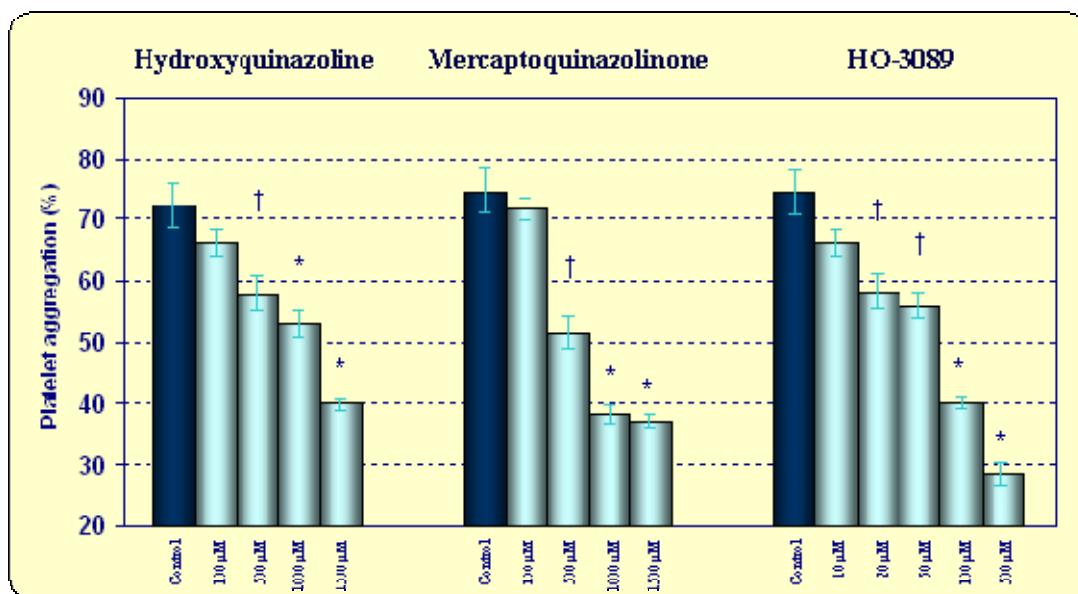
#### 4.3.3 Effect of PARP inhibitors on ADP-, collagen-, and epinephrine-induced platelet aggregation

The commercially available PARP inhibitors, 4-hydroxyquinazoline and 2-mercapto-4(3H)-quinazolinone markedly decreased platelet aggregation induced by either 5 or 10µM ADP at a final concentration of 500µM. At the same time, the experimental molecule, HO-3089 provided a remarkable inhibition even when applied at 20µM (Figures 4.3.4 and 4.3.5). Each compound exerted an incremental antiplatelet efficacy as the administered concentration increased and could markedly reduce the effect of ADP when applied at higher concentrations. While 4-hydroxyquinazoline did not affect significantly collagen- (2µg/ml) and epinephrine- (10µM) induced platelet

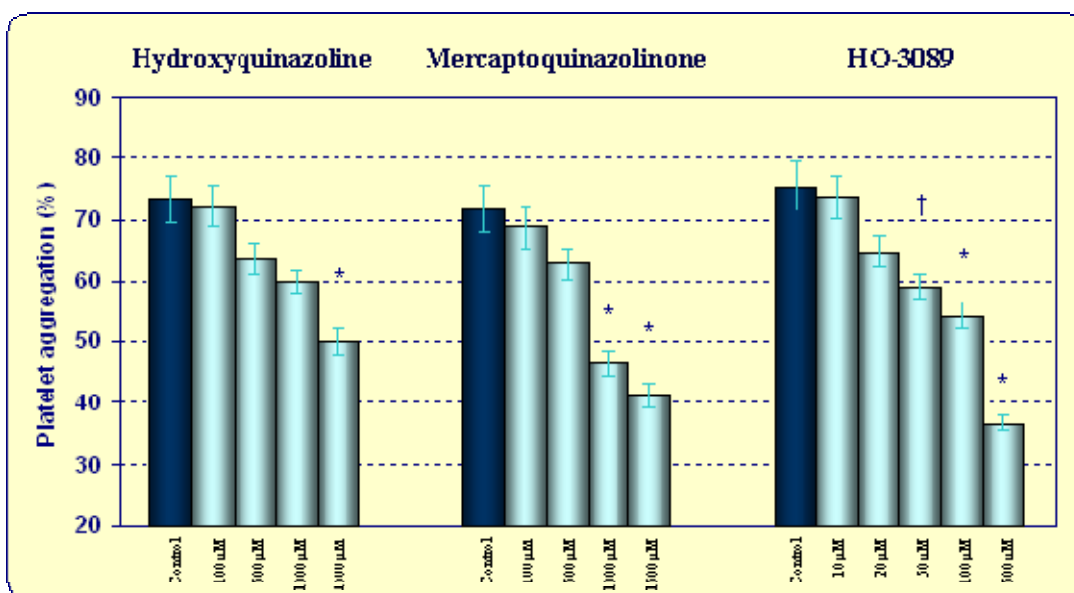
aggregation, 2-mercapto-4(3*H*)-quinazolinone and HO-3089 were able to impede it, but merely at the highest examined concentrations (1500µM and 500µM, respectively) (data not shown).



**Figure 4.3.3:** Dose-response curves of human platelets obtained when using ADP (5µM and 10µM), collagen (2µg/ml) and epinephrine (10µM) as agonists in the presence of increasing concentrations of eptifibatid.



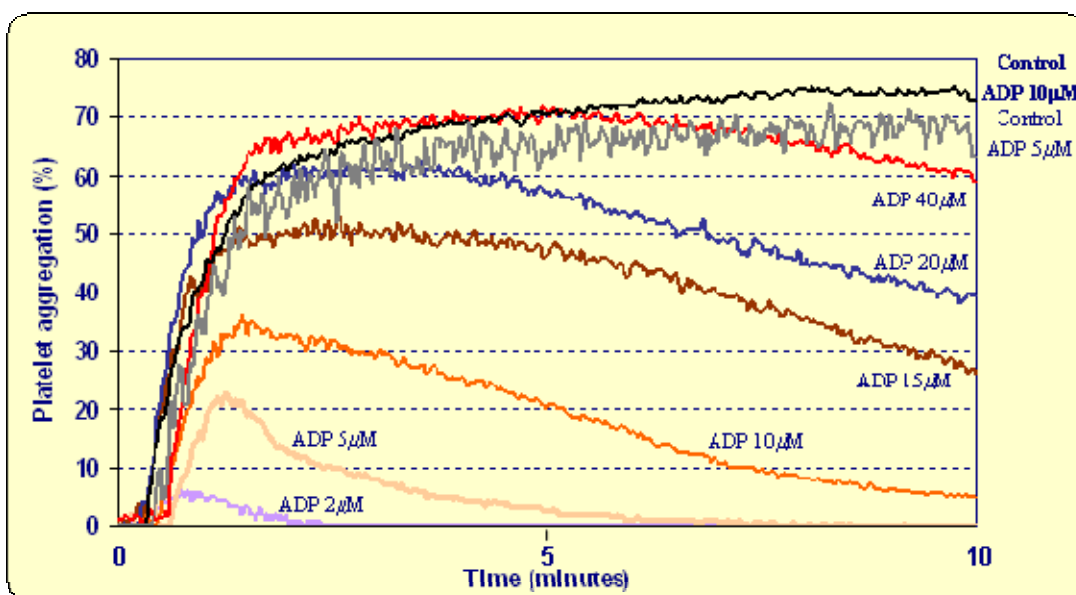
**Figure 4.3.4:** Response of human platelets to 5µM ADP as agonist in the presence of increasing concentrations of PARP inhibitors (mean±SEM) (†p<0.05, \*p<0.01). All experiments were repeated 15 times.



**Figure 4.3.5:** Response of human platelets to 10μM ADP as agonist in the presence of increasing concentrations of PARP inhibitors (mean±SEM) (†p<0.05, \*p<0.01). All experiments were repeated 15 times.

#### 4.3.4 Antiplatelet activity of PARP inhibitors in the presence of incremental ADP concentrations

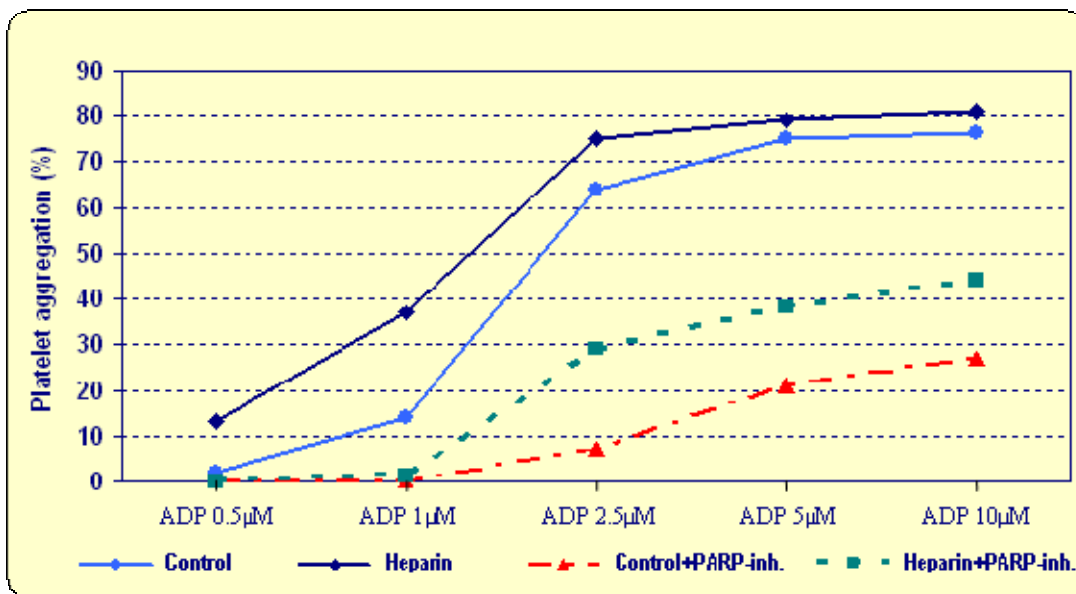
As anticipated, we found PARP inhibitors to primarily antagonize ADP-induced platelet aggregation in our experiments. Considering the fact, that a particular PARP inhibitor concentration had reduced impact on platelets when higher levels of ADP (5μM vs. 10μM) were applied, the dependence of their antiplatelet efficacy on the applied inductor concentration was evaluated. As we can note on Figure 4.3.6, maximum aggregation indices achieved by 5μM and 10μM ADP are essentially the same in case of control measurements as 5μM ADP is sufficient to induce a complete aggregatory response. In contrast, platelet reactions accomplished with the same agonists were reduced significantly in the presence of PARP inhibitors. The figure also demonstrates that parallel to the increasing ADP concentrations, the hindrance of HO-3089 on platelet aggregation waned and finally disappeared at 40μM. Similar phenomena were documented when applying any of the other two commercially available PARP inhibitors (data not shown). The level of maximum aggregation in case of the highest ADP concentration - despite the presence of high PARP inhibitor concentrations - was found to be similar to that of control measurements.



**Figure 4.3.6:** Representative response curves of human platelets using incremental concentrations of ADP (2-40 $\mu$ M) in the presence of HO-3089 (1000 $\mu$ M, a concentration that caused complete blockade of aggregation at the lowest ADP concentration). Similar phenomenon was observed with the application of 4-hydroxyquinazoline or 2-mercapto-4(3*H*)-quinazolinone (both in 2000 $\mu$ M, a concentration which caused complete blockade of aggregation) (data not shown). All experiments were repeated 15 times

#### 4.3.5 Heparin-induced platelet hypersensitivity and the efficacy of PARP inhibitors

In accordance with previous studies, our findings confirmed that human platelets show hypersensitivity to ADP in the presence of unfractionated heparin. As expected, ADP stimulated platelet aggregation in a dose dependent manner, but the concentration required to initiate the process was significantly lower than in the absence of heparin. Furthermore, an almost complete aggregation response was recorded even when applying the agonist well below its commonly utilized concentration (1 $\mu$ M vs. 10 $\mu$ M). Most importantly, this heparin-evoked sensitization of platelets was markedly reduced in the presence of the experimental PARP inhibitor molecules (Figure 4.3.7).



**Figure 4.3.7:** The mean response of human heparin-sensitized platelets (5U/ml) to increasing concentrations of ADP (0.5-10 $\mu$ M) in the presence of high concentrations of 4-hydroxyquinazoline, 2-mercapto-4(3*H*)-quinazolinone or HO-3089 (2000, 2000, 1000 $\mu$ M, respectively).

#### 4.4 Discussion

Pathological platelet aggregation is widely accepted to play an indispensable step in the pathogenesis of cardio- and cerebrovascular diseases and antiplatelet therapy has become a useful means of preventing and treating atherothrombotic events [136]. Numerous compounds have been developed to interfere with clot formation either at a receptorial (GP IIb/IIIa blockers, thienopyridine derivatives) or enzymatic level (ASA), although they do not exert any additional impact on cell survival. In our in vitro study we evaluated the effect of various PARP inhibitors on platelet sensitivity in autologous platelet-rich plasma.

Previous studies demonstrated that poly(ADP-ribose) polymerase inhibitors can exert remarkable protection in experimental models of cardiac and brain ischemia-reperfusion, AZT- and anticancer agent-induced cardiomyopathy, ultraviolet radiation-induced skin lesion, various forms of shock, chronic heart failure, diabetic endothelial dysfunction, and diabetes [163,36,45,110,111,118,145,61]. The inhibition of

the PARP enzyme contributes to the preservation of intracellular nicotinamide adenine dinucleotide and adenosine triphosphate pools in oxidatively challenged cells and tissues. Due to their structural resemblance, the most efficacious PARP inhibitors can compete with ADP-ribose (natural substrate of PARP) for binding to the enzyme [163,22]. This obvious similarity raised the possibility that some PARP inhibitors may also bind to and thus block the ADP receptors present on platelets surface. We tested our hypothesis by utilizing three different experimental molecules that have been shown to promote the survival of H9c2 cardiomyocytes exposed to hydrogen peroxide-induced oxidative insult. In this widely used setting of oxidative stress, 4-hydroxyquinazoline ( $IC_{50}$  for PARP enzyme inhibition:  $7\mu M$ ), 2-mercapto-4(3*H*)-quinazolinone ( $IC_{50}=35\mu M$ ), and HO-3089 ( $IC_{50}=46nM$ ) protected the cardiomyocytes in a concentration of 100, 100, and  $0.1\mu M$ , respectively, and above. These data are also in accordance with the results obtained in an isolated heart perfusion system, where 4-hydroxyquinazoline and 2-mercapto-4(3*H*)-quinazolinone could preserve myocardial high-energy phosphate levels during ischemia-reperfusion cycle when applied in a concentration of  $100\mu M$  [61]. HO-3089 also promoted significantly the recovery of high-energy phosphate intermediates tested in the same system, even in a much lower concentration ( $10\mu M$ ) [82]. Consequently, these PARP inhibitors indeed render protection for oxidatively challenged myocardial cells.

Our initial findings with well-known, widely administered antiplatelet agents validated our in vitro method for the investigation of experimental molecules using the same model. As expected, ASA decreased the collagen- and epinephrine-induced platelet aggregation in a concentration dependent manner, while eptifibatide completely blocked the aggregation - well below its in vivo serum concentration - stimulated by any of the agonists.

All three PARP inhibitors exerted marked antiplatelet activity in our in vitro model when ADP was employed as the agonist. However, on a molar basis HO-3089 had superior platelet inhibitory efficacy (significant inhibition could be observed even at  $20\mu M$ ) over 4-hydroxyquinazoline and 2-mercapto-4(3*H*)-quinazolinone (remarkable antiplatelet effect at  $500\mu M$  and above). We can note an obvious inverse correlation between the applied dose of the compounds and the platelet sensitivity to ADP. Although the concentrations required to abolish platelet aggregation completely proved to be significantly higher than that of delivering protection in case of the H9c2 cell

culture by inhibiting the PARP enzyme. Considering the relevance of oxidative stress-induced thrombocyte activation under various pathological conditions, even partial hindrance of platelet function attained by lower serum concentrations of PARP inhibitors might have an additional beneficial effect upon tissue ischemia-reperfusion.

The observed antiplatelet efficacy of the tested PARP inhibitors seems to be specific on ADP receptors. 4-hydroxyquinazoline did not have any effect on either collagen- (2 $\mu$ g/ml) or epinephrine- (10 $\mu$ M) induced platelet aggregation and 2-mercapto-4(3*H*)-quinazolinone and HO-3089 were able to impede it only at the highest examined concentrations (data not shown).

In order to elucidate the hypothetical mechanism regarding the antiplatelet activity of PARP inhibitors, we applied incremental amounts of ADP so as to induce platelet aggregation. In accordance with our initial theory, increasing concentrations of ADP gradually neutralized the antiplatelet properties of the selected PARP inhibitor molecules with maximal aggregation indices reaching similar values to that of untreated samples when applying ADP at 40 $\mu$ M. This observation might confirm an eventual competitive antagonism as the possible underlying mechanism behind the antiplatelet properties of PARP inhibitors. As the newly described phenomenon represents a receptorial mechanism, it is not surprising that the minimal concentration of the tested molecules required to abolish platelet aggregation was higher than that inhibiting the PARP enzyme. In addition, all the examined agents were capable of hindering thrombocyte aggregation even if those were sensitized to ADP by the pre-administration of heparin. These findings suggest that beyond their myocardial protective effect, our PARP inhibitor molecules may also be useful in the prevention and treatment of occlusive thrombotic events.

It might be important to note that Vacutainer<sup>®</sup> tubes containing sodium citrate were utilized throughout our experiments. The use of this anticoagulant might be of great interest when assessing the antiplatelet efficacy of novel molecules with unknown mechanism of action. It is well documented that decreasing the extracellular Ca<sup>2+</sup> concentrations in vitro might activate platelet thromboxane formation subsequent to the stimulation by adrenaline or ADP. As a consequence, a “secondary response” might be noted on the recorded aggregation curve. Inhibiting this excessive generation of thromboxane A<sub>2</sub> subsequent to ADP or epinephrine stimulation may lead to an overinterpretation of the antiplatelet efficacy of both thromboxane synthesis inhibitors

and thromboxane receptor antagonists [14,164]. It has also been documented previously that artificially added sodium citrate might alter the physiological function of the GP IIb/IIIa complex enhancing the binding of some antagonists to this receptor [125]. According to our present study, PARP inhibitors are thought to compete with ADP to occupy its receptor on the platelet surface and this process is thought to arise independent of the low extracellular  $\text{Ca}^{2+}$  concentrations [60]. In addition - as discussed previously - the examined agents did not have any effect on either epinephrine- or collagen-induced platelet aggregation confirming that PARP inhibitors are not likely to influence platelet thromboxane  $\text{A}_2$  biosynthesis. We might conclude that the observed antiplatelet effect of PARP inhibitors is certainly not influenced significantly by the use of sodium citrate as the anticoagulant.

#### **4.5 Conclusions**

This is the first report on the ability of certain poly(ADP-ribose) polymerase inhibitors to interfere with ADP-induced platelet aggregation. As this agonist has a major role in the physiological thrombocyte activation, our findings may be of crucial relevance in the eventual future therapeutic applications of PARP inhibitors. Besides preserving the cellular energy stores and protecting the functionality of vascular endothelial cells, the revealed beneficial “side-effect” of the selected agents may contribute to the survival of ischemic tissues by hindering intravascular thrombus formation. These results depict an exciting novel feature of selected, ADP- or adenine-mimicking PARP inhibitors that further adds to our understanding on the future therapeutic potential of these molecules.



## 5. AN AUTOMATED CAPILLARY TUBE VISCOMETER: VALIDATION STUDIES

### 5.1 Introduction

The rapidly expanding science of hemorheology concerns the flow properties of blood and its relationship to normal and abnormal physiology. As a major determinant of microvascular and tissue perfusion, rheology received considerable attention in the last decades. Increasing evidence from several investigations indicated marked changes in the rheologic behavior of pathologic blood, including elevated whole blood viscosity, increased RBC aggregation and more pronounced non-Newtonian flow behavior [23,30,75,80,92,124,128].

A basic principle of hemorheology relies on the Hagen-Poiseuille equation describing the association between the volumetric flow rate through a tube/vessel, pressure head, viscosity of the fluid and the diameter and length of the tube/vessel (Figure 5.1.1). As we can note, vessel diameter represents the principal determinant of blood flow under normal conditions. In contrast, under pathological circumstances such as atherosclerosis, whole blood viscosity turns into a dominant factor affecting volumetric flow rate.

$Q = \frac{\Delta P \pi r^4}{8 l \eta}$	Q = volumetric flow rate	r = tube diameter
	$\Delta P$ = pressure head	l = tube length
	$\eta$ = blood viscosity	

**Figure 5.1.1:** Hagen-Poiseuille equation.

Numerous clinically important laboratory tests have been developed to study hemorheological parameters, most notably whole blood viscosity profiles. Identifying elevated whole blood viscosity is of clinical importance since it has a predictive value for increased resistance to blood flow in the general circulation and for abnormal blood flow dynamics in the microcirculation [92,90,91,124,143] that are identified as the central pathophysiologic features of several diseases. They further promote and contribute to atherosclerotic plaque formation, shear stress damage at the endothelial

wall and also facilitate plasma protein interaction with the endothelium in post-stenotic recirculation zones (Figure 1.1.1). Note that in addition to the well-known conventional risk factors, elevated blood and plasma viscosities have been found to be independent predictors of initial and recurrent myocardial infarction, stroke and all-cause mortality [30]. Furthermore, assessing whole blood viscosity provides essential information in various hematological disorders associated with the “hyperviscosity syndrome” and can be a useful diagnostic tool to optimize and monitor therapeutic procedures such as hemodilution and exchange transfusions [39,81].

Numerous viscometers have been developed aiming the accurate measurement of whole blood viscosity. Most devices can be classified as rotational or capillary tube systems.

### *5.1.1 Rotational viscometers*

In a rotational viscometer system the fluid sample is sheared in a narrow gap between two surfaces, usually one rotating while the other is stationary. The two most frequently utilized geometries are the coaxial cylinder (Couette) and the cone-and-plate devices (Figure 5.1.2).

- The inner cylinder of a Couette viscometer is often referred as the bob; the external one is called the cup. The shear rate is determined by the speed of rotation and also the fixed geometrical dimensions. The latter parameter and the torque can be utilized to calculate the shear stress. Torque is a product of a rotating force and the distance of its action from the centre of rotation [23]; the same torque can be a result of a large force acting near, or a small force acting far from the centre. Thus, the uniformity of the shear field is of great importance. The collection of viscosity data over various shear rates is only possible by repeating the measurements at various speeds of the rotating component. The most sensitive coaxial cylinder viscometer is the Contraves LS-30 (Contraves AG, Zurich, Switzerland). While the cup rotates at preset frequencies covering shear rates of  $10^{-3}$  to  $10^2 \text{ s}^{-1}$ , the torque required to keep the bob in a steady position is measured by an opto-electromagnetic feedback circuit in this device [141].
- The common feature of cone-and-plate viscometers is that the fluid is sheared within the narrow gap between a flat plate and a cone with low angle (typically not exceeding  $4^\circ$ ). A nearly uniform shear rate is produced, which is determined

by the angle of the gap and also the linear rotational speed of the plate. The most reliable, commercially available cone-and-plate device is the Wells-Brookfield viscometer (Brookfield Engineering Labs, Middleboro, MA). The cone is coupled by a spring to a motor with adjustable rotational speeds. The strain of the spring is proportional to the torque exerted on the sample and can be read directly on a digital display [167].

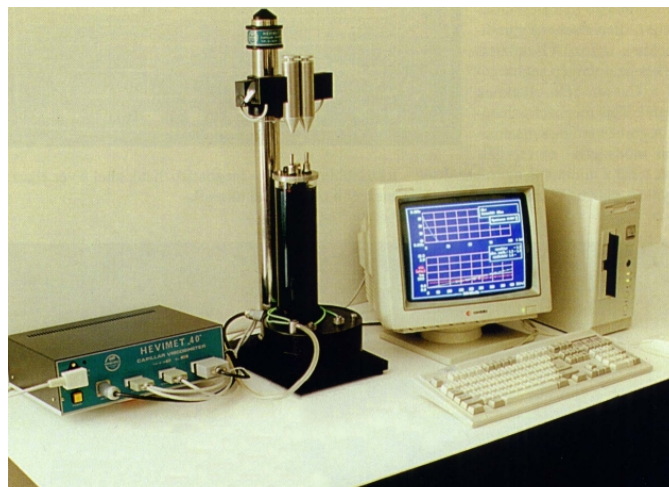


**Figure 5.1.2:** The most widely used rotational viscometer systems: Contraves LS-30 (left photo) and Wells-Brookfield (right picture).

Despite their popularity, rotational viscometers can be subject to artifacts limiting their routine clinical applicability. They are associated with denatured protein films, surface tensions, RBC sedimentation, limited range of reliability and phase separation leading to a cell-poor layer at the torque-measuring element [27,128,23]. These difficulties are especially evident when measuring whole blood viscosity at low to medium flow rates in order to obtain a shear rate-viscosity profile. Commercially available instruments tend to work only over a limited shear rate range, so that collecting data over a wide range of physiologically relevant flow rates (to create clinically appropriate viscosity profiles) often requires two separate viscometer systems and extended periods of time. Furthermore, blood samples have to be removed and the test section needs to be cleaned manually following each test posing a potential risk for contacting contaminated sample.

### 5.1.2 Capillary tube viscometers

The principle of capillary tube viscometers relies on the Hagen-Poiseuille equation detailed previously (Figure 5.1.1). In order to accurately assess whole blood viscosity using these systems, it is inevitable to independently determine the pressure drop and flow rate along the cylindrical capillary. The shear stress is zero at the axis of the capillary and increases linearly with the distance, reaching its greatest value at the tube wall. Thus, it is essential to know the exact dimensions of the capillary in order to determine the functional dependence between the volumetric flow rate and the pressure drop due to friction. Also, the sample flow has to be regarded as steady-state, isothermal and laminar. Most of the commercially available capillary viscometers produce accurate measurements only at a specified, pre-determined shear rate at a time. However, for non-Newtonian fluids - since the viscosity varies with shear rate - it is vital to repeat the test by varying the pressure in the reservoir tank in order to change the flow rate and to obtain physiologically relevant whole blood viscosity-shear rate profiles. This makes measurements complicated and extremely time consuming. Other common difficulties associated with traditional tube viscometers include their limited shear rate range, the “end effect” and the “carry-over” phenomena [23]. “Hevimet 40” is the only available capillary viscometer system that is free from most of the difficulties mentioned above (Figure 5.1.3). Following the injection of 0.5ml sample into the device, blood flow is endorsed by gravity and is monitored continuously using an optoelectronic system. Shear rate and shear stress are calculated by a computer program and viscosity values are determined according to the Casson's principle.

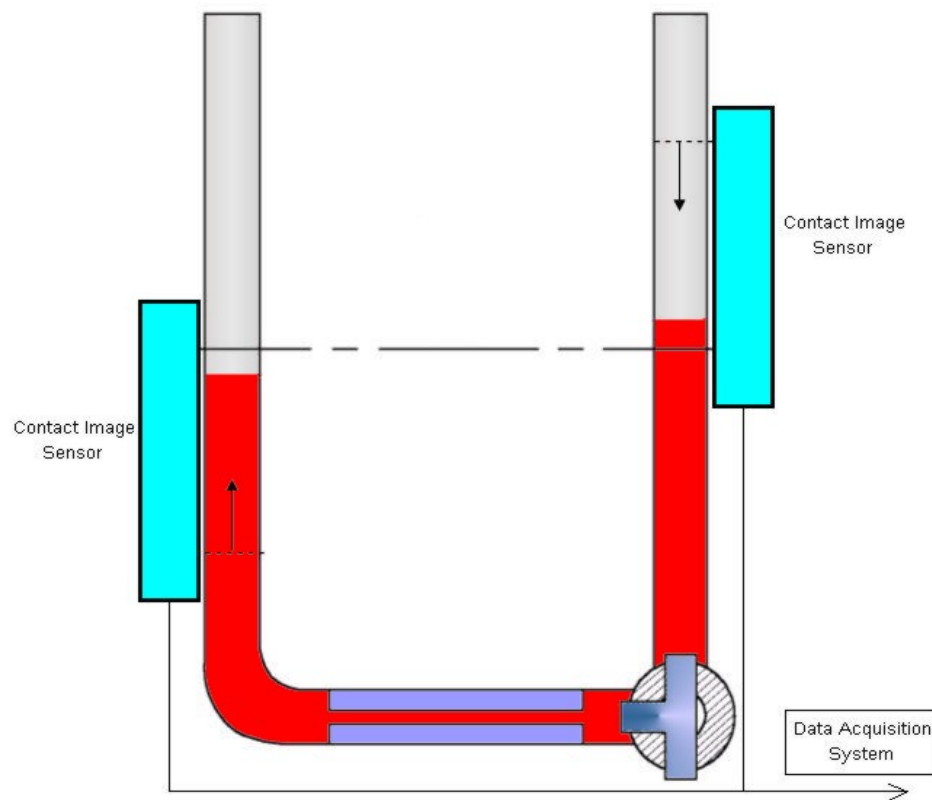


**Figure 5.1.3:** Hevimet 40 capillary viscometer system.

Despite its significant clinical value, whole blood viscosity is not measured routinely, principally because of the difficulties mentioned before and the logistical complications associated with conventional viscometer systems. To address these problems a computerized scanning capillary rheometer (Rheolog™, Rheologics, Inc., Exton, PA) was developed to provide fast sample testing and data analysis over a wide range of shear rates [24]. The viscometer utilizes a U-shaped disposable assembly consisting of a horizontal, pre-calibrated capillary with vertical “riser” tubes at each end and a stopcock (Figure 5.1.4). The geometry of the capillary is the key component of the test as it defines the pressure gradient between the riser tubes. Also, its relatively large inside diameter (800µm) minimizes the tendency of erythrocytes to move towards the center of the tube, leaving a plasma-rich zone at the capillary wall (Fahraeus-Lindqvist effect) [44]. A further essential feature of the scanning capillary viscometer is the use of optical detectors (CCD sensors and LED arrays) in order to accurately assess fluid level variations in the riser tubes every 0.02s. Since 1mm is represented by 12 pixels of the sensor, the actual changes of the sample height can be determined with an accuracy of 0.083mm. Three milliliter of blood is drawn into a Vacutainer® tube and introduced into the device via a standard extension kit. Initially the sample is directed into the left vertical tube until it reaches a pre-determined height. Then the stopcock rotates and allows the blood to flow through the capillary and into the right riser tube. When it fills-up to a certain, lower level as detected by the contact image sensor, the stepper motor repositions the stopcock thus isolating the sample completely. Following a three second stabilization period the analysis begins as the valve turns to a position allowing blood flow from the left vertical tube to the right one through the capillary. As the process is driven purely by the pressure gradient created by the initially unequal heights of blood in the riser tubes, flow occurs rapidly when the test starts and - as a result of the decreasing pressure differential - with gradually lower and lower rates as the volumes in the vertical tubes equalize. The pressure drop caused by the friction at the capillary can be obtained by continuously monitoring the volume of the samples in the riser tubes. This parameter is used to derive the velocity, viscosity and yield stress of the sample employing a data reduction algorithm based upon the Casson relation between shear rate and shear stress [27,128]. Viscosity results over the shear rate range of 1-1500s<sup>-1</sup> are graphically available through the computerized interface in less than 5 minutes from the introduction of the sample into the device. The

scanning capillary viscometer is temperature controlled; all measurements are performed at 37°C. Components are disposable, requiring no blood contact and cleaning, further reducing biohazard risk.

Previous reports have described the operating principle of the Rheolog™ in detail [24] and its application in studies of interindividual variability of whole blood viscosity with hydration [165], the effect of low-density lipoproteins on blood viscosity [103], and temporal variations of blood viscosity over a 14-day period [166]. To date, however, trials specific to validity and reproducibility of the measurements under various conditions affecting whole blood viscosity have not been published. The present study was thus designed to provide direct comparisons between results obtained by the Rheolog™ over a shear rate range of 1-1500s<sup>-1</sup> with data collected using cone-and-plate and Couette viscometers. Furthermore, the effects of storage time and storage temperature on whole blood viscosity were assessed at various shear rates.



**Figure 5.1.4:** Schematic drawing of the disposable assembly employed by the Rheolog™ system. The heights of the blood columns are monitored continuously via two contact image sensors, thereby providing the pressure gradient and flow data used to compute blood viscosity-shear rate results.

## 5.2 Methods

### 5.2.1 Viscometer systems

Results from three separate viscometer systems were analyzed in the present study.

1) Wells-Brookfield micro cone-plate viscometer model 1/2RVDTCP-200 equipped with a 0.8 degree cone and a shear rate range of  $75\text{-}1500\text{s}^{-1}$ . The cone-plate separation was adjusted per the manufacturer's instruction and the viscometer calibrated using Newtonian oils of known viscosity (Cannon Instrument Co, State College, PA). One ml of blood was used per test and the sample was allowed to equilibrate to  $37^{\circ}\text{C}$  for five minutes at a constant shear rate of  $75\text{s}^{-1}$  (Figure 5.1.2).

2) Contraves Couette viscometer model LS-30 equipped with a 1T-1T bob and cup system (i.e., bob diameter = 11mm, cup diameter = 12mm, gap = 0.5mm). The viscometer was calibrated with standard Newtonian oils (Cannon Instrument Co, State College, PA) and operated at shear rates of 1.29, 3.23, 8.11, 15.0, 37.7 and  $51.2\text{s}^{-1}$ . One ml of blood sample was used per test and was allowed to equilibrate to  $37^{\circ}\text{C}$  for five minutes at a constant shear rate of  $15\text{s}^{-1}$ . Note that at the lower rates of steady shear where torque-time phenomena can lead to incorrect torque values, an extrapolation to zero time method was used to obtain the correct torque at each shear rate (Figure 5.1.2) [28].

3) Rheolog<sup>TM</sup> automated scanning capillary viscometer (Rheologics, Inc, Exton, PA). For the present study, all the capillary tubes had an internal diameter of 0.79mm and a new assembly was used for each test (Figure 5.1.4). Calibration data for the disposables were supplied by the manufacturer. As the automated measurement starts as soon as the blood is introduced into the device, the Rheolog<sup>TM</sup> samples were pre-heated to  $37^{\circ}\text{C}$  for five minutes in a water bath immediately prior to testing to assure temperature stability. Note that since the Rheolog<sup>TM</sup> system employs a computer for data analysis, it is possible to compute viscosity values at any shear rate within the useful range of the instrument (i.e.,  $1\text{-}1500\text{s}^{-1}$ ). In the present study, shear rates of 1.29, 3.23, 8.11, 15.0, 37.7, 51.2, 75, 150, 300, 750, and  $1500\text{s}^{-1}$  were utilized for device comparisons and 1, 2, 5, 10, 50, 100, 150, 300, and  $1000\text{s}^{-1}$  were used for reproducibility studies.

### 5.2.2 Blood samples

Blood samples were drawn by sterile antecubital venipuncture into EDTA (1.5mg/ml) vacuum tubes from healthy adult donors. Unless otherwise indicated, samples were stored at room temperature ( $22\pm 1^\circ\text{C}$ ) and tested within four hours. The study was approved by the Institutional Review Board at the University of Southern California. No inclusion criteria were employed for the study and exclusion criteria were only investigational drug treatment or recent surgery. Blood and RBC-plasma suspension hematocrits were determined using the microhematocrit method (i.e., centrifugation at 12000g for 5 minutes).

### 5.2.3 Reproducibility studies

Fifty ml of blood was collected from five healthy volunteers (3 males, 2 females, mean age:  $31\pm 4$  years). Samples were processed via centrifugation (1750g, 10 minutes), and then RBC and plasma were recombined to achieve 15-20ml per donor of low and of high hematocrit suspensions (27% and 58% on average, respectively). Using the Rheolog™, ten replicate measurements in rapid sequence were performed for each low and high hematocrit suspension. No direct comparisons were employed with other viscometer systems in this part of the study.

### 5.2.4 Comparison studies

Comparison studies were conducted using two separate protocols:

- Measurements performed at native hematocrit: Blood samples were collected into three K<sub>2</sub>EDTA Vacutainer® tubes (5ml each) from 44 apparently healthy adult donors (26 males, 18 females, mean age:  $37\pm 4$  years). One tube was utilized for the measurements with the Brookfield and Contraves viscometer systems, with the other two used for two consecutive Rheolog™ tests termed “Rheolog A” and “Rheolog B”. Each tube was sampled only once for the experiments with the scanning capillary rheometer.
- Measurements performed at adjusted hematocrit values: Six vacuum tubes of five ml each were collected from eight subjects (4 males, 4 females, mean age:  $33\pm 2$  years) in order to prepare low (i.e., less than 30%) and high (i.e., greater than 55%) hematocrit suspensions via centrifugation/recombination. The viscosity of each suspension was measured once in the Brookfield and Contraves



systems and twice using the Rheolog™ device (i.e., “Rheolog A” and “Rheolog B” tests).

### *5.2.5 Storage Studies*

The effects of storage time and storage temperature on whole blood viscosity profiles were assessed using the Rheolog™ viscometer in this part of the study. Samples were collected from eight healthy subjects (4 males, 4 females, mean age: 30±5years) into K<sub>2</sub>EDTA Vacutainer® tubes. Three tubes from each donor were maintained at room temperature (22±1°C) and viscosity was measured at 10 minutes, at 4 and 8 hours following venipuncture. Two tubes were incubated in a water bath at 37°C for 3 or 6 hours then tested. Five tubes were refrigerated immediately and measurements were performed seven hours subsequent to blood draw and on the consecutive four days. Except for the tubes stored at 37°C, all samples were incubated for five minutes at 37°C in a water bath prior to testing. Samples stored at 4°C were removed from the refrigerator 60 minutes before performing the measurements. Note that for all storage periods and temperatures, the tubes were maintained in a vertical position and were not inverted or mixed until selected for testing.

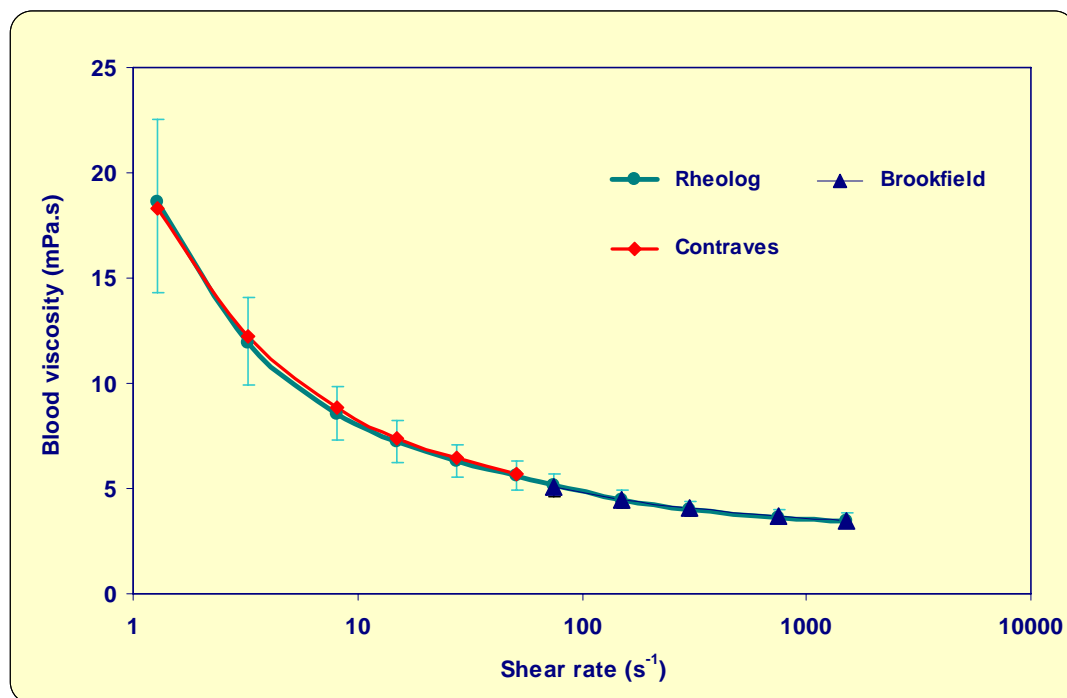
## **5.3 Results**

### *5.3.1 Reproducibility tests*

Results of reproducibility studies with the Rheolog™ (i.e., ten replicate measurements of low and high hematocrit suspensions from five donors) are presented in Table 5.3.1. Means, standard deviations (SD) and coefficients of variation [CV] are shown for shear rates of 1-1000s<sup>-1</sup>. Upon the inspection of these results we can note that the mean CV levels are within 5% or less, except for the high hematocrit data at 1000s<sup>-1</sup> where the mean CV reached 8%. CV values were independent of shear rate ( $p > 0.2$  for linear regression of mean CV versus shear rate at either hematocrit), but tended to be slightly lower for the high hematocrit suspensions in the range of 5-100s<sup>-1</sup>. These data indicate that the Rheolog™ provided reliable and reproducible viscosity data in all donors independent of the hematocrit and shear rate.

### 5.3.2 Comparison studies

Results of the comparison tests performed between the Rheolog™, Brookfield and Contraves viscometers are shown in Figure 5.3.1 and Table 5.3.1. In these studies, blood from 44 donors was tested at native hematocrit (mean hematocrit = 41.2%), and hematocrit-adjusted suspensions from 8 donors were tested at low and high hematocrit values. As anticipated from the results presented in Table 5.3.1, replicate measurements with the Rheolog™ indicated very close agreement between the means of the two separate tests (i.e., “Rheolog A” and “Rheolog B”): at native hematocrit, the maximum difference was 2% at  $1.29\text{s}^{-1}$  that decreased to less than 1% at all other shear rates (Table 5.3.2).



**Figure 5.3.1:** Comparison of mean blood viscosity data for blood at native hematocrit from 44 donors. Contraves results are for shear rates of  $1.29$  to  $51.2\text{s}^{-1}$  and Brookfield data are for  $75$  to  $1500\text{s}^{-1}$ . The Rheolog™ was programmed to provide viscosity data at shear rates corresponding to those obtained by the Brookfield and Contraves systems. Solid line represents smoothed fit of Rheolog™ data. Error bars indicate +SD for Rheolog™ data and -SD for the Brookfield and Contraves results.

Over the shear rate range used for these tests (i.e.,  $1.29$ - $1500\text{s}^{-1}$ ), blood viscosity measured with the Rheolog™ compared very favorably with the results obtained with

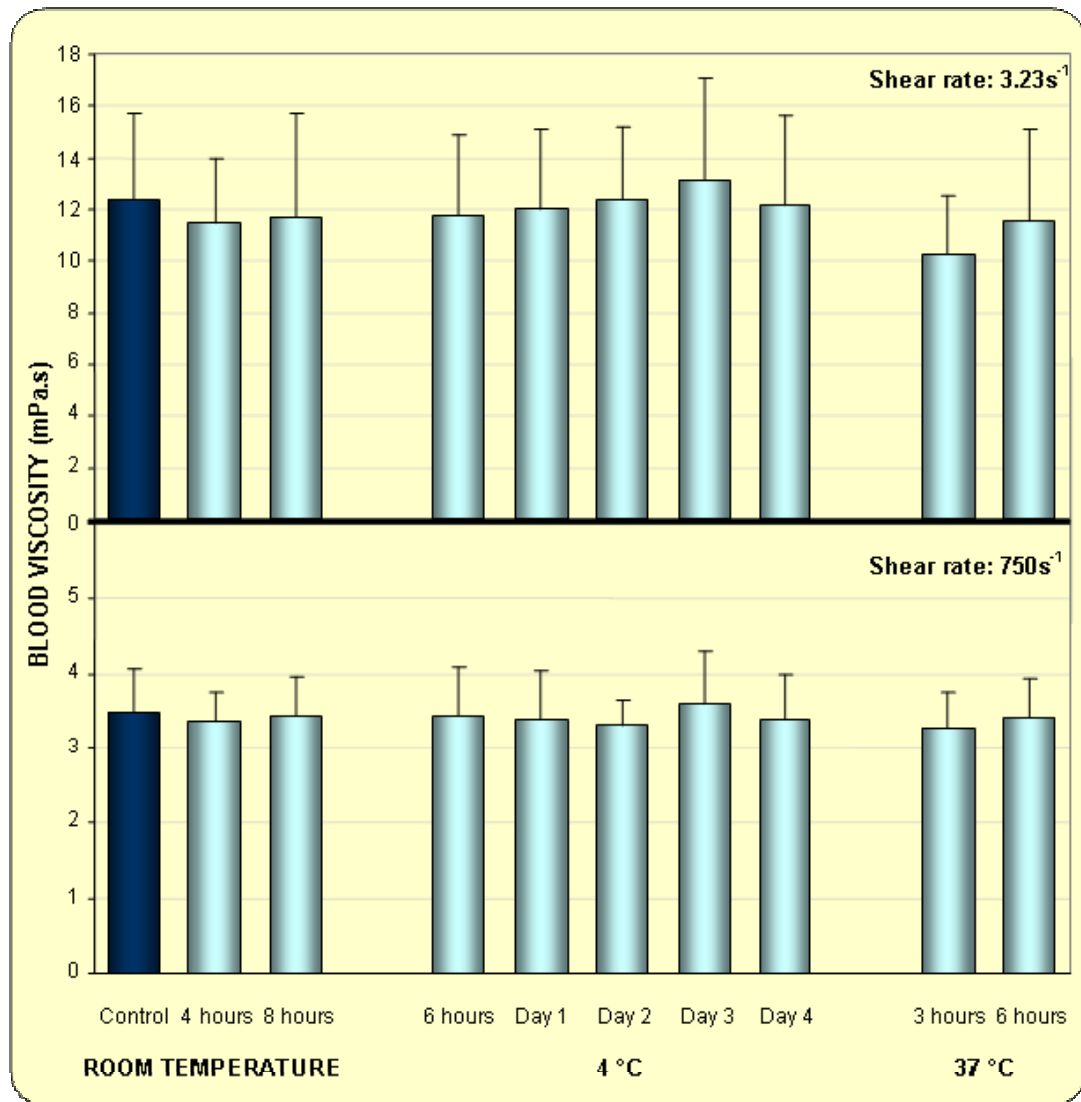
the Brookfield and Contraves instruments: 1) for blood at native hematocrit, the mean difference was  $-1.4 \pm 1.5\%$ ; 2) for the low hematocrit suspensions (average hematocrit = 27.5%), the mean difference seemed to be  $-0.31 \pm 2.8\%$ ; 3) for the high hematocrit suspensions (average hematocrit = 56.3%), the mean difference was  $1.0 \pm 4.9\%$ . We were unable to reveal any significant associations between the differences in viscosity and shear rate at native, low or high hematocrit levels. The average whole blood viscosity values obtained by the three viscometer systems could easily be represented by a single curve (Figure 5.3.1).

**Table 5.3.1:** Rheolog™ reproducibility measurements. Data for each donor are for 10 replicate tests at low and at high hematocrit levels. In each cell, the top value represents the mean, the value in “( )” is the standard deviation, and the number in [ ] is the coefficient of variation (i.e., mean/SD times 100).

Shear rate (s <sup>-1</sup> )	Whole blood viscosity (mPa.s)									
	Donor 1		Donor 2		Donor 3		Donor 4		Donor 5	
	Hct=27%	Hct=58%	Hct=28%	Hct=57%	Hct=29%	Hct=54%	Hct=27%	Hct=57%	Hct=27%	Hct=57%
1	6.42 (0.05) [0.78]	42.71 (2.03) [4.76]	6.38 (0.04) [0.63]	35.72 (2.45) [6.86]	6.53 (0.25) [3.83]	41.34 (2.04) [4.93]	6.46 (0.06) [0.93]	39.27 (2.39) [6.09]	6.60 (0.22) [3.33]	40.37 (2.03) [5.03]
2	5.40 (0.07) [1.29]	27.69 (1.05) [3.79]	5.50 (0.09) [1.64]	23.58 (1.16) [4.92]	5.47 (0.22) [4.02]	26.47 (1.14) [4.31]	5.36 (0.08) [1.49]	25.33 (1.33) [5.25]	5.35 (0.19) [3.55]	26.23 (0.95) [3.62]
5	4.56 (0.16) [3.51]	17.08 (0.43) [2.52]	4.77 (0.17) [3.56]	14.92 (0.39) [2.61]	4.61 (0.26) [5.64]	16.04 (0.57) [3.55]	4.47 (0.18) [4.03]	15.51 (0.65) [4.19]	4.35 (0.21) [4.83]	16.23 (0.31) [1.91]
10	4.17 (0.20) [4.80]	12.70 (0.25) [1.97]	4.42 (0.20) [4.52]	11.30 (0.24) [2.12]	4.21 (0.29) [6.89]	11.77 (0.36) [3.06]	4.05 (0.23) [5.68]	11.47 (0.42) [3.66]	3.88 (0.23) [5.93]	12.10 (0.18) [1.49]
50	3.38 (0.15) [4.44]	7.87 (0.20) [2.54]	3.59 (0.18) [5.01]	7.26 (0.36) [4.96]	3.39 (0.19) [5.60]	7.10 (0.20) [2.82]	3.28 (0.21) [6.40]	7.08 (0.28) [3.95]	3.18 (0.17) [5.35]	7.53 (0.28) [3.72]
100	3.04 (0.10) [3.29]	6.89 (0.21) [3.05]	3.18 (0.16) [5.03]	6.40 (0.35) [5.47]	3.03 (0.10) [3.30]	6.16 (0.19) [3.08]	2.97 (0.16) [5.39]	6.14 (0.23) [3.75]	2.95 (0.13) [4.41]	6.61 (0.30) [4.54]
150	2.89 (0.08) [2.77]	6.37 (0.26) [4.08]	3.01 (0.16) [5.32]	5.87 (0.32) [5.45]	2.87 (0.07) [2.44]	5.68 (0.17) [2.99]	2.84 (0.15) [5.28]	5.68 (0.19) [3.34]	2.85 (0.13) [4.56]	6.06 (0.23) [3.79]
300	2.71 (0.08) [2.95]	5.57 (0.40) [7.18]	2.79 (0.16) [5.73]	5.20 (0.32) [6.15]	2.68 (0.06) [2.24]	5.01 (0.15) [2.99]	2.67 (0.13) [4.87]	5.10 (0.24) [4.70]	2.73 (0.13) [4.76]	5.31 (0.24) [4.52]
1000	2.52 (0.09) [3.57]	4.76 (0.55) [11.55]	2.56 (0.16) [6.25]	4.52 (0.35) [7.74]	2.48 (0.09) [3.63]	4.33 (0.17) [3.93]	2.49 (0.11) [4.42]	4.50 (0.35) [7.78]	2.59 (0.15) [5.79]	4.54 (0.32) [7.05]

**Table 5.3.2:** Comparison of blood viscosity data obtained by the Rheolog™, Contraves and Brookfield systems. Data are mean and SD for 44 donors tested at native (mean = 41.2%) and 8 donors at low and high hematocrits.

Shear Rate (s <sup>-1</sup> )	Device	Whole blood viscosity (mPa.s)		
		Native Hct	Low Hct	High Hct
1.29	Rheolog A	18.77 (3.82)	7.25 (0.92)	39.67 (4.60)
	Rheolog B	18.41 (4.07)	7.06 (1.30)	39.70 (3.87)
	<b>Rheolog Average</b>	<b>18.59</b> (3.94)	<b>7.15</b> (1.11)	<b>39.68</b> (4.23)
	<b>Contraves</b>	<b>18.29</b> (3.96)	<b>7.24</b> (1.20)	<b>39.18</b> (6.18)
3.23	Rheolog A	12.04 (2.06)	5.33 (0.56)	23.43 (2.34)
	Rheolog B	11.88 (2.21)	5.27 (0.72)	23.58 (2.11)
	<b>Rheolog Average</b>	<b>11.96</b> (2.13)	<b>5.30</b> (0.64)	<b>23.50</b> (2.22)
	<b>Contraves</b>	<b>12.27</b> (2.37)	<b>5.32</b> (0.72)	<b>24.13</b> (3.01)
8.11	Rheolog A	8.61 (1.26)	4.29 (0.38)	15.48 (1.42)
	Rheolog B	8.54 (1.32)	4.29 (0.44)	15.68 (1.31)
	<b>Rheolog Average</b>	<b>8.57</b> (1.29)	<b>4.29</b> (0.41)	<b>15.58</b> (1.36)
	<b>Contraves</b>	<b>8.81</b> (1.52)	<b>4.20</b> (0.48)	<b>16.46</b> (1.84)
14.98	Rheolog A	7.24 (0.97)	3.85 (0.32)	12.41 (1.13)
	Rheolog B	7.21 (0.99)	3.88 (0.34)	12.61 (1.02)
	<b>Rheolog Average</b>	<b>7.22</b> (0.98)	<b>3.86</b> (0.33)	<b>12.51</b> (1.07)
	<b>Contraves</b>	<b>7.41</b> (1.19)	<b>3.76</b> (0.38)	<b>12.78</b> (1.30)
27.7	Rheolog A	6.31 (0.80)	3.55 (0.28)	10.36 (0.98)
	Rheolog B	6.30 (0.78)	3.60 (0.27)	10.56 (0.83)
	<b>Rheolog Average</b>	<b>6.30</b> (0.79)	<b>3.57</b> (0.27)	<b>10.46</b> (0.90)
	<b>Contraves</b>	<b>6.49</b> (0.93)	<b>3.45</b> (0.32)	<b>10.24</b> (1.00)
51.2	Rheolog A	5.66 (0.69)	3.33 (0.25)	8.98 (0.89)
	Rheolog B	5.65 (0.64)	3.39 (0.23)	9.17 (0.71)
	<b>Rheolog Average</b>	<b>5.65</b> (0.66)	<b>3.36</b> (0.24)	<b>9.07</b> (0.80)
	<b>Contraves</b>	<b>5.68</b> (0.75)	<b>3.38</b> (0.29)	<b>8.46</b> (0.76)
75	Rheolog A	5.14 (0.58)	3.23 (0.24)	8.05 (0.79)
	Rheolog B	5.13 (0.55)	3.29 (0.22)	8.17 (0.69)
	<b>Rheolog Average</b>	<b>5.13</b> (0.56)	<b>3.26</b> (0.23)	<b>8.11</b> (0.74)
	<b>Brookfield</b>	<b>5.11</b> (0.51)	<b>3.32</b> (0.37)	<b>7.31</b> (0.66)
150	Rheolog A	4.47 (0.45)	3.08 (0.23)	6.84 (0.69)
	Rheolog B	4.45 (0.46)	3.16 (0.20)	6.88 (0.68)
	<b>Rheolog Average</b>	<b>4.46</b> (0.45)	<b>3.12</b> (0.21)	<b>6.86</b> (0.68)
	<b>Brookfield</b>	<b>4.47</b> (0.43)	<b>3.19</b> (0.27)	<b>6.45</b> (0.56)
300	Rheolog A	4.02 (0.39)	2.86 (0.19)	6.05 (0.65)
	Rheolog B	4.00 (0.43)	2.92 (0.16)	6.04 (0.69)
	<b>Rheolog Average</b>	<b>4.01</b> (0.41)	<b>2.89</b> (0.17)	<b>6.04</b> (0.67)
	<b>Brookfield</b>	<b>4.07</b> (0.40)	<b>3.04</b> (0.21)	<b>5.91</b> (0.51)
750	Rheolog A	3.64 (0.36)	2.65 (0.18)	5.39 (0.63)
	Rheolog B	3.62 (0.41)	2.70 (0.14)	5.34 (0.70)
	<b>Rheolog Average</b>	<b>3.63</b> (0.38)	<b>2.67</b> (0.16)	<b>5.36</b> (0.66)
	<b>Brookfield</b>	<b>3.72</b> (0.40)	<b>2.78</b> (0.21)	<b>5.42</b> (0.47)
1500	Rheolog A	3.46 (0.35)	2.55 (0.19)	5.07 (0.62)
	Rheolog B	3.43 (0.41)	2.59 (0.13)	5.00 (0.71)
	<b>Rheolog Average</b>	<b>3.44</b> (0.38)	<b>2.57</b> (0.16)	<b>5.03</b> (0.66)
	<b>Brookfield</b>	<b>3.50</b> (0.35)	<b>2.61</b> (0.15)	<b>5.13</b> (0.44)



**Figure 5.3.3:** Effects of storage time and temperature on blood viscosity measured with the Rheolog™. Data are mean +SD for 8 donors at native hematocrit; for clarity, only results at 3.23s<sup>-1</sup> and 750s<sup>-1</sup> are shown. The control values in the left-most columns were obtained 10 minutes following venipuncture.

### 5.3.3 Storage

The effects of storage time and temperature on whole blood viscosity profiles are presented in Figure 5.3.3; for clarity only results obtained at a representative low (3.23s<sup>-1</sup>) and high (750s<sup>-1</sup>) shear rates are shown. Note that in this figure, the vertical “error” bars were calculated as the standard deviation for the measured eight donors,

but, as in Figure 5.3.2, primarily reflect differences in the hematocrit of native blood among the subjects rather than measurement variability (Table 5.3.1).

In general, data in Figure 5.3.3 seem remarkable for the absence of major changes of blood viscosity from the control (i.e., 10 minutes post venipuncture) values. In fact, paired t-test analyses indicated that only the 3 hour 37°C results at shear rates of 1.29 and 3.23s<sup>-1</sup> had p values less than 0.05. However, some trends were evident: 1) changes from control tended to be larger at lower shear rates (e.g., for room temperature storage, a 7% average decrease at 3.23s<sup>-1</sup> versus only 3% average decrease at 750s<sup>-1</sup>); 2) with the exception of the day three results, changes due to storage at either room temperature or 4°C were essentially independent of storage time; 3) the effect of storage at 37°C was the greatest at 3 hours (i.e., -17% at 3.23s<sup>-1</sup> and -7% at 750s<sup>-1</sup>) with smaller changes noted at 6 hours (i.e., -7% at 3.23s<sup>-1</sup> and -3% at 750s<sup>-1</sup>).

## 5.4 Discussion

The complex measurement of whole blood viscosity profiles summarizes the effect of numerous rheological parameters affecting blood flow, such as plasma viscosity, red blood cell deformability, erythrocyte aggregation and aggregability. Besides the well-known conventional risk factors, hyperviscosity has been proved to play a major role in the pathophysiology of ischemic heart disease, stroke and various hematological disorders as well. However, the measurement of clinically relevant whole blood viscosity profiles with either commercial or prototype rheometers can be associated with several artifacts that are often not recognized, especially at lower rates of shear. The main sources of error are: 1) Formation of a rigid, denatured protein film at the blood-air or plasma-air interface. Such a film can transfer torque or provide additional drag on a torque sensing section (e.g., bob of a Couette viscometer, cone of a cone-and-plate viscometer), with the effects particularly important at low torque or shear rate levels. This film artifact has previously led to erroneous reports of non-Newtonian behavior for normal human plasma [27,128]. However, for viscometers in which the torque-sensing element remains essentially stationary, the use of a “guard ring” can eliminate this problem [128], whereas a guard ring is not usable in cone-and-plate systems where the torque-sensing element rotates (e.g., Brookfield

viscometer). Note that this film can also affect tube viscometers in which pressure differences are measured through an air-blood interface. 2) Phase separation leading to lower RBC concentration at the torque-sensing element. This phenomenon has been described in detail by Cokelet [28] for a Couette viscometer operating at shear rates less than about  $10\text{s}^{-1}$ . Under these conditions, the observed torque at steady shear exhibits a decay with time due to the formation of a cell-poor, lower-viscosity layer at the wall of the cup and bob. Use of a torque extrapolation to zero time technique, rather than the lower, incorrect final steady state torque, has been shown to yield the proper torque value [28,128]. 3) Sedimentation of RBCs in capillary or rotational viscometer systems at low flow rates. This phenomenon is most evident in blood samples or RBC suspensions having a high degree of erythrocyte aggregation. Sedimentation in capillary viscometers can arise in the tube itself or the connecting tubing as well resulting in an increased pressure drop for a given flow rate [1]. The effects of sedimentation in rotational viscometers depend primarily on their design: in Couette or Mooney systems elevated hematocrit at the bottom of the cup can increase the measured torque, whereas sedimentation away from the cone of a Brookfield-type viscometer will decrease the measured torque. The recently used viscometer systems are further complicated with several limitations: measurements are time consuming, require a well-trained, full time technician, have a potential biohazard risk and are incapable of measuring whole blood viscosity over a wide range of clinically relevant shear rates.

Based upon the findings obtained in the present study, where Rheolog™ results were compared to cone-and-plate and Couette viscometers operated to avoid artifacts, the Rheolog™ system appears to be completely free from the instrumental problems mentioned above. Data presented in Table 5.3.1 indicate good reproducibility, with those in Table 5.3.2 and Figure 5.3.2 demonstrating close agreement between the Rheolog™ and the two other viscometer systems over a wide range of shear rates. In particular, good agreement was noted at the lower rates of shear where surface film and RBC sedimentation can often cause difficulties [28,128]. Based on these findings it is evident, that the reproducibility and accuracy of the new scanning capillary rheometer is acceptable for clinical use. We should note though, that our conclusions are constrained by the scope of the parameters used in the present study (average hematocrit range: 28%-56%, shear rate range:  $1\text{-}1500\text{s}^{-1}$ , temperature =  $37^{\circ}\text{C}$ ) and that extending our conclusions beyond this range may not be warranted. In particular, pilot studies

indicated that the present software algorithms and capillary tube diameter are not appropriate for hematocrits less than about 20% or for cell-free plasma. However, discussions with the Rheolog™ manufacturer have indicated that testing low hematocrit suspensions and cell-free plasma are possible with modified software algorithms.

Results presented in Figure 5.2.3 indicate that whole blood anticoagulated with EDTA exhibits quite small changes of blood viscosity when stored for up to 8 hours at room temperature or for up to 4 days at 4°C. Conversely, storage at 37°C causes marked changes within 3 hours, with these alterations partially reversed at 6 hours. This phenomenon might be explained by certain changes occurring at 37°C in the red blood cell membrane affecting erythrocyte aggregation and aggregability. Literature reports on blood viscosity and storage conditions appear to be limited, and often deal with blood containing added preservatives intended for transfusion [106], or do not fully define the storage conditions [175]. We are thus unable to compare our findings to those reported previously.

The rapidity of whole blood viscosity testing over a wide range of shear rates using the Rheolog™ (i.e., less than 5 minutes for testing and data printout) suggests possible “real time” applications. For example, monitoring of therapy for rheumatoid arthritis often involves performing an erythrocyte sedimentation rate (ESR) test [169], with results only available after the usual one-hour sedimentation period. Rapidly accessible blood viscosity data at low shear rates could serve as a surrogate for ESR tests, and, if appropriate, would provide guidance for medication changes. The results obtained for refrigerated storage at 4°C (Figure 5.3.3) may also have clinical implications, in that blood could be sampled at various sites and then transferred to a central laboratory for testing. Finally, and perhaps of greatest importance, is the possibility that easily performed and rapidly available wide-shear blood viscosity data may promote epidemiological studies evaluating associations between blood rheology and various diseases. Trials to date have started to explore such associations [142,171], but have generally been limited to either measurements of plasma viscosity or blood viscosity at a single, high shear rate.



## 6. SUMMARY

1. Our study investigating the efficacy of routine antiplatelet medication confirmed the existence of both aspirin and thienopyridine non-responder individuals in the general population. ASA monotherapy proved to be ineffective in more than one third of our patients; however, we could not note a direct correlation between the prescribed daily drug dose and the ratio of appropriate treatment. Ticlopidine and clopidogrel resistance was less frequent, even though previously documented ASA non-response was the principal indication for the thienopyridine derivatives. Our study also demonstrated a positive association between the regularity of patient follow-up and the efficacy of routine antiplatelet drugs. These findings emphasize the importance of tight medical visits and the significance of aggregometry controlled, individually adjusted antiplatelet therapy.
2. Our follow-up study revealed that the inhibition of platelet function achieved by a fixed daily dose of ASA progressively decreased in a significant number of subjects over the 24-month trial period. The observed decline was in accordance with the administered aspirin dose, remarkably higher number of individuals converted to non-responder in the patient group on a low daily dose of the drug. In contrast, the antiplatelet efficacy of thienopyridine derivatives remained constant in most cases for the entire follow-up period. Our results highlight the inevitable value of platelet function tests throughout the secondary prevention of ischemic vascular diseases.
3. Our preliminary data documented a highly significant association between the individual responsiveness to the prescribed platelet inhibitory medication evaluated *ex vivo* and the occurrence of undesirable ischemic episodes. By improving the efficacy of the applied antiplatelet therapy throughout the secondary prevention, the incidence of recurrent clinical episodes might be reduced by 40%. Further, prospective studies are mandatory to confirm these initial findings in a large patient population.

4. All the examined PARP inhibitor compounds reduced ADP-induced platelet aggregation in a dose dependent manner. However, on a molar basis the novel HO-3089 molecule was proved to have superior antiplatelet efficacy over the commercially available agents. The effect of the experimental PARP inhibitors was specific on ADP receptors as they were unable to antagonize collagen- or epinephrine stimulated thrombocyte responses. In addition, all the examined agents were capable of hindering platelet aggregation even if those were sensitized to ADP by the pre-administration of heparin. Our findings strongly suggest that beyond their myocardial protective effect, the administration of PARP inhibitors might be of great value in the treatment of ischemia-reperfusion injuries.
  
5. Replicate measurements with the newly developed scanning capillary rheometer indicated an outstanding reproducibility independent of hematocrit and shear rate. Results obtained using the Rheolog™ compared favorably with those recorded with cone-and-plate and Couette viscometer systems. Storing the anticoagulated sample at room temperature for up to 8 hours or at 4°C up to 4 days had merely minimal effect on blood viscosity; whereas notable changes were observed when stored for 3 hours at 37°C. Our results further indicate that the Rheolog™ provides rapid, accurate and reproducible blood viscosity data over a wide range of clinically important shear rate profile suggesting its inevitable value for basic research and clinical studies as well.

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## 8. PUBLICATIONS OF THE AUTHOR

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