

**IMPAIRED HEMORHEOLOGICAL PARAMETERS AND  
RESISTANCE TO ROUTINE ANTIPLATELET THERAPY**

**Ph.D. thesis**

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## 1. INTRODUCTION

Cardio- and cerebrovascular diseases and their complications are the leading cause of morbidity and mortality in the developed world. In the development of these diseases several factors are involved that interact with each other. While the role of “classic” risk factors (e.g., smoking, hypertension, hypercholesterolemia, diabetes mellitus, etc.) has been well known for a long time, the importance of hemorheologic parameters (hematocrit, fibrinogen, red blood cell deformability, red blood cell aggregation, plasma and whole blood viscosities) in the development of arteriosclerotic disorders has been accepted only in the last two decades.

It is generally agreed that a rise in hemorheological factors leads to a state of hypoperfusion which results in impaired microcirculation (1-3). Several studies confirmed that hemorheological parameters are independent risk factors of ischemic heart disease (IHD) (4-7). While the role of “classic” risk factors has been noted a long time ago, recent clinical and epidemiological studies have provided compelling evidence that altered hemorheological parameters are also primary risk factors for the development and progression of cardio- and cerebrovascular diseases.

Platelets are vital components of normal hemostasis and key participants in pathologic thrombosis by virtue of their capacity to adhere to injured blood vessels and to accumulate at sites of injury (8). Although platelet adhesion and activation should be viewed as a ‘physiological’ response to the sudden fissuring or rupture of an atherosclerotic plaque, eventually contributing to its repair, uncontrolled progression of such a process through a series of self-sustaining amplification loops may lead to intraluminal thrombus formation, vascular occlusion and transient ischemia or infarction (9).

Aspirin works by irreversibly acetylating the cyclooxygenase (COX)-1 enzyme, thus suppressing thromboxane A<sub>2</sub>. Thromboxane A<sub>2</sub> serves as a potent agonist of platelet aggregation, and aspirin prevents thrombus formation via this mechanism (8). Clopidogrel is a prodrug that is converted in the liver by cytochrome P450 3A4 (CYP3A4) into its active metabolite. It inhibits platelet aggregation by irreversibly binding to the P<sub>2</sub>Y<sub>12</sub> ADP receptor on the platelet surface (9). Similar to aspirin, clopidogrel therapy may fail owing to patient non-compliance or because physicians may not prescribe it. In addition, there may be variability in absorption with associated under-dosing in patients and possible drug–drug interactions (8,9).

As the mechanisms of aspirin and clopidogrel resistance are becoming clearer, defining these clinical entities remains a challenge. Despite the lack of a standard definition of resistance as well as the lack of a standard diagnostic modality aspirin and clopidogrel resistance were associated with worsening clinical outcome based on the results of recently published meta-analysis (10,11).

## **2. AIMS OF THE INVESTIGATIONS**

1. The aim of our first study was to examine the relationship of hemorheological parameters to the “classic” risk factor, advancing age.

2. The aim of our second study was to compare some parameters of patients with effective platelet inhibition by aspirin (ASA) (risk profile, previous diseases, medication, and hemorheological parameters) to patients with ineffective ASA treatment.

3. The aim of our third study was to compare some parameters of patients with effective platelet inhibition by clopidogrel (CLP) (risk profile, previous diseases, medication, hemorheological parameters, plasma von Willebrand factor and soluble P-selectin levels) to patients with ineffective clopidogrel treatment.

### 3. HEMORHEOLOGICAL PARAMETERS AND AGING

Cardio- and cerebrovascular diseases and their complications are the leading cause of morbidity and mortality in the developed world. While the role of “classic” risk factors (e.g. smoking, hypertension, hypercholesterolemia, diabetes, etc.) has been well known for a long time, the importance of hemorheologic parameters in the development of coronary artery disease (CAD) has been accepted only in the last two decades. „Classic” risk factors can also influence hemorheological parameters. A positive correlation between body mass index (BMI) and blood viscosity and its determinants has been demonstrated in several studies. Arterial hypertension is in association with increased blood viscosity. Smoking increases in a reversible way plasma and whole blood viscosity, partly by increasing hematocrit and fibrinogen. The relationships of these rheological variables to cardiovascular events are at least as strong as those of conventional risk factors (smoking habit, diastolic blood pressure, and low-density lipoprotein cholesterol).

The aim of our present study was to determine the association between hemorheological parameters (hematocrit, fibrinogen, red blood cell aggregation, plasma and whole blood viscosity) and increasing age, because a relatively few number of studies examined the association between these parameters and increasing age, and their results are controversial.

#### 3.1 Patients and methods

##### 3.1.1 Patients

Between January 1999 and May 2004 the blood samples of 6236 cardio- and cerebrovascular patients (3774 males, mean age 59.8±13.2 years and 2462 females, mean age 60.9±12.8 years) were examined in our laboratory. Both sexes were divided into three categories: A. young patients: <45 years. B, middle-aged patients: 45-65 years and C, old patients: >65 years. To evaluate the possible effect of risk profile, 623 patients from the study population with matching parameters were divided into the three age groups as mentioned before. They had the same prevalence of risk factors including body mass index, smoking habits, diabetes mellitus, dyslipidemia, hypertension), previous diseases (pulmonary embolism, deep vein thrombosis, ischemic heart disease (IHD), acute myocardial infarction (AMI), stenosis of the carotid artery, transient ischemic attack (TIA), stroke and peripheral arterial disease and took the same medication beta-blockers, alpha blockers, angiotensine converting enzyme (ACE) inhibitors, angiotensine II (AT II) receptor antagonists, calcium (CA) channel blockers, nitrates, statins, fibrates, diuretics, H2 receptor blockers, proton pump inhibitors (PPI), acetylsalicylic acid (ASA), clopidogrel (CLP), trimetazidine (TMZ), vinpocetine and pentoxifylline.

##### 3.1.2. Methods

###### 3.1.2.1 Hemorheological measurements

Blood samples were taken from the cubital vein; routine blood chemistry and hemorheological parameters - hematocrit, plasma fibrinogen level, plasma and whole blood viscosity, red blood cell (RBC) aggregation and deformability - were determined.

#### *Hematocrit:*

Venous blood collected into lithium-heparin coated Vacutainer tubes was used to determine hematocrit. Hematocrit was measured by centrifuging hematocrit capillaries (80  $\mu$ l, containing heparin) at 12000 rpm for five minutes in microhematocrit centrifuge (Hemofuge, Heraeus Instr., Germany). Measurements were performed at room temperature ( $22 \pm 1$  °C).

#### *Plasma fibrinogen:*

4.5 ml blood sample was drawn into a Vacutainer tube containing sodium citrate (0.129 M, 1:10 dilution) and plasma fibrinogen concentration was determined by using Clauss's method (12).

#### *Plasma and whole blood viscosity:*

Venous blood samples were collected into lithium-heparin coated Vacutainer tubes for viscosity measurements. Plasma was prepared by centrifuging samples at 1500 g for ten minutes. Plasma and whole blood viscosities were determined in Hevimet 40 capillary viscosimeter (Hemorex Ltd., Hungary). 1.0-1.0 ml of plasma or whole blood was injected into the capillary tube of the device. In this viscosimeter the flow of the fluid is detected optoelectronically along the capillary tube and a flow curve is drawn. Shear rate and shear stress are calculated from this curve, viscosity values are determined as a function of these parameters according to Casson's principle (13). For the presentation of our results, apparent whole blood viscosity values calculated at  $90 \text{ s}^{-1}$  shear rate are given. Measurements were carried out at  $37.0 \pm 0.5$  °C within 2 hours after venepuncture.

#### *RBC aggregation:*

RBC aggregation measurements were carried out from venous blood samples collected into lithium-heparin coated Vacutainer tubes. RBC aggregation was measured in Myrenne aggregometer (MA-1 Aggregometer, Myrenne GmbH, Germany) applying the light transmission method. In this study the aggregometer was used in low shear (M1) mode. In this mode blood sample (30  $\mu$ l) is first sheared at  $600 \text{ s}^{-1}$  to disperse all pre-existing aggregates, then shear rate decreases rapidly to low shear ( $3 \text{ s}^{-1}$ ). The extent of aggregation is characterized by the aggregation index (AI), calculated from the surface area below the light intensity curve in a 10 s measurement period (13,14). Measurements were performed at room temperature ( $22 \pm 1$  °C) within 2 hours after venepuncture.

#### *3.1.2.2. Statistical analysis*

Data were evaluated as means  $\pm$  S.E.M. (standard error of mean) by Student's t test and chi square test.

## **3.2 Results**

### *3.2.1 Whole population*

#### *3.2.1.1 Hematocrit*

Hematocrit level slightly, but significantly increased with advancing age ( $p < 0.01$ ) in the whole population as well as in males and females. Analyzing the data of the different age subgroups we found that hematocrit increased with age in the young and middle-aged males ( $p < 0.05$ ), while it decreased significantly in the old patients ( $p < 0.01$ ). On the other hand, in females a significant positive correlation could only be found in young patients ( $p < 0.01$ ). Young patients had significantly lower hematocrit levels ( $p < 0.01$ ) compared to the other two age groups. There was no difference between middle-aged and old patients in either sexes.

### 3.2.1.2 Plasma fibrinogen

Plasma fibrinogen significantly increased with age ( $p < 0.01$ ) in the studied population and also in both genders ( $p < 0.05$ ). In the different age subgroups positive correlation could be found in old males and middle-aged females ( $p < 0.01$ ). Young patients had significantly lower plasma fibrinogen levels ( $p < 0.01$ ) compared to the other two age groups.

### 3.2.1.3. Red blood cell aggregation

There was a significant correlation between red blood cell aggregation and advancing age in both males and females ( $p < 0.05$ ). Analyzing the subgroups, we found a significant positive correlation in young males and significantly decreasing values in the case of old males ( $p < 0.05$ ). Young patients had significantly lower red blood cell aggregation level compared to the other groups ( $p < 0.001$ ).

### 3.2.1.4. Plasma viscosity

Plasma viscosity significantly increased with age when both sexes were considered together ( $p < 0.01$ ). This finding remained significant in males, but not in females ( $p < 0.01$ ). Examining the age groups we could find a significant correlation only in young men ( $p < 0.01$ ). Lower plasma viscosity values could be found in young patients compared to the other groups ( $p < 0.05$ ).

### 3.2.1.4. Whole blood viscosity

Whole blood viscosity significantly increased with age in the whole population ( $p < 0.01$ ), but analyzing the sex groups we could find a significant negative correlation only in old males ( $p < 0.01$ ). Young patients had lower whole blood viscosity values than middle-aged and old patients ( $p < 0.01$ ).

## 3.2.2 Selected population

All the measured parameters did not correlate with increasing age neither in the selected population nor in the subgroups. No significant difference could be found in the mean values of the different subgroups

## DISCUSSION

Since 1993, results of a meta-analysis of six prospective epidemiological studies, including 14 988 persons for a total of 92 147 person-years investigated, showed that plasma fibrinogen is a powerful independent predictor of myocardial infarction and stroke (7). Afterwards, several prospective trials confirmed that increased whole blood viscosity, primarily dependent upon hematocrit value and fibrinogen concentration, is a major risk factor for ischemic heart disease and stroke (15). The relationships of these rheological variables to cardiovascular events are at least as strong as those of conventional risk factors (smoking habit, diastolic blood pressure, and low-density lipoprotein cholesterol) (15).

On the other hand, the role of hemoheological parameters still remains unclear. A number of clinical studies have demonstrated significant positive correlation between the severity of arterial hypertension (AHT) and whole blood viscosity. Red blood cell aggregation has also been associated with AHT especially in the severe form of

the disease. The main possible cause of increased red blood cell aggregation is fibrinogen which can be found in a significantly higher concentration in patients with AHT than in healthy controls. On the other hand, blood pressure reduction with angiotensin-converting-enzyme inhibitors, calcium-channel-blocking agents, beta or alpha-receptor blocking drugs leads to a significant improvement of blood rheology. It can be presumed that abnormal hemorheology and AHT are not directly linked but they share the same inductive genetic and/or environmental factors like obesity, chronic mental stress, physical inactivity and cigarette smoking. Regarding this hypothesis, the appropriate question is not whether hemorheological factors are causes or results of AHT but what their common origins are. Further studies are needed to clarify this hypothetical link between hemorheology and AHT (16).

We examined the possible connection between increasing age and hemorheological parameters in 6234 cardio- and cerebrovascular patients first in the whole study population and later in the different subgroups. We found a very weak but statistically significant positive correlation between increasing age and hemorheological parameters in the whole population, but not in all subgroups. In the case of old males whole blood viscosity and its main determinants were negatively correlated with advancing age. These results suggest that these positive correlations are probably just statistically, but not clinically significant. In the second part of our study, in the selected population we did not find any significant correlation, not even in the subgroups. Our results suggest that these parameters are mostly independent of aging, increased values are not associated with older age but the more frequently occurring diseases.

#### **4. THE POTENTIAL BACKGROUND(S) OF ASPIRIN RESISTANCE**

There is no debate that long term aspirin use attenuates the risks of myocardial infarction, stroke, and vascular related deaths in patients with cardiovascular disease, but a significant number of patients prescribed aspirin as antithrombotic therapy have major adverse vascular related events each year. Consequently other antiplatelet agents in addition to aspirin have been prescribed for certain patients (17-19).

The major controversy about aspirin therapy is why particular patients do not benefit from such therapy and how they might be identified. It has been suggested that some patients require a higher dose of aspirin than is normally recommended to achieve the expected antiplatelet effect - for example, inhibition of platelet function or inhibition of platelet thromboxane A<sub>2</sub> synthesis. It is unclear whether these patients simply receive too low aspirin dose, are not compliant, have differing abilities to absorb aspirin, or have an underlying genetic disposition that renders aspirin ineffective. Such patients have been labelled aspirin "resistant" - that is, their platelets are not affected in the same way or are affected differently from the platelets of those who seem to benefit from aspirin therapy (aspirin "sensitive" patients with no subsequent adverse cardiovascular event). Little consistency exists about which measure should be used to identify patients who seem to be resistant to aspirin. Also, few studies have assessed the effect of aspirin resistance on clinically important outcomes (17,19).

In the following experiments we examined the potential factors of aspirin resistance (ASA).

## **4.1 Patients and methods**

### *4.1.1 Patients*

Between March 2004 and April 2005, 99 consecutive chronic cardio- and cerebrovascular patients took part in our study. These patients were commenced on standard antiplatelet therapy with aspirin 100–325 mg/day. Patients were excluded from the study if they were taking any other antiplatelet or anticoagulant medications, had a history of other nonvascular diseases (e.g. hematological, endocrinological disorders or had recently experienced an acute vascular event (AMI, stroke or peripheral embolisation 3 months prior to the study).

### *4.1.2 Methods*

#### *4.1.2.1 Platelet aggregation*

Epinephrine-, ADP- and collagen-stimulated aggregation of platelets was analyzed. 450 µl platelet rich plasma (PRP) was measured against 450 µl platelet poor plasma (PPP) to determine spontaneous platelet aggregation. 50 µl of ADP (5 and 10 µM), epinephrine (10 µM) or collagen (2 µg/ml) was added to PRP so as to measure stimulated platelet aggregation. Platelet aggregation was measured using a Carat TX4 optical platelet aggregometer (Carat Diagnostics Ltd., Hungary). Platelet aggregation index has a “normal range” (mean ± 2 SD) to each stimulants in 68 untreated healthy persons (30 males, 38 females, mean age 38 ± 6 years). If a platelet aggregation inhibitor drug is effective, it decreases the aggregation index induced by the appropriate agonists (55,56). ADP stimulation is used to estimate the effect of thienopyridines (ticlopidine and clopidogrel). In our study antiplatelet medication was considered to be effective if the aggregation index to the appropriate agonists (collagen 2 µg/ml and epinephrine 10 µM) were lower than the range of untreated persons (both < 60 %) (20).

#### *4.1.2.2. Hemorheological measurements*

Blood samples were taken from the cubital vein; routine blood chemistry and hemorheological parameters - hematocrit, plasma fibrinogen level, plasma and whole blood viscosity, RBC aggregation and deformability - were determined. Details can be seen under 3.1.2 section.

#### *4.1.2.3. Risk profile and previous diseases*

Risk profile and investigated previous diseases included body mass index (BMI), smoking habits, diabetes, hypertension, dyslipidemia, deep vein thrombosis, pulmonary embolism, ischemic heart disease (IHD), acute myocardial infarction (AMI), carotid stenosis, transient ischemic attack (TIA), stroke, and peripheral arterial disease. Exclusion criteria included previous acute myocardial infarction or stroke < 3 months, or documented IHD or cerebrovascular disease lasting < 1 year previous to our investigation, and any other than vascular disorders. Hypertension was diagnosed in the case of elevated blood pressure values (>140/90 mm Hg measured twice in a resting position) and in subjects with normal blood pressure (<140/90 mm Hg) but on antihypertensive therapy. Dyslipidemia was defined according to the following criteria: serum total cholesterol level >200 mg/dL, serum high density lipoprotein cholesterol level <40 mg/dL, or serum triglyceride level >195 mg/dL. The term “current smoker” was applied if the patient smoked at least 10 cigarettes a day or consumed an equivalent daily dose of tobacco. Obesity was defined as a body mass index (BMI) >30 kg/m<sup>2</sup>. Diabetes mellitus was defined as fasting glucose > 130 mg/dL or patients with normal fasting blood glucose level but on antidiabetic medication.

#### *4.1.2.4. History of medication*



History of medication was related to beta-blockers, alfa-blockers, angiotensine converting enzyme (ACE) inhibitors, angiotensine (AT) II receptor blockers, calcium (Ca) channel blockers, statins, fibrates, diuretics, nitrates, H2 receptor blockers, proton pump inhibitors (PPIs), trimetazidine, benzodiazepines, selective serotonin reuptake inhibitors (SSRIs), phosphodiesterase inhibitors (vinpocetine, pentoxyphilline), and nonsteroidal anti-inflammatory drugs (NSAIDs). Exclusion criteria included clopidogrel/dipyridamole use or anticoagulation with warfarin.

#### *4.1.2.5. Statistical analysis:*

Data were evaluated as means  $\pm$  SD (standard deviation) by Student's t-test and the chi square test. Logistic regression analysis was used to determine the significance of the different parameters as independent risk factors in the development of clopidogrel resistance. The analysis was performed with appropriate adjustments for differences in risk factors and medication usage. For all odds ratios, an exact CI of 95% was constructed in our study. Logistic regression analyses were performed using the statistical package of SPSS 11.0 (SPSS, Chicago, IL, USA).

## **4.2 Results**

### *4.2.1 Risk profile and previous diseases*

The prevalence of hypertension was higher in the group of patients with effective inhibition (80 % vs. 62 %,  $p < 0.05$ ). There was not any other difference in risk profile and previous diseases between the two groups. On the other hand, after the setup of logistic regression analysis between risk profiles and ASA resistance, hypertension did not seem to be an independent predictor

### *4.2.2 History of medication*

In the case of effective platelet aggregation beta-blockers (75 % vs. 55 %,  $p < 0.05$ ) and ACE inhibitors (70 % vs. 50 %,  $p < 0.05$ ), while in the group of ineffective platelet aggregation statins were taken more frequently (52 % vs. 38 %,  $p < 0.05$ ) (see Table 6). After the setup of logistic regression analysis between medications and ASA resistance, only statins remained independent factors of ASA resistance (OR 5.92; 95% CI 1.83 to 16.9;  $p < 0.001$ ).

### *4.2.3 Hemorheological variables*

Patients with effective ASA inhibition had significantly lower plasma fibrinogen level (3,8 g/l vs. 3.3 g/l,  $p < 0.05$ ) and red blood cell aggregation values (28.2 vs. 24.3,  $p < 0.01$ ).

## **DISCUSSION**

The term of ASA resistance has been used to describe a number of different phenomena, including the inability of ASA to accompanish the followings: 1. to protect individuals from thrombotic complications, 2. to cause a prolongation of bleeding time, 3. to reduce TXA<sub>2</sub> production, or 4. to produce an anticipated effect on one or more in vitro tests of platelet function (21). Approximately one in eight high-risk patients will experience a recurrent atherothrombotic vascular event in the subsequent two years despite taking ASA, while it also fails to prevent 81 % of recurrent serious vascular episodes among high-risk patients. Resistance to this drug is the only one explanation as

to why may not be absolutely effective in preventing recurrent vascular events. Other possible reasons include an incorrect diagnosis or noncompliance with the prescribed dosage of medication (22). There are other parameters which play an important role of ASA resistance.

Although several studies examined a set of different factors in ASA resistance by in vitro test of platelet inhibition, in our knowledge this was the first study which analysed the possible role of risk factors, previous diseases medication and hemorheological parameters together.

Poor compliance may be the most important factor of ASA resistance. The higher prevalence of hypertension in the case of effective platelet inhibition may refer this, and it can explain the difference in the prevalence of ACE-inhibitors and beta-blockers. These patients can be tightly controlled by their general practitioners and it can cause better drug-taking compliance. A recent review showed the hazards of discontinuing aspirin. They concluded non-compliance or withdrawal of aspirin treatment has ominous prognostic implication in subjects with or at moderate-to-high risk for CAD. Aspirin discontinuation in such patients should be advocated only when bleeding risk clearly overwhelms that of atherothrombotic events (23).

On the other hand, a recent study showed the potential platelet inhibitory effect of beta-blockers on collagen- and epinephrine-induced platelet aggregation (16), thus an additive effect of these drugs may be involved in the effective antiplatelet therapy. Other publications showed that the benefits of ASA and ACE inhibitors may be attenuated when both agents are used together, although other studies showed no effect of ACE-inhibitors on platelet aggregation (24-26).

Statins are widely used in cardiovascular diseases and their favourable effects were proven by many large-population studies, and they may partially be independent from plasma cholesterol levels, combining lipid-lowering with positive effects on hemorheological conditions and endothelial function (27,28). Statins are also reported to decrease platelet aggregation (29). Our results suggest that statins may interfere with ASA.

Several studies confirmed that hemorheological parameters are independent risk factors of IHD (7,15), but this is the first study which showed impaired hemorheological parameters as potential background of ASA resistance. When plasma fibrinogen level increases red blood cells adhere and release ADP, which is a potential agonist of platelet aggregation. On the other hand, the aggregated red blood cells migrate in the center of blood flow displacing other cells (platelets) in small vessels, so they can easily contact to the endothelium (16). In the case of effective platelet inhibition we found significantly lower plasma fibrinogen levels compared to the other group. This may be caused by the higher rate of ACE inhibitors, because their favourable effect on hemorheological parameters (especially fibrinogen and plasma viscosity) was shown in a recent study (27). Furthermore, platelets from aspirin-resistant patients appeared to be more sensitive and activable by ADP. This hypersensitivity could provide a possible explanation for the so-called aspirin resistance, and this could justify therapeutic improvement with alternative antiplatelet agents (21).

A recent meta-analysis showed that patients who are "resistant" to aspirin are at greater risk of clinically important adverse cardiovascular events, regardless of the assay used to measure aspirin resistance. Not only did aspirin resistance have an effect on clinical outcomes but this risk was not ameliorated by currently used adjunct antiplatelet therapies. Patients who were classified as aspirin resistant were at about a fourfold increased risk of non-fatal and fatal cardiovascular, cerebrovascular, or vascular events while taking aspirin than their aspirin sensitive

counterparts. This risk can be generalised to a wide variety of patient populations with cardiovascular or cerebrovascular diseases (10,11). Prospective randomized studies are warranted to elucidate the optimal aspirin dosage for preventing ischemic complications of atherothrombotic diseases.

So, aspirin resistance seemed to be associated with worsening clinical outcome. Our study showed the role of hemorheological parameters in aspirin resistance. We also found antiplatelet effects beyond traditional antiplatelet agents, which may affect the efficacy of aspirin therapy.

## **5. THE POTENTIAL BACKGROUND(S) OF CLOPIDOGREL RESISTANCE**

Platelets have a central role in the development of arterial thrombosis and subsequent cardiovascular events. An appreciation of this has made antiplatelet therapy the cornerstone of cardiovascular disease management. Resistance to clopidogrel both in vitro and in vivo has also been described (18). Up to 5-11% of clopidogrel-treated patients were found to be non-responders, while 9-26% were semi-responders (17,18), but the possible mechanisms are still unclear.

### **5.1 Patients and methods**

#### *5.1.1 Patients*

157 chronic cardio- and cerebrovascular patients taking 75 mg clopidogrel daily (not combined with aspirin) as antiplatelet agent were involved in our study (83 males, mean age  $61 \pm 11$  yrs, 74 females,  $63 \pm 13$  yrs).

#### *5.1.2 Methods*

##### *5.1.2.1 Platelet aggregation*

##### *5.1.2.2. Hemorheological measurements*

##### *5.1.2.3 Risk profile and previous diseases*

##### *5.1.2.4 History of medications*

All these parameters can be seen undersection 4.1.2.

##### *5.1.2.5 Measurement of von Willebrand factor*

A quantitative direct enzyme immunoassay (Shield Diagnostics Ltd., U.K.) was used for the detection of von Willebrand factor (vWf) activity in human citrated plasma. The wells of the microtitre strips are coated with a preparation of purified monoclonal antibody which recognizes a functional epitope on the vWf antigen. During first incubation the specific antigen in diluted plasma will bind to the antibody coating. The wells are then washed to remove unbound plasma components. A conjugate of horseradish-peroxidase-labelled mouse anti-human monoclonal anti-vWf conjugate binds to surface-bound antigen in the second incubation. After a further washing step, specifically-bound antibody is traced by the addition of substrate solution. Addition of stop solution terminates the reaction. The amount of conjugate bound is measured in absorbance units. The activity of vWf in unknown sample can be estimated by interpolation from a dose-response curve prepared from calibrator set according to the 4th International Standard (30).

#### 5.1.2.6 Measurement of sP-selectin factor

Determination of the level of sP-selectin was performed by using a sandwich ELISA procedure according to manufacturer's instructions (Bender MedSystems, Austria) using the previously mentioned steps. sP-selectin was determined from platelet poor plasma prepared as described above.

#### 5.1.2.7 Statistical analysis

All these parameters can be seen under section 4.1.2.

## 5.2 Results

### 5.2.1 Platelet aggregation

Among 157 chronic cardio- and cerebrovascular patients involved in our study we found ineffective platelet aggregation in 35 patients (22 %). There was no significant difference in the mean age, the male/female ratio, the dosage of CLP and the duration of therapy between the two groups.

### 5.2.2 Risk profile and previous diseases

Patients with effective inhibition had lower BMI (28,8 kg/m<sup>2</sup> vs. 26,1 kg/m<sup>2</sup>,  $p < 0.05$ ). There was not any other difference in the prevalence of different risk factors and previous diseases between the two groups. After the setup of logistic regression analysis between risk profiles and CLP resistance, BMI remained to be independent factor of CLP resistance (OR 2.62; 95% CI: 1.71 to 3.6;  $p < 0.01$ ).

### 5.2.3 History of medication

In the case of ineffective platelet inhibition, benzodiazepines (25 % vs. 10 %,  $p < 0.05$ ) and selective serotonin reuptake inhibitors (28 % vs. 12 %,  $p < 0.05$ ) were taken more frequently. After the setup of logistic regression analysis between risk profiles and CLP resistance, they remained to be independent factors of CLP resistance (benzodiazepines: OR 5.83; 95% CI: 2.53 to 7.1;  $p < 0.05$  and SSRIs: OR 5.22; 95% CI: 2.46 to 6.83;  $p < 0.05$ ).

### 5.2.4 Hemorheological variables and adhesive molecules

There were no significant differences in the hemorheological parameters and in the plasma level of adhesive molecules between the two groups.

## DISCUSSION

The concept of clopidogrel resistance has emerged in the medical literature to reflect the failure to inhibit platelet function in vitro, although its existence and definition remain to be established. It has been proposed that the term resistance encompasses patients for whom clopidogrel does not achieve its pharmacological effect, and failure of therapy reflects patients who have recurrent events on therapy (17). The prevalence of clopidogrel nonresponse in patients is evaluated between 4% and 30% 24 h after administration (11,17,18). The reported rates vary between

studies because of the technique used to measure the extent of platelet aggregation and the presence of factors contributing to greater baseline platelet reactivity. Furthermore, the definition of nonresponders is not standardized.

We defined clopidogrel resistance as an inadequate decrease of the aggregation index induced by the appropriate agonists compared to healthy volunteers. We found that higher BMI is an independent factor of clopidogrel resistance, which is in concordance with the results of Angiolillo et al. (31,32). High dose of clopidogrel (600 mg loading dose) was shown to be more effective compared with 300 mg (33). Our results suggest that long term treatment should be weight-adjusted.

There were no other differences in cardiovascular risk profile or previous disease history between the two groups, although smoking might play an important role of clopidogrel metabolism by the way of P 450 1A2 (18). Many drugs have an increasing effect of platelet function (17), thus they may be associated with platelet inhibition.

Certain statins were reported to may decrease the efficacy of clopidogrel treatment by the way of CYP3A4 (17,18). In our study statins did not interfere with the efficacy of clopidogrel, but SSRIs and benzodiazepines did. Recent studies showed that benzodiazepines, especially alprazolam, triazolam and midazolam are metabolised and eliminated by CYP3As. It is also shown that certain SSRIs, as fluvoxamine are potent inhibitors of CYP1A2 and nefazodone is a potent inhibitor of CYP3A4, which isoenzymes play an important role in clopidogrel metabolism (34-36). CYP3A4 activity plays a role in clopidogrel resistance and metabolic interactions on this isoenzyme may decrease the metabolism and antiplatelet effect of clopidogrel (37). On the other hand, diazepam and clonazepam in a concentration-dependent manner inhibited thrombin, ADP or AA-stimulated platelet aggregation and the thrombin-induced increase in free intracellular  $Ca^{2+}$  (38). Thus receptorial interactions may also play a role in this phenomenon.

The severity of atherosclerosis may be associated with resistance to antiplatelet therapy in patients treated with aspirin (17). The level of hemorheological parameters, von Willebrand factor and P-selectin were shown to be correlated with the progression of atherosclerosis (38). Our findings suggest that clopidogrel resistance may not be associated with the progression of atherosclerosis.

In summary, our study is among the first studies which examined the potential background(s) of clopidogrel resistance. Clopidogrel resistance is a clinical entity with serious outcome. Monitoring and adjusting the antiplatelet therapy may be associated with decreased recurrent vascular events. We showed that BMI is associated with low response to clopidogrel and psychotropic agents (benzodiazepines and SSRIS) but not statins may inhibit the metabolism of clopidogrel possibly by the way of CYP3A4 and CYP1A2.

## **6. SUMMARY**

Although we have the data of several large trials, the role of hemorheological parameters and their possible relation to age is still under-represented in circulation research and in the clinical practice. „Classic” risk factors can also influence hemorheological parameters. A positive correlation between BMI and blood viscosity and its determinants has been demonstrated in several studies. Arterial hypertension is in association with increased blood viscosity. Smoking increases in a reversible way plasma and whole blood viscosity, partly by increasing hematocrit

and fibrinogen. Many drugs have potential effect on hemorheological parameters. Relatively few study examined the associations between hemorheological parameters and increasing age. They reported a not very pronounced increase and these reports depended on the populations studied and are controversial. To evaluate the effect of risk profile, previous diseases and medication, 623 patients were selected from the examined group with the same parameters. Our results suggest, that in a homogenous population, hemorheological parameters are independent of aging, thereby altered hemorheological parameters are much more associated with different diseases and the severity of atherosclerosis, than with aging alone.

Our study investigating the efficacy of routine antiplatelet medication confirmed the existence of both aspirin and thienopyridine non-responder individuals in the general population. Many different ways to perform aggregometry have been published. All tests have in common that their widespread clinical use is substantially limited due to complex preanalytic factors, reduced specificity and reproducibility. The results of these tests may become more comparable after the standardization of the different methods. On the other hand, despite of methodological problems, antiplatelet resistance seemed to be associated with worsening clinical outcome based on the result of recent meta-analysis.

The mechanisms underlying antiplatelet resistance can be multifactorial. Impaired hemorheological parameters (especially plasma fibrinogen) seemed to be associated only with aspirin, but not with clopidogrel resistance. When plasma fibrinogen level increases red blood cells adhere and release ADP, which is a potential agonist of platelet aggregation. On the other hand, the aggregated red blood cells migrate in the center of blood flow displacing other cells (platelets) in small vessels, so they can easily contact to the endothelium, releasing ADP, which is a strong agonist of platelet activation.

In the case of effective platelet inhibition we found significantly lower plasma fibrinogen levels compared to the other group.

Increasing BMI was associated with lower response to clopidogrel therapy. We found that higher BMI is an independent factor of clopidogrel resistance, which is in concordance with the results of previous studies. Our results suggest that long term treatment should be weight-adjusted.

Statins remained an independent predictor of aspirin resistance and benzodiazepines and SSRIs remained an independent predictor of clopidogrel resistance even after adjustment for risk factors and medication use. The COX-1 enzyme dependent antiplatelet effect of statins has been previously showed. They may interfere with the COX-1 inhibitory effect of aspirin. Recent studies showed that benzodiazepines, especially alprazolam, triazolam and midazolam are metabolised and eliminated by CYP3As. It is also shown that certain SSRIs, as fluvoxamine are potent inhibitors of CYP1A2 and nefazodone is a potent inhibitor of CYP3A4, which isoenzymes play an important role in clopidogrel metabolism. CYP3A4 activity plays a role in clopidogrel resistance and metabolic interactions on this isoenzyme may decrease the metabolism and antiplatelet effect of clopidogrel. Our result showed the potential antiplatelet effect of different (not antiplatelet) agents, which may affect the efficacy of routine antiplatelet therapy.

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