EFFECTS OF IMPAIRED GLUCOSE METABOLISM, AGING AND METEOROLOGICAL FACTORS ON HEMORHEOLOGICAL PARAMETERS AND PLATELET AGGREGATION

Ph.D. thesis

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1. Introduction

Hemorheology is the science of blood flow that deals with the deformation and flow properties of blood and its elements under mechanical forces. Blood is a non-Newtonian fluid, a suspension of cells, chylomicrons, carbohydrates, proteins, electrolytes in an aqueous medium.

Numerous studies have investigated the effects of aging on different hemorheological parameters. Age-associated changes have been described among others in the rheological properties of erythrocytes, plasma proteins, fibrinogen level, fibrin degradation products and blood viscosity. It is suspected that the increased incidence of cardio-and cerebrovascular diseases in the elderly may partially be due to age-related hemorheological alterations, i.e. hyperviscosity due to elevated fibrinogen level and altered erythrocyte properties may negatively influence hemodynamics and impair tissue oxygenisation. Fibrinogen is a high-molecular-weight (340.000) glycoprotein, a main determinant of plasma viscosity, and it also plays a role in erythrocyte and platelet aggregation as well as in the activation of the coagulation system. A significant rise was found in plasma viscosity and fibrinogen associated with aging in several studies. Bauer et al. [1] found higher concentrations of fibrinopeptide A in elderly subjects that refers to a more frequent activation of prothrombin and also to a more rapid production of fibrinogen in older subjects. In a study by Avellone et al. on 600 individuals a significant increase in plasma viscosity and fibrinogen was found due to aging [2]. In a study by Tarallo et al. fibrinogen was found to be higher in children than in adults (age between 20-30 years). However, in adults older than 30 years a gradual increase in fibrinogen levels was observed in both sexes. Ott et al. studied a group of asymptomatic cerebrovascular patients and found that older patients (mean age 59 years) exhibited significantly higher values of fibrinogen than younger individuals (mean age: 48 years) [3].

The Bezafibrate Infarction Prevention study [4] also showed a significant increase in fibrinogen levels both in men and women. In this study, correlations were found for plasma viscosity and fibrinogen (r=0.81) and triglycerides (r=0.42). Hager et al. reported a significant increase with age for erythocyte sedimentation rate, plasma cholesterol and triglycerides and a significant decrease in albumin in a group of 156 subjects aged 23-96 years [5]. De Simone et al. [6] found a correlation between age and plasma viscosity and globulin fraction in 127 normotensive subjects. A study by Sharp et al. [7] on 3571 elderly Japanese American men showed a significant correlation between age, elevated fibrinogen and chances of having coronary heart disease. A study by Banerjee et al. [8] found that fibrinogen was the best single discriminator for myocardial infarction in an elderly population. A rise of 1 g/l increased the probability of death by 2-fold in the next 6 years. Feher et al. found a weak but statistically significant correlation between selected hemorheological parameters (plasma viscosity, fibrinogen) and aging in 6234 cardio-and cerebrovascular patients, however, in a subgroup of old males whole blood viscosity and its main determinants were negatively correlated with advancing age. Also in a subgroup of 623 patients with matching parameters (risk factors, previous diseases, and medication) no significant association was found between hemorheological parameters and aging and the observed increase in the examined parameters in the whole population were contributed to a higher number of concomittant diseases in the elderly.

Platelet aggregation basically influences coagulation and blood flow dynamics. A study of 116 hypertensive and 142 normotensive individuals showed an increase with age in platelet aggregation [9]. ADP-induced platelet aggregation showed a more pronounced increase compared to epinephrine induced platelet aggregation in this study.

In a study of 18 smokers and 34 nonsmokers collagen-induced platelet aggregation was found to increase significantly with age in the nonsmoker group [10].

Diabetes mellitus is a clinical term denoting a group of metabolic impairments which affect glucose utilization and lead to hyperglycemia. The importance of diabetes is reflected by the fact that the worldwide prevalence of the disease is projected to double to 300 million by 2025. Type 1 diabetes is characterized by the complete absence of insulin, while type 2 diabetes is characterized by hyperinsulinemia and insulin resistance which precedes the development of hyperglycemia. The latter form of diabetes accounts for at least 90% of diabetic cases worldwide and it is associated with modern lifestyle characterized by abundant nutrient supply and reduced physical activity. As vascular disease represents the main etiology for death, and accounts for a great percent of morbidity in diabetic patients, poorly controlled diabetes is considered to be a vascular disease. Hyperglycemia may influence hemorheological parameters through enhanced advanced glycation end product (AGE) formation. Reducing sugars may react non-enzimatically with the amino groups in proteins or lipids that ultimately leads to the formation of stable covalent adducts, the AGEs. AGEs can bind to biological membranes in a nonspecific manner, but they also induce specific cellular responses including the release of profibrogenic and proinflammatory cytokines by interacting with RAGE (receptor for AGE), a cellular surface receptor that binds AGE-modified proteins with high affinity. Several other receptors and cell surface molecules that are capable of binding AGE-modified proteins have been identified recently. The consequence of AGE-RAGE interaction is the generation of reactive oxygen species (ROS), partially caused by the activation of NADPH oxidase. AGE-RAGE interaction induces the expression of vascular endothelial growth factor (VEGF) in endothelial cells. AGEs also inhibit prostacyclin production and stimulate plasminogen activator inhibitor-1 (PAI-1) synthesis by endothelial cells. AGEs thus stimulate the growth of microvascular endothelial cells, leading to angiogenesis on one hand and to a pro-thrombotic state on the other hand [11-14].

Obesity is both a cause and a consequence of type-2 diabetes mellitus. The adipose tissue synthesizes and releases numerous bioactive substances, several of which are known to have an effect on blood rheology. PAI-1 and interleukin-6, important regulators of plasma fibrinogen level, are also produced in significant amounts by adipocytes. Low-grade inflammation is present in adiposity as reflected by elevated levels of C-reactive protein, interleukin-6 and tumor necrosis factor–alpha in obese individuals. The low-grade, chronic inflammatory state may contribute to insulin resistance and endothelial dysfunction. Adipose tissue also releases leptin, a cytokine that circulates at high concentrations in the plasma of obese, insulin resistant patients. This hormone has been recently shown to stimulate proliferation and migration of myocytes in the vessel wall, and thus been suggested to be a possible pathogenic factor for atherosclerosis. Positive correlations have been reported between leptin and both plasma viscosity and RBC partial disaggregation threshold measured by laser backscattering [15].

Accelerated atherosclerosis in type-2 diabetic patients is multifactorial and includes very complex interactions, including hyperglycemia, hyperinsulinemia, hyperproinsulinemia, dyslipidemia, oxidative stress, accelerated aging, platelet hyperreactivity as well as alterations in coagulation and fibrinolysis and changes in hemorheological parameters. The initial lesion of atherosclerosis is manifested in changes of endothelial cell function. Endothelial dysfunction has been shown to be manifested in patients with type-2 diabetes as well as in type-1 diabetic patients early in the course of the disease, especially when microalbuminuria is present. Moreover, endothelial dysfunction has been shown to be present in insulin resistant patients with impaired glucose tolerance (IGT) and in individuals with former gestational diabetes [16]. There is a growing body of evidence showing that poly(ADP) ribose polymerase (PARP) activation has an important role in the pathophysiology of endothelial dysfunction in diabetes. In animal models, destruction of pancreatic islet cells with

streptozotocin leads to PARP activation and endothelial dysfunction. Studies on wild-type and PARP deficient mice showed that elevated blood glucose levels represented a very strong stimulus for PARP activation. These studies led to the conclusion that PARP activation due to hyperglycemia is influenced by the genesis of superoxide species; O2 free radicals may be produced in endothelial cells exposed to hyperglycemia.

Hemorheological parameters

Glycemic control seems to be a major factor for determining the hemorheological consequences of diabetes. Type-1 diabetic patients with poor glycemic control exhibit increased plasma and whole blood viscosity when compared to normoglycemic individuals; blood viscosity is also negatively correlated with insulin sensitivity. Treatment of insulin resistance by exercise training specifically improves plasma viscosity due to the close association between plasma viscosity and insulin sensitivity. Positive associations have also been found between parameters of glycemic control (HbA1C, fructoseamine), fibrinogen levels and red blood cell aggregation; fibrinogen levels are also closely correlated to insulin resistance. A single hyperglycemic spike increases red blood cell aggregation in both type-1 and type-2 diabetic patients and alters fibrinogen concentration and activity in type-1 diabetic patients.

Several studies have reported that insulin improves hemorheological abnormalities in diabetes, and when studied in vitro, incubation of red cells obtained from diabetic patients with insulin resulted in improved cellular deformability as measured by micropore filtration. Interestingly, this observation could not be reproduced when utilizing washed red blood cells. These ex vivo results suggest a direct effect of insulin on red cell membrane fluidity, although beneficial hemorheological effects related to insulin treatment in vivo may be indirectly mediated by metabolic improvements. Nevertheless, it now seems that insulin affects red cell

rheology via direct effects on the membrane, including alterations of the lipid membrane bilayer composition and microviscosity and changes in membrane Na/K ATPase function. It is interesting to note that supra-physiological levels of insulin can have adverse effects on red cell deformability, with very high in vitro levels decreasing red blood cell deformability.

Both type-1 and type-2 diabetes are associated with diabetic thrombocytopathy, a condition related to increased platelet adhesiveness and aggregability. Enhanced platelet aggregation is present in diabetes early in the course of the disease, well before the development of diabetic vascular lesions. Several biochemical abnormalities play a role in diabetic platelet hyperreactivity:

1) membrane fluidity is reduced due to changes in the lipid composition of the membrane or glycation of membrane proteins;

2) increased intracellular Ca^{2+} levels and decreased magnesium concentrations reduce membrane fluidity and increase platelet adhesiveness;

3) platelets from diabetic individuals produce less NO and prostacyclin, agents which normally promote endothelium-mediated vasodilation and inhibit platelet-endothelium interactions;

4) increased platelet arachidonic acid metabolism leads to enhanced TXA2 production, a possible underlying cause for platelet hyperreactivity;

5) platelets from diabetic subjects contain reduced levels of antioxidant molecules which might also contribute to their hyperaggregability.

The binding of fibrinogen to the GP IIb-IIIa receptor is increased in diabetic patients, and they also have a higher ratio of platelets expressing activation-dependent adhesion molecules such as activated GP IIb-IIIa, lysosomal Gp53, thrombospondin and P-selectin; plasma fibrinogen levels are also increased in both types of diabetes. Platelets may interact with

glycosylated low density lipoproteins, von Willebrand factor or immune complexes, and platelet turnover may be shortened in diabetes, thereby contributing to the observation that antiplatelet agents such as aspirin and clopidogrel have a diminished effect in these patients. Chronically poor metabolic control is associated with increased platelet activation and aggregation. Hyperglycemia has been described to result in enhanced platelet aggregation, with reduced sensitivity to aspirin observed in type-2 diabetic patients having poor metabolic control. Soluble P-selectin is a widely accepted marker for platelet activation: elevated plasma levels have been proved to be significantly associated with diabetes mellitus [17-19].

Polymorphonuclear leukocytes derived from diabetic patients were found to be more rigid as measured by filtration techniques, suggesting an activated stage of these cells. Leukocytes are larger and much more rigid than erythrocytes, thus they can strongly influence microvascular blood flow. Moreover, polymorphonuclear leukocytes (PMN), the largest fraction of leukocytes, are capable of causing microvascular damage by the release of proteases and toxic oxygen radicals. Comparing PMN of normoglycemic and diabetic patients, the rigidity of leukocytes obtained from individuals with diabetes is significantly elevated both in their basal stage and following their activation with the bacterial polypeptide fMLP. Diabetic and hypertensive individuals have less deformable PMN than patients with diabetes and normal blood pressure [20-22].

Chronobiology is the „scientific discipline concerned with the definition, mechanisms, and significance of the so-called time structure of life forms" [23]. While homeostasis presumes a relatively constant internal state, chronobiology presumes „human bioprocesses and functions exhibit predictable variability in time, biological rhythms, at every level of organization" [23].

Variations in the annual per capita death rates in different countries are well documented. Less well known are seasonal variations in death rates with the highest levels occurring

during the colder winter months as it has been described in many countries. This phenomenon is referred to as excess winter mortality. A seasonal variation in the incidence of MI, cardiac death, and stroke is consistently described. CVD-related deaths account for the majority of excess winter deaths (up to 70% in some countries) [24,25], while about half of the remaining are due to increases in respiratory diseases. Correlation between seasons and attacks of coronary thrombosis was first noted in 1926 [26]. Seasonal variations in cardiovascular mortality have been documented in both northern and southern hemispheres [27-30], while seasonal changes are absent in equatorial regions [30]. The mechanisms underlying this variation, which holds true in both cold and milder climates, are not fully understood. Seasonal deaths from MI may partially be due to endogenous physiological rhythms in cardiovascular risk factors. Large annual blood presssure amplitude has been observed with a peak in winter and a through in the summer [31,32]. Circannual rhythms have been described in parameters as fibrinogen , hematocrit, platelet count [33] hormones [34,35], serum lipids, and glucose [36,37]. Total cholesterol, HDL-C and LDL-C showed seasonal alterations in many studies with a peak in winter and a through in the summer months [38]. Several findings may explain this phenomenon. Higher intake of total fat in winter was shown in a Danish study [39]. Seasonal variation in intake of fat, beta-carotene and vitamins A, C and E was found in a study by Woodhouse et al [40]. Beta-carotene and vitamin A intakes peaked in autumn and winter while vitamin C and E intakes reached their highest level in summer. Glycemic parameters as hemoglobin A_{1C} values showed a seasonal rhythm in diabetic patients [41]. Fluctuations in glucose and insulin sensitivity were demonstrated in several studies, with higher levels in the fall or winter in most studies [42-44]. Microvascular disease related complications also follow a seasonal pattern in diabetes. Initiation of dialysis for patients with end-stage renal disease is more common in January and least common in August [45]. Data concerning a possible seasonal variation in endothelial function is controversial. Widlansky et

al. observed in the Framingham Offspring Cohort that brachial artery flow-mediated dilatation was lowest in winter [46]. Conduit artery endothelial function was most strongly related to season. On the other hand, Klein-Wiegel et al. found no seasonal variation in flow-mediated dilatation of the brachial artery either in women with primary Raynaud's phenomenon or in healthy controls [47].

In developed countries, death rates for cardiovascular disease (CVD) have decreased nearly 60 % from their peaks in the 1960s and 1970s. Hungary, with one of the highest mortality rates in Europe has shown a delayed start from the mid-1990s in this decline [48-50]. Exogenous factors such as climate, diet, physical activity levels, heating and air conditioning may directly or indirectly influence endogenous rhythms such as blood pressure, coagulation factors or platelet count, rendering the body more vulnerable to cardiovascular events. MI mortality has been shown to increase both with low and high temperatures, therefore climatic trends and changes to indoor environments may influence its seasonal variation [51,52]. Increased use of central heating and air conditioning alleviates temperature stress in winter and summer respectively and may interfere with seasonal variation of cardiovascular risk factors. Seretakis et al. [53] reported a sharp decline in seasonal variation for coronary deaths in the US from 1937-1970, followed by a reversal of this trend in the period between 1970-1991. The authors proposed that the increase in the second time frame of their study resulted from the increased use of air conditioning, which blunted the effects of heat waves on coronary mortality, thus the increase in seasonal variation was due to a decrease in summer deaths.

Several other studies found a negative correlation between MI mortality and mean monthly temperatures [54,55]. A Northern Irish study of MI mortality between 1979-1990 found that death rate for both sexes for MI decreased by 2.2 % for each 1 °C [56]. The same

study examined the effect of hours of sunshine per month and found no significant relationship between the level of sunshine and mortality.

While improvements in lifestyle, diet, housing and medical care may have contributed to decreased mortality rates, seasonal fluctuations remain a significant problem.

2. Aims

1. Rheological factors and increased platelet aggregation are convincingly implicated in the development of micro- and macrovascular diseases associated with diabetes mellitus. The presented examination has been designed to describe the effects of a standard oral glucose load on hemorheological parameters, platelet activation and aggregation in patients with normal and pathologic glucose tolerance.

2. In the second part of my work I aimed to investigate the association between the effectiveness of the most widely used antiplatelet therapies (100 and 300-325 mg acetylsalicylic acid (ASA), 75 mg clopidogrel, 500 mg ticlopidine and the combination of 100 mg aspirin and 75 mg clopidogrel), fibrinogen levels and aging.

3. In the third part of my work I aimed to investigate whether hemorheological parameters and platelet aggregabiliy show seasonal differences in a population with established vascular disease. A possible association with certain meteorological factors as ambient temperature, daily amount of sunshine hours, relative humidity and simultaneous changes in air pressure was also investigated.

3. The effect of blood glucose levels on hemorheological parameters, platelet activation and aggregation in oral glucose tolerance tests

3.1. Background

Cardio- and cerebrovascular diseases and their complications are the leading cause of morbidity and mortality in the world. Hemorheological parameters were proved to be independent risk factors [57-67], however, many „classic" risk factors have an influence on blood rheology [68]. Rheological factors are also convincingly implicated in the development of diabetic microvascular diseases [69]. Several studies have proved that a chronic poor metabolic control is associated both with pathologic alterations of hemorheological parameters [11,13,70-72] and with increased platelet activation and aggregation [73,74]. Reduced sensitivity to aspirin observed in type 2 diabetic patients has been ascribed to poor metabolic control [75]. Several in vitro studies have been performed to clarify the effect of acute changes in glucose concentration on hemorheological parameters and platelet aggregation [76,77]. At the same time much less data are available on how glucose consumption influences these factors in vivo, and the precise time course of events has not yet been fully elucidated. Hyperglycemia has been described to result in enhanced platelet aggregation, however, very little data can be found whether there is a relationship between platelet activation and aggregation and blood glucose levels within the normoglycemic range. Soluble P-selectin (sP-selectin) is taken as a plasma marker of platelet activation by many investigators [78,79]. sP-selectin levels have been proved to be significantly associated with diabetes mellitus [80]. The present study has been designed to clear whether or not acute changes in blood glucose levels have an immediate effect on hemorheological parameters, platelet activation and aggregation in oral glucose tolerance tests (OGTT).

3.2. Patients and methods

Study population

Oral glucose tolerance test was performed in 30 patients (12 males, 18 females, mean age: 58 ± 10 years) suspected to have diabetes mellitus. OGTT was in all cases medically indicated (high cardiovascular risk, previously measured fasting glucose levels were between 6.0-7.0 mmol/l). The study was approved by the regional ethics committee. All participants agreed to participate and signed an informed consent. Exclusion criteria were any inflammatory or malignant conditions, surgical or invasive procedure, or infarction within 6 months before the recruitment. Mean body mass index (BMI) was 31.5 ± 5. kg/m^2.

OGTT

After an overnight fast, fasting glucose levels were measured, then patients were loaded with 75 g glucose. Blood samples were taken 1, 2, and 3 hours after the oral glucose load. Based on the 2-hour result of the tests, patients were divided into a normal glucose tolerance (NGT) group (glucose levels below 7.8 mmol/l at the 2-hour sampling) and an impaired glucose tolerance/diabetes mellitus (IGT/DM) group, where 2-hour glucose values were above the normal limits.

Hemorheological measurements

Hematocrit, erythrocyte aggregation, red blood cell filtration and P-selectin measurements were performed at room temperature (22±1°C), whole blood and plasma viscosity and platelet aggregation measurements at 37 °C within two hours after venepuncture.

Hematocrit:

Venous blood collected into lithium-heparin coated Vacutainer tubes was centrifugated in hematocrit capillaries at 12000 rpm for five minutes in a microhematocrit centrifuge (Hemofuge, Heraeus Instr., Germany).

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 of Schmid-Schonbein et al. [81,82]. The aggregometer has two modes of operation: M and M1. For both modes blood sample (30 μl) is first sheared at 600 s^{-1} to disperse all pre-existing aggregates, then shear rate decreases rapidly to zero (M0 mode) or low shear (M1 mode). The extent of aggregation is characterized by the aggregation index (AI_M, AI_{M1}), calculated from the surface area below the light intensity curve in a 10 s measurement period.

Plasma and whole blood viscosity:

Plasma and whole blood viscosity were determined in Hevimet 40 capillary viscosimeter (Hemorex Ltd., Hungary) from venous blood samples collected into lithium-heparin coated Vacutainer tubes. Plasma was obtained by centrifugating samples at 1500 g for ten minutes. 1.0-1.0 ml plasma or whole blood was injected into the capillary tube of the viscosimeter. In this device 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. For the presentation of our results, apparent whole blood viscosity values calculated at 90 s^{-1} shear rate are given.

RBC filtration:

RBC filterability, a variable refering to cell deformability was measured in a Carat FT-1 Filtrometer (Carat Diagnostics Ltd., Hungary) using the St George's technique. In this apparatus RBC suspension flows through a Nucleopore filter with 5 μm pores. Filtration rate is calculated from the data measured at four pairs of light sources and detectors. The device is interfaced to a computer, which automatically analyses sequential flow rates and thus distinguishes the cell transit time (relative cell transit time) and the pore clogging rate. In our experiments, filtrating pressure was set to 4 cm of water. All measurements were repeated three times for each sample.

Platelet aggregation:

Platelet-rich plasma (PRP) was separated by centrifugation at 150g for 10 minutes. After carefully removing PRP, the remaining samples were further centrifugated at 2500g for 10 minutes to obtain platelet-poor plasma (PPP). Platelet aggregation was measured by Carat TX-4 optical platelet aggregometer (Carat Diagnostics Ltd., Hungary) at 37 °C within two hours after vein puncture. 10, 5 and 2,5 μM ADP, 2 and 1 μg/ml collagen, 10 and 5 μM epinephrine inducers were used.

sP-selectin:

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

Statistical analysis

Patients were divided into two groups based on the result of OGTT. The IGT/DM group consisted of 14, either diabetic or IGT patients, the NGT group consisted of 16 non-diabetic patients. All data in the text and tables are expressed as means ± standard error of the

mean (S.E.M.). Student's t test for paired data was used to calculate differences between baseline results and measurements at other time-points. Pearson's linear correlation coefficients were calculated between variables. Student's t test for unpaired data was used to determine differences between the IGT/DM and the NGT group in the case of hematocrit, whole blood and plasma viscosity, red blood cell aggregation and filtration and P-selectin measurements. The minimum value used to establish statistical significance was $p< 0.05$.

3.3. Results

Diagnosis of diabetes mellitus was confirmed if the 2-hour glucose level was more than 11.1 mmol/l, IGT was diagnosed if the 2-hour result was between 7.8 and 11.1 mmol/l. Blood glucose levels in the different groups are represented in Table 1. BMI was significantly higher in the IGT/DM group ($p<0.05$). Red blood cell aggregation showed a significant elevation in the course of time in all groups, however, in IGT/DM patients the elevation was more expressed and could already be observed in the 2nd hour of the examination (Table 1).

Both the M0 and M1 indices correlated significantly with BMI in the IGT/DM group ($p<0.01$), but no significant association could be found between these parameters in patients with normal glucose tolerance (Table 2).

			Fasting	1 hour	2 hours	3 hours
Glucose (mmol/l)		**NGT** (n=16)	5.29 ± 0.49	9.20 ± 1.82‡	6.67 ± 1.30‡	4.20 ± 1.24†
		IGT/DM (n=14)	6.07 ± 1.25 *	12.58 ± 2.12‡ ‡	11.96 ± 3.44‡ ‡	7.43 ± 3.51 †
Hematocrit (%)		**NGT** (n=16)	43.1 ± 3.2	42.9 ± 3.2	42.9 ± 3.9	42.6 ± 3.6*
		IGT/DM (n=14)	44.2 ± 3.9	43.7 ± 3.3	43.0 ± 4.7*	42.8 ± 3.1*
Red blood cell aggre-gation	**M0**	**NGT** (n=16)	13.87 ± 2.93	13.47 ± 2.83	14.62 ± 2.36	14.85 ± 2.35
		IGT/DM (n=14)	13.99 ± 4.52	15.16 ± 3.86	16.38 ± 3.54*	16.77 ± 4.34†
	M1	**NGT** (n=16)	25.51 ± 3.57	25.75 ± 3.66	26.22 ± 3.44 *	27.01 ± 3.68† *
		IGT/DM (n=14)	27.15 ± 5.71	27.94 ± 5.20	29.96 ± 5.23†	30.81 ± 4.07‡
Plasma viscosity (mPas)		**NGT** (n=13)	1.30 ± 0.09	1.29 ± 0.06	1.28 ± 0.14	1.28 ±0.17
		IGT/DM (n=13)	1.33 ± 0.12	1.25 ± 0.13†	1.28 ± 0.07†	1.25 ± 0.09†
Whole blood viscosity (mPas)		**NGT** (n=13)	4.45 ± 0.57	4.41 ± 0.52 *	4.24 ± 0.75	4.30 ± 0.61
		IGT/DM (n=13)	4.62 ± 0.53	4.79 ± 0.59	4.59 ± 0.70	4.54 ± 0.48
Red blood cell filtration		**NGT** (n=13)	6.9 ± 0.49	6.88 ± 0.53	7.11 ± 0.58	7.03 ± 0.55
		IGT/DM (n=12)	6.9 ± 0.49	7.01 ± 0.50	7.24 ± 0.46	7.15 ± 0.65
sP-selectin (ng/ml)		**NGT** (n=13)	6.4 ± 2.9	4.5 ± 2.7 *	4.9 ± 2.4	6.5 ± 2.36
		IGT/DM (n=12)	9.6 ± 9.9	14.9 ± 20.8	12.1 ± 14.4	12.3 ± 12.6

*p<0.05 †p<0.01 ‡p<0.001

Table 1. Blood glucose levels, hemorheological parameters compared to fasting values in oral glucose tolerance tests (values are represented as mean ± SEM)

		BMI
NGT	**M0 (n=16)**	r=0.0957 NS
	M1 (n=16)	r=0.3581 NS
IGT/DM	**M0 (n=14)**	r=0.7740 p<0.01
	M1 (n=14)	r=0.7407 p<0.01

Table 2. Correlation coefficient and its significance between M and M1 indices and BMI in NGT and IGT/DM patients

M0 and M1 indices did not correlate significantly with coinstantaneous blood glucose levels. The M1 index was significantly higher in the IGT/DM group than in the NGT group at the 2- and 3-hour samplings ($p<0.05$).

Plasma viscosity in the IGT/DM group showed significant decrease in all samplings compared to baseline values, while no statistically significant changes could be observed in the NGT group (Table 1).

Whole blood viscosity was significantly higher in the IGT/DM group at the one-hour measurements ($p<0.05$), but not at the other tested time-points. However, it did not change significantly in the course of time in either groups.

Hematocrit decreased statistically significantly in the IGT/DM group in the 2nd hour, while in the NGT group only in the 3rd hour and to a lesser extent (Table 1).

Red blood cell filtration did not show significant changes at any time points in this study, and no significant difference was found between the two groups either (Table 1).

Platelet aggregation showed statistically significant decrease at the 1-hour measurements compared to baseline values both in the non-diabetic and in the diabetic/IGT group in the case

of higher ADP concentrations, but no significant changes could be observed with other inducers (Table 3).

		Fasting	1 hour	2 hours	3 hours
NGT	**ADP 10µM**	72.5 ± 6.9	67.9 ± 14.2	71.1 ± 9.8	67.3 ± 13.0
	ADP 5 µM	69.1 ± 9.1	64.7 ± 13.5 *	67.8 ± 8.8	68.6 ± 11.3
	ADP 2.5 µM	60.4 ± 14.1	60.9 ± 16.3	57.9 ± 18.6	56.5 ± 21.0
	Coll 2 µg/ml	56.8 ± 23.8	54.1 ± 23.8	58.2 ± 24.2	57.9 ± 24.1
	Coll 1 µg/ml	47.9 ± 23.8	49.0 ± 26.6	43.1 ± 26.4	45.3 ± 27.9
	Epi 10 µM	53.9 ± 23.9	45.7 ± 24.3	53.0 ± 28.5	49.2 ± 28.1
	Epi 5 µM	55.1 ± 21.1	45.4 ± 24.4	45.6 ±28.5	47.5 ± 24.0
	Spont. aggr.	4.0 ± 3.6	9.2 ±16.5	5.4 ± 5.0	4.5 ± 6.5
IGT/DM	**ADP 10 µM**	75.5 ± 7.7	69.8 ± 9.7 †	71.8 ± 9.2	72.3 ± 11.7
	ADP 5 µM	66.9 ± 12.1	69.2 ± 9.7	69.5 ± 10.5	69.8 ± 13.6
	ADP 2.5 µM	59.7 ± 16.1	59.2 ± 15.7	56.7 ± 23.3	58.9 ± 20.7
	Coll 2 µg/ml	64.9 ± 12.2	64.7 ±14.6	61.9 ± 20.3	55.9 ± 25.3
	Coll 1 µg/ml	50.1 ± 22.5	49.5 ± 26.8	54.4 ± 27.7	52.4 ± 26.5
	Epi 10 µM	61.0 ± 21.2	54.9 ± 26.6	58.1 ± 27.6	56.1 ± 35.5
	Epi 5 µM	47.6 ± 26.0	52.7 ± 29.0	57.1 ± 27.6	56.1 ± 32.3
	Spont. aggr.	14.4 ± 23.1	9.6 ±17.8	10.2 ± 19.2	8.5 ± 16.7

*p<0.05 †p<0.01

Table 3. Platelet aggregation measured in optical aggregometer during OGTT in the different groups (values are represented as mean ± SEM)

Difference between platelet aggregation in IGT/DM patients and the nondiabetic group could not be tested because of the lack of homogenous antiplatelet therapy. sP-selectin concentrations did not show significant changes in either examined groups in the course of time. However, IGT/DM patients showed significantly higher sP-selectin concentrations at the 1-hour measurements ($p<0.05$) than the NGT group (Table 1).

3.4. Discussion

Long-term effects of poor metabolic control have been proved to result in erythrocyte hyperaggregability [72] and hyperviscosity [11,71]. However, much less information could be found on the immediate effects of changes in blood glucose levels on hemorheology and platelet aggregation in vivo, and results are often controversial. Although whole blood viscosity did not change significantly in this study, plasma viscosity decreased significantly in IGT/DM patients, while it showed no changes in non-diabetics. These results are partially in accordance with the results of Khodabandehlou et al. [83], who reported a significant decrease in both plasma- and whole blood viscosities in type-1 diabetic patients during a hyperglycemic spike. However, in their study hematocrit levels remained unchanged, while our investigation showed a clinically minor, although statistically significant decrease in hematocrit levels that was more pronounced in the IGT/DM group. These results implicate that increased blood osmolality due to elevated blood glucose levels may lead to a fluid influx from the interstitial space into the circulation.

Previous studies have already shown association between BMI and erythrocyte aggregation [16,84]. Interestingly, this study shows a strong correlation between BMI and erythrocyte aggregation in IGT/DM patients, but no association between these parameters in patients with normal glucose tolerance. Elevated M1 values seen in the IGT/DM group might be associated with enhanced levels of fibrinogen and CRP, known to be present in impaired glucose tolerance and diabetes mellitus. Our unpublished data of 106 type-2 diabetic, vascular patients show significant association between CRP and BMI ($p<0.01$); fibrinogen was found to be significantly associated to BMI ($p<0.05$), while the M1 aggregation index was related to both CRP ($n=191$, $p<0.01$) and BMI ($p<0.05$). A recent study found body fat to be an important predictor of plasma fibrinogen level [85]. These relationships between fibrinogen, CRP and BMI/body fat may support the theory that erythrocyte aggregation is related to BMI through

low-grade inflammation. Data on the correlation between blood glucose levels and erythrocyte aggregation are controversial in the literature. A recent study found that erythrocyte aggregation elevated with the increase of glucose concentration [70]. Other investigators reported significant decrease in erythrocyte aggregation during short-term hyperglycemia in type-1 diabetic patients [83]. Our results showed a significant increase in erythrocyte aggregation in both M0 and M1 modes from the 2nd hour sampling on in the IGT/DM group. Red blood cell aggregation in NGT patients also elevated in the course of time, however, to a lesser extent than in patients with abnormal glucose tolerance. Interestingly, the increase in erythrocyte aggregation did not follow immediately the elevation of glucose levels. The correlation between glucose levels and the M1 aggregation index tended to be stronger -although never significant- when M1 levels were compared to glucose levels measured an hour earlier. These results may suggest that the increase in erythrocyte aggregation following glucose consumption is due to the elevation of insulin levels [86], and a marked elevation of erythrocyte aggregation during OGTT might be a sign of hyperinsulinemia. This would also explain the differences between our findings and the decreased erythrocyte aggregation seen in type-1 diabetic patients lacking insulin during hyperglycemia [75]. Nevertheless, a further study is needed to prove this hypothesis.

Poor metabolic control on the long term is known to affect red blood cell filterability [87]. In this study, however, red blood cell filterability did not change significanly after the oral glucose load, although a tendency to increase –most recognisable at 2 hours after glucose consumption – was present.

Very few data are available in the literature on the immediate effects of glucose consumption on soluble P-selectin levels. In this study, results are controversial, as two patients showed a very high rise in sP-selectin levels in the course of time, which could not be explained with any of the investigated parameters, while the great majority of the participants did not show

any significant changes in sP-selectin levels. A further study with more functional and morphological parameters of platelets, and with more participants with homogenous medication could possibly clarify this question.

It has not been cleared whether or not, and how glucose consumption influences platelet activation and aggregation in the course of time. In a recent in vitro study platelet activation proved to be increased after exposure to high concentrations of both glucose and mannitol, proving the role of increased osmolarity in enhanced platelet aggregation and activation [77]. Our in vivo results, however, showed no significant changes in platelet aggregation at the tested time points compared to baseline values with most tested inducer concentrations. Platelet aggregation induced by higher concentrations of ADP showed significant alterations at the 1 hour sampling, but these results were inconsequent inasmuch only one tested concentration resulted in a significant change in both groups. Results obtained in this investigation are also contradictory to data seen in the in vitro studies. Our hypothesis to explain the lack of increase in platelet aggregation in this study is an interference with the circadian pattern of platelet aggregability, which is highest in the morning hours [88]. It is also possible that blood glucose levels seen in IGT/DM patients in this study were not elevated enough to increase platelet aggregation in vivo. A further study with separate subgroups of type-2 diabetic and IGT patients with uniform antiplatelet medication would be needed to clarify this question.

3.5. Conclusion

Glucose consumption influences hematocrit, plasma viscosity and red blood cell aggregation, however, a direct association between actual glucose levels and these parameters could not be shown in this study. Red blood cell aggregation is associated with BMI, possibly through

low-grade inflammation known to be present in obesity. Erythrocyte aggregation after glucose consumption might be influenced by changing insulin levels in the course of time. Changes in plasma viscosity and hematocrit levels might be caused by an influx from the interstitial space into the circulation due to elevated osmolality.

4. Relation of platelet aggregation and fibrinogen levels to advancing age in aspirin and thienopyridine treated patients

4.1. Background

Increasing life expectancy has led to a growing population of elderly patients with ischemic heart diseases taking antiplatelet medication. Antiplatelet therapy has an established role in the primary and secondary prevention of cardiovascular diseases based on the results of large-scale clinical trials and meta-analyses that have demonstrated a significant reduction of risk of acute myocardial infarction, cardiovascular death and stroke. Aspirin is effective in reducing the risk of serious vascular events by 25 % [89]. However, several in vitro and clinical studies have shown interindividual differences in ASA responsiveness [89]. Neither is the effect of thienopyridines uniform in all patients. Current available data show that 4-30% of patients treated with clopidogrel fail to display adequate antiplatelet response [90]. Previous studies have reported a link between advancing age and prothrombotic changes [91,92]. Measurement of blood tissue plasminogen activator (t-PA) and plasminogen activator inhibitor-1 (PAI-1) levels suggest that thrombolytic activity is also reduced with aging [93,94]. The relation of platelet reactivity to aging has also been studied with platelet function tests in a few studies, however, findings were contradicting [93,95,96]. In spite of these facts, only few data can be found on the efficiency of antiplatelet regimens in the elderly. In our present study we aim to describe differences between different age groups in *in vitro* platelet aggregation and fibrinogen levels in a large number of vascular patients treated with different antiplatelet regimens.

4.2. Materials and methods

Patients

Between 2001 and 2005, platelet aggregation was checked in 5026 patients with clinical evidence of vascular diseases receiving antiplatelet therapy in our laboratory. Platelet aggregation tests were performed to analyze whether the chosen antiplatelet medication was effective. Age, sex, and antiplatelet agent taken were recorded in all patients. Patients were under antiplatelet therapy for at least 4 weeks at the time of testing. 3389 patients received aspirin therapy (100, 300 or 325 mg per day, mean age: 63 ± 11 years, 1661 males, 1728 females). 970 patients took 75 mg clopidogrel (mean age: 64 ± 10, 539 males, 431 females). 362 patients took 500 mg ticlopidine (mean age: 63 ± 10, 212 males, 150 females). We also investigated 305 patients who received a combination of 100 mg ASA and 75 mg clopidogrel (mean age: 62 ± 10, 201 males, 104 females.) Patient compliance to antiplatelet treatment was assessed by interview. Fibrinogen level was measured in 3243 patients by the method of Clauss.

Platelet aggregation tests

Blood samples anticoagulated with sodium citrate were taken between 8 a.m. and 10 a.m. from each patient after an overnight fast. Platelet-rich plasma (PRP) was separated by centrifugation at 150g for 10 minutes. After carefully removing PRP, the remaining samples were further centrifugated at 2500g for 10 minutes to obtain platelet-poor plasma (PPP). Platelet aggregation was measured by Carat TX-4 optical platelet aggregometer (Carat Diagnostics Ltd., Hungary) at 37 °C within two hours after vein puncture according to Born's method. Platelet aggregation was evaluated considering the maximal percentage of platelet aggregation in response to 5 and 10 μM ADP, 2 μg/ml collagen, and 10 μM epinephrine inducers.

Statistical analysis

Continuous variables are presented as means ± SD. The Kolmogorov-Smirnov and Shapiro-Wilk tests were used to investigate normal distribution of data. Subjects were divided by age decade. Age groups containing more than 5 individuals were evaluated. In the case of normal distibution Student's t test for unpaired data was used to compare age groups. In the case of non-normal ditribution the robust Welch test [97] was used. Pearson's linear correlation coefficients were calculated between variables in the case of normally distributed data, Spearman's correlation was assessed in the case of non-normal distribution. A p value <0.05 was considered statistically significant. Analyses were performed using SPSS 11.0 (SPSS Inc. Chicago, Ill.) and Microsoft Excel.

4.3. Results

In patients treated with 100 and 300-325 mg ASA platelet aggregation increased significantly across age decades, in the case of the former dosage with all but the collagen inducer, while in the latter with all used inducers. Patients aged ≥80 years exhibited significantly higher aggregation indices than individuals aged less than 40 years ($p<0.001$) (Table 4-5).

Age (years)	<40 (n=62)	40-49 (n=263)	50-59 (n=673)	60-69 (n=824)	70-79 (n=683)	≥80 (n=124)	p value*
ADP 5 μM	58.6 (14.5)	61.8 (14.0)	62.2 (13.8)	63.2 (12.2)	64.1 (11.2)	66.6 (12.7)	<0.001
ADP 10 μM	63.6 (11.9)	66.6 (12.1)	66.6 (11.7)	67.6 (10.7)	68.2 (10.4)	68.4 (10.6)	<0.001
Collagen 2 μg/ml	47.4 (28.2)	47.2 (26.1)	45.8 (26.0)	46.2 (23.8)	45.1 (22.6)	48.0 (21.5)	0.122
Epinephrine 10 μM	45.9 (28.5)	45.8 (28.3)	45.0 (27.2)	48.6 (25.1)	49.3 (23.4)	55.8 (24.1)	<0.001

Table 4. Platelet aggregation induced with different stimulants in patients treated with 100 mg ASA stratified by age
Data are presented as means (SDs)
**Assessed linear trend across age*

Age (years)	<40 (n=21)	40-49 (n=73)	50-59 (n=213)	60-69 (n=254)	70-79 (n=171)	≥80 (n=28)	p value*
ADP 5 μM	53.9 (11.0)	61.0(11.6)	63.2 (12.1)	63.0 (10.7)	64.7 (10.3)	66.2(10.2)	<0.001
ADP 10 μM	61.8 (9.4)	65.1 (10.9)	68. 1(9.9)	68.2 (9.9)	68.5 (9.1)	70.3 (7.5)	0.005
Collagen 2 μg/ml	27.8 (17.4)	33.7 (23.2)	37.4 (23.6)	37.5 (21.6)	42.4 (20.0)	48.1(18.7)	<0.001
Epinephrine 10 μM	24.8 (15.9)	30.0 (18.9)	38.6 (23.8)	40.0 (20.8)	45.2 (22.1)	54.8(21.3)	<0.001

Table 5. Platelet aggregation induced with different stimulants in patients treated with 300-325 mg ASA stratified by age
Data are presented as means (SDs)
**Assessed linear trend across age*

At the same time we found no association between platelet aggregation and aging either in patients treated with 75 mg clopidogrel or with 500 mg ticlopidine (Table 6-7).

Age (years)	<40 (n=19)	40-49 (n=64)	50-59 (n=246)	60-69 (n=344)	70-79 (n=259)	≥80 (n=38)	p value*
ADP 5 µM	45.4 (24.8)	49.8 (23.3)	50.2 (21.4)	52.5 (20.3)	53.7 (19.5)	47.8 (21.1)	0.198
ADP 10 µM	46.4 (25.4)	53.9 (20.0)	55.1 (20.8)	56.0 (19.0)	57.7 (17.8)	54.3 (22.5)	0.470
Collagen 2 µg/ml	68.5 (19.6)	62.2 (23.1)	63.9 (22.0)	65.9 (18.2)	63.1 (21.1)	61.3 (21.2)	0.366
Epinephrine 10 µM	68.3 (32.8)	75.5 (22.8)	73.4 (22.4)	77.1 (19.6)	75.7 (19.4)	73.6 (16.9)	0.309

Table 6. Platelet aggregation induced with different stimulants in patients treated with 75 mg clopidogrel stratified by age
Data are presented as means (SDs)
**Assessed linear trend across age*

Age (years)	40-49 (n=28)	50-59 (n=108)	60-69 (n=118)	70-79 (n=94)	≥80 (n=13)	p value*
ADP 5 µM	47.9 (23.0)	45.2 (19.9)	42.3 (18.8)	44.6 (17.7)	45.5 (24.9)	0.709
ADP 10 µM	50.2 (22.5)	48.9 (22.4)	45.7 (18.8)	48.6 (18.3)	51.5 (21.7)	0.611
Collagen 2 µg/ml	73.2 (11.4)	65.0 (18.1)	60.7 (21.3)	62.0 (19.6)	58.0 (27.2)	0.420
Epinephrine 10 µM	80.1 (17.8)	77.4 (20.3)	74.2 (20.9)	76.7 (18.6)	71.7 (29.6)	0.503

Table 7. Platelet aggregation induced with different stimulants in patients treated with 500 mg ticlopidine by age decades
Data are presented as means (SDs)
**Assessed linear trend across age*

However, in the case of combination therapy with 100 mg aspirin and 75 mg clopidogrel the association between platelet aggregation and age was similar to that seen in patients treated only with ASA ($p<0.001$) (Table 8).

Age (years)	40-49 (n=37)	50-59 (n=88)	60-69 (n=93)	70-79 (n=79)	p value*
ADP 5 µM	40.2 (16.6)	45.9 (14.9)	47.6 (13.0)	52.4 (15.5)	0.001
ADP 10 µM	47.0 (17.6)	52.0 (15.7)	53.0 (14.1)	56.9 (15.3)	0.001
Collagen 2 µg/ml	28.4 (23.7)	32.7 (25.1)	33.0 (21.3)	33.0 (23.9)	0.566
Epinephrine 10 µM	36.7 (26.2)	41.7 (23.5)	48.3 (21.9)	52.9 (23.4)	0.001

Table 8. Platelet aggregation induced with different stimulants in patients treated with a combination of 75 mg clopidogrel and 100 mg ASA by age decades
Data are presented as means (SDs)
**Assessed linear trend across age*

Fibrinogen levels increased significantly across age decades in ASA treated patients, and significant difference was found between the oldest age group and patients aged less than 40 years ($p<0.001$). In patients treated with thienopyridines no association was found between fibrinogen levels and aging. Neither was any difference found between different age groups in patients taking ASA–clopidogrel combination therapy (Table 9).

Age (years)	<40	40-49	50-59	60-69	70-79	≥80	p value*
ASA 100 mg n=1873	3.26 (0.89)	3.46 (0.96)	3.50 (0.81)	3.61 (0.83)	3.70 (0.84)	3.84 (0.85)	<0.001
ASA 300-325 mg n=580	3.33 (0.69)	3.51 (0.63)	3.67 (0.79)	3.66 (0.79)	3.63 (0.78)	3.71 (0.68)	<0.001
ticlopidine 500 mg n=292	-	3.23 (0.64)	3.26 (0.90)	3.20 (0.79)	3.07 (0.63)	3,06 (0.93)	0.099
clopidogrel 75 mg n=345	-	3.50 (0.77)	3.62 (0.92)	3.53 (0.80)	3.47 (0.80)	3.58 (0.51)	0.533
ASA 100 mg + clopidogrel 75 mg n=153	-	3.64 (0.97)	3.31 (0.90)	3.61 (0.86)	3.65 (0.71)	-	0.059

Table 9. Fibrinogen levels in the case of different antiplatelet regimens by age decades
Data are presented as means (SDs)
**Assessed linear trend across age*

Fibrinogen levels were significantly lower in the case of clopidogrel and ticlopidine treatment than in patients treated with ASA ($p<0.001$), and ticlopidine treated patients had significantly lower fibrinogen levels than patients treated with clopidogrel ($p<0.001$). Individuals treated with a combination of ASA and clopidogrel exhibited fibrinogen levels which did not differ significantly from results detected in either ASA or clopidogrel monotherapy, but that were significantly higher than in the case of ticlopidine treatment (Figure 1).

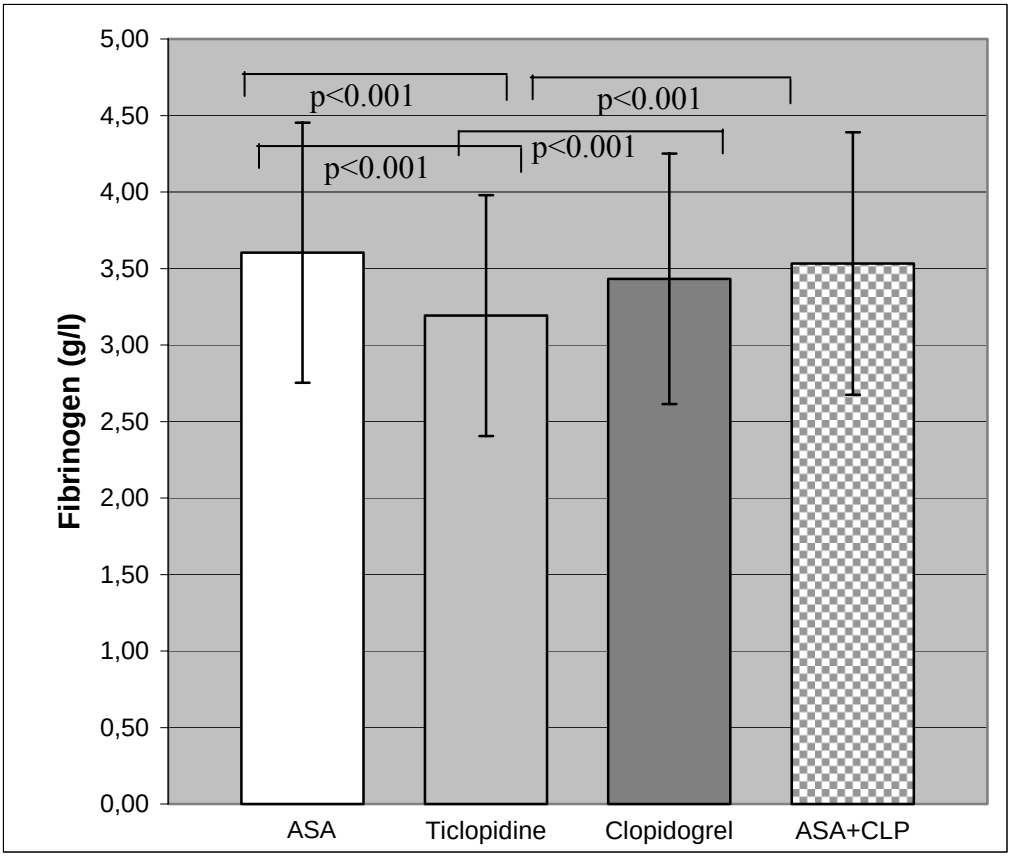

Figure 1. Fibrinogen levels in patients treated with different antiplatelet agents Data are represented as means ± SDs

4.4. Discussion

According to clinical investigations, antiplatelet therapies are not uniformly effective in all patients. There is a huge body of data available showing that 5-45% of patients treated with aspirin do not achieve the inhibitory response anticipated on the basis of different laboratory measurements of platelet activation and aggregation [98-103]. In vitro resistance to aspirin has been found to be significantly associated with an increase of arterial thrombotic events [104,105]. Resistance to thienopyridines both in vitro and in vivo has also been described [90,106]. Up to 5-11% of clopidogrel-treated patients were found to be non-responders, while 9-26% were semi-responders [107]. 8.7% of ticlopidine treated patients were found to be non-responders [108]. Very few data could be found on the association

between platelet aggregability, the efficiency of antiplatelet regimens and aging [109,110], in spite of the fact that elderly patients are known to be at an increased risk of cardio- and cerebrovascular events [89]. In a study of 3230 individuals without evidence of cardiovascular disease Tofler et al. found that fibrinogen, von Willebrand factor and measures of impaired fibrinolytic potential as plasminogen activator inhibitor and tissue plasminogen activator antigens were significantly associated with advancing age indicative of a prothrombotic state [92]. Significantly reduced spontaneous thrombolytic activity has also been proved in older patients [93,111]. Similarly to these results an age associated increase was found in fibrinogen level in ASA treated patients in our present study. Fibrinogen has been identified as an important risk factor of cardio- and cerebrovascular events which plays an important role in platelet aggregation [91,112,113]. Age associated increase in fibrinogen level may play a crucial role in increased platelet aggregation in elderly patients found in this study. Independence of fibrinogen levels of age in both clopidogrel and ticlopidine treated patients might be contributed to the beneficial effect of these drugs on fibrinogen levels [114] also seen in our study. Platelet aggregability may be negatively influenced by several factors in the elderly. Elder patients tend to have more co-morbidities and take more drugs that raise the potential of drug interactions. NSAIDs have been shown to interact with aspirin's antiplatelet effect. A higher incidence of chronic heart failure may contribute to the decrease in aspirin's antiplatelet effect in the older population, as the prevalence of aspirin resistance in this population has been shown to be more than double than in patients with stable general vascular disease [111]. A higher prevalence of other chronic diseases as diabetes, hypertension and dyslipidemia may further increase the propensity for increased platelet activation and aspirin resistance [115]. An other possible cause of increased *in vitro* platelet aggregation in older people may be due to long-lasting aspirin treatment, as this has been

shown to be associated with a progressive reduction in platelet sensitivity to this drug [108,116,117].

Aspirin is a potent inhibitor of collagen and epinephrine induced platelet aggregation in *in vitro* tests, but has only a moderate effect on ADP induced platelet aggregation. Therefore, interindividual differences in ASA sensitivity lead to a wider distribution of values in the case of collagen and epinephrine inducers, hence the larger standard deviation in these cases. The present study has a number of limitations. This was a non-randomized, observational study. Several factors that may further explain the differences observed in platelet aggregation and fibrinogen levels in the different age and drug groups (plasma lipid levels, concomittant medication, case history) were not investigated. The type and dosage of antiplatelet agent(s) taken were documented based on medical records and interview. However, compliance was not checked with direct methods. Further tests exist to investigate platelet aggregability (urinary thromboxane measurements, arachidonic acid induced aggregation etc.) that were not used in this study and might lead to different results. Data of patients less than 40 and more than 80 years could not be evaluated in the case of certain regimens because of low number of cases. Prospective, randomized further examinations are needed to confirm our results in a multivariate analysis.

4.5. Conclusions

Effective antiplatelet therapy is of particular importance in older patients being at increased risk of vascular events. Age-related increase in platelet aggregability may contribute to higher risk of cardio- and cerebrovascular diseases in this age group. Therefore, effective antiplatelet therapy in the elderly should be more carefully checked and perhaps individually determined.

Elevation of fibrinogen levels that occurs with aging may contribute to the observed increase in *in vitro* platelet aggregation in ASA treated patients.

5. Investigation of seasonal differences in hemorheological parameters and platelet aggregability and their association with meteorological factors

5. 1. Patients and methods

Patients

Between 2001 and 2005, platelet aggregation was checked in our laboratory in 2693 patients (1417 males, 1276 females, age: 62.9 ± 11.2 years) with clinical evidence of vascular disease receiving a daily dose of 100 mg aspirin. Platelet aggregation tests were performed to analyze whether the chosen antiplatelet medication was effective. Patients were under antiplatelet therapy for at least 4 weeks at the time of testing. In a subgroup of 1873 patients fibrinogen level was measured by the method of Clauss. In 1260 patients plasma and whole blood viscosity and hematocrit were also tested. In a third subgroup of 724 patients we assessed erythrocyte aggregation. Patients are resindents of an area within 40 km-s from the local station of the Hungarian Meteorological Service.

Materials and methods

Hematocrit and erythrocyte aggregation, whole blood and plasma viscosity were assesed as described previously (page 16-17). Platelet aggregation measurements were performed as described on page 27 using 5 and 10 μM ADP, 2 μg/ml collagen and 10 μM epinephrine inducers.

External temperature at 8 a.m., daily maximal and minimal temperatures, air pressure at 8 a.m. and 10 a.m., relative humidity and daily amount of precipitation were assessed in the local station of the Hungarian Meteorological Service. Daily temperature variation (difference between daily minimal and maximal temperatures) was assessed. Difference between air

pressure at 8 and 10 a.m. (Δp) was calculated as an indicator of meteorological front activity at the time-point of sampling.

Statistical analysis

Continuous variables are presented as means ± SD. The Kolmogorov-Smirnov test was used to investigate normal distribution of data. Data were divided into 12 groups according to the months of the year based on the time-point of the examination. To compare means of data in different months we used one-way ANOVA. Peak and trough monthly values were compared by Student's t-test or the Mann-Whitney U test. Pearson's linear correlation coefficients were calculated between variables in the case of normally distributed data, Spearman's correlation was assessed in the case of non-normal distribution. A p value <0.05 was considered statistically significant. Analyses were performed using SPSS 11.0 (SPSS Inc. Chicago, Ill.) and Microsoft Excel.

5.2. Results

Similarly to other investigators we found significantly higher fibrinogen levels in the winter than in the summer with a peak in December and a trough in July ($p<0.01$) (Figure 2).

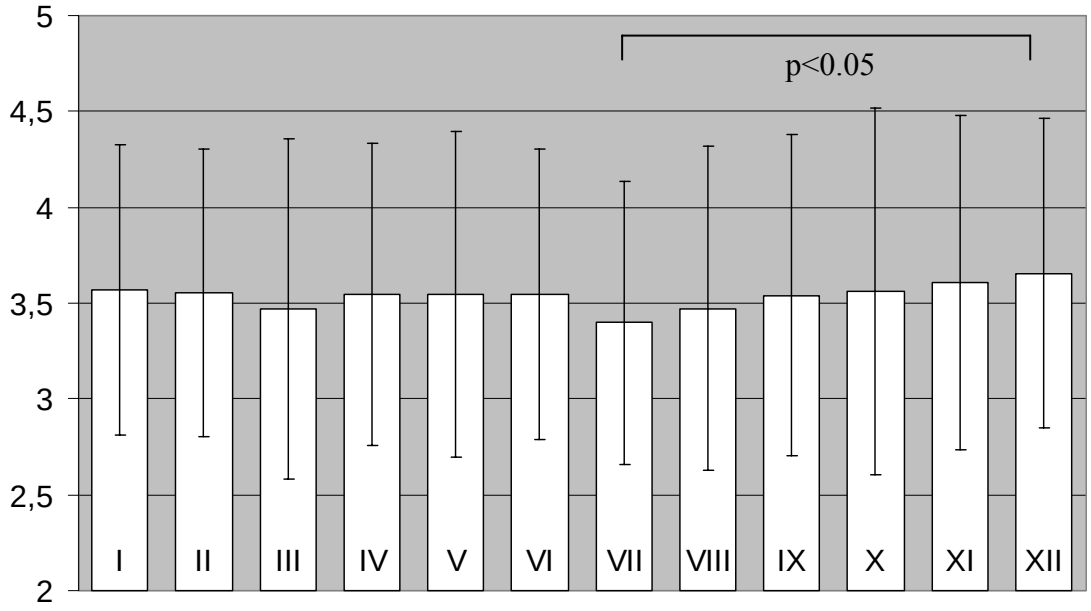

Figure 2. Monthly alterations in plasma fibrinogen levels

Both the M0 and M1 indices proved to be significantly higher in the winter than in the summer months ($p<0.0001$) with a peak in December (M0) and January (M1) and a through in July and June, respectively (Figure 3-4).

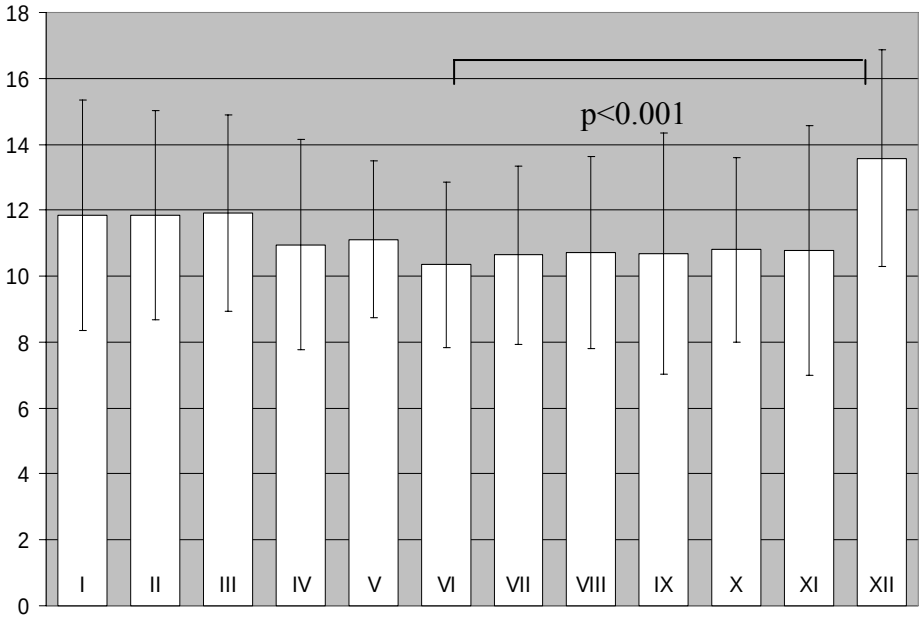

Figure 3. Seasonal changes in M0 index

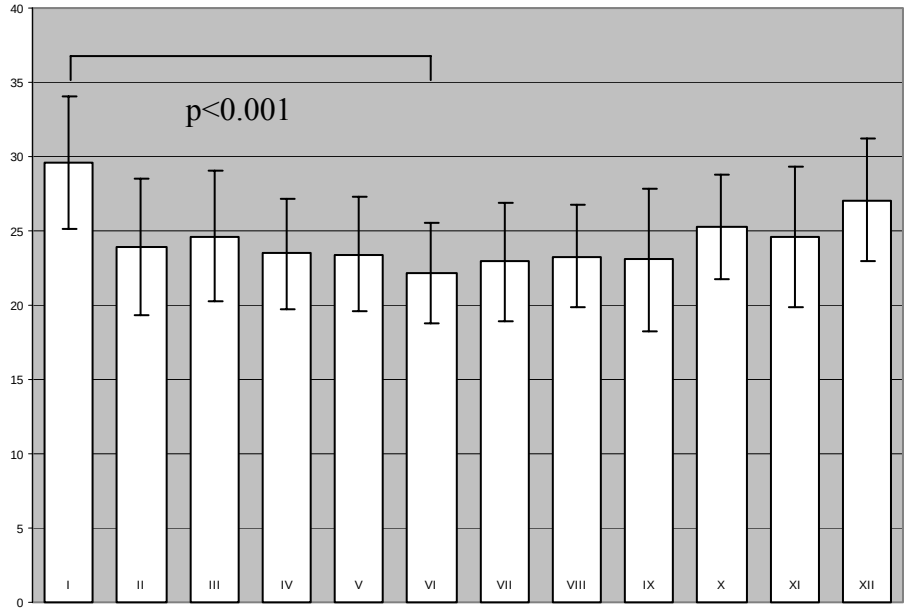

Figure 4. Seasonal changes in M1 index

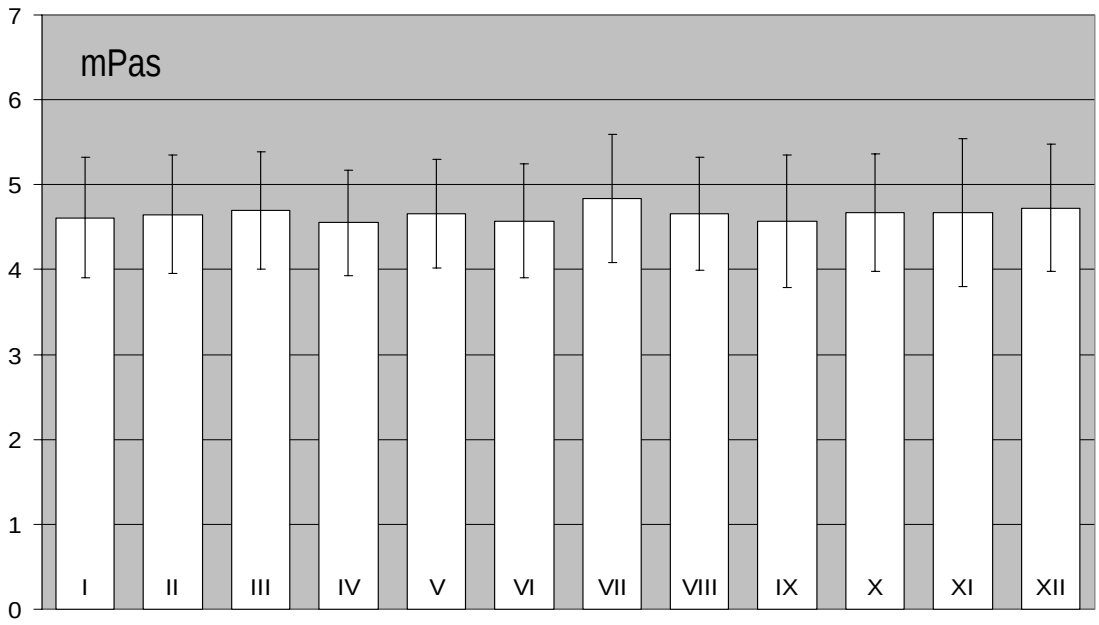

Figure 5. Monthly changes in whole blood viscosity

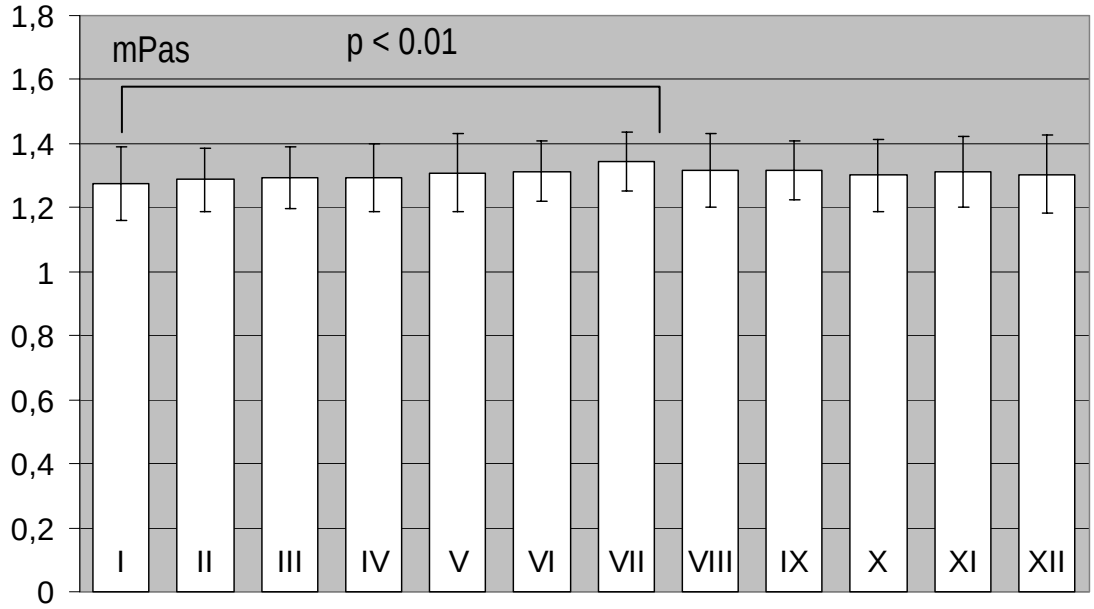

Figure 6. Monthly changes in plasma viscosity

Differences between winter-and summer results of whole blood viscosity measurements were not significant. Plasma viscosity showed significantly higher values in the hottest months than in the winter (Figure 5-6).

Epinephrine-induced platelet aggregation was highest in April and May, while its minimum was in August and September ($p<0.001$) (Figure 7).

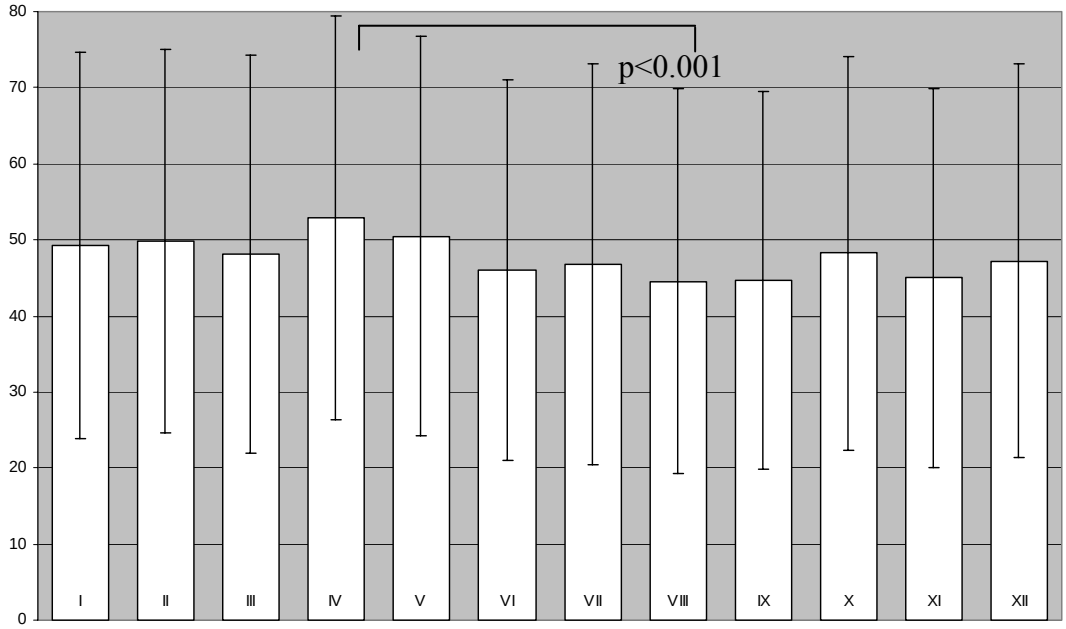

Figure 7. Seasonal changes in epinephrine induced platelet aggregation

Significant negative correlation was found between external temperature measured at 8 a.m. as well as daily minimal and maximal temperatures and M0 and M1 indices ($p<0.001$) (Figure 9). Plasma viscosity showed a positive correlation with daily minimal temperatures ($p<0.001$). A weak, but statistically significant correlation was found between platelet aggregation induced with ADP 10 and all examined temperature parameters ($p<0.01$), however, no significant association was found with either the smaller dose of ADP inducer or with the other applied inducers. Fibrinogen and hematocrit exhibited a weak negative association with ambient temperature ($p<0.05$). Neither of the examined hemorheological and platelet aggregation parameters were associated with daily temperature variability or with changes in

air pressure between 8 and 10 a.m. Air pressure measured at 8, 9, and 10 a.m. showed a significant positive correlation with M0 ($p<0.001$) and M1 ($p<0.01$) indices. Plasma- and whole blood viscosity were negatively correlated to air pressure ($p<0.01$ and $p<0.05$, respectively). Daily precipitation and humidity at 8 and 10 a.m. were not associated with any examined hemorheological or platelet aggregation parameters.

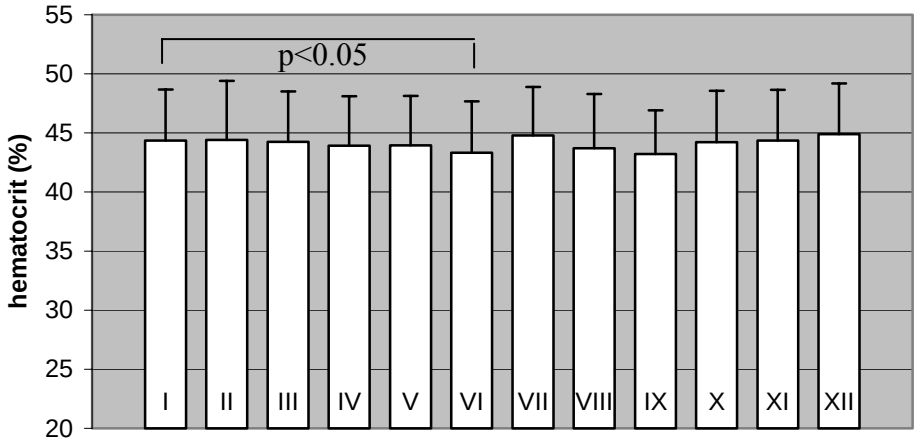

Figure 8: Monthly variation of hematocrit

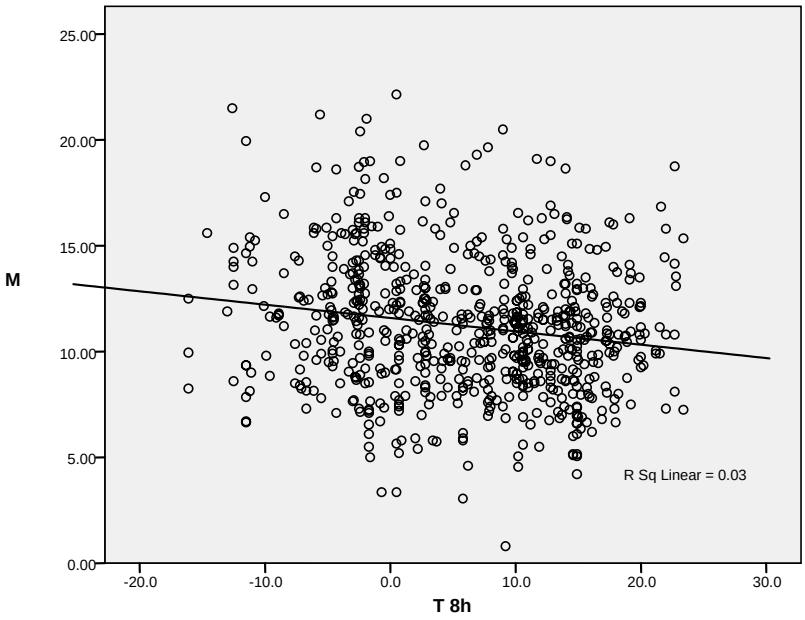

Figure 9: Association between ambient temperature and red blood cell aggregation

5.3. Discussion

Higher cardio- and cerebrovascular mortality in the winter in areas of the world where ambient temperatures show huge seasonal differences has been observed in large-scale analyses [24,27]. Exogenous factors such as climate, diet, activity levels, heating and air conditioning, upper respiratory infections have been described to influence directly or indirectly the homeostasis and have an effect on hemorheological and hematological rhythms [118,119]. Some authors have suggested that an acute phase response triggered by upper respiratory tract infections was responsible for the higher incidence of acute coronary syndromes during the winter months [36]. However, data on seasonal variation of major acute-phase reactant CRP levels are contradicting. In two studies, seasonal variation of CRP was investigated on an intraindividual basis. Woodhouse el al. reported higher CRP concentrations in winter with a peak in March [36]. Crawford et al. found a significant seasonal variation of CRP with a peak in late February, but detected no seasonal rhythm in white cell count or IL-6 [120]. No evidence for seasonal variation of CRP was found in large populations in a study by Fröhlich et al [29] and an investigation by Stout et al. in elderly people [121]. This data in a way contradicts results showing a seasonal pattern in a variety of acute-phase proteins as PAI-1, plasminogen or fibrinogen [38], as also seen in the present study for the latter. However, in contrast to CRP, coagulation proteins are not exclusively related to the acute phase response.

Fibrinogen is an independent risk factor for cardiovascular diseases [122]. Fibrinogen levels showed a high-amplitude yearly variation in a study by Maes et al. [33]. Seasonal differences in fibrinogen levels have been described to be higher in older age groups [121,123,124]. In contrast to previous investigators, differences between winter- and summer fibrinogen levels were relatively small in our study. This might be the consequence of a younger mean age of

patients in our study. Also, patients in our investigation were homogenously treated with a 100 mg daily dose of aspirin that might influence seasonal patterns in fibrinogen levels. We found a weak negative correlation between fibrinogen levels and both daily minimal and maximal temperature values as well as temperature measured at 8 a.m. This finding is in concordance with the results of Stout et al., they found that core body temperature showed a statistically significant seasonal variation and a peak corresponding with those for ambient temperatures, fibrinogen and MPV [30]. Maes and De Meyer reported seasonal rhythms in MPV and fibrinogen together with their association with climatic data [33]. They propose that the seasonal rhythms observed in immune and hematologic variables may be entrained by the seasonal rhythms in ambient temperature [58].

A strong seasonal variation was found for plasma viscosity with a maximum in January and a minimum in July in a study by Fröhlich et al. [38]. Significant, however, less pronounced seasonal differences for whole blood viscosity were also found by the same group. Otto et al. found a significant reduction in native blood viscosity in their study of 14 healthy, middle aged individuals over 6 months. However, they found no alterations concerning hematocrit, plasma viscosity and fibrinogen concentration [125]. Opposite to these results, we found a statistically significant but clinically minor difference between winter and summer values with a peak in July and a through in January in plasma viscosity values that might be indicative of inadequate fluid intake in the summer months in the examined population. Ambient temperature was found to be negatively correlated to plasma viscosity and erythrocyte deformability of *ex vivo* samples in a study by Cinar et al. [126]. However, in the present study neither plasma nor whole blood viscosity were associated with ambient temperature. Surprisingly, we observed a weak negative correlation between whole blood and plasma viscosity and air pressure measured at 8 and 10 a.m. We suspect that ambient air

pressure might have a minor influence on the result of capillary viscosimetry, however, this theory needs further investigation.

Hematocrit has been found to show a „group-seasonal" effect in the population in general. However, the results of studies from different geographical areas indicate a climatic effect: a study from Iceland did not show any significant effect [127], while a study from Israel showed significantly lower hematocrit levels in the summer [128]. A third, large-scale study from the U.K. showed a „slight seasonal trend" [124]. Our results are in accordance with this pattern, as we found a statistically significant, but clinically minor difference between winter and summer values.

Hemoglobin levels were significantly associated with the maximum daily temperature in a study by Hoekstra et al. of 130000 blood donors in the Netherlands [129]. In a recent paper Sebok et al. examined the extent to which environmental temperature contributes to the seasonal trend in deferral rates of presenting donors due to low hematocrit at the American Red Cross Blood Services in a very large population. Hematocrit deferral rates showed a strong seasonal trend with a peak in the summer months and demonstrated a strong association with environmental temperature, which accounted for 77 percent of the variation in hematocrit deferral rates [130]. The area with the highest temperature variability during the year demonstrated the strongest relationship between Htc deferral rate and temperature, which is in concordance with the European results [124,127,129]. Our study showed a statistically significant but weak negative correlation between ambient temperature and hematocrit which is in accordance with these results, as Hungary is located in the continental climate zone characterized by relatively high annual temperature variability. Decrease in hematocrit during the summer might be explained by hemodilution occurring in warm weather due to increased plasma volume [131]. Also, significant hemoconcentration as a response to extreme cold has been described in male Antartic workers with a mean hematocrit value of 52.5 % at -38 °C

and 48.0 % at 0 °C [132]. These results support the finding that blood and plasma play an important role in thermoregulatory responses [133].

In our study both M0 and M1 indices showed significant differences between summer and winter values. This is in contrast with the results of Fröhlich et al. who found no statistically significant seasonal alteration in red blood cell aggregation. The contrast between results may arise from differences in the study population: Fröhlich et al. studied 16 young (20-41 years old), healthy subjects, while our patients were substantially older and suffering from vascular diseases. Fibrinogen, an important determinant of red blood cell aggregation has been shown to exhibit an age-dependent seasonal rhythm present in the elderly but lacking in young individuals [123,134]. We suspect that seasonal variance in red blood cell aggregability may follow a similar pattern in different age groups. To our knowing this is the first study to show a negative correlation between ambient temperature and red blood cell aggregation. However, the exact mechanism by which outdoor temperature influences the result of red blood cell aggregometry is unknown.

Epinephrine-induced platelet aggregation also showed a seasonal fluctuation in the present investigation. We are unaware of any previous study to show a circannual variation in platelet aggregability in ASA treated vascular patients. Previous studies have shown significant seasonal variation in mean platelet volume and platelet distribution width [33]. Von Willebrand factor has also been proved to show seasonal variation [33,134]; similarly to our results, it peaked in April while it reached its minimum in October. Platelet aggregation induced with 10 μM ADP showed a very weak association with outdoor temperature in our study, but this result is questionnable as neither aggregation induced with the smaller dose of ADP nor that induced with collagen and epinephrine showed similar correlations.

Meteorological front activity can be characterized by rapid changes in air pressure. As neither positive nor negative association was found between the examined hemorheological and

platelet aggregation parameters and the extent of changes in air pressure at the time point of sampling, cold or warm fronts do not seem to have an instant effect on these parameters. On the other hand, it needs further examinations whether meteorological fronts have a delayed effect on these variables.

Diet may substantially influence several hemorheological parameters and platelet aggregation [135,136]. Winter-related difficulties in achieving weight loss relative to the summer have been described [137]. Small seasonal fluctuations were observed in body weight and time spent on various physical activities [39], factors that have been shown to be related to hemorheological factors and platelet aggregability [138,139]. It has been shown both cross-sectionally and longitudinally that relatively higher levels of physical activity are associated with lower levels of fibrinogen, CRP, vWF, t-PA and platelet aggregability. Seasonal variations in physical activity may thus contribute to the seasonal variations observed.

Seasonal variation in intake of fat and vitamin C and E was found in a study by Woodhouse et al [40]. Fat intake peaked in the winter while vitamin C and E intakes reached their highest level in summer.

Tseng et al. detected seasonal patterns in monthly hemoglobin A_{1C} values in a large cohort of diabetic patients with higher levels in the winter than in the summer. Regions with colder winter temperatures had larger winter-summer contrasts than did those with warmer winter temperatures [41]. Previous studies of populations and healthy volunteers have demonstrated fluctuations in glucose and insulin sensitivity, with higher levels in the fall or winter in most studies [42,43,44]. Seasonal metabolic alterations, differences in food intake and physical activity may play an important role in the observed seasonal differences of hemorheological parameters and platelet aggregability in our study, however, further investigation is needed to prove this theory.

5.4. Conclusion

Our study showed that certain hemorheological parameters and platelet aggregability exhibited seasonal differences in aspirin treated vascular patients. The observed differences might contribute to the seasonal fluctuation in the mortality of cardiovascular diseases known from the literature. Our results might also be of importance in the case of longitudinal investigations when consecutive samplings take place in different periods of the year. In these cases seasonal alterations may interfere with study results. Our results support the theory that beside seasonal variations in diet and physical activity, annual fluctuation in ambient temperature may contribute to seasonal variation in certain hemorheological parameters. Meteorological front activity do not seem to have an immediate effect on the examined parameters.

6. List of abbreviations

ADP - adenosine-5'-diphosphate
ASA - acetylsalicylic acid
BMI- body mass index
CRP - C-reactive protein
Coll - collagen
Epi - epinephrine
IGT - impaired glucose tolerance
NGT- normal glucose tolerance
NSAID - non-steroid anti-inflammatory drug
OGTT - oral glucose tolerance test
PAI-1 - plasminogen activator inhibitor-1
PPP- platelet poor plasma
PRP- platelet rich plasma
RBC - red blood cell
S.E.M.- standard error of the means
Spont. aggr. - spontaneous aggregation
sP-selectin - soluble P-selectin
t-PA - tissue plasminogen activator
vWF-von Willebrand factor

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