Evaluation of Specific Methods in Hemorheology and Angiology

Ph.D. dissertation

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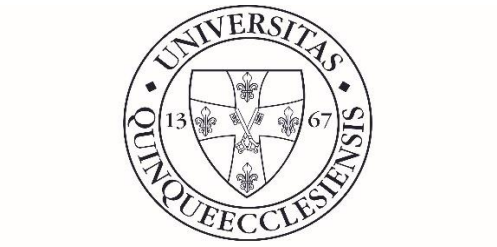

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2018

List of abbreviations

6MWT	6-minute walk test
ABI	ankle-brachial index
ACC	American College of Cardiology
AHA	American Heart Association
BMI	body mass index
CLI	critical limb ischemia
CTA	computed tomography angiography
CV	coefficient of variation
DPA	dorsal pedal artery
DSA	digital subtraction angiography
DUS	Doppler ultrasonography
EDTA	ethylenediaminetetraacetic acid
IC	intermittent claudication
LDF	laser Doppler flowmetry
LEAD	lower-extremity arterial disease
MWD	maximum walking distance
NA	not applicable
NO	nitric oxide
p	significance level
PAD	peripheral artery disease
PFWD	pain-free walking distance
PTA	posterior tibial artery
PU	perfusion unit
PV	plasma viscosity
RBC	red blood cell
ROC	receiver operating characteristics
SD	standard deviation
SLI	severe limb ischemia
TBI	toe-brachial index
$tcpO_2$	transcutaneous partial oxygen pressure
WBV	whole blood viscosity

1. Prologue

1.1. Importance of clinical hemorheology and blood viscosity

Hemorheology is the science of flow properties of blood and its elements. Clinical hemorheology describes the unique behavior of blood with such measurable parameters like hematocrit, plasma and whole blood viscosity, red blood cell aggregation and deformability. Numerous vascular, hematological and pulmonary diseases are associated with increased plasma (PV) and whole blood viscosity (WBV), both identified as primary cardiovascular risk factors as well as variables with prognostic significance in certain clinical conditions. The blood plasma behaves as a Newtonian fluid (its viscosity does not depend on shear rate) while the whole blood behaves as a non-Newtonian pseudoplastic fluid. WBV is an important determinant of blood flow resistance, its elevated level may cause a disturbance in tissue perfusion due to decreased flow rate. Plasma viscosity is an important factor of flow resistance in the microcirculation, mediating shear stress toward the endothelium as a consequence of the axial migration of RBCs, therefore it plays a role in the mechanism of vasodilation. The altered hemorheological variables in association with atherosclerosis are elevated plasma and whole blood viscosity, plasma fibrinogen concentration and impaired red blood cell deformability.

1.2. Relevance of peripheral artery disease

Peripheral artery disease is a clinical manifestation of atherosclerosis of the abdominal aorta and arteries of the extremities with a high prevalence (3-10%). Pathologically altered arteries contribute to several clinical conditions: asymptomatic disease, intermittent claudication, atypical leg pain, acute and chronic critical limb ischemia. To estimate the prevalence of asymptomatic disease among the general population, hand-held Doppler ultrasound is widely used. The derived ankle-brachial index (ABI) ≤ 0.9 as a hemodynamic criterion of PAD is generally accepted. The typical symptomatic manifestation of PAD is termed as intermittent claudication (IC): the exertional leg pain does not exist at rest, it involves the calf causing reduction or stoppage of walking and it relieves by rest (Rose criteria). The prevalence of IC increases with age (2% in age group 50-54 vs. 6% in age group 65-69) and there is a higher prevalence in men than in women. The pitfall of searching typical IC is the fact that most patients with PAD do not have typical symptoms. These patients have one or more of the following: exertional leg pain that are not met the Rose criteria of IC (atypical leg pain), exertional leg pain with walking through phenomenon, leg pain at rest and asymptomatic PAD. Identifying of latter patients' groups is very challenging as well as the underlying causes of asymptomatic PAD can be diverse: peripheral neuropathy (silent ischemia in diabetic patients), sedentary lifestyle, habitual slow walking speed to avoid exertional pain and truly asymptomatic PAD.

Regardless of the symptomatology, PAD is a progressive disease without early diagnosis and treatment: the end-stage manifestation, namely critical limb ischemia (CLI) and major amputation can develop in 5-10% and 1-2% of cases over 5 years, respectively. CLI is the end-stage manifestation of PAD with a 1-year outcome to amputation in 30% of cases.

2. Focuses

2.1. Viscometer validation studies for routine and experimental hemorheological measurements

Despite the usefulness of viscosity measurement in various clinical cases, blood viscosity is not a routinely measured macrorheological parameter because of its troublesome implementation: 1. Whole blood is a non-Newtonian fluid, its viscosity is shear dependent, thus one viscosity value is insufficient to characterize a sample, therefore a shear rate – viscosity profile should be attained. This profile is affected by several factors including RBC aggregation and deformability. 2. Blood plasma is a Newtonian fluid, therefore it can be more easily measured, although in certain clinical conditions (e.g. hematological disorders), measurement settings or surface film artifacts may alter the results. 3. Viscometers, depending on their construction, are sensitive to artifacts to different extents; moreover, device-specific artifacts may also be present.

Our laboratory has recently acquired a Brookfield DV-III Ultra LV programmable rotational viscometer *(Brookfield Engineering Labs; Middleboro, USA)*. Starting to work with a different type of instrument is always challenging due to the above-mentioned reasons. Before any experimental or clinical measurements can be done, several calibration and validation measurements are required.

2.2. Toe-brachial index and exercise test can improve the exploration of peripheral artery disease

All epidemiological data highlighted the importance to identify patients with PAD with appropriate staging. Though angiography is considered as a gold standard diagnostic procedure, its use is limited because of its invasive nature as well as the application of ionizing radiation and potentially nephrotoxic contrast agent. Exercise testing has been almost neglected in the diagnostic evaluation of peripheral artery disease, although some consensus guidelines and scientific papers mentioned its role in the diagnostic approach of vascular patients. Nevertheless, there is no evidence on its routine application or it is limited to assess improvement in claudicants and/or to differentiate vascular claudication from neurogenic one.

We hypothesized that ABI at rest was not a sufficient parameter in the staging of lower-extremity arterial disease (LEAD). Our diagnostic procedure was set up including ABI, toe-brachial index (TBI) and transcutaneous partial oxygen pressure ($tcpO_2$) measurements before and after exercise provided by a well-trained technician in a vascular laboratory. Our aim was to evaluate these non-invasive methods and the impact of exercise testing on their sensitivity in PAD patients and control subjects.

3. Methodology

3.1. Capillary viscometer

Various types of instruments have been developed to measure viscosity, though capillary and rotational viscometers are the most frequently used ones.

The Hevimet 40 capillary viscometer *(Hemorex Ltd.; Budapest, Hungary)* consists of a capillary connected to a vertical glass tube surrounded by high specific heat capacity oil maintaining stable 37°C temperature. Next to the vertical tube 40 diodes are set which register the height of the fluid column against time. Shear stress and shear rate are calculated from intrinsic attribution of viscometer (tube length and radius) and from the flow velocity of the injected fluid (pressure drop, flow rate). The injected sample is exposed to a range of shear stress therefore the software only calculates the apparent viscosity and then it is inter/extrapolated between 10-240 s^{-1} by the application of Casson equation. The values are displayed by the measurement program. 620 µl of blood is injected into the system and released to flow out.

3.2. Rotational viscometer

The Brookfield rotational viscometer is equipped with a cone-plate configuration, using a CP40 spindle. 500 µl sample size is required for a single measurement. The cone and plate are two concentric surfaces: the gap between them must be precisely adjusted to achieve ideal chamber geometry. The cone rotates at a constant speed generating shear rate and measures shear stress simultaneously. Shear stress is determined by the measured torque and the geometry. Every viscosity values are directly obtained as a single data at a given shear rate. The useful shear rate-range is between 50-600 s^{-1}, depending on blood viscosity. The operating temperature is maintained by an external circulating bath *(TC650-MX, Brookfield Engineering Labs; Middleboro, USA)*. Samples are pre-incubated in the external bath before injecting the samples into the instrument.

3.3. Hand-held Doppler ultrasound and ankle-brachial index

According to several consensus guidelines, the first-line non-invasive method to detect peripheral arterial flow as well as to diagnose lower-extremity artery disease is the hand-held continuous-wave Doppler ultrasound. It was operated with an 8 MHz probe and a manual sphygmomanometer to measure systolic blood flow in posterior tibial and dorsal pedal artery of both legs as well as in the brachial artery of both arms following the same sequence of measurement (counterclockwise right arm – right ankle – left ankle – left arm). To calculate the ankle-brachial index (ABI), the higher systolic blood pressure between both arms was used as the denominator while the higher pressure from the posterior tibial and dorsal pedal arteries at each ankle was considered as the numerator. The threshold of ABI value for diagnosing LEAD was ≤0.9. ABI 0.9-0.71 was considered as mild, 0.7-0.41 as moderate and ≤0.4 as severe LEAD while ABI >1.3 was regarded as false high value due to media sclerosis; ABI between 0.91-1.00 was considered as borderline. The ankle-brachial index has great reliability and validity to detect stenosis ≥50% in lower limb arteries compared with angiography but a recent review reported that specificity of ABI ≤0.9 ranged from 83% to 99% while sensitivity ranged from 15% to 79%.

3.4. Laser Doppler flowmetry, toe pressure and toe-brachial index

Laser Doppler flowmetry (LDF) is a non-invasive, real-time method based on Doppler-effect to detect and measure blood flow in nutritive and thermoregulatory capillaries. LDF instrument *(PeriFlux System 5000, Perimed, Stockholm, Sweden)* uses optical fibers (distance between fibers: 0.25 mm) to carry (illuminating fiber) and to detect (detecting fiber) laser light (wavelength 780 nm). An attached computer displays the continuous wave in function of Doppler shift which is proportional with the amount and the velocity of moving red blood cells. The measured tissue volume is small and the total local blood perfusion is detected including capillaries, arterioles, venules and shunts. The LDF probe was attached to the skin by a double-sided adhesive tape provided by the manufacturer. The detected flux signal is expressed as perfusion unit (PU) which is a manufacturer dependent arbitrary unit. PU is calculated by the sum of the number of moving red blood cells in the given volume and the mean velocity of moving red blood cells. Several functional tests can be conducted with LDF to evaluate skin perfusion and microcirculatory blood flow: thermal challenge, linear pressure deflation and post-occlusive reactive hyperemia. Performing linear pressure deflation, the toe pressure can be measured. The toe-brachial index (TBI) can be calculated dividing the absolute toe pressure (nominator) by the higher systolic blood pressure between both arms (denominator). Although the application of TBI is recently limited to cases with vessel stiffness, several guidelines consider TBI $\leq$0.70 as the cut-off value for diagnosing PAD while $\leq$0.25 is used to identify the severe PAD.

3.5. Transcutaneous partial oxygen pressure measurement

Transcutaneous partial oxygen pressure measurement ($tcpO_2$) is a non-invasive, real-time monitoring electrochemical method to detect oxygen concentration of tissues and to assess the function of microcirculation of the related tissues. The $tcpO_2$ device consists of a main computing unit and several oxygen sensors with Clark-type electrodes. The sensors are placed on the skin surface by a self-adhesive probe holder (fixation ring) provided by the manufacturer. Between the sensor and the skin surface there are some drops of contact liquid in the fixation ring as a medium which ensures as well as facilitates the diffusion of oxygen molecules from the tissue towards to the electrode. The sensors are made of an oxygen-permeable membrane and a platina-silver electrode while between them there are a phosphate puffer solution. The continuous polarizing voltage generates electrical potential differences which is proportional with the oxygen concentration and the partial oxygen pressure of the related tissue. This value is displayed then by the main unit in mmHg. To achieve maximal vasodilation and increase the permeability of the related skin surface to oxygen, the sensor is heated up to 44°C. The detected diffused oxygen molecules are originated from the nutritive capillaries of the measuring site therefore this method ensures a real-time information about the oxygen supplies of related tissue. There is a consensus guideline to assess the measured $tcpO_2$ values: on the foot >50 mmHg is considered as physiologic, <40 mmHg is viewed as impaired value or hypoxia while $tcpO_2$ <30 mmHg is viewed as a threshold for the diagnosis of severe PAD (at normobaric air). This method can also be completed by several functional tests to enhance its sensitivity (elevation or depression of lower limb, breathing hyperbaric oxygen, local subcutaneous injection of pharmacological agent).

4. Viscometer validation studies for routine and experimental hemorheological measurements

4.1. Study design

4.1.1. Subjects, blood samples

Blood was obtained by sterile antecubital venipuncture into EDTA (6.0 mL/ 10.8 mg) containing Vacutainer® tubes from healthy, non-smoker volunteers between the ages of 18 and 40 in the early morning. The measurements were performed at native hematocrit values. Prior to each measurement, the samples were incubated for 5 minutes in the external bath (T= 37°C) and after injecting 500 µl of it into the chamber, for further 3 minutes (T= 37°C) at 50 s^{-1} constant shear rate.

4.1.2. Torque stability time

The viscosity of a Newtonian calibration fluid was measured at 5, 10, 25, 50, 100, 150 and 200 s^{-1} shear rates. The shear rate was rapidly increased to the desired value from zero while the measurement program obtained viscosity value in every second. Stability was considered when the fluctuation of the value stopped.

4.1.3. Temperature effect

6 ml of blood from 8 donors (4 females, 4 males) was used to measure WBV at 50, 75, 100, 200 and 400 s^{-1} shear rates. The system was cooled down to 20°C, the cone-plate distance was adjusted, sample was injected. Then the system was heated up to 40°C without the alteration of calibration or change of sample (the sample was sheared constantly at 50 s^{-1} to avoid sedimentation of RBCs), then WBV was measured again. After that the sample was removed, the chamber was cleaned, the geometry was re-set at 40°C, a new sample was injected and viscosity values were acquired.

4.1.4. Reproducibility

From 7 volunteers (1 female, 6 males) 30 ml of blood was drawn. 10 replicate WBV (at 50, 75, 90, 100, 150, 200, 300, 500 s^{-1} shear rates) and PV (at 500 s^{-1} shear rate) measurements were carried out on each sample. After each measurement, the sample was immediately replaced with a new one from the same blood pool, gently mixed before being injected into the system.

4.1.5. Storage

30 ml of blood from 9 donors (2 females, 7 males) was collected to perform WBV (at 50, 100, 200, 500 s^{-1}) and PV (500 s^{-1}) measurement at the following time points and temperatures: baseline (22°C), 2 hours (22°C), 3 hours (37°C), 4 hours (22°C), 6 hours (37°C), 8 hours (4 and 22°C), 24 hours (4°C) and 48 hours (4°C). Prior to each measurement, the samples – pending their testing in vertical position – were gently mixed.

4.1.6. Comparison

Comparison studies were carried out to compare the Brookfield DV-III Ultra LV and Hevimet 40 viscometers. 12 ml of blood from 26 donors (9 females, 17 males) was drawn, WBV at 50, 100, 150, 200 s^{-1} and PV at 500 s^{-1} shear rates were measured within 2 hours from sampling.

4.2. Results

4.2.1. Torque stability

Torque stability is demonstrated at 10 and 200 s^{-1} shear rates (Figure 1). Because of oscillation, the device required 8 seconds at 10 s^{-1} and 10 seconds at 200 s^{-1} to achieve stable viscosity values.

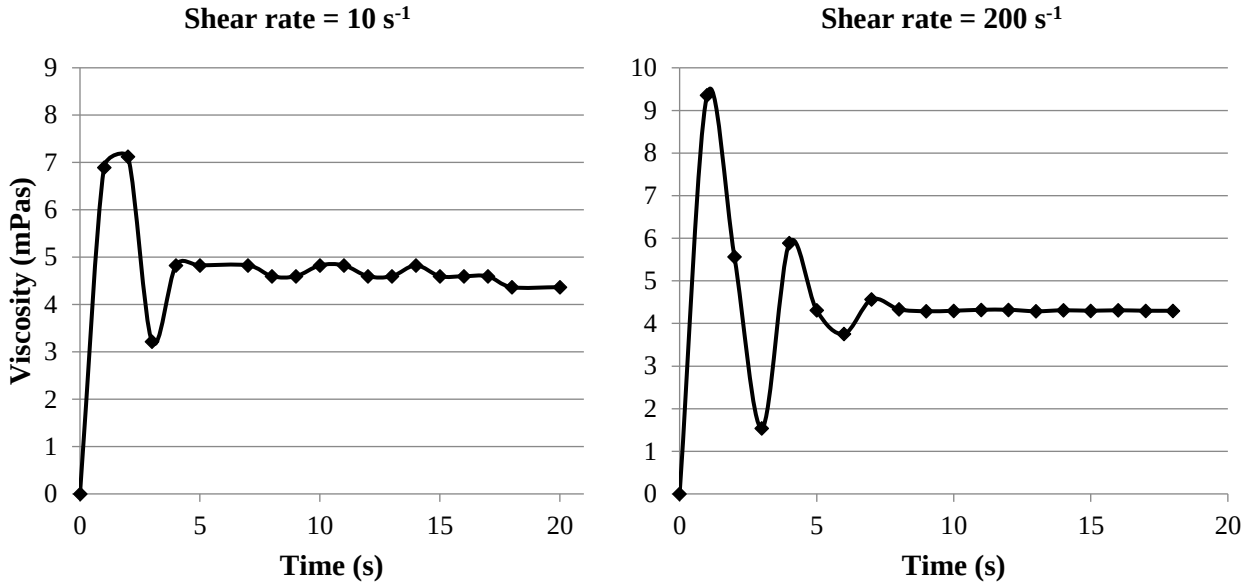

Figure 1. Time to torque stability at 10 and 200 s^{-1} shear rates (37°C).

4.2.2. Temperature effect

The observed viscosity values with unchanged and re-set cone-plate distance are shown in Table 1. There were no significant statistical differences between the two setups.

Table 1. Temperature effect on cone-plate geometry and viscosity values (mean ± SD).

	WBV (mPas) at 40°C				
Shear rate (s^{-1})	**50**	**75**	**100**	**200**	**400**
Calibrated at 20°C	4.81 ± 1.99	4.29 ± 1.48	3.97 ± 1.21	3.45 ± 0.78	3.12 ± 0.57
Calibrated at 40°C	4.29 ± 0.63	3.92 ± 0.54	3.71 ± 0.49	3.33 ± 0.41	3.09 ± 0.37
Difference	0.52 (12.2%)	0.37 (9.3%)	0.26 (6.9%)	0.12 (3.7%)	0.03 (1.1%)
p	0.36	0.36	0.40	0.46	0.74

4.2.3. Reproducibility

Results of reproducibility studies are presented in Figure 2. Mean CV levels were less than 5% at all shear rates. In Donor 1, 2 and 4 there was significant negative correlation between shear rate

and CV values (Donor 1: -0.891; Donor 2: -0.753, Donor 4: -0.765). Mean CV level of plasma viscosity at 500 s^{-1} shear rate was 2.74 ± 0.73.

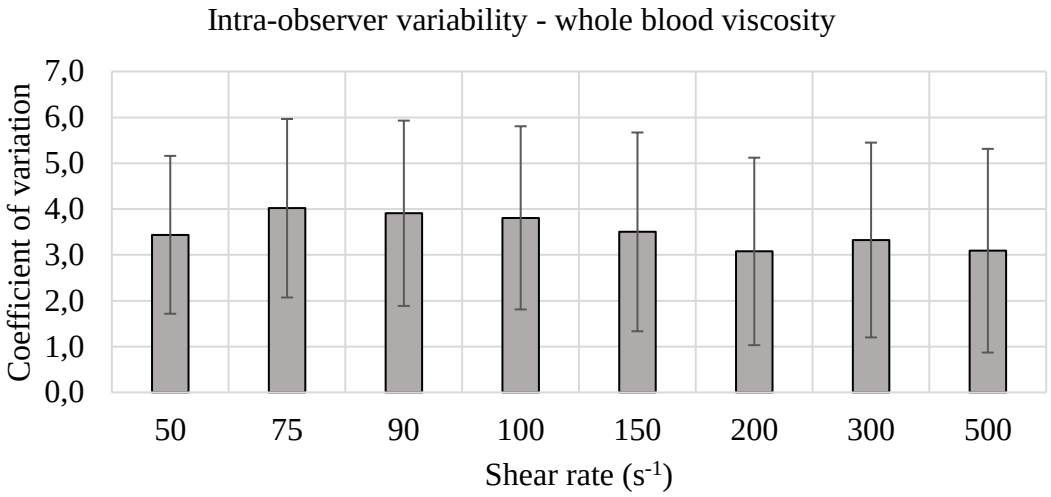

Figure 2. Coefficient of variation on 10 replicate measurements of whole blood viscosity. Data are shown as a mean ± standard deviation

4.2.4. Storage

The effects of storage are shown in Figures 3 and 4. The hematocrit of the samples was not adjusted; thus the shown standard deviations reflect differences in hematocrit (44.3% ± 2.9%) rather than errors of measurements. WBV after 3 hours at 37°C was significantly lower at 50 and 100 s^{-1} shear rates ($p<0.05$). In all other cases, no significant difference was observed. PV remained constant at all temperatures.

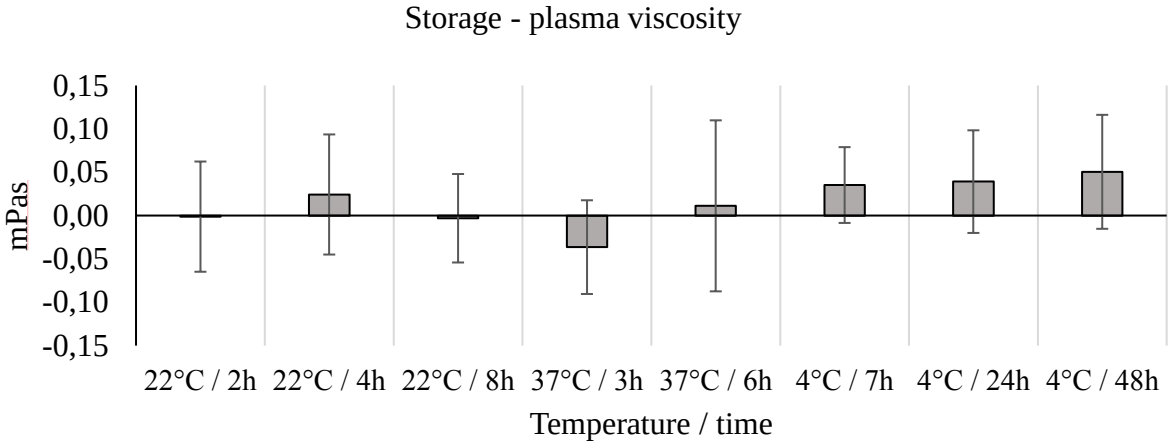

Figure 3. Effect of storage on plasma viscosity at different temperatures (absolute changes compared to baseline). Data are shown as a mean ± standard deviation.

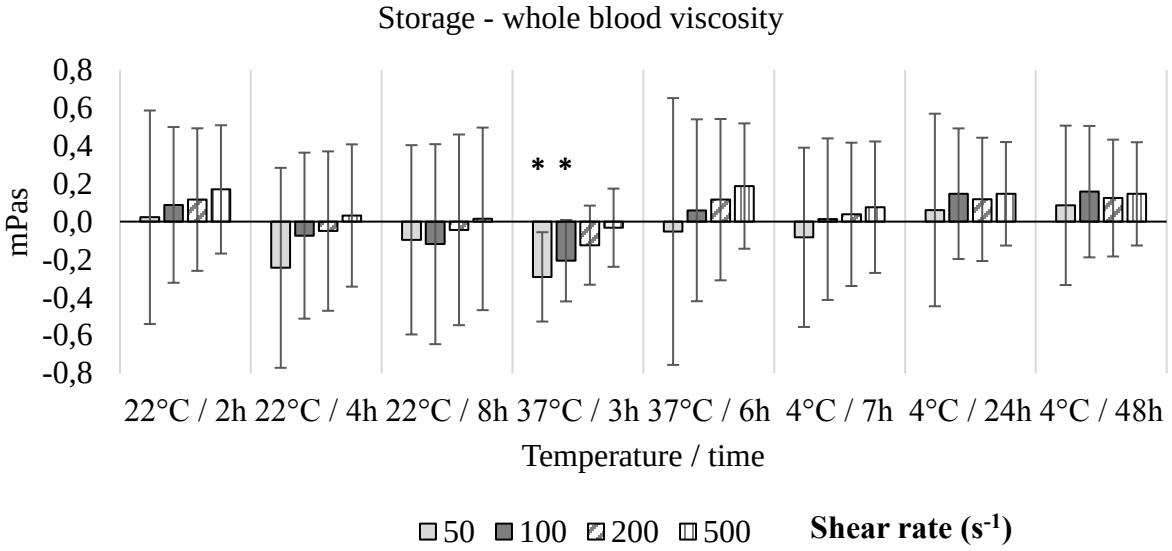

Figure 4. Effect of storage on whole blood viscosity at different temperatures (absolute changes compared to baseline). Data are shown as a mean ± standard deviation. * $p<0.05$

4.2.5. Comparison

The results are presented in Table 2. The capillary viscometer measured around 7% higher WBV and 10% higher PV values compared to the rotational one. At lower shear rates the difference in WBV was higher. At 50 s^{-1} shear rate correlation value was 0.67, while at the higher shear rates it was above 0.8 (100 s^{-1}: 0.82, 150 s^{-1}: 0.84, 200 s^{-1}: 0,81).

Table 2. Whole blood and plasma viscosity values measured by Hevimet 40 and Brookfield DV-III Ultra viscometers (37°C) (mean ± SD).

Shear rate (s^{-1})	**Hevimet 40**	**Brookfield**
Whole blood (mPas)		
50	4.81 ± 0.67	4.52 ± 0.52
100	4.30 ± 0.50	3.95 ± 0.39
150	4.01 ± 0.45	3.58 ± 0.30
200	3.94 ± 0.42	3.54 ± 0.33
Plasma (mPas)		
500	1.28 ± 0.12	1.14 ± 0.08

4.3. Conclusion

Installing a new device is always very challenging, beneath the general aspects, device specific problems should be addressed. Our results indicate that the rotational viscometer has a good reproducibility. The torque stability – depending on the magnitude of shear rate change – requires around 10 seconds, which needs to be taken into consideration. The device is able to measure accurate viscosity at clinically relevant temperatures and measures slightly lower values compared to our other tested instrument. Samples can be stored up to 48 hours without affecting measured values, but storage at 37°C is not recommended for several hours.

5. Toe-brachial index and exercise test can improve the exploration of peripheral artery disease

5.1. Patients and Methods

5.1.1. Subjects and baseline characteristics

120 patients were enrolled as a patient group. They met the following inclusion criteria: adult (≥18 years) and patients with diagnosed lower-limb arterial disease (based on previous positive imaging, endovascular/surgical intervention, ABI <0.9 or >1.3). Exclusion criteria were the following: patient did not sign written informed consent or patient had ischemic rest pain and/or ulcer/gangrene. 30 volunteers without any known arterial diseases (negative history and 1.00 < ABI < 1.3) were randomly chosen into the control group. Patients' self-reported symptoms and the baseline characteristics of the study population are reported in Table 3.

Table 3. Characteristics of the study population.

	Patient group (n= 120)	*Control group (n= 30)*	*p*
Age (mean ± SD, years)	66 ± 10	61 ± 10	0.045
Male sex (No., %)	55 (46%)	15 (50%)	0.689
BMI (mean ± SD, kg/m^2)	28 ± 6	27 ± 4	0.272
Symptoms of lower limb (No., %)			
Non-ischemic rest pain	5 (4%)	0 (0%)	<0.005
Intermittent claudication	84 (70%)	0 (0%)	<0.005
Atypical claudication	11 (9%)	0 (0%)	<0.005
Asymptomatic (No., %)	20 (17%)	30 (100%)	<0.005
Self-reported claudication distance (mean ± SD, m)	220 ± 239	N.A.	

Before any instrumental investigations, the participants had at least 5 minutes of rest period to acclimate to room temperature (20-22°C). The index limb was defined according to participants' complaints or the more deteriorated pre-test absolute ankle pressures. The workflow of procedure detailed below is presented in the Figure 5.

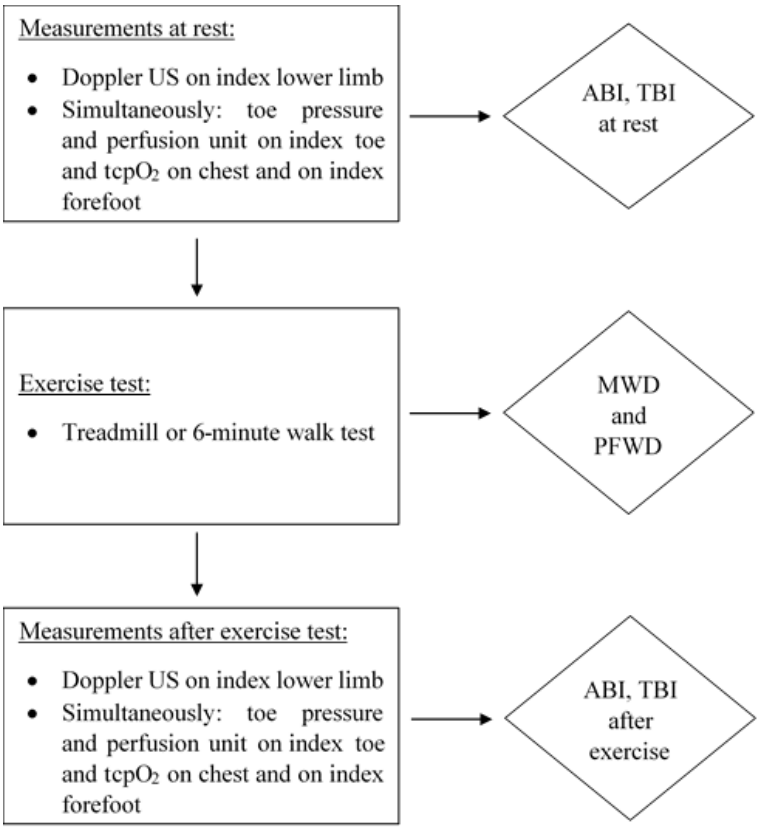

Figure 5. The workflow of procedure

5.1.2. Hand-held Doppler ultrasound, ankle-brachial index

Ankle pressures were measured by using hand-held Doppler ultrasound *(MultiDoppy, Medicad, Hungary)*. It was operated with an 8 MHz probe and a manual sphygmomanometer to measure systolic blood flow in posterior tibial and dorsal pedal artery of both legs as well as in the brachial artery of both arms following the same sequence of measurement. The absolute ankle pressures were measured at rest and after exercise. The threshold of ABI value for diagnosing LEAD was ≤0.9. ABI 0.9-0.71 was considered as mild, 0.7-0.41 as moderate and ≤0.4 as severe LEAD while ABI >1.3 was regarded as false high value due to media sclerosis; ABI between 0.91-1.00 was considered as borderline.

5.1.3. Toe pressure and toe-brachial index

The absolute toe systolic pressure was measured by laser Doppler flowmetry (LDF) with linear deflation pressure method *(PeriFlux System 5000, Perimed, Stockholm, Sweden)*, which was analyzed by a software *(PeriSoft v2.50)*. Three sequential toe pressure measurements were averaged. The toe pressure of index limb was measured at rest and after exercise.

5.1.4. Transcutaneous partial oxygen pressure measurement

Pre-calibrated Clark electrode *(Tina TCM 4000 oximeter, Radiometer, Denmark)* was positioned on the second intercostal space of anterior chest wall as a reference probe and at the dorsum of the

index feet near the first and second toes with a self-adhesive fixation ring that was filled by two drops of contact liquid. A steady-state in a supine position of the index limb was obtained for 15 minutes with heating of probes to 44°C to achieve maximal vasodilation. During the exercise the fixation rings remained at the same position covered with an adhesive tape, the electrodes were reset to the instrument. After the exercise they were attached back to the fixation rings with new drops of contact liquid and $tcpO_2$ values of the index limb were obtained at 5, 10 and 15 minutes. $TcpO_2$ values <40 mmHg were considered as a cut-off value for LEAD.

5.1.5. Exercise testing

Following the initial procedure, participants were asked to perform treadmill test at a 10% slope and a speed of 3.2 km/h. The treadmill test lasted until any symptoms (e.g. cramp, dyspnea) occurred or it was intermitted by patient's request or at the end of maximal exercise duration, which was 5 minutes. Those patients who were unable to perform the treadmill test (e.g. joint disorders, spinal diseases) conducted the 6-minute walk test (6MWT) as an alternative exercise test. The measurements were repeated after the exercise.

5.2. Results

5.2.1. Exercise testing

57 (48%) patients could walk on the treadmill, 63 patients performed 6MWT; 21 (70%) volunteers walked on the treadmill, and 9 (30%) random volunteers performed 6MWT. Out of the 84 patients with a history of intermittent claudication, 54 (64%) patients had claudication provoked by the exercise; 46 diabetic patients reported claudication, of whom 44 (96%) had claudication during the test. There were no statistically significant differences in the results of non-invasive tests between patients performed treadmill or 6MWT.

5.2.2. Absolute ankle pressures, ankle-brachial index

Absolute ankle pressures and ankle-brachial indices are presented in Table 4. In the patient group, the absolute dorsal pedal and posterior tibial artery pressures as well as the ABI decreased significantly while in the control group the pressures increased and ABI remained unchanged due to the exercise ($p<0.005$). 22% of patients had definitely low pre-exercise ankle pressure (<50 mmHg); which increased to 40% after the exercise ($p=0.002$).

5.2.3. Absolute toe pressures, toe-brachial index and microcirculatory perfusion

Absolute toe pressures and TBI values at rest and after exercise are presented in Table 4. Following the exercise, absolute toe pressure and TBI reduced significantly in the patient group ($p=0.003$ and $p<0.005$). Absolute toe pressure <30 mmHg was detected in 14% of patients at rest and 24% after exercise ($p=0.049$). At rest, very low (≤0.25) TBI could be found in 24%, low TBI in 64%, and normal (>0.70) TBI in 12% of the patients. After exercise, these percent values were 39%, 55%, and 6%, respectively (increment of patients' ratio having TBI ≤0.25 was significant, $p=0.018$). The pre- and post-exercise mean perfusion (PU) did not differ significantly among the patients; the control group had significantly higher pre- and post-exercise PU values than the patient group (Table 4).

5.2.4. Transcutaneous partial oxygen pressure measurement

$TcpO_2$ values of the patients were significantly lower than those of the controls both at rest and after exercise (Table 4). Low (<30 mmHg) $tcpO_2$ could be detected in 18% of patients at rest and 38% after the exercise ($p<0.005$). 19% of patients belonged to the intermediate range (30-40 mmHg) at rest and 24% after the exercise; while the ratio of patients in the normal range (>40 mmHg) reduced from pre-test 63% to post-test 38% ($p<0.005$).

Table 4. Ankle pressures, ABI, toe pressures, TBI, laser Doppler perfusion unit and $tcpO_2$ at rest and after exercise (mean ± SD; median in parentheses). [a], [b]: the difference was statistically significant within the group compared to the value measured at rest ([a]: $p< 0.005$, [b]: $p< 0.05$).

	Patient group (n= 120)	*Control group (n= 30)*	*p*
6MWT, m (mean ± SD)	n= 63	n= 9	
MWD	228 ± 88 (220)	610 ± 137 (600)	<0.005
PFWD	112 ± 104 (94)	610 ± 137 (600)	<0.005
Treadmill, m (mean ± SD)	n= 57	n= 21	
MWD	161 ± 77 (130)	267 ± 0 (267)	<0.005
PFWD	124 ± 80 (90)	267 ± 0 (267)	<0.005
Ankle pressure at rest (mm Hg)			
DPA	89 ± 51 (85)	135 ± 29 (130)	<0.005
PTA	97 ± 47 (90)	140 ± 28 (140)	<0.005
ABI at rest	0.75 ± 0.34 (0.63)	1.07 ± 0.17 (1.00)	<0.005
Ankle pressure after exercise (mm Hg)			
DPA	75 ± 57 (65)[a]	141 ± 31 (140)[a]	<0.005
PTA	82 ± 56 (70)[a]	149 ± 29 (150)[b]	<0.005
ABI after exercise	0.59 ± 0.38 (0.53)[a]	1.02 ± 0.13 (1.05)	<0.005
Absolute toe pressure (mm Hg)			
At rest	62 ± 28 (60)	101 ± 23 (101)	<0.005
After exercise	56 ± 34(50)[a]	105 ± 22 (103)	<0.005
TBI			
At rest	0.43 ± 0.20 (0.40)	0.78 ± 0.18 (0.78)	<0.005
After exercise	0.35 ± 0.22 (0.31)[a]	0.73 ± 0.16 (0.72)	<0.005
Perfusion (PU)			
At rest	177 ± 90 (170)	223 ± 89 (215)	0.045
After exercise	174 ± 91 (165)	242 ± 55 (225)	0.001
$TcpO_2$ on chest (mm Hg)			
At rest	53 ± 12 (54)	60 ± 11 (58)	0.002
5 min after exercise	54 ± 16 (56)	62 ± 12 (63)	0.030
10 min after exercise	54 ± 14 (54)	61 ± 13 (64)	0.019
15 min after exercise	54 ± 14 (56)	62 ± 13 (61)	0.006
$TcpO_2$ on index forefoot (mm Hg)			
At rest	42 ± 15 (44)	55 ± 9 (55)	<0.005
5 min after exercise	33 ± 20 (36)[a]	57 ± 9 (56)	<0.005
10 min after exercise	40 ± 17 (43)	59 ± 10 (57)[a]	<0.005
15 min after exercise	42 ± 17 (45)	60 ± 11 (60)[a]	<0.005

5.2.5. Comparing the diagnostic value of the non-invasive tests

Examining the diagnostic performance of the studied non-invasive tests, ROC curve analysis was performed (Figure 6). Based on the post-exercise ABI ≤0.4, patients were grouped into LEAD with or without severe limb ischemia (SLI); the prevalence of SLI was 51 (42.5%). The ROC curve of TBI at rest differed significantly from the ROC curve of $tcpO_2$ on forefoot at rest (the difference between areas: 0.193; p=0.0014). The curve of TBI after exercise differed significantly from the curves of $tcpO_2$ at rest (0.267; p<0.005) and after exercise (0.140; p=0.0024). The curve of $tcpO_2$ at 5 minutes after exercise was significantly different from the curve at rest (0.127; p=0.0032).

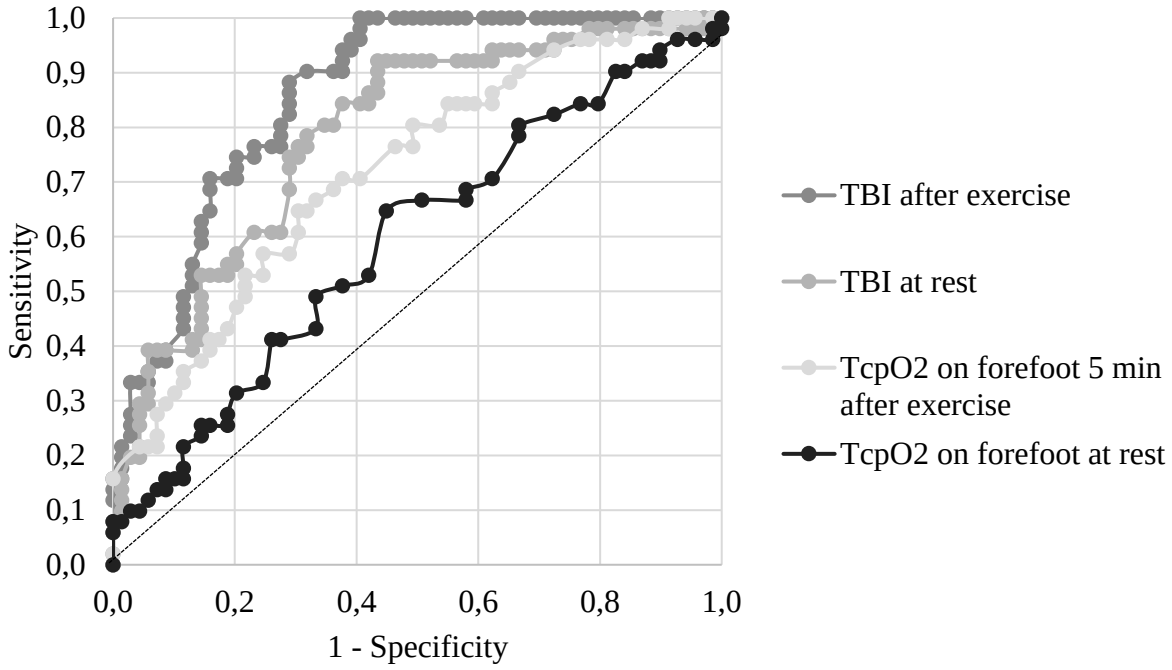

Figure 6. Receiver operating characteristic curves of TBI and $tcpO_2$ (n=120). The dotted line represents the line of no-discrimination. Diagonal segments of affected ROC curve are produced by ties.

5.3. Conclusion

Our study design was unique because of the several parameters investigated in parallel. These pre- and post-exercise non-invasive tests have the ability to identify lower limb ischemia as the underlying cause of complaints. The staging of peripheral artery disease could be performed and the severe limb ischemia could be revealed to select those patients whose radiological imaging is inevitable. The tests can differentiate lower limb pain of arterial and non-arterial origin. We imply that one non-invasive test (usually DUS and ABI) at rest should not be sufficient to diagnose or exclude peripheral artery disease and the exercise testing as well as the toe-brachial index could become routine procedures in the vascular work-up.

6. Summary of the new scientific results

6.1. Viscometer validation studies for routine and experimental hemorheological measurements

1. This was the first study which compared Hevimet 40 capillary and Brookfield DV-III Ultra LV rotational type viscometers.
2. From this study, the systematic variances of Hevimet 40 and Broofield DV-III Ultra LV viscometers were obtained.
3. Based on acquired results, we may suggest that Brookfield DV-III Ultra LV should not be applied to measure plasma viscosity.
4. The torque stability of Brookfield DV-III Ultra LV requires some time (8-10 s) which should be taken into consideration.
5. The Brookfield DV-III Ultra LV was able to measure accurate viscosity at clinically relevant temperatures without resetting the cone-plate geometry.
6. Our results of storage study agreed with previous findings in the literature.

6.2. Toe-brachial index and exercise test can improve the exploration of peripheral artery disease

1. This was the first study that compared several non-invasive diagnostic approaches at rest as well as after exercise to discover and classify lower extremity artery disease.
2. Non-invasive functional tests are rarely performed in peripheral artery disease. Detection of lower limb ischemia could result in early diagnosis; ischemic and non-ischemic origin of leg complaints could be differentiated.
3. One non-invasive test at rest should not be sufficient to diagnose or exclude peripheral artery disease.
4. Exercise test should get a greater role in angiology: diagnosis in patients with silent leg ischemia or masked LEAD, functional capacity in patients with typical leg pain could be established by functional measurements before and after exercise.
5. Our study demonstrated that 6-minute walk test can be used as an alternative of treadmill to provoke lower limb ischemia.
6. The severity of intermittent claudication should be assessed by exercise tests because many claudicants have a compensated lower limb circulation at rest.
7. Severe lower limb ischemia could be provoked by exercise test in patients with normal or moderately lower ankle-brachial index.
8. This was the first study that evaluated the post-exercise toe-brachial index in patients with peripheral artery disease.
9. Post-exercise toe-brachial index was the most sensitive parameter to detect severe limb ischemia among patients having different severity of peripheral artery disease. We may suggest that pre- and post-exercise toe-brachial index should be a part of the angiological examination.

7. Acknowledgement

These studies were carried out at the Division of Cardiology & Angiology and Hemorheological Research Laboratory of the 1st Department of Medicine, Medical School, University of Pecs between 2015 and 2018. The scientific contributions were dedicated to the 650th anniversary of the foundation of the University.

I am grateful to my program leader Professor Kalman Toth for the provided support throughout my scientific work. I would like to express my thanks to my project leader Gabor Kesmarky for the guidance in my undergraduate and Ph.D. research. I express my special thanks to Katalin Biro and Andras Toth for the methodological support and consultations.

I am also thankful to Beata Csiszar, Dora Endrei, Istvan Juricskay, Peter Kenyeres, Katalin Koltai, Mariann Pesti, Dora Praksch, Miklos Rabai, Aniko Ronaky, Barbara Sandor, Kinga Totsimon.

I am thankful to all the physicians, medical students of undergraduate research society, nurses, and technicians for their professional help and counselling throughout my scientific work, especially to Kornelia Tapasztone Fazekas and Szilvia Kovacsne Levang.

I am very grateful to my whole family for their patience and encouraging support.

I am thankful to 1st Department of Medicine, School of Medicine, University of Pecs for the financial support of this booklet.

This research was supported by a European Union grant: EFOP - 3.6.1-16-2016-00004.

8. Publications of the author

8.1. Topic related journal articles

1. **Kovacs D**, Csiszar B, Biro K, Koltai K, Endrei D, Juricskay I, Sandor B, Praksch D, Toth K, Kesmarky G. Toe-brachial index and exercise test can improve the exploration of peripheral artery disease. Atherosclerosis. 2018; 269: p. 151-58.
   Impact factor: 4.239
2. **Kovacs D**, Totsimon K, Biro K, Kenyeres P, Juricskay I, Kesmarky G, Toth K, Toth A. Viscometer validation studies for routine and experimental hemorheological measurements. Clin Hemorheol Microcirc. 2018 (in press). DOI: 10.3233/CH-170301.
   Impact factor: 1.679
3. **Kovacs D**, Biro K, Koltai K, Endrei D, Toth K, Kesmarky G. Reply to: Exercise oximetry in patients with arterial claudication. Atherosclerosis. 2018; 272: p. 245-246.

8.2. Other journal articles

1. Koltai K, Biro K, **Kovacs D**, Csiszar B, Toth K, Kesmarky G. Cilosztazol szerepe a perifériás verőérbetegség kezelésében. Lege Artis Medicinae 2015, 25 (4-5): p. 177-81.
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3. Biro K, Sandor B, **Kovacs D**, Csiszar B, Vekasi J, Totsimon K, Toth A, Koltai K, Endrei D, Toth K, Kesmarky G. Lower limb ischemia and microrheological alterations in patients with diabetic neuropathy. Clin Hemorheol Microcirc. 2018; 69: p. 23-35.
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8.3. Book chapter

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8.4. Published abstracts

1. Kesmarky G, **Kovacs D**, Csiszar B, Biro K, Koltai K, Endrei D, Battyani I, Menyhei G, Toth K. A noninvaziv angiológiai vizsgálatok szerepe a döntéshozatalban perifériás ütőérbetegnél: esetismertetés. A Magyar Kardiológusok Társasága 2015. évi Tudományos kongresszusa. Balatonfüred, Magyarország. Cardiologia Hungaria. 2015, 45: (Suppl. D): p. D57.
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3. Koltai K, Biro K, **Kovacs D**, Csiszar B, Toth K, Kesmarky G. A transcutan parciális szöveti oxigéntenzió mérés és a lézer-doppler-áramlásmérés szerepe diabeteses betegekben. Magyar Belgyógyász Társaság Dunántúli Szekciójának LVIII. Vándorgyűlése. Kaposvár, Magyarország: 2015.06.18 - 20. Magyar Belorvosi Archivum. 2015, 68:(Suppl. 1) p. 18.
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9. **Kovacs D**, Csiszar B, Biro K, Koltai K, Praksch D, Totsimon K, Endrei D, Toth K, Kesmarky G. Perifériás ütőérbetegek végtag ischaemiájának terheléses vizsgálata hemoreológiai aspektusból. Magyar Haemorheologiai Társaság XXIII. Magyar Mikrocirkulációs és Vaszkuláris Biológiai Társaság Magyar Szabadgyök-Kutató Társaság V. Közös Kongresszusa, Balatonkenese, 2016. április 22-23. Érbetegségek. 2016, 23: p. 30.
10. Praksch D, **Kovacs D**, Sandor B, Totsimon K, Mezey B, Petrovics P, Wilhelm M, Kesmarky G, Toth K, Szabados E. Ambuláns és otthoni fizikai tréning hatásának vizsgálata magas kardiovaszkuláris rizikójú nőbetegek körében. Magyar Haemorheologiai Társaság XXIII. Magyar Mikrocirkulációs és Vaszkuláris Biológiai Társaság Magyar Szabadgyök-Kutató Társaság V. Közös Kongresszusa, Balatonkenese, 2016. április 22-23. Érbetegségek. 2016, 23: p. 30.
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