Evaluation of Specific Methods in Hemorheology and Angiology

Ph.D. dissertation

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1. List of Abbreviations

6MWT	6-minute walk test
ABI	ankle-brachial index
ACC	American College of Cardiology
AHA	American Heart Association
CLI	critical limb ischemia
CTA	computed tomography angiography
CV	coefficient of variation
DSA	digital subtraction angiography
DUS	Doppler ultrasonography
EDTA	ethylenediaminetetraacetic acid
IC	intermittent claudication
LDF	laser Doppler flowmetry
LEAD	lower-extremity arterial disease
NO	nitric oxide
p	significance level
PAD	peripheral artery disease
PRIMA	pattern recognition by independent multicategory analysis
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

2. Prologue

2.1. 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) (1), both identified as primary cardiovascular risk factors (2-8) as well as variables with prognostic significance in certain clinical conditions (9). Blood is a fluidized suspension of elastic cells therefore blood has a viscoelastic behavior. 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 (its viscosity depends on shear rate). WBV is an important determinant of blood flow resistance, its elevated level may cause a disturbance in tissue perfusion due to decreased flow rate (10-11), which is associated with deteriorated vascular compensation mechanism (e.g. in severe arterial stenosis), facilitated cardiovascular remodeling and accelerated atherosclerosis (9). 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 (11-12), therefore it plays a role in the mechanism of vasodilation (11).

Atherosclerosis as a progressive disease is related to altered hemorheological parameters considering the underlying mechanisms are the endothelial dysfunction and plaque formation resulting in turbulent blood flow. Inversely, some deleterious hemorheological conditions play a role as a primary cause in atherosclerotic plaque formation (9). The altered hemorheological variables in association with atherosclerosis are elevated plasma (13) and whole blood viscosity

(5), plasma fibrinogen concentration (13-14) and impaired red blood cell deformability (15). Apparently, peripheral artery disease (PAD) is a disorder of the larger arteries; however, the microcirculation can also be affected by the altered hemorheological conditions. Blood viscosity and red blood cell deformability are the main determinant factors of microcirculatory blood flow and capillary resistance (2,16).

2.2. 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%) (17-20). 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. Because the conduction of hand-held Doppler ultrasound examination, the calculation of ABI and the threshold of an impaired ABI have been varied in several studies, there is no consistence in the prevalence of asymptomatic PAD. The ratio of the symptomatic and the asymptomatic PAD patients can be in the range of 1:3 to 1:4 (17). The typical symptomatic manifestation of PAD is termed as intermittent claudication (painful walking impairment). The underlying pathophysiological finding is the leg muscle ischemia during exercise caused by mismatch of blood perfusion and metabolic demands. The presented symptoms as intermittent claudication (IC) must be met the criteria defined by Rose: the exertional leg pain does not exist at rest, it involves the calf causing reduction or stoppage of walking and it relieves by rest. There are several epidemiological questionnaires to identify these symptoms (e.g. Rose, Edinburgh, Walking Impairment Questionnaire) (21-23). 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 (17). The pitfall of searching typical IC is the fact that most patients with PAD do not have typical symptoms (30-60% in community and primary care) (Lipid Research Clinical Study (24), Edinburgh Artery Study (25), PARTNERS study (26), Get ABI Study (27)). 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 (without critical limb ischemia) 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 (WALCS Chicago Cohort) (28). On the other hand, several comorbidities can mimic the exertional leg symptoms in the absence of PAD like spinal stenosis, lumbar degenerative diseases, peripheral neuropathy and chronic venous insufficiency.

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 (17). CLI is the end-stage manifestation of PAD with a 1-year outcome to amputation in 30% of cases (17). PAD patients have a three- to four-fold increased risk of cardiovascular morbidity and mortality compared to individuals without PAD (29). Non-fatal cardiovascular (CV) events (acute myocardial infarct or stroke) can occur in 20% of patients with PAD, while all-cause mortality is 10-15% over 5 years (17).

Smoking and PAD have the strongest causative relationship in several large epidemiological studies (30-33). Diabetes mellitus is also a great risk factor for PAD whereas the severity and

duration of diabetes is strongly associated to PAD causing a worse outcome: higher lower limb amputation and mortality rates (34). Concomitance of PAD and diabetes is highly predisposing to non-healing ulcer with or without infection. Other risk factors are hypertension, dyslipidemia and altered hemorheological variables.

3. Focuses

3.1. 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.

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 (35). Blood plasma is a Newtonian fluid, therefore it can be more easily measured, although in certain clinical conditions (e.g. hematological disorders) or measurement settings surface film artifacts may alter the results.

Beyond the natural complexity of blood, several artifacts may complicate the measurements. These artifacts are generated by surface tension, plasma proteins, phase separation and RBC aggregation (35). Bias of artifacts is more visible at low shear rates where one tries to observe low intrinsic forces. These instruments, depending on their construction, are sensitive to artifacts to different extents; moreover, device-specific artifacts may also be present. Hence, different instruments aiming to measure the same parameter may produce different results.

Difficulties of viscosity assessment originate from both the nature of blood and the properties of the various available viscometer systems. 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.

3.2. 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 (36), its use is limited because of its invasive nature as well as the application of ionizing radiation and potentially nephrotoxic contrast agent (37). 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 (38-43). Nevertheless, there is no evidence on its routine application (17) or it is limited to assess improvement in claudicants and/or to differentiate vascular claudication from neurogenic one (18). The newest AHA/ACC guideline recommends performing exercise treadmill ABI testing in patients with PAD and an abnormal resting ABI ($\leq$0.9) to assess functional status (44).

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.

4. Methodology

4.1. Measurement of viscosity

Various types of instruments have been developed to measure viscosity, though capillary and rotational viscometers are the most frequently used ones (45).

4.1.1. Capillary viscometer

The Hevimet 40 capillary viscometer (*Hemorex Ltd.; Budapest, Hungary*) (Figure 1) 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.

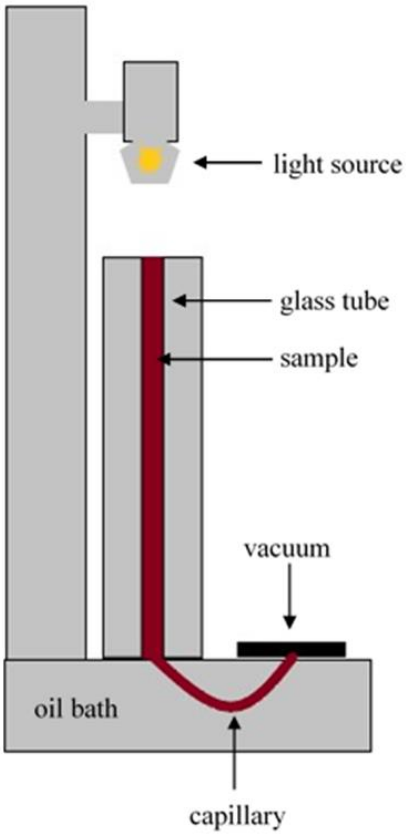

Figure 1. Hevimet 40 capillary viscometer

4.1.2. Rotational viscometer

The Brookfield rotational viscometer (Figure 2) 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.

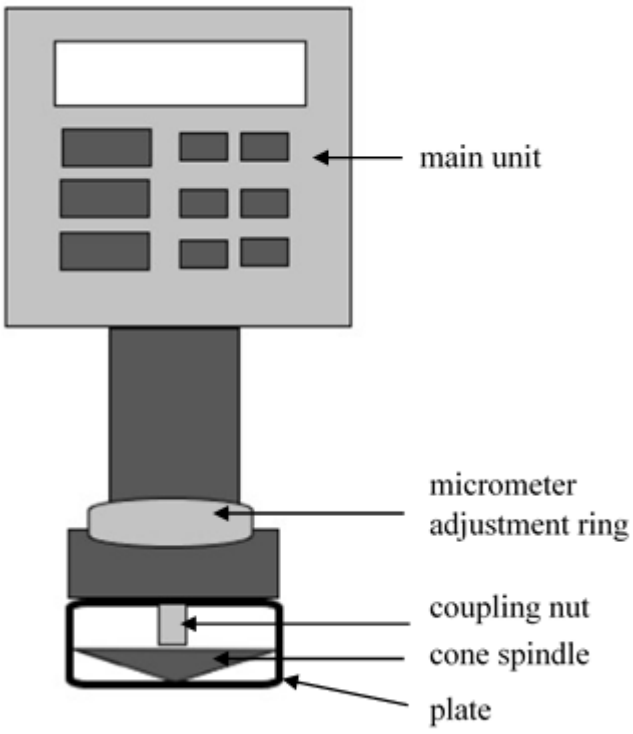

Figure 2. Brookfield rotational viscometer

The computer-controlled potential of Brookfield rheometer (*Rheocalc v3.3. Build 49-1, Brookfield Engineering Labs; Middleboro, USA*) allows creating automatic measuring algorithm. Due to the structure of *Brookfield DV-III Ultra LV* rheometer the same element generates shear rate and measures shear stress simultaneously, thus after changing the rotational speed of the spindle, some time is required until the torque of the coiled spring reaches a steady-state. Viscosity for a given shear rate should be measured when fluctuation of torque terminates. This effect is especially relevant using LV type of Brookfield rheometer, where the spring is quite weak.

Both the capillary and the rotational viscometers were calibrated with the same Newtonian calibration fluid to ensure the precise comparison and were securely mounted, leveled on a vibration-free table.

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

According to several consensus guidelines, the first-line non-invasive method to detect peripheral arterial flow (ankle pressure) 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) (38). The cuff was placed around the ankle approximately 1 cm above the medial malleolus with parallel wrapping (38). The measurements were carried out in a supine position. To calculate the ankle-brachial index, 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 ensuring better specificity than using the lower ankle

pressures (83% vs. 64%) (38,46-47). The threshold of ABI value for diagnosing LEAD was ≤0.9 (38). ABI 0.9-0.71 was considered as mild, 0.7-0.41 as moderate and ≤0.4 as severe LEAD (48-51) while ABI >1.3 was regarded as false high value due to media sclerosis (52-54); ABI between 0.91-1.00 was considered as borderline (38,55). The ankle-brachial index (ABI) 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% (37-38,56). In the background of lower sensitivity there may be two main cause: first, the ABI can reflect those stenosis that are situated on the aorto-ilio-femoro-distal axis (57). Second, there are some clinical conditions (advanced age, diabetes mellitus, chronic renal insufficiency) when ABI is not a reliable parameter to detect PAD because of severe calcification (37). Moreover, approximately 30% of patients with intermittent claudication have a normal ABI at rest (58). Aboyans et al. reported that the sensitivity of ABI could be increased by measuring it after treadmill exercise due to augmentation of the ankle-brachial pressure gradient (38). Although there can be a mild decrease in the ABI in healthy subjects after exercise, the decrease is more prominent in patients with PAD (38). The pitfall of the post-exercise ABI is that ankle pressures reach their pre-exercise value within minutes after cessation of exercise. Yet an impaired post-exercise ABI is associated with increased mortality (38,59). Recently, it is stated that a post-exercise drops in ankle pressure >30 mmHg or in ABI >20% is diagnostic for PAD (38). The American Heart Association (AHA) recommends that the post-exercise ABI or another non-invasive test should be used when the ABI >0.9 but there is suspicion of PAD (38).

4.3. 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 optical fiber separation, the emitted wavelength and the optical properties of tissue (melanosomes – pigmentation) influence the depth of blood flow detection, which is approximately 0.5-1 cm. The measured tissue volume is small and the total local blood perfusion is detected including capillaries, arterioles, venules and shunts. LDF instrument requires calibration process which was carried out before each measurement. The LDF probe was attached to the skin by a double-sided adhesive tape provided by the manufacturer. The probe must be placed onto a hairless, not bony and not inflamed or edematous skin surface. 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. LDF method has some technical limitations. 1. Currently it is not possible to express flux signal as an absolute tissue perfusion unit (milliliter blood flow per minute through 100 gram). 2. The PU has a temporal and spatial variability because the detected PU is influenced by several – yet not fully understood – factors (e.g. great variability of microcirculation due to heterogenous distribution of capillaries, gender, pharmacological effects, sympathetic vs. parasympathetic activity) (60). 3. LDF measuring can cause motion artefacts generated by movement of the probe, local muscle contraction (e.g. tremor) or inappropriate probe fixation. The motion artefacts cause different signal from the

perfusion related one therefore these artefacts can be eliminated from the register. 4. LDF detects flux signal even if there is no blood perfusion (i.e. biological zero, usually when PU < 10) (60). In the background several factors are assumed: Brownian motion of cells, thermal motion of particles, vasomotion causing Doppler shift and electrical noise (60). Vasomotion is a regular and fine oscillation in tone of capillary beds: their smooth muscles rhythmically dilate and contract; it has an important physiological role: it reduces the flow resistance. Vasomotion has a great correlation with endothelial function (61). Under pathological conditions vasomotion pattern can be altered: the impact of rhythmical oscillation can be reduced in diabetes or it can be increased in hypertension.

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. Following the baseline skin perfusion detection (when the temperature of probe is equal to the temperature of skin), the heatable body of the probe can be set at 44°C.

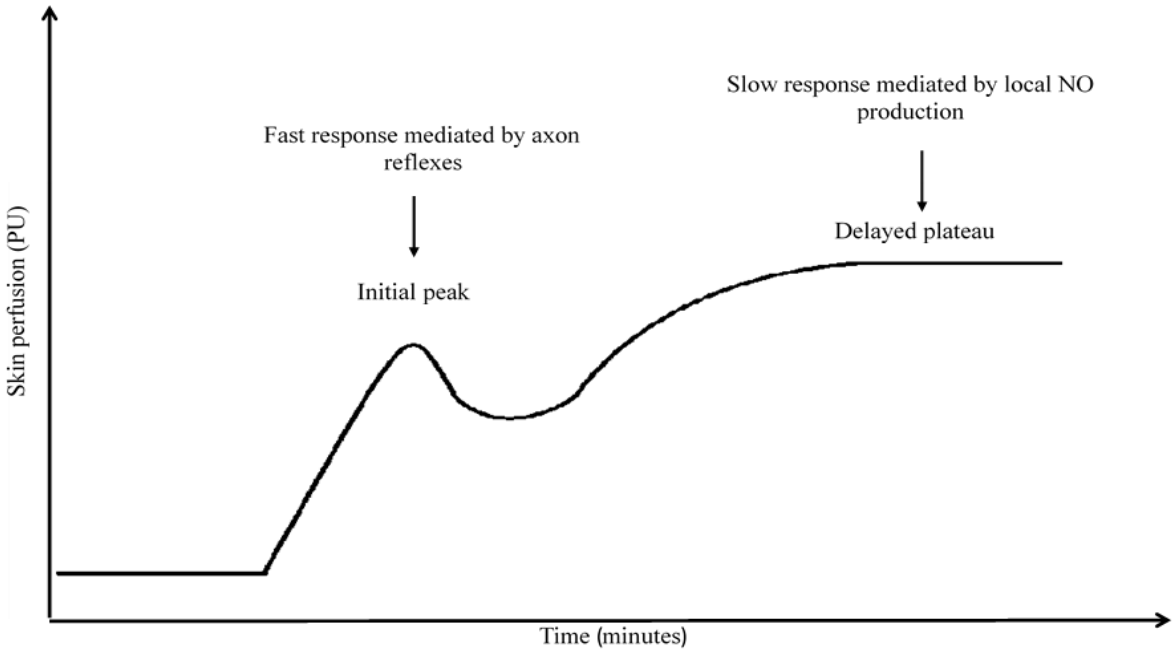

Figure 3. Heat challenge (*PU,* perfusion unit; *NO,* nitric oxide)

Due to the heat provocation, capillaries situated under the probe vasodilate which is proportional with the increase of perfusion unit. Heat provocation can result in a maximal vasodilation of related capillaries in two phases: the initial peak of PU increments due to fast response (approximately 10 minutes) is mediated by axon reflexes while the delayed plateau of PU due to slow response (approximately 20 minutes) is mediated by local nitrogen-monoxide production (Figure 3).

The percent change of baseline and heat-provoked PU can indicate the local reserve capacity of related capillaries which can be deteriorated in case of endothelial dysfunction. Furthermore, the reduced response for heat provocation can predict a poor outcome of wound healing as well as it is linked with skin perfusion disturbances (i.e. ischemia).

Performing linear pressure deflation, the toe pressure can be measured. During this procedure, an occlusive cuff (of which size depends on diameter of toe + 20%) is placed around the proximal portion of the great toe (or in absence of it on the second toe). The probe is applied to the plantar surface of the distal portion of cleaned great toe with a double-sided adhesive tape without compressing. The cuff *(Hokanson, Bellevue, WA, USA)* is inflated by a built-in inflator 20 mmHg above the systolic blood pressure then it is gradually (by 2 mmHg) decreased by LDF device. The definite increment of PU implies the returning of blood perfusion. Because of the variability of LDF and methodological factors, several toe pressure measurements must be averaged. Toe pressure is normally 30 mmHg less then systolic pressure determined above the ankle and it is not affected by arteriolar calcification which is the main advantage of this methods in longstanding diabetes, renal insufficiency, advanced age and conditions with hypercalcemia (62). Moreover, the LDF method for measuring toe pressure is sensitive at low-flow conditions (blue toe syndrome, severe ischemia) (63). 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 ≤0.70 as the cut-off value for diagnosing PAD (44,64) while ≤0.25 is used to identify the severe PAD (64). Absolute ankle (<50 mmHg or <70 mmHg in case of ischemic lesion) and great toe pressures (≤30 mmHg) are used as cut-off points for critical limb threatening ischemia as well as they are prognostic markers for wound healing (18,52,64-66). However, TBI is more sensitive for small-vessel disease as far as a decreased ABI is a marker of large-vessel disease therefore patients with normal ABI and deteriorated TBI can reflect small-vessel disease (64,67-68).

4.4. 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. Over the past decades, the $tcpO_2$ measurements had several indications: 1. evaluating and following ischemia in diabetic foot syndrome and PAD, 2. assessing wound healing, 3. determining the level of lower limb amputation, 4. plastic surgery, 5. hyperbaric oxygen therapy. 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. One of the cornerstone of $tcpO_2$ measurement to place the fixation ring on a suitable surface: not over large vessel, bony, hairy, inflamed or edematous skin. 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. The equilibrium (i.e. delivered oxygen molecules towards and from the electrode) is evolved within 15 minutes. A reference sensor is placed on the chest to rule out systemic arterial hypoxemia. Calibration is required before every measurement. Similarly to LDF, the $tcpO_2$ measurements can also show spatial and temporal variability as well as it can be influenced by several outer circumstances (e.g. altitude). 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) (17,44,52,69-70). However, in the background of low $tcpO_2$ values atherosclerosis, impaired microcirculation, barrier to oxygen diffusion, high consumption of oxygen (inflammation), cardiopulmonary mismatch and/or hypobaric condition can be found. In these cases, checking the oxygen saturation is recommended. The averaged $tcpO_2$ at the same measuring site have a better predictive value. 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).

5. Viscometer validation studies for routine and experimental hemorheological measurements

5.1. Study design

5.1.1. 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 venipuncture was performed in accordance with the latest hemorheological guideline (71). None of the volunteers had regularly taken any medications or had surgery within 1 month. The study was approved by the Regional Ethics Committee of the University of Pecs (approval No.: 5336) and undertaken with the understanding and consent of each subject. 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.

5.1.2. 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 (the shortest interval available by the program). Stability was considered when the fluctuation of the value stopped.

5.1.3. 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 same (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.

5.1.4. 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.

5.1.5. 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.

5.1.6. Comparison

Comparison studies were carried out to compare the Brookfield DV-III Ultra LV and Hevimet 40 viscometers. Both the capillary and rotational viscometers were calibrated with the same Newtonian calibration fluid (polyhydric alcohol, viscosity: 3.56 ± 3%, No. 130703) and were securely mounted and leveled on a vibration-free table.

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.

5.1.7. Statistical procedures

Shapiro-Wilk test was used to check normality, f-test for equivalence of variation between analyzed groups. Data were compared by two independent samples t-test and paired samples t-test. A two-tailed p value below 0.05 was considered statistically significant. To assess the agreement between the viscosity data obtained by the instruments, Pearson correlation and Bland-Altman method (confidence interval: 95%) was used. Analyses were carried out on IBM© SPSS© Statistics v23.

5.2. Results

5.2.1. Torque stability

Torque stability is demonstrated at 10 and 200 s^{-1} shear rates. 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 4).

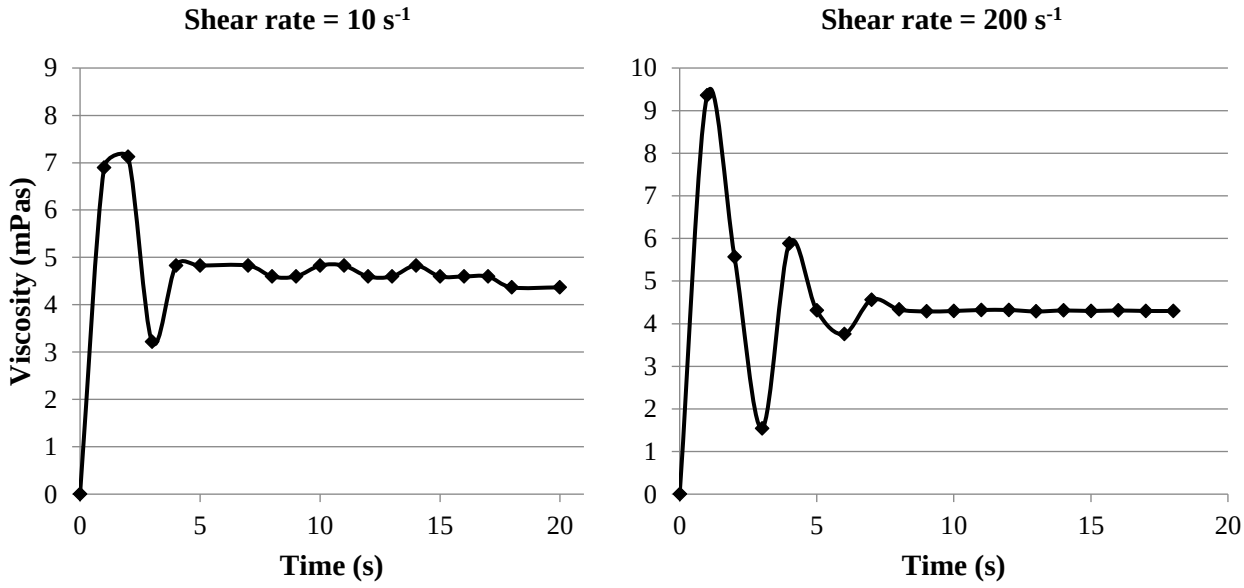

Figure 4. Time to torque stability at 10 and 200 s^{-1} shear rates (37°C).

5.2.2. 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, although at lower shear rates the device measured – not statistically – higher viscosity values if cone-plate geometry was not set at the appropriate temperature.

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

SD, standard deviation; *WBV*: whole blood viscosity

5.2.3. Reproducibility

Results of reproducibility studies are presented in Figure 5. 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.

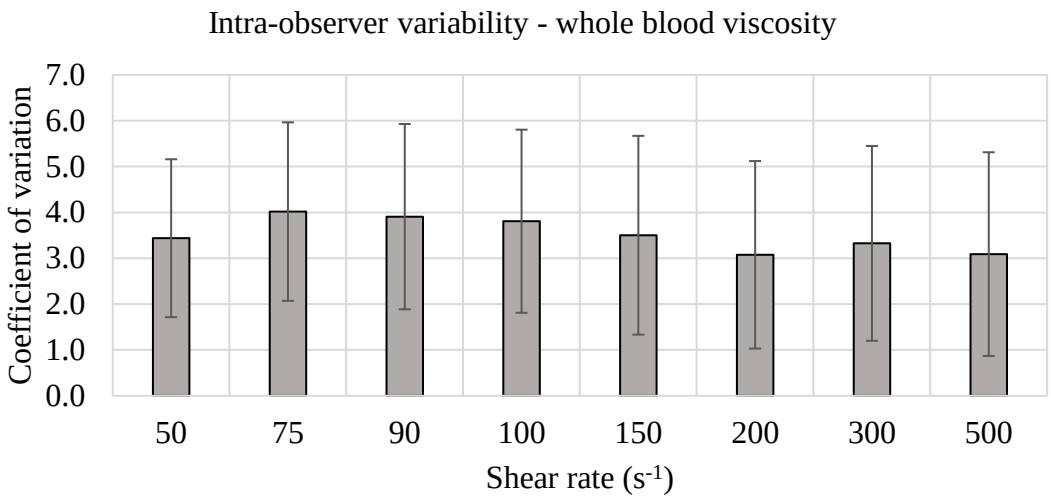

Figure 5. Coefficient of variation on 10 replicate measurements of whole blood viscosity. Data are shown as a mean ± standard deviation

5.2.4. Storage

The effects of storage are shown in Figures 6 and 7. 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.

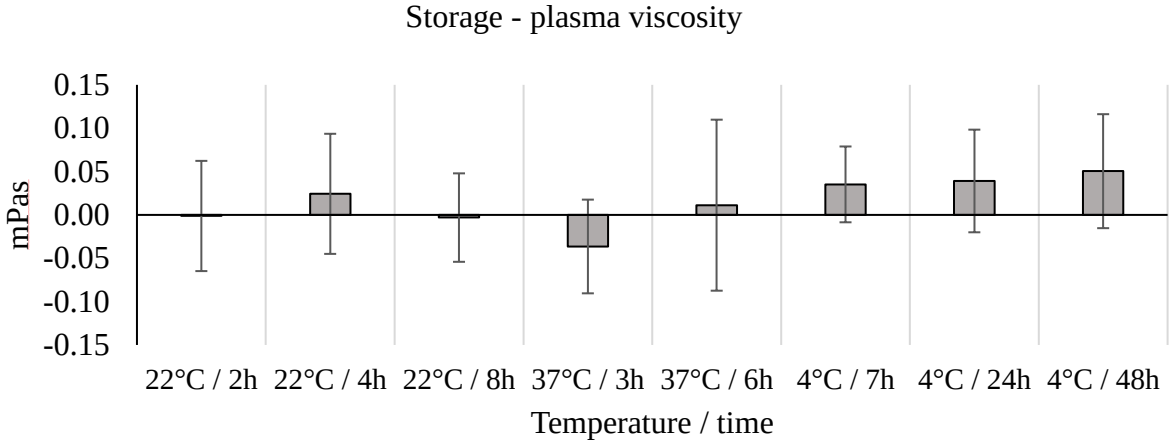

Figure 6. Effect of storage on plasma viscosity at different temperatures (absolute changes compared to baseline). Data are shown as a mean ± standard deviation.

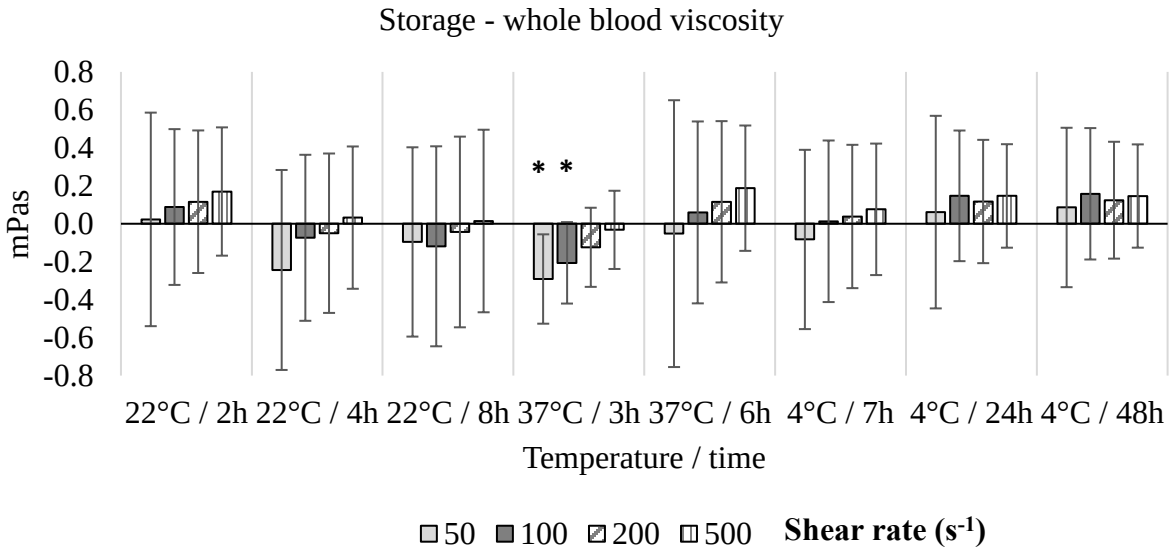

Figure 7. Effect of storage on plasma viscosity at different temperatures (absolute changes compared to baseline). Data are shown as a mean ± standard deviation. * $p<0.05$

5.2.5. Comparison

The results are presented in Table 2 and Figure 8. 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). Bland-Altman analysis shows the above described systematic difference, but no visible trends can be seen regarding viscosity values (Figure 9).

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

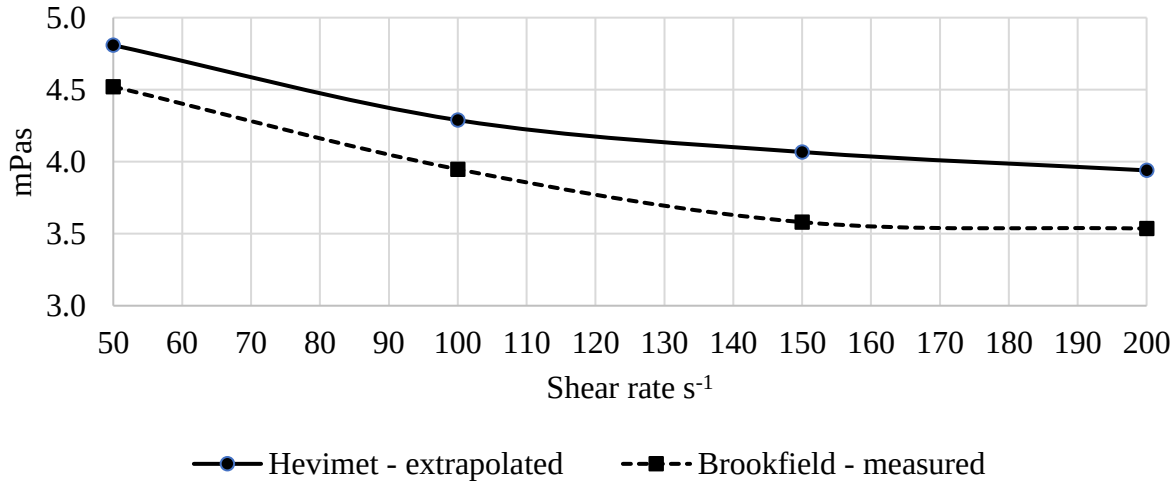

Figure 8. Whole blood viscosity values measured by Hevimet 40 and Brookfield DV-III Ultra viscometers (37°C).

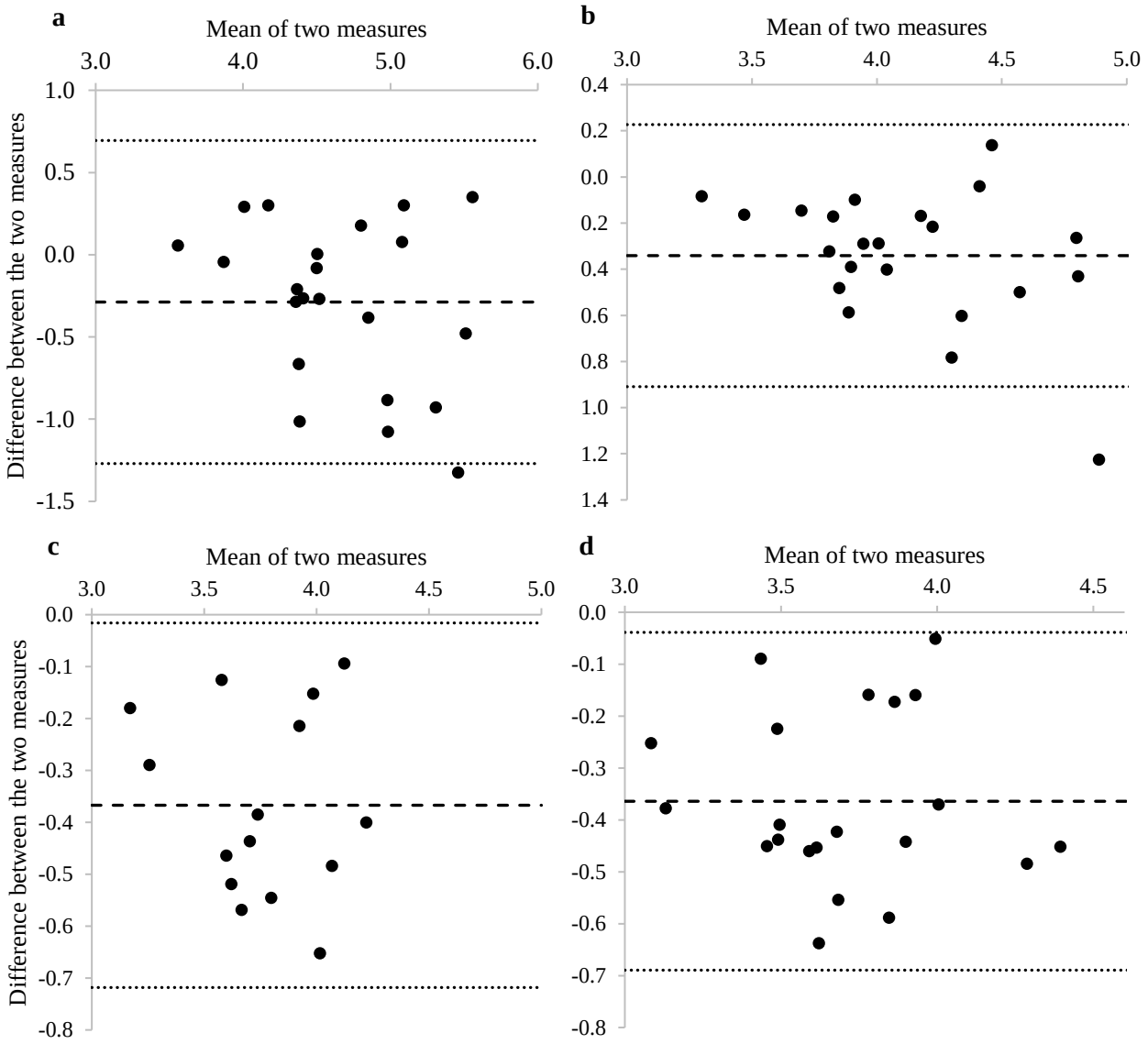

Figure 9. Bland-Altman plots; at shear rate **a**: 50 s^{-1}, **b**: 100 s^{-1}, **c**: 150 s^{-1}, **d**: 200 s^{-1}. Dotted lines represent 95% confidence interval. Dashed line represents mean bias.

5.3. Discussion

Due to the structure of the cone-plate system – as it was expected – some time is required to reach a steady torque value after a shear rate change. According to our data – depending on the magnitude of this change – around 10 seconds is required before viscosity values can be obtained. This finding should be taken into consideration when an automated measuring

algorithm is created. Measuring viscosity at several shear rates will increase the measurement time significantly.

Starting temperatures of the device had no statistically significant effect on viscosity values, presumably it is due to the small sample size. It should be emphasized that this study only examined the 20-40°C temperature range (most likely in clinical conditions); if greater temperature ranges were necessary, a new test should be done. Several factors can alter viscosity values during temperature changes: (1) thermal expansion of sample, (2) thermal expansion of chamber components, (3) sedimentation of RBCs during the time until new temperature is achieved. Thermal expansion can change the distance between the cone and the plate, thus altering chamber geometry. Before this experiment we cooled down the empty instrument to 10°C, set the distance and heated up to 37°C. At 37°C the micrometer adjusting ring had to be turned one scale to achieve the original distance (distance increased due to heating). According to the user's manual, one scale division is equivalent to 0.0127 mm movement of the plate relative to the cone. Heating up the instrument from 20°C to 40°C took around 21 minutes (in the opposite direction it is about 30 minutes), after which time RBC sedimentation must be taken into consideration, which can be reduced by continuous shear. Although no significant changes were observed, it is recommended to calibrate the device after a temperature change.

PV and WBV presented in Figure 4 demonstrate good reproducibility over a wide range of shear rates. In 3 cases (out of 7) there was a negative correlation between shear rate and coefficient of variation (CV). Originating from the instrument's design, the rotational viscometer is more accurate at higher shear rates or in case of higher viscosity samples (when torque value is higher). The accuracy of measurements depends on several variables, e.g. sample handling,

device setting and environmental conditions (e.g. vibration, dust, temperature, humidity, etc.), thus their precise control is inevitable to conduct valid measurements.

Despite the calibration of both viscometers with the same non-Newtonian fluid, there were different WBV and PV values in the two viscometers. In the background, we assume that surface tension has a different effect on the two instruments. In Hevimet capillary viscometer the sample is exposed to a range of shear rates making it hard to define a single shear rate at which the particular viscosity is measured and the pressure gradient and blood flow will decrease over time. From the gained data the software will define apparent viscosity values at shear rates from 10-240 s^{-1} based on the Casson's equation. In a cone-plate system viscosity is directly measured, but it is sensitive to surface film artifacts. At higher shear rates – where shear stress is high – film tension causes negligible extra force, but at low shear rates this tension force becomes more prominent compared to the total shear stress. Antonova et al. described similar finding at studied shear rate range (0.017 s^{-1} - 128.5 s^{-1}). They compared a Couette type rotational viscometer to a tube capillary one where the obtained plasma viscosity values measured by the capillary viscometer were lower (72). Wang et al. reported that the whole blood and plasma viscosity values obtained in capillary type viscometer were lower than those obtained with the Couette type rotational viscometer but there was a linear relationship. The relative difference was found greater at lower shear rates. They explained the obtained difference by the viscoelastic, thixotropic behavior of the blood and the systematic errors of the instrument (73). Marinakis et al. compared the cone-plate type rotational viscometer to a capillary type one. They found that the cone-plate rotational viscometer obtained smaller whole blood viscosity values and the relative difference was greater at lower shear rate range (74). These findings suggest the whole blood and plasma viscosity values could vary depending on the method applied. The relative

difference between cone-plate rotational and capillary viscometers at higher shear rates is not considerable and systematic errors of the chosen method are assumed in the background.

WBV and PV are stable at room temperature up to 8 hours and at 4°C up to 48 hours allowing to postpone the measurements. The significant difference in WBV was detected at 37°C after 3 hours which result meets a previous finding (75). This should be taken into consideration when designing studies with long incubation times.

5.4. 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.

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

6.1. Patients and Methods

6.1.1. 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) (17-18,39). Exclusion criteria were the following: patient did not sign written informed consent or patient had ischemic rest pain and/or ulcer/gangrene. All patients were consecutively included from the vascular outpatient referrals by several physicians. 30 volunteers without any known arterial diseases (negative history and 1.00 < ABI < 1.3) were randomly chosen into the control group. Each participant signed written informed consent. The regional ethics committee of the University of Pecs approved the study (No. 5909). All instrumental tests were processed by one independent operator. Our prospective study lasted from September 2015 to March 2017.

The demographics and baseline characteristics of the study population are reported in Table 3 based on the available history and documentation.

6.1.2. General diagnostic approach

Medical history, risk factors, co-morbidities and regular medication were obtained from every participant. Claudication history was assessed according to the Edinburgh claudication questionnaire (22). The term ‘intermittent claudication’ was used according to the Rose criteria (15).

Table 3. Characteristics of the study population.

	Patient group (n= 120)	*Control group (n= 30)*	*p*
Age (mean ± SD, y)	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
Co-morbidities and risk factors (No., %)			
Coronary artery disease	40 (33%)	0 (0%)	<0.005
Cerebral events	15 (13%)	0 (0%)	0.042
Carotid artery disease	41 (34%)	0 (0%)	<0.005
Renal artery disease	7 (6%)	0 (0%)	0.346
Hypertension	102 (85%)	10 (33%)	<0.005
Diabetes mellitus*	62 (52%)	0 (0%)	<0.005
Dyslipidemia	92 (77%)	6 (20%)	<0.005
Smoker (current)	39 (33%)	5 (17%)	<0.005
Smoker (former)	51 (43%)	1 (3%)	<0.005
Sedentary lifestyle	26 (22%)	1 (3%)	0.003
Current medication (No., %)			
Antiplatelet	91 (76%)	4 (13%)	<0.005
Anticoagulant	22 (18%)	0 (0%)	0.005
ACE-I	83 (69%)	6 (20%)	<0.005
ARB	23 (19%)	2 (7%)	0.079
Beta-blocker	63 (53%)	7 (23%)	0.003
CCB	56 (47%)	3 (10%)	<0.005
Statin	92 (77%)	6 (20%)	<0.005
Fibrate	20 (17%)	0 (0%)	0.008

y, year; *SD*, standard deviation; *BMI*, body mass index; *ACE-I*, angiotensin-converting enzyme inhibitor; *ARB*, angiotensin receptor blocker; *CCB*, calcium channel blocker; *m*, meter. *Mean duration of diabetes: 14.1±1.4 yrs, hemoglobin A_{1c}: 7.4±1.7%; 63% of diabetic patients had polyneuropathy.

Systematic physical examination was performed including palpation of the pulses of the lower limbs and measurement of skin temperature of the feet with a non-contact infrared skin thermometer. Patients' self-reported symptoms and physical findings are presented in Table 4. Before any instrumental investigations, the participants had at least 5 minutes of rest period to acclimate to room temperature (20-22°C). The measurements were performed in supine position. The index limb was defined according to participants' complaints or the more deteriorated pre-test absolute ankle pressures. The temperature of the index toe was 26.6±2.7 (median: 26.7)°C in the patient group which was not different significantly in the control group 26.5±3.0 (25.9)°C. The workflow of procedure detailed below is presented in the Figure 10. There were no adverse events during the measurements, which took approximately one hour.

Table 4. Symptoms and physical findings.

	Patient group (n= 120)	*Control group (n= 30)*	*p*
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.	
Absent pulse (No., %)			
Dorsal pedal artery	64 (53%)	0 (0%)	<0.005
Posterior tibial artery	66 (55%)	0 (0%)	<0.005

SD, standard deviation; *m*, meter; *N.A.*, not applicable

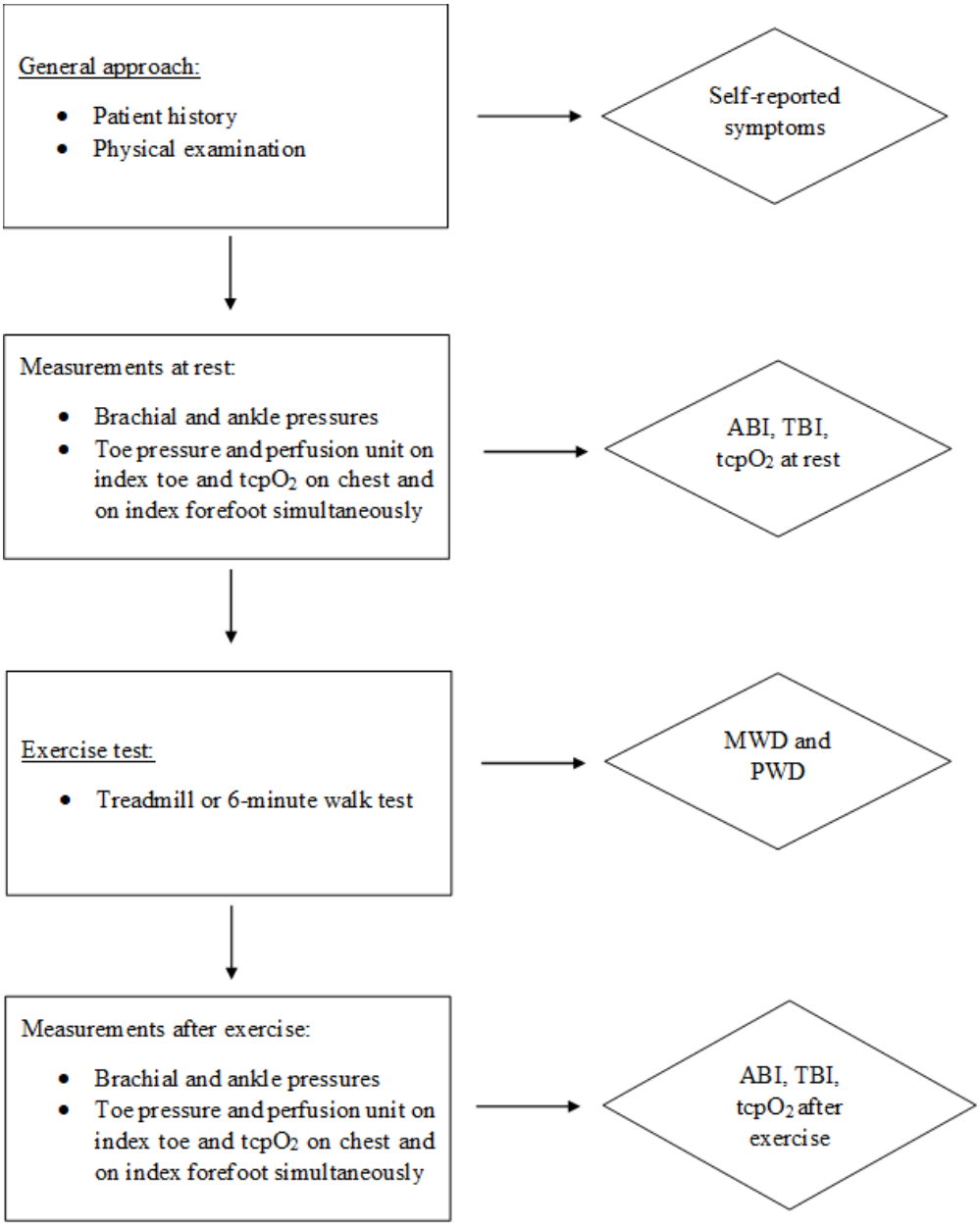

Figure 10. The workflow of procedure (*ABI*, ankle-brachial index; *TBI*, toe-brachial index; *tcpO₂*, transcutaneous partial oxygen pressure; *MWD*, maximal walking distance; *PWD*, pain-free walking distance).

6.1.3. 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 (38). The cuff was placed around the ankle approximately 1 cm above the medial malleolus with parallel wrapping (38). To calculate the ankle-brachial index, 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 absolute ankle pressures were measured at rest and after exercise. The threshold of ABI value for diagnosing LEAD was ≤0.9 (38). ABI 0.9-0.71 was considered as mild, 0.7-0.41 as moderate and ≤0.4 as severe LEAD (48-51) while ABI >1.3 was regarded as false high value due to media sclerosis (52-54); ABI between 0.91-1.00 was considered as borderline (38,55).

6.1.4. 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. Pre-test heating was not applied, the measurements were obtained under standard room temperature (20-22°C). The toe pressure of index limb was measured at rest and after exercise. The calibration of laser Doppler device and measurement of toe pressure were performed according to the manufacturer's users' manual. The temperature of the index toe was recorded by the temperature unit of LDF device.

6.1.5. Transcutaneous partial oxygen pressure measurement

Pre-calibrated Clark electrode (*Tina TCM 4000 oximeter, Radiometer, Denmark*) was positioned on cleaned, hairless skin at 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 (fixation rings and contact

liquid were provided by the manufacturer). 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. The calibration was conducted according to the manufacturer's guideline (76), the measurement was performed according to the consensus guideline (70). $TcpO_2$ values <40 mmHg were considered as a cut-off value for LEAD (69-70).

6.1.6. 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 (17,43). 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.

6.1.7. Statistical procedures

The population size was estimated based on relative standard deviations of our retrospective clinical data, alpha level (0.05), power (80%) and enrollment ratio (0.25). The assumption of normality was assessed by Kolmogorov-Smirnov test. As the vast of our studied data violated this assumption (i.e. the data did not distribute normally), non-parametric statistical tests were applied to analyze the differences between groups (Mann-Whitney U test, Kruskal-Wallis H test) and related groups (Wilcoxon signed-rank test, Friedman test). To compare the categorical variables between groups, either Pearson's chi-square test or Fischer's exact test was used. Some

data were missed at random and they were handled with pairwise deletion method. Discriminant factor analysis and pattern recognition by independent multicategory analysis (PRIMA) were applied to classify patients into two arbitrary groups (77). The receiver operating characteristic (ROC) curves were created to study the diagnostic value of the non-invasive tests applying a cut-off point of post-exercise ABI ≤0.4 as severe limb ischemia (SLI). Data are presented as a mean ± standard deviation (SD) if it is not marked otherwise; the median values are placed after the mean ± SD in the parenthesis. The data acquisition and figuring were carried out by Microsoft Excel (v. 1706). The statistical procedures were performed by IBM SPSS Statistics (v. 23.0).

6.2. Results

6.2.1. 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. The participants' maximal and pain-free walking distances are shown in Table 5; the measured pain-free walking distance was significantly lower than the self-reported walking distance previously shown in Table 4 ($p<0.05$). There were no statistically significant differences in the results of non-invasive tests between patients performed treadmill or 6MWT.

Table 5. Participants' maximal and pain-free walking distances (mean ± SD; median in parentheses).

	Patient group	*Control group*	*p*
6MWT, m (mean ± SD)	n= 63	n= 9	
MWD	228 ± 88 (220)	610 ± 137 (600)	<0.005
PWD	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
PWD	124 ± 80 (90)	267 ± 0 (267)	<0.005

SD, standard deviation; *m*, meter; *6MWT*, 6-minute walk test; *MWD*, maximal walking distance; *PWD*, pain-free walking distance

6.2.2. Absolute ankle pressures, ankle-brachial index

Absolute ankle pressures and ankle-brachial indices are presented in Table 6. 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 ($p=0.002$).

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

Absolute toe pressures and TBI values at rest and after exercise are presented in Table 6. 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 6). The diabetic patients had significantly lower PU at rest compared to the non-diabetic patients ((159 ± 93 (146) vs. 198 ± 84 (189), p=0.028).

6.2.4. 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 6). Changes in $tcpO_2$ due to exercise comparing to baseline are displayed in Figure 11. 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% ($p<0.005$). Diabetic patients had significantly lower $tcpO_2$ at rest and 15 minutes after exercise (diabetic: 38± 16 (42) vs. non-diabetic: 46 ± 13 (46) mmHg, p=0.011); diabetic: 39 ± 18 (42) vs. non-diabetic: 47 ± 15 (49) mmHg, p=0.010, respectively).

Table 6. Ankle pressures, ABI, toe pressures, TBI, laser Doppler perfusion unit and $tcpO_2$ at rest and after exercise (mean ± SD; median in parentheses)

	Patient group (n= 120)	*Control group (n= 30)*	*p*
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

SD, standard deviation; *mm Hg*, millimeter of mercury; *DPA*, dorsal pedal artery; *PTA*, posterior tibial artery; *ABI*, ankle-brachial index; *TBI*, toe-brachial index; *$TcpO_2$*, transcutaneous partial oxygen pressure. [a, b]: the difference was statistically significant within the group compared to the value measured at rest ([a]: $p< 0.005$, [b]: $p< 0.05$).

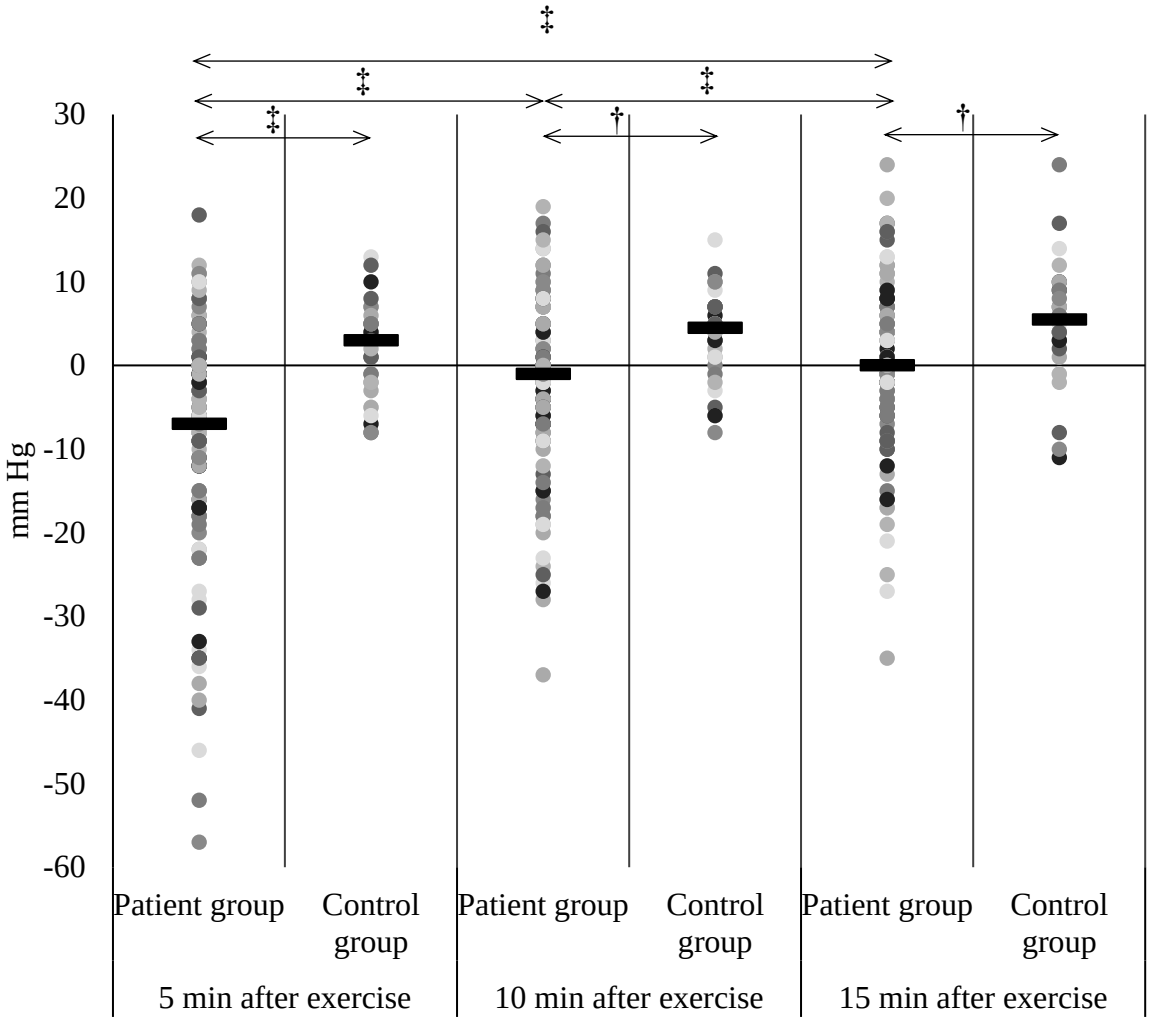

Figure 11. The scatterplot represents the difference between pre- and post-exercise $tcpO_2$ measured on forefoot in the two groups (the black thick lines represent the median values; *mmHg*, millimeter of mercury*; min*, minutes; *†*, $p<0.001$; *‡*, $p<0.01$).

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

Examining the diagnostic performance of the studied non-invasive tests, ROC curve analysis was performed. Based on the post-exercise ABI ≤0.4 (38), patients were grouped into LEAD with or without severe limb ischemia (SLI); the prevalence of SLI was 51 (42.5%). The ROC curves are displayed in Figure 12. 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).

Conducting the non-invasive vascular measurements routinely, we assume it is necessary to distinguish the patients with severely impaired parameters. Therefore, we used the cut-off values of instrumental tests for critical limb ischemia (CLI) written in the guidelines to establish the term 'severe limb ischemia (SLI)' without the clinical symptoms of CLI (ischemic rest pain, ulcer, gangrene). SLI is defined as ABI ≤0.40, TBI ≤0.25 and $tcpO_2$ measured on forefoot <30 mmHg. Based on any cut-off values of SLI, the patients were classified into two groups (whether they have or do not have SLI). To determine which non-invasive method stratifies the patients better with and without SLI, a discriminant model was created and a pattern recognition by independent multicategory analysis (PRIMA) was performed.

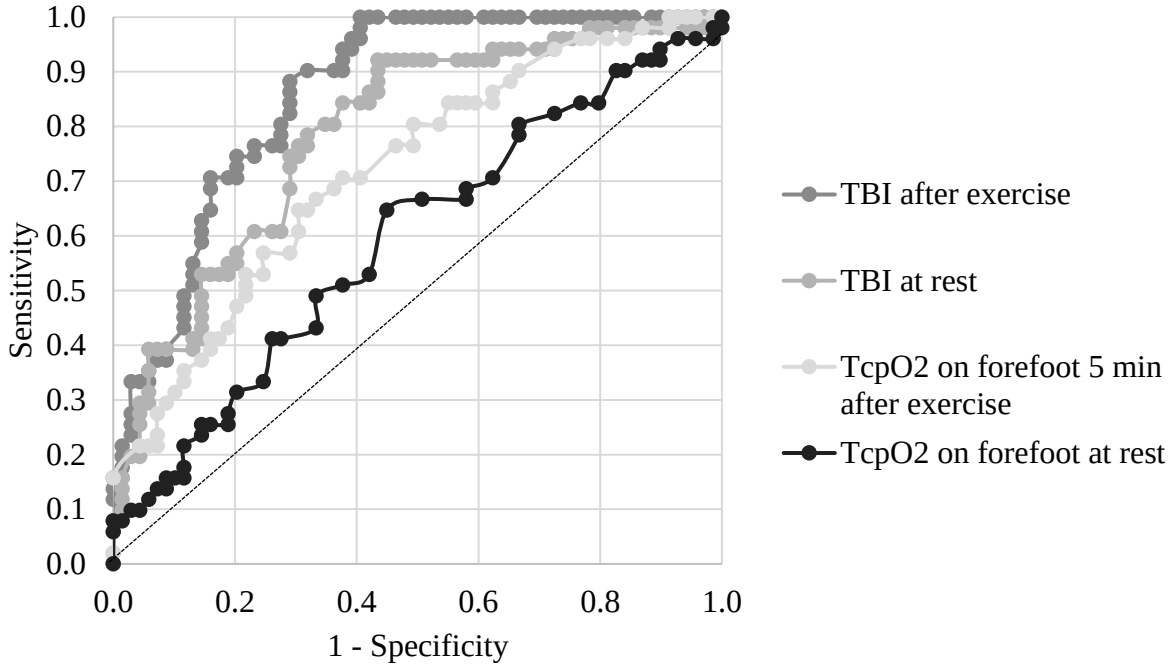

Figure 12. Receiver operating characteristic (ROC) curves of TBI and $tcpO_2$ (n=120). The dotted line represents the line of no-discrimination. Diagonal segments of affected ROC curve are produced by ties (*TBI*, toe-brachial index; *$tcpO_2$*, transcutaneous partial oxygen pressure).

The discriminant factor analysis was run with a stepwise and Wilks' lambda selection method. Our discriminant model equation is expressed as D= -2.901 + 2.797 (TBI after provocation) + 1.200 (ABI after exercise) + 0.037 ($tcpO_2$ on forefoot 5 minutes after exercise). Evaluating our model, a classification test was carried out. 85% of the originally grouped cases were correctly classified with sensitivity of 87.5% [95% CI: 77.6-94.1%], specificity of 80.9% [95% CI: 66.7-90.9%], diagnostic accuracy of 84.9% [95% CI: 76.9-90.6%] and odds ratio of 30 [95% CI: 11-81].

Since our discriminant factor analysis was violated because of inhomogeneity, patients with and without SLI were classified by a pattern recognition called PRIMA. This analysis resulted in

the following discriminant scores (≥1 is better than <1): TBI after exercise (1.815), $tcpO_2$ on forefoot 5 minutes after exercise (1.693), ABI after exercise (1.393), TBI at rest (1.110) ($p<0.005$; CI 95%); ABI at rest (0.656) and $tcpO_2$ at rest (0.653) (n.s.; CI: 95%). PRIMA resulted in sensitivity of 89% [95% CI: 79.5-95.2%], specificity of 85% [95% CI: 71.7-93.8%] with diagnostic accuracy of 88% [95% CI: 79.9-92.6%] and odds ratio of 46 [95% CI: 16-138].

6.3. Discussion

The studied non-invasive vascular tests have been known for decades, yet – except for DUS and ABI – they are not widely used in the vascular diagnostic and prognostic approach (64,78-80). Exercise testing is widely accepted in cardiology to reveal myocardial ischemia and to determine patients' functional capacity as well as their prognosis (81). Although there is a lot common in these fields, exercise testing has played a marginal role in the diagnostic work-up of vascular medicine. Non-invasive diagnostic tests with and without exercise were not accentuated in the international consensus guidelines (79). This could be attributed to the lack of large, randomized, multi-center studies on the role of non-invasive methods in peripheral vascular diseases. On the other hand, the main disadvantages of digital subtraction angiography (DSA) and computed tomography angiography (CTA) are well-known, i.e. contrast agent, ionizing radiation, more morphological and less functional information, costs. CTA or DSA is not optimal for patients' regular follow-up and it could be omitted when revascularization is out of consideration.

Doppler ultrasound and ankle-brachial index have great reliability and validity to detect stenosis ≥50% in lower limb arteries. Nevertheless, this non-invasive standard method characterizes the macrocirculation of the lower limb and it is mainly used at rest. The deterioration of microcirculation of the skin and the muscular blood flow evolves parallel with the impairment

of macrocirculation in the early stage of LEAD. The impaired microcirculation of the muscles can be provoked by exercise resulting in lower limb ischemia with clinical manifestations of claudication or silent ischemia. Exercise induced changes cannot be characterized by resting examinations. We think that exercise should get a greater role in the clinical work-up of PAD. Calcified lesions in the calf arteries could result in misleading (overestimated) ABI values. Henni et al. have recently mentioned that post-exercise ABI is not an adequate method to assess the origin of lower limb complaints at some situations since the lesions of the internal iliac artery, the deep femoral artery and the distal foot are not located on the axis measured by DUS and ABI (57). We assumed that the studied non-invasive tests at rest and after exercise can characterize the peripheral vascular status better than resting ABI alone. Furthermore, there are several patients with different severity of LEAD who have normal non-invasive parameters at rest due to compensated resting blood flow. Two different types of exercise (6-minute walk test, treadmill) were applied in our study which seemed to be a reasonably practical approach. There are several patients presented in the vascular lab with lower limb complaints of different origins. Many patients have joint, vertebral or neurological disorders (beside PAD), who are unable to perform the treadmill test. Therefore, the 6-minute walk test served as an alternative to provoke lower limb ischemia. We supposed the necessity of a control group to investigate the post-exercise physiological changes in the non-invasive hemodynamic parameters.

In the whole population the self-reported claudication distance was significantly longer than the pain-free walking distance under controlled clinical conditions. Exercise test performed either on treadmill or as a 6-minute walk test could provide more objective information on functional capacity of a peripheral vascular patient similarly to patients with heart disease. Moreover, exercise can increase the sensitivity of lower limb measurements. Out of the 25 symptom-free

patients, seven had severely impaired ABI, TBI, or $tcpO_2$ at rest; following the exercise, sixteen had at least one severely impaired value, which was a remarkable tendency especially from a clinical viewpoint (silent ischemia could be detected). Pre-test ABI was positive in less than 2/3 of the patients that increased to more than 3/4 after exercise and similar re-classifications could be performed based on TBI and $tcpO_2$. It implies that compensated circulation at rest turned into decompensation (ischemia) of the lower limb, which could be measured by these non-invasive methods.

The dynamics of $tcpO_2$ measured on forefoot due to exercise was different between the control and patient subjects. The post-exercise $tcpO_2$ measured on forefoot showed a gradual increase in the control subjects in contrast with the patients in whom it decreased significantly after 5 minutes followed by gradual normalization. Our results corresponded well with the results of Abraham et al. (82), Byrne et al. (80) and Mahe et al. (42).

Performing all the studied non-invasive tests in every patient could be time-consuming. Therefore, the diagnostic value of these methods was evaluated by ROC curve analysis, discriminant function analysis, and PRIMA. The post-exercise TBI was shown to have the best capacity to differentiate patients with and without severe limb ischemia. As Høyer et al. and Shishehbor et al. reported earlier, not only post-exercise TBI but also TBI at rest had a neglected role in the vascular diagnostic approach (64,79). The recent society guideline (18,83) recommends the use of toe pressure and TBI only in the case of non-compressible arteries as a surrogate test of the ABI. The sensitivity of toe pressure for CLI and predicting limb loss is better than ABI and ankle pressures as Shishehbor et al. (79) and Vallabhaneni et al. (84) have reported. Moreover, exercise testing can improve the sensitivity of TBI. Therefore, our findings underline the importance of wider application of TBI combined with exercise testing and their

incorporation in the non-invasive staging of peripheral artery disease. It could fill the diagnostic gap between basic examinations and radiological imaging.

6.4. 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.

6.5. Study limitations

Our study was limited because of the use of both the treadmill and the 6-minute walk test, although it was practical from a clinical viewpoint. Our $tcpO_2$ unit could detect not more than two channels, thus proximal limb ischemia was not studied; $tcpO_2$ was not recorded continuously during the exercise for safety reasons. Our study had one independent operator.

7. Summary of the new scientific results

7.1. 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.

7.2. 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.

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10. Publications of the author

10.1. 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.

10.2. 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.
2. Papp J, Barbara S, Toth A, Rabai M, Botor D, **Kovacs D**, Csernus Z, Toth K, Kesmarky G. Altered microrheological parameters in Raynaud's phenomenon. Clin Hemorheol Microcirc. 2017, 65 (1): p. 23-29.

   Impact factor: 1.679

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. DOI: 10.3233/CH-189103.
   Impact factor: 1.679

10.3. Book chapter

1. Biro K, **Kovacs D**, Koltai K, Toth K, Kesmarky G. Haemorherological Aspects of Peripheral Vascular Diseases. In: Mariella Catalano et al. (eds) VAS European Book on Vascular Medicine/ Angiology. Aracne editrice 2018, p. 89-97 (in press).

10.4. Published abstracts

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