Title:
Influence of Rivaroxaban for Intermittent Claudication and Exercise Tolerance in Patients With Symptomatic PAD

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SUMMARY

This study aims to assess the efficacy of rivaroxaban (trade name of the medicinal product Xarelto) administered together with acetylsalicylic acid (ASA) in comparison with the efficacy of ASA itself with respect to peripheral artery disease (PAD) severity and cardiological capacity in patients with PAD. Current COMPASS results (Cardiovascular Outcomes for People Using Anticoagulation Strategies) have shown that rivaroxaban at a vascular dose (2.5 mg 2x/day) combined with ASA (75-100 mg 1x/day) provides more effective cardiovascular protection (defined collectively as cardiovascular death, myocardial infarction, and stroke) compared to ASA alone. Basic and clinical studies indicate that rivaroxaban not only has a positive effect on thrombotic and embolism processes, but also probably causes pleiotropic effects, which are associated with the reduction of vascular damage resulting from cardiovascular risk factors, and has the potential to inhibit the development of atherosclerotic changes.

However, so far, no clinical trials have been conducted on the protective effects of rivaroxaban in relation to the progress of peripheral atherosclerosis and exercise tolerance in patients with PAD in short-term observation.

The studies will be based on the prospective assessment of the clinical condition of PAD patients hospitalized in the Clinical Hospital of Transfiguration, Department of Vascular, Endovascular Surgery, Angiology and Phlebology of the Poznan University of Medical Sciences or monitored in the adjacent vascular Outpatient Clinic where anticoagulants are used in thrombotic events prevention. The expected observation period is three months. The proposed pharmacotherapy does not exceed the standard of medical care in patients with PAD. The assessment of intermittent claudication (IC) distance will be performed on the basis of a treadmill test, according to Gardner protocol. The assessment of exercise tolerance will be performed on the basis of exercise duration, questionnaire, and biochemical exponents. The individual oxygen ceiling value (VO2max) and energy expenditure expressed (metabolic equivalent MET-s) will be also estimated. Fatigue levels change will be independently assessed using the Borg subjective scale. At rest and after exercise, the intensity of metabolic exercise acidosis will be assessed (a measurement of lactic acid in blood before and after exercise).

An additional effect of the research will be the collection of biological material for further genetic research and research on vascular biomarkers. It is assumed that the genetic characterization of patients with respect to functional polymorphisms of genes affecting the function of blood vessels (e.g., *MTHFR, ApoE, PON1, HIF1A, VEGF*) will be performed.

The obtained results will be the basis for applying for external funding for research (NCN, NCBiR) and further multi-center research..

EXISTING KNOWLEDGE AND MAIN ASSUMPTIONS OF THE PROJECT

Atherosclerosis is the most common cause of peripheral artery disease (PAD), the first symptom of which is intermittent claudication (IC). IC is characterized by the occurrence of pain, cramps, numbness, and discomfort in the lower limb muscles. They gradually intensify while walking, forcing the patient to stop. The ailments are caused by continuous, specific physical effort and quickly disappear when it is stopped. The limitation of the possibility of walking leads to a decrease in the quality of life of patients. It adds to other dangerous consequences of

atherosclerosis, increased risk of cardiovascular incidents (myocardial infarction, stroke, acute lower limb ischemia) (Micker M et al. 2006).

Treatment of patients with IC is aimed at modifying cardiovascular risk factors and improving the quality of life by relieving pain and prolonging the IC distance. (Goszcz et al., 2009)

1. New possibilities of pharmacotherapy for patients with symptomatic peripheral artery disease

According to the adopted TASC (The Trans-Atlantic Inter-Society Consensus), and its TASC II supplement concerning aspects of management and treatment of patients with chronic limb ischemia, drugs used in PAD patients were divided into several groups because of their justified and documented therapeutic efficacy in multicenter clinical trials. These include: drugs with documented clinical effect used in intermittent claudication (cilostazol and naphthydrofuryl), drugs with probable clinical effect used in intermittent claudication (carnitine and propyl-L-carnitine), lipid-lowering drugs (statins), drugs with insufficiently proven clinical effects (penthoxyphilin), anticoagulants, L-arginine, 5-hydroxytrypatamine (ketanserin) antagonism, buflomedil, and defibrotide.

Antiplatelet drugs play an essential role in the therapy of PAD patients. They are used to prevent thrombotic complications in the course of myocardial infarction, stroke and thrombi in peripheral arteries. Acetylsalicylic acid (ASA, aspirin) at doses of 75-325 mg per day, ticlopidine at doses of 250 mg 2x/day and clopidogrel at doses of 75 mg 1x/day (CAPRIE, 1996) are proven to be effective in PAD patients. Recently there have been reports of beneficial effects of policosanol in patients with peripheral circulation disorders. This drug lowers cholesterol levels but also has an antiplatelet effect, demonstrating in clinical trials a significant prolongation of the claudication distance after ten weeks of treatment (10 mg 1x/day), while the results after ASA (100 mg daily) were inconclusive (Illnait et al., 2008).

The group of new antiplatelet drugs, used in PAD patients, also includes rivaroxaban (trade name of the medicinal product Xarelto). Currently, the results of the COMPASS study have shown that rivaroxaban in a vascular dose (2.5 mg 2 x/day) combined with ASA (75-100 mg 1 x/day) provides more effective cardiovascular protection (defined together as cardiovascular death, myocardial infarction and stroke) compared to ASA alone (Anand SS et al. 2018; Olinic DM et al. 2018; Connolly SJ, 2018). Other studies with the acronyms MACE and MALE, conducted in the PAD patients of nearly 5,000 cases in total, confirm that the use of rivaroxaban provides comprehensive vascular protection in this group of patients. The former showed a 28% reduction in the risk of cardiovascular death/myocardial infarction/brain stroke, the latter a 44% reduction in the risk of acute lower limb ischemia and a 46% reduction in the risk of acute/chronic limb ischemia and a 70% reduction in the risk of large amputations. This is the only anticoagulant that, in combination with ASA, reduces mortality in patients with coronary artery disease (CAD) and PAD. At the same time, Xarelto, in a vascular dose in combination with ASA, does not cause a significant increase in the risk of major bleedings compared to ASA itself. So far, this drug has not been tested in the context of prolongation of the claudication distance

2. Pleiotropic effects of rivaroxaban on arteriosclerosis

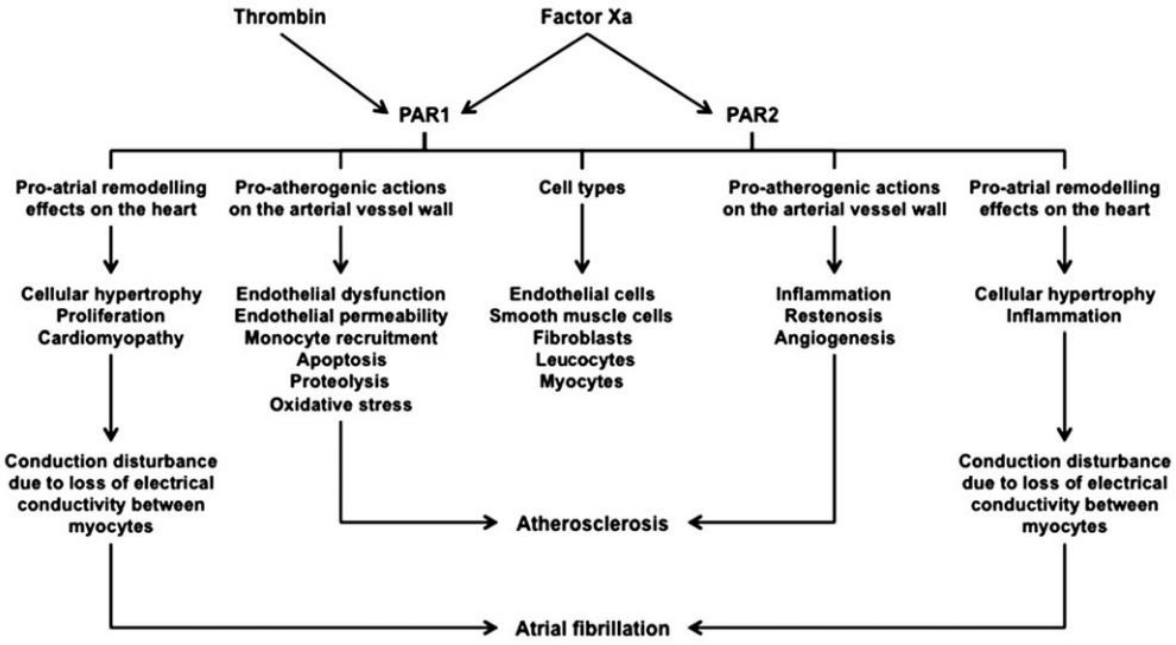
Rivaroxaban inhibits the formation of thrombin and clots by directly inhibiting factor Xa (activated factor X), thus preventing the transformation of prothrombin into thrombin and interrupting the final common path of the blood clotting cascade. This drug **competitively inhibits**

factor Xa with 10,000 times the selectivity of related serine proteases such as thrombin and factors VIIa, IXa, and XIa.

Numerous **experimental data suggest the role of clotting system components in regulating atherosclerosis progression** (Borensztajn K et al., 2008; Borissoff et al., 2009). This is particularly expressed in the case of platelets, which not only play a role in the process of hemostasis, but their action is also important in proinflammatory states, such as atherosclerosis. Moreover, many clotting proteins are involved in atherosclerotic processes such as endothelial damage, oxidative stress, recruitment of leukocytes, inflammation, migration, and proliferation of vascular smooth muscle cells, activation of the immune system, platelet apoptosis and angiogenesis (Platek and Szymański, 2019). **These activities are mediated by various factors of the coagulation cascade, and the broadest spectrum of action concerns the effect of the complex of factor Xa and thrombin precisely.**

After activation, factor Xa initiates intracellular signaling in different types of cardiovascular cells, preferentially through type 2 protease-activated receptors (PAR-2); or both PAR receptors (-1 and -2).

Figure 1. Pleiotropic mechanism of rywaroxaban in relation to atherosclerosis. Representation of cellular locations of PAR1 and PAR2 and the potential effects of thrombin- or factor Xa-mediated PAR activation on the arterial vessel wall and the heart, and the resulting contribution to atherosclerosis and AF (according to Spronk H.M. i wsp. *Cardiovasc. Res.* 2014; 101: 344–351) .



PAR-1 and/or -2 receptors are present in large numbers on endothelial cells, leukocytes, smooth muscle cells, fibroblasts, and dendritic cells. The signal transmitted through PAR contributes to the production of proinflammatory cytokines (e.g., IL-6) and expression of cellular adhesion molecules (E-selectin, ICAM-1, VCAM-1), together with tissue factor growth regulation, smooth muscle cell proliferation and release of growth factors. All these factors may contribute to atherosclerotic plaque progression, including inflammation, leukocyte transmigration, restenosis, and angiogenesis. It should also be noted that vascular wall remodeling and the formation of new atherosclerotic plaque can be reduced by administering non-specific Xa factor inhibitors (low

molecular weight heparin and low molecular weight heparin) conjugated to the anti-fibrin antibody (Borissoff JI et al., 2009).

Scientific research confirms the pleiotropic effects of rivaroxaban on the cardiovascular system. However, most of the currently available observations come from basic research whose results need to be confirmed in clinical trials. The results of in vitro studies using human cells indicate that **rivaroxaban** reduces the expression of proinflammatory cytokines in the vascular endothelium and may contribute to the **stabilization of atherosclerotic plaques**. On the other hand, it has been shown in animal models that selective inhibition of factor Xa ensures a reduction of restenosis after balloon angioplasty of the arteries, and this drug is used to weaken atherosclerotic plaque progression and its destabilization in mice with ApoE gene excluded. Rivaroxaban **positively influences neovascularization and angiogenesis**, especially in hyperglycemic conditions, increasing the concentration of endothelial nitric oxide synthesis and expression of vascular endothelial growth factor (VEGF). The beneficial effects of rivaroxaban under hypoxia-reperfusion and periodic hypoxia conditions were also observed.

Among the planned clinical trials that may establish pleiotropic rivaroxaban actions, the **PRE ¬FER-AF study** (PREvention oF thromboembolic events - European Registry in Atrial Fibrillation) is mentioned as the most important. This study aims to determine the efficacy of oral anticoagulants other than vitamin K antagonists (NOAC) in preventing endothelial dysfunction and atherosclerosis progression **in patients with atrial fibrillation**. Patients with no previous clinically apparent history of CAD, PAD, or stroke will be randomly assigned to the NOAC (dabigatran or rivaroxaban) or placebo-taking group. It is planned to determine vascular endothelial function in long-term therapy after 12 and 24 months. The thickness of the intima-media complex in the carotid arteries will also be measured. This study will assess whether long-term treatment with rivaroxaban brings measurable benefits in relation to the first stages of atherogenesis and atherosclerosis development. **However, it will not provide information on the effect of the drug on the progress of PAD or cardiological capacity of patients.**

3. The objective

The study aims to assess the protective efficacy of rivaroxaban (trade name of the medicinal product Xarelto), administered together with acetylsalicylic acid (ASA), in comparison with the effectiveness of ASA alone, with respect to intermittend claudication and cardiological capacity in patients with PAD over a 3-month period.

4. Type of study

Prospective, observational, unblinded (open), randomized, clinical-control, single-centre.

5. Description of the study group (age, gender, health status, size of the group)

The study will include patients with symptomatic peripheral artery disease (PAD), hospitalized in the The Lord's Transfiguration Clinical Hospital of PUMS, the Department of Vascular, Endovascular, Angiology and Phlebology, monitored in the adjacent Vascular Outpatient Clinic, or referred by cooperating physicians who will use a different protocol of anticoagulation

pharmacotherapy to prevent thrombotic events. The recommended pharmacotherapy does not exceed the standard of treatment in patients with PAD.

5.1 Size of the study group

At least **60 patients with symptomatic PAD**, i.e., those with symptoms of intermittent claudication, will be included in the study. In the case of outflow of patients from the study due to an unexplained course (lack of information about the reason for not attending the follow-up visit after three months) or lack of statistically significant effects, the possibility of increasing the recruitment of patients for the study is assumed. The maximum group size will be increased to 100 patients. **The collection and observation of the study group will take place until the scientific goal of the study is achieved**.

5.2 Characteristics of the study group and collected material

Researchers will perform a clinical evaluation of patients with PAD. A clinical history of the inclusion/exclusion criteria, medical history, and cardiovascular risk factors will be performed. Patients will be subjected to the objective assessment of exercise tolerance, assessment of claudication distance, implementation of anticoagulation pharmacotherapy protocol, and prospective study protocol.

The examined persons will be taken venous blood samples in the volume of 9-18 ml to perform necessary diagnostic tests (including blood morphology, lipid profile, glucose level, biochemistry) and molecular (genetic and biomarkers in the blood). Capillary and venous blood will also be collected to assess the intensity of metabolic exercise acidosis and other biochemical indicators

The costs of blood collection and diagnostic tests will be covered from the budget of the Poznan University of Medical Sciences from the pool of funds allocated for scientific activities. The costs of biochemical and genetic research will be covered by the Poznan University of Physical Education and Institute of Human Genetics, Polish Academy of Sciences, respectively.

5.3 Inclusion and exclusion criteria

The primary selection criteria for all the subjects are the presence of PAD, Caucasian origin, inhabitants of the Great Poland region. Patients who do not have clinical contraindications to performing the exercise test will be included in the study. Detailed inclusion criteria are given in Table 1. In Table 2, exclusion criteria are collected.

In order to qualify for inclusion in the study, patients with PAD of Fountain grade IIa or IIb will be included (with intermittent claudication). The disease will be confirmed by the ankle-brachial pressure index (ABI), with a score of less than 0.90. In all subjects, the ABI will be measured at the start of the study; the result will be calculated from the ratio of the highest systolic limb blood pressure to the highest systolic arm blood pressure.

Table 1. Inclusion criteria

Parameter	**Study**
Age 50-70 years old	This study
Sex: women and men	This study
PAD with advancement on the Fountain IIa or IIb scale	This study

Table 2. Exclusion criteria

LP	Parameter	Study
1	Severe heart failure with known ejection fraction <30% or NYHA class III or IV symptoms	COMPASS
2	High risk of bleeding	COMPASS
3	Stroke with 1 month or any history of hemorrhaging or lacunar stroke	COMPASS
4	Estimated glomerular filtration rate <15 ml/ml	COMPASS
5	Need for dual antiplatelet therapy, other non-aspirin antiplatelet therapy/, oral anticoagulant therapy/	COMPASS
6	Known non-cardiovascular disease that is associated with poor prognosis (i.n, metastatic cancer)	COMPASS
7	History of hypersensitivity or known contraindication for rivaroxaban, aspirin	COMPASS
8	Systemic treatment with strong inhibitors of CYP 3A4 as well as p-glycoprotein or strong inducers of CYP 3M	COMPASS
9	Any known hepatic disease associated with coagulopathy	COMPASS
10	[Subjects who are pregnant, breastfeeding, or are of childbearing potential, and sexually active]	[COMPASS-not used in this study]
11	Concomitant participation in another study with investigational drug	COMPASS
12	Known contraindication to any study-related procedures* Concerns: Absolute contraindications to perform an exercise test	COMPASS
13	Respiratory failure	This study
14	BMI ≥ 40	This study
15	Musculoskeletal dysfunction preventing walking (e.g. amputations)	This study

*- myocardial infarction within 2 days, unstable coronary artery disease, unmanaged by pharmacological treatment; symptomatic arrhythmia, severe, symptomatic aortic valve stenosis; unmanaged heart failure; fresh pulmonary embolism or myocardial infarction; acute pericarditis or myocardial infarction; acute aortic dilation.

5.4 Pharmacotherapy

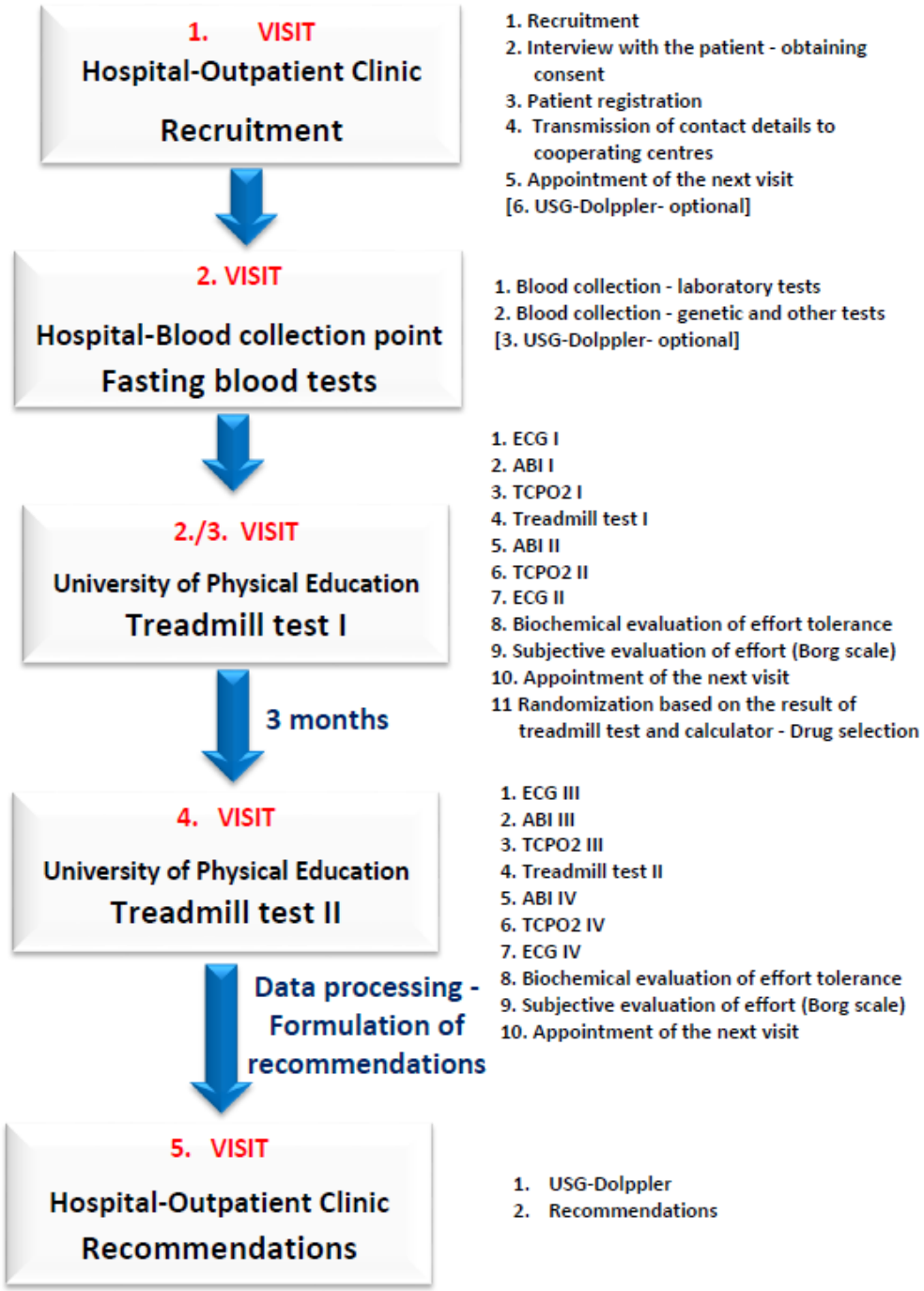
Patients will be selected according to inclusion and exclusion criteria and randomly allocated to two groups with the different anticoagulation treatment protocol. The following treatment profile is planned:

1. ASA
2. ASA + rivaroxaban
Vascular doses of drugs will be used, including in group 1: 1 x/day ASA 75-100 mg and in group 2: 1 x/day ASA 75-100 mg + 2 x/day 2.5 mg Xarelto. **Treatment costs will not be reimbursed by the researchers, patients pay for the drugs themselves**.

6. Methods

The main plan of the research is shown in Figure 2.

Figure 2. The research plan.



6.1 Clinical characteristics of the studied population

The following clinical data will be collected using existing clinical documentation, and/or commissioned laboratory tests (performed under contract with the hospital), and based on data obtained from the patient (see Figure 3 for a clinical history):

1. Results of aortic and lower limb arteries imaging (angiography, computed tomography, ultrasounds; which will assess the severity and location of atherosclerosis in lower limb arteries)
2. Results of heart function tests (echocardiography, electrocardiography- ECG)
3. Assessment of the occurrence and severity of atherosclerotic lesions in blood vessels (including ankle-brachial index -ABI: at rest and after exercise, the degree of thickening of the intima-media complex; IMT-ultrasound-Doppler examination; blood supply and oxygenation of tissues by percutaneous oximetry (Precise 8008 device for measurement of TcPO2)
4. Results of fasting venous blood laboratory tests (Table 3) glucose metabolism: glucose (GLU), glycated hemoglobin (HbA1C), diabetes mellitus, lipid metabolism (TC, HDL, LDL, TG), uric acid concentration (URCA), CRP, renal function: glomerular filtration rate (eGFR); creatinine (CREA); urea (UREA), liver function: aspartate aminotransferase (AST), arginine aminotransferase (ALT), homocysteine (Hcy), folic acid (FA), peripheral blood morphology, blood coagulation parameters: fibrinogen, prothrombin time (PT), international normalized ratio (INR)
5. Blood pressure values and the presence of hypertension.
6. Results of virological and bacteriological tests
7. Anthropometric data (weight, height, BMI, waist and hip circumference, WHR)

(8) Interview on smoking (estimation of the "peak years" index),

9. Pharmacotherapy (drugs: antiplatelet, lipid-lowering, anti-hypertensive: angiotensin-converting enzyme inhibitors, angiotensin receptor blockers, beta-blockers, proton pump inhibitor)
10. Coexisting diseases
11. Severity of pain
12. Family history of cardiovascular disease and cancer

Tabela 3. Laboratory blood tests

No.	Parameter
1	Lipid profile (TC, HDL, LDL, TG)
2	Glucose (GLU)
3	Glycated hemoglobin (HbA1C)
4	Uric acid (URCA)
5	Glomerular filtration rate (eGFR)
6	Creatinine (CREA)
7	Urea (UREA)
8	Aspartate aminotransferase (AST)
9	Arginine aminotransferase (ALT)
10	Peripheral blood morphology
11	Fibrinogen
12	Prothrombin time (PT)
13	International Normalized Ratio (INR)
14	Homocysteine (Hcy)
15	Folic acid (FA)

Figure 3. Sample questionnaire for a clinical interview

NO.	

Date:.....

AN INTERVIEW OF THE PATIENT'S HEALTH

All information provided is subject to medical confidentiality. Please answer the following questions carefully. If you find it difficult to answer, please skip the question and clarify your doubts with your doctor.,

PESEL

Surname, name of the patient .....

Phone .....

Address.....

1. Have you been treated in a hospital in the last 2 years? YES ☐ NO ☐

If YES, then for what reason: .....

2. Have you ever had vascular surgeries? YES ☐ NO ☐

If YES, then for what reason: .....

3. Do you have diabetes? YES ☐ NO ☐

If YES, since when (how long .....?): .....

4. Do you have hypertension? YES ☐ NO ☐

If YES, since when (how long .....?): .....

5. Are you taking any drugs? YES ☐ NO ☐

If YES, what kind of: .....

.....

6. After passing what distance your lower extremities are in pain:

I'm walking without any restrictions ☐ up to 200m ☐

over 200m ☐ already hurts at rest ☐

7. Do you smoke cigarettes?: NEVER ☐ IN THE PAST ☐ NOW ☐

If YES how long do you smoke ..... (starting age for smoking.... ...../ since when you don't smoke .....)

how many cigarettes a day: .....

8. Do you consume alcohol? NEVER ☐ ONCE A WEEK ☐ MORE THAN ONCE A WEEK ☐

9. Do you have or have you had any of the following illnesses (if YES, which)?

Heart disease (MI ☐/ CAD/IHD☐ / HF☐ / CRM ☐ / other ☐)..... YES ☐ NO ☐

Vascular diseases (VV☐ / AO☐ / PAD☐ / AIOD☐/ AAA☐/TAA ☐)..... YES ☐ NO ☐

Stroke YES ☐ NO ☐ (ischemic ☐ / hemorrhagic ☐/ TIA☐ / other ☐)

Lung diseases (COPD☐ / cancer ☐ / asthma ☐ / other☐)..... YES ☐ NO ☐

Gastrointestinal diseases ..... YES ☐ NO ☐

Liver diseases ..... YES ☐ NO ☐

Urinary diseases ..... YES ☐ NO ☐

Thyroid diseases (hyperactivity ☐/ hypothyroidism ☐/ Hashimoto ☐).....YES ☐ NO ☐

Diseases of the osteoarticular system .....YES ☐ NO ☐

Rheumatic disease .....YES ☐ NO ☐

Other health problems? .....

10. Was there a disease in the family: Heart..... YES ☐ NO ☐

Vascular ..... YES ☐ NO ☐

AAA .....YES ☐ NO ☐

TAA.....YES ☐ NO ☐

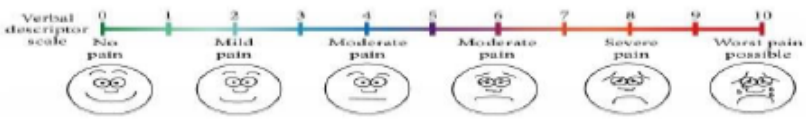
PAD.....YES ☐ NO ☐

Cancer..... .....YES ☐ NO ☐

11 Anthropometric data :

Hight [cm] ..... Weight [kg].....BMI.....	
Arterial blood pressure [mmHg]	SBP.....DBP..... Heart rate .....
Waist to hip ratio [WHR]	Waist [cm]..... Hip [cm]..... W/H.....
Lower limb wounds? Description	Right leg Left leg

For wounds: 11-point Pain Numbered Rating Scale (NRS-11)



Legend to Questions Nos 9 and 10:

Heart disease

MI – myocardial infarction

CAD/IHD - coronary arteriosclerosis / ischaemic heart disease

HF-heart failure

CRM - cardiomyopathy

Vascular diseases

VV – varicose veins

AO - aortic atherosclerosis

PAD - peripheral arterial disease

AIOD- aorto-iliac atherosclerosis

AAA - abdominal aortic aneurysm

TAA - thoracic aortic aneurysm

Stroke

TIA - transient ischaemia of the brain

Lung diseases

COPD - obstructive pulmonary disease

6.2. Treadmill test

The test will be carried out in the Laboratory of Functional Research of the Department of Physiology and Biochemistry of the University of Physical Education in Poznań; in the absence of contraindications. The subjects will be tested at each of the test dates to assess the claudication distance on the Katana S30p medical treadmill (Lode B.V., Netherlands). Before performing the test, patients will undergo an initial, minimum 6-minute implementation of the principles of research, primarily to familiarize them with the way of moving on the treadmill (Mika et al. 2009). Additionally, they will be instructed not to support themselves on treadmill railings during the march. The total time of each test will not exceed 16 minutes 40 seconds, and the regeneration time will be at least 5 minutes.

6.2.1. Qualification for the treadmill test

Patients will be qualified for the treadmill test based on the result of an electrocardiographic examination (ECG), and the test on a treadmill will be carried out by authorized investigators under the supervision of the doctor.

6.2.2 The evaluation of the claudication distance

The treadmill test will be carried out according to Gardner protocol (Gardner et al., 1991). Patients will march on a treadmill at a constant speed of 3.2 km/h. The initial angle of inclination will be 0% and will be raised by 2% every 2 minutes (Table 4). When the patient reports pain (i.e., grade 2 on a subjective pain assessment scale), the time of occurrence of the claudication will be recorded, from which the claudication distance will be calculated (DG_{CH}). The test will be interrupted when very severe pain occurs that prevents further walking (step 5 corresponding to maximum walking time), and the maximum walking time will then be recorded. The maximum walking time will be used to estimate the maximum walking distance (DG_{MAX}).

Table 4. Parameters of the treadmill test according to Gardner protocol

Time	Level	Speed [mph]	Speed [kph]	Distance [m]	Slope [%]
00:00	1	2,0	3,2	0	0,0
02:00	**2**	**2,0**	**3,2**	**107**	**2,0**
04:00	3	2,0	3,2	213	4,0
06:00	4	2,0	3,2	320	6,0
08:00	5	2,0	3,2	427	8,0
10:00	6	2,0	3,2	533	10,0
12:00	7	2,0	3,2	640	12,0
14:00	8	2,0	3,2	747	14,0
16:00	9	2,0	3,2	853	16,0

Abbreviations: mph- miles/hour; kph-km/hour

6.2.3. Evaluation of exercise tolerance.

Evaluation of exercise tolerance will be carried out based on effort duration and a survey and biochemical exponent.

Based on the duration of the effort, the individual oxygen ceiling value (VO_2max) and the energy expenditure expressed as metabolic equivalent MET-s will be estimated. Table 5 gives the MET-s values calculated from the parameters for the cardiological treadmill test, according to Ramped Bruce Protocol; (Ramped Bruce Protocol; Bires AM et al., 2013).

Table 5. Results of the treadmill test depending on the severity of ischemia in the Fountaine scale. Energy expenditure expressed as metabolic equivalent of MET-s is given. The values of MET-s were estimated based on parameters of cardiological treadmill test according to Bruce's ramp protocol.

Time	Level	Speed [mph]	Speed [kph]	Distance [m]	Slope [%]	MET-s	Fountaine scale
00:00	1	2	3,2	0	0,00	2,1	-
00:20				18			IIb
00:40				36			IIb
01:00				54			IIb
01:20				71			IIb
01:40				89			IIb
02:00	2	2	3,2	107	2,00	3,2	IIb
02:20				125			IIb
02:40				143			IIb
03:00				161			IIb
03:20				178			IIb
03:40				196			IIb
04:00	3	2	3,2	213	4,00	4,3	IIa
04:20				231			IIa
04:40				249			IIa
05:00				267			IIa
05:20				284			IIa
05:40				302			IIa
06:00	4	2	3,2	320	6,00	5,3	IIa
06:20				338			IIa
06:40				356			IIa
07:00				374			IIa
07:20				391			IIa
07:40				409			IIa
08:00	5	2	3,2	427	8,00	6,4	IIa
08:20				445			IIa
08:40				463			IIa
09:00				481			IIa
09:20				498			IIa
09:40				516			IIa

10:00	6	2	3,2	533	10,00	7,5	IIa
10:20				551			IIa
10:40				569			IIa
11:00				587			IIa
11:20				604			IIa
11:40				622			IIa
12:00	7	2	3,2	640	12,00	8,5	IIa
12:20				658			IIa
12:40				676			IIa
13:00				694			IIa
13:20				711			IIa
13:40				729			IIa
14:00	8	2	3,2	747	14,00	8,5	IIa
14:20				765			IIa
14:40				783			IIa
15:00				801			IIa
15:20				818			IIa
15:40				836			IIa
16:00	9	2	3,2	853	16,00	9,6	IIa

Abbreviations: mph- miles/hour; kph-km/hour; MET-s - metabolic equivalent.

The degree of intensity of fatigue changes after the completed effort will also be assessed using a subjective scale to evaluate the degree of fatigue (Borg scale; Smarż et al. 2015). At rest and 3 minutes after the exercise, the intensity of metabolic exercise acidosis will be assessed based on lactate concentration in capillary blood taken from the fingertip. At the same time, venous blood will also be collected for biochemical and genetic tests.

6.2.4. Post-treadmill test examination

After completing the treadmill tests, patients will undergo a control ECG examination to exclude ischemic changes in the myocardium and cardiac arrhythmias..

6.3. Length of prospective observation

The effects of the treatment will be assessed within three months. It is assumed that the observation period can be extended to a maximum of 12 months.

6.4. Endpoints of the study

The endpoints of this study and the complications were developed in accordance with the results of the COMPASS study. Parameters related to the results of the treadmill test were added. The study endpoints are summarized in Table 6. The complications related to possible bleeding risk for PAD patients are presented in Table 7.

Table 6. Endpoints of the study

Parametr	Study
Primary outcomes	
Rate of stroke	COMPASS
Rate of myocardial infarction	COMPASS
Rate of cardiovascular death	COMPASS
Rate of death	COMPASS
Prespecified limb outcomes	
Rate of acute limb ischaemia	COMPASS
Rate of major amputation	COMPASS
Rate of all vascular amputations	COMPASS
Tredmill tests outcomes	
Change in the distance of intermittent claudication in the test according to Gardner's protocol ($\Delta D_{ch;} \Delta D_{max}$).	This study
Change in the value of oxygen uptake (Δ VO2max)	This study
Change in energy expenditure expressed as the metabolic equivalent of MET-s (Δ MET-s)	This study

Table 7. Safety outcomes for patients with PAD

Parametr	Study
Rate of major bleeding	COMPASS
Rate of fatal bleeding	COMPASS
Rate of non-fatal symptomatic intracranial haemorrhage	COMPASS
Rate of non-fatal, non-intracranial haemorrhage symptomatic bleeding into a critical organ	COMPASS
Rate of other major bleeding (surgical site bleeding requiring reoperation or bleeding leading to hospitalization)	COMPASS
Rate of fatal or symptomatic bleeding into a critical organ	COMPASS
[Rate of fatal or symptomatic bleeding into a critical organ or surgical site bleeding leading to re-operation]	[COMPASS- not used in this study]
Rate of ISTH major bleeding	COMPASS
Sites of bleeding (frequency of occurrence)	COMPASS
Gastrointestinal	COMPASS
Intracranial	COMPASS
Genitourinary	COMPASS
Ocular	COMPASS
Skin	COMPASS
Respiratory	COMPASS
Other	COMPASS
Minor bleeding	COMPASS

6.5. Biological samples

6.5.1. Biological material collection - blood samples

For standard diagnostic and molecular tests, fasting venous blood samples will be taken from the ulnar vein in the volume of 9-27 ml, from a single puncture. Samples will be collected after the patient is qualified for tests (**1 collection during the study**).

Subsequent samples of venous blood from the ulnar vein (10 ml) and capillary blood from the fingertip (1 ml) will be collected during treadmill tests before and after the exercise to determine objective metabolic markers associated with the exercise (**a total of 2 venous and 2 capillary blood collections during the tests**).

Blood collection from the ulnar vein is a standard invasive test that causes about 1 minute of discomfort and pain, and the risk of bruising, redness, or skin infection is very low. Whenever possible, to reduce the discomfort, additional blood samples will be taken when other routine laboratory diagnostic tests are carried out.

Blood samples may be taken: at the wards of the Department of Vascular and Endovascular Surgery, Angiology and Phelbology of the PUMS at the blood collection point of the The Lord's Transfiguration Clinical Hospital of PUMS or the Department of Physiology and Biochemistry of the University of Physical Education by researchers or collaborators. As compensation, the examined persons will receive free of charge the results of additional laboratory tests conducted by the investigators. If it is not possible to collect blood from the ulnar vein, the blood will be collected from another location (wrist, hand) with the consent of the examined person. The capillary blood from the pad also causes short (30 s) pain and discomfort to the patient, and the risk of bruising, redness, or skin infection is very low in this case.

6.5.2. Preparation and storage of blood samples

Blood samples will be used to perform laboratory tests (9 ml, The Lord's Transfiguration Clinical Hospital Central Laboratory) and to obtain material for further biochemical and molecular analyses: plasma/serum and genomic DNA (residual blood; **Table 8).**

Table 8. Use of biological material

LP	Biological material	**Analyses**
1	Plasma / peripheral fasting blood serum	• Assessment of blood biomarker concentrations
2	Morphotic elements of peripheral blood	• Isolation of genomic DNA, genetic analysis
3	Plasma / peripheral blood serum before and after exercise	• Biochemical analysis - effort markers
4.	Krew kapilarna przed i po wysiłku	• Capillary blood before and after exercise

Serum samples (fasted venous blood) will be collected in serum-separating tubes containing a serum accelerator (SST; proposed: S-Monovette Serum Z, manufacturer of SARSTEDT), left to clot for 15 minutes at room temperature or overnight at 4°C and then centrifuged at 1000 x g for 15 minutes. The serum will be divided into 500 µl samples, placed in 1.5 ml Eppendorf tubes and stored at -80°C.

Blood samples for plasma collection (venous blood collected on an empty stomach) will be collected using the sodium version, EDTA, as an anticoagulant (S-Monovette EDTA,

manufacturer of SARSTEDT), centrifuged for 15 minutes at 1,000 x g at 2-8°C within 30 minutes of collection. Part of the plasma will then be centrifuged for 15 minutes at 4000 x g (for blood micro RNA levels). The plasma will be divided into 500 µl samples, placed in 1.5 ml Eppendorf tubes and stored at -80°C. The remainder of the blood sample taken on EDTA, which contains all cellular elements, will be used for DNA extraction.

Twice during each test, i.e., at rest and after exercise, approximately 10 ml of blood will be taken from the ulnar vein using an S-Monovette syringe without anticoagulant (made by SARSTEDT, Germany), then centrifuged (1500 g, 4°C, 4 minutes) to separate the serum and plasma. The samples will be frozen at -80°C pending biochemical analyses. Similarly, twice during each test

Capillary blood samples will be analyzed immediately after collection.

All samples (blood, fractions obtained) will be stored in the Department of Vascular Surgery, Endovascular Surgery, Phlebology and Angiology of the Medical University of Poland, Department of Physiology and Biochemistry of the University of Physical Education and the Institute of Human Genetics of the Polish Academy of Sciences in Poznań.

6.5.3. Material and methods for laboratory testing of patient blood

The results of laboratory tests will be performed in the Lord's Transfiguration Clinical Hospital Central Laboratory. These results will come from available clinical documentation or will be performed in the above mentioned Laboratory based on a separate agreement with the Hospital. It is also acceptable for the patient to pay for the tests themselves. The analyses will be carried out using standard laboratory techniques used in the Lord's Transfiguration Clinical Hospital Central Laboratory.

6.5.4. Biochemical blood tests

The concentration of lactate in capillary blood before and after exercise (assessment of the metabolic severity of exercise acidosis) will be tested using EDGE Blood Lactate Test Strip, ApexBio, (Taiwan) based on the analysis of blood taken from the fingertip. Similarly, venous blood will be collected before and after exercise for biochemical tests, including gasometry.

6.5.5. Material and methods for testing peripheral blood biomarkers

The level of selected proteins in the blood will be assessed by the immunoenzymatic method (ELISA). The assays of selected circulating miRNAs will be performed using TaqMan (Life Technologies) using the 7900HT Fast Real-Time PCR System (Life Technologies). These tests will be performed at Institute of Human Genetics PAS.

Further analysis of biomarkers present in the blood, to obtain funds for testing, assumes the possibility of using high-pass transcriptomic (use of expressive microarrays) and proteomic (using mass spectrometry) methods.

6.5.6. DNA analysis

DNA extraction will be performed chemically using guanidine isothiocyanate, phenol, and chloroform to remove proteins.

Analysis of genotypes for selected SNPs, including genes with proven role in cardiovascular susceptibility (related to: response to hypoxia, angiogenesis, antioxidant barrier, lipid metabolism, homocysteine and hypertension). Among others, functional polymorphisms of genes *MTHFR, ApoE, HIF1A, VEGF, PON1*) will be assessed using allele discrimination tests (TaqMan SNP Genotyping Assays, Life Technologies) and 7900HT Fast Real-Time PCR System (Life Technologies) in at Institute of Human Genetics PAS.

Material is to be collected for mutation evaluation, whole exome/genome sequencing and epigenome studies. These studies will be carried out based on the DNA sequencing method using the apparatus available in Poznan University of Medical Sciences / Institute of Human Genetics PAS or external services.

6.7. Equipment

In the course of the research project, the equipment of the Poznan University of Medical Sciences, the University of Physical Education in Poznań, the Institute of Human Genetics of the Polish Academy of Sciences in Poznań and cooperating units will be used.

7. Use of results

The results will be published in scientific journals and presented at conventions and scientific conferences.

8. Statements

The research is carried out in compliance with personal data protection. The prospective assessment will be carried out during the Clinic's inspection visits. Additionally, to conduct prospective research, contact with the patient will be made by phone and e-mail based on the submitted statement.

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