

Division of Internal Medicine
Department of Vascular Diseases

Zaloška cesta 7
1525 Ljubljana
T 01/522 80 32
F 01/522 80 70
E ales.blinc@kclj.si
W www.kclj.si

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Study Protocol and Statistical Analysis Plan

Arterial Function and Atherosclerosis in Patients With JAK2 V167F Positive Essential Thrombocythemia

Principal Investigator: Aleš Blinc, MD, PhD,
Professor of Medicine,
Head, Department of Vascular Diseases

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1. Overview and Aim of the Study

We will study patients with JAK2 V617F positive essential thrombocythemia (ET) without clinically apparent atherosclerotic vascular disease in comparison to age-and sex-matched, apparently healthy control subjects and measure changes in vascular function and morphology at baseline and after a 4-year observation period. In addition, the JAK2 V617F allele burden, i.e., the percentage of mutated alleles in leukocytes, will be determined and correlated with vascular function and morphology. The study protocol has been approved by the Committee for Medical Ethics of the Republic of Slovenia, approval number: 0120-428/2017/4.

Our aim is to test (a) whether changes in vascular function and morphology in a 4 year observation period differ between patients with JAK2 V617F positive ET in comparison with control subjects, and whether (b) the JAK2 V617F allele burden correlates with vascular function and morphology.

2. Patients and Control Subjects

Patients with JAK2 V617F positive ET have been selected from the database of the Department of Hematology of the University Medical Centre Ljubljana, Slovenia, after having given their informed consent. Only patients without a personal history of clinically manifest atherosclerotic vascular disease (myocardial infarction, angina pectoris, peripheral arterial disease, aortic disease, transient ischemic attack or ischemic stroke) were eligible. The control group has been selected among apparently healthy employees of the University Medical Centre Ljubljana and their relatives. The groups have been matched for age and sex distribution and classical risk factors for cardiovascular disease. Forty patients (14 male and 26 female) with JAK2 V617F positive ET without clinically apparent cardiovascular disease were enrolled in the study in 2014 - 2015 for the first examination and 36 (12 male and 24 female) of them are expected to participate also in 2018-19. Forty two control subjects (16 male and 26 female) participated in the first examination in 2014-2015 and at least 38 (14 male and 24 female) subjects are expected to participate in the second examination.

Inclusion criteria for patients are: clinically confirmed ET with genetically confirmed JAK2 V617F mutation, age above 18 years, absence of clinically suspected or overt atherosclerotic vascular disease. For the control subjects, the inclusion criteria are being apparently healthy, matching the patients in age and sex, age above 18 years, and absence of clinically suspected or overt atherosclerotic vascular disease.

Exclusion criteria for both groups are: clinically suspected or overt atherosclerotic vascular disease, chronic kidney disease stage 3 and above, known cancer, chronic inflammatory disease, autoimmune disease, and pregnancy.

All subjects will be examined twice, the first time in 2014 - 2015 and the second time in 2018 – 2019. All participants will have signed the informed consent form before enrolment. The subjects will complete a structured questionnaire about personal and family medical history, risk factors for cardiovascular disease and their medication. They will be physically examined and will donate blood samples for laboratory tests. Ultrasound examination of the extracranial carotid arteries will be done, and echo-tracking of the common carotid arterial wall will be used to assess the β-stiffness index and to estimate the pulse wave velocity. EndoPat plethysmography will be used to assess the endothelial function via the reactive hyperemia response of the digital arteries. Coronary artery calcium scanning will be performed to evaluate the extent of coronary artery calcification.

3. Baseline Measurements

Every participant will be examined physically, including measurements of height, weight, waist circumference, systolic and diastolic blood pressure. Blood will be taken for laboratory analyses - electrolytes, complete blood cell count, serum lipids, liver function tests, urea, creatinine, inflammatory markers and DNA

isolation for measurement of JAK V617F allele burden. A structured questionnaire about family history of cardiovascular diseases, personal medical history, smoking status and medications will be completed. The 10-year risk of coronary heart disease (CHD) and general cardiovascular disease (CVD) will be calculated using the Framingham risk equation.

4. Vascular Imaging

4.1. Carotid Artery Ultrasound Examination

For examination of the extracranial carotid arteries an ultrasound machine Aloka Prosound a7 (Hitachi Aloka Medical, Ltd., Japan) will be used with a linear vascular probe working at a frequency of 5-13 MHz. The common, internal and external carotid arteries on both sides will be examined. At each examination, the measurements will be done twice and their average values will be calculated. The ultrasound examination will be done by the same ultrasonographer at both visits of all participants.

The intima-media thickness will be measured 2 cm proximal to the bulb of common carotid artery on both sides. Screening of the extracranial carotid arteries for atherosclerotic plaques will be performed, with a plaque definition of a focal lesion, exceeding the intima-media thickness for at least 50% or reaching an absolute thickness of at least 1.5 mm in two orthogonal projections. Scoring of atherosclerotic plaques is done according to the methodology from the Rotterdam Study. The presence of at least one plaque in each segment of the extracranial carotid arterial bed, (divided into the common carotid artery and the bulb, the internal carotid artery and the external carotid artery) on either sides is scored 1 point. Thus, the carotid plaque score ranges from 0 (absence of plaques) to 6 (plaques present in all segments on both sides).

Echo-tracking of the common carotid artery will be used to assess arterial stiffness expressed by the β -stiffness index and the pulse wave velocity. Measurements will be done at the common carotid artery 2 cm proximal to the bulb on both sides. The β -stiffness index will be calculated as: $\beta = \ln(P_{\max} / P_{\min}) / [(D_{\max} - D_{\min}) / D_{\min}]$, where P_{\max} = the systolic blood pressure, P_{\min} = the diastolic pressure; D_{\max} = the maximum arterial diameter and D_{\min} = the minimal arterial diameter. Pulse wave velocity (PWV) will be calculated as: $PWV = \sqrt{((\beta \times P_{\min}) / 2\rho)}; \rho = 1050 \text{ kg/m}^3$.

4.2. Measurement of the Coronary Artery Calcium Burden

The calcium burden of coronary arteries will be measured with a Biograph M 128-row PET-CT scanner (Siemens, Erlangen, Germany). A non-contrast protocol with sequential prospective ECG triggering will be used. Post-processing will be done on the Syngo Leonardo workstation. The coronary calcium burden will be expressed as the Agatston score.

5. Assessment of Endothelial Function of the Digital Arteries

Endothelial function of the digital arteries will be measured by digital plethysmography with EndoPat 2000, Itamar Medical REF, Caesarea, Israel, Software Version 3.3.x and expressed as the Reactive Hyperaemia Index (RHI) and the Augmentation Index (AI). Changes in arterial tone are elicited by creating a downstream hyperemic response induced by a standard 5-minute occlusion of the brachial artery (using a blood pressure cuff, inflated to 60 mmHg above the arterial blood pressure). When the cuff is released, the surge of blood flow causes endothelium-dependent flow mediated dilatation (FMD) which is manifested as reactive hyperemia. A post-

occlusion to pre-occlusion ratio is calculated by EndoPAT software and the and A) are determined. The results are normalized to a heart rate of 75/min.

6. Measurement of the JAK2 V617F/G1849T Allele Burden

The ipsogen JAK2 MutaQuant Kit, Qiagen (ZDA) (Ref: No. 673523) will be used for the detection and quantification of JAK2 V617F/G1849T allele in genomic DNA extracted from peripheral blood of patients and of control subjects. JAK2 V617F allele burden will be calculated and expressed as a percentage of JAK2 V617F mutated alleles throughout the whole JAK2 record.

7. Statistical Analysis

All sets of data will be tested for normality of distribution using the normal-quintile plot, calculating the correlation coefficient and checking it for the critical value that would warrant rejection of normal distribution with an α -error probability of 0.05.

Normally distributed data will be presented as mean and standard deviation, while non-normally distributed data will be presented as median and range between the 1st and 3rd quartile.

Differences between subjects with ET and control subjects at the first and second examination will be tested by the chi-square test for discrete variables, for normally distributed continuous variables by the paired Student's t-test for independent samples, and for non-normally distributed continuous variables by the Mann-Whitney test for independent samples. P-values of < 0.05 will be considered significant with a correction for multiple comparisons.

Changes in vascular function and morphology in the 4-year observation period will be compared between the two groups by the log rank test. P-values of < 0.05 will be considered significant.

The association between the parameters of vascular function / morphology and the JAK2 V617F allele burden will be assessed by the Pearson correlation coefficient.