STUDY PROTOCOL

Genetic Determinants of the Coronary Microvascular Obstruction in Percutaneous Coronary Interventions

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Study Rationale

For decades, ischemic heart disease (IHD) has been the leading cause of mortality in developed countries. Currently, percutaneous coronary intervention (PCI) is the primary method for restoring blood flow in the coronary artery (CA) in IHD, significantly reducing mortality in myocardial infarction (MI) and improving quality of life in stable angina. However, in 5–10% of cases, PCI leads to coronary microvascular obstruction (CMVO, or "no-reflow")—a phenomenon characterized by the absence of adequate myocardial perfusion despite successful restoration of epicardial coronary artery patency [1]. CMVO substantially worsens prognosis and can be a direct cause of death [1].

The pathogenesis of CMVO is complex and involves multiple concurrent mechanisms with varying degrees of contribution [1]. The most significant clinically established predictors of CMVO include: age, time from symptom onset to reperfusion, severity of acute heart failure (Killip classification), ineffective prehospital thrombolytic therapy, collateral blood flow (Rentrop classification) [6], coronary artery thrombus burden (TIMI thrombus grade) [7], baseline coronary flow (TIMI flow grade) [8], implantation of ≥3 stents during PCI, direct stenting of the target artery, blood neutrophil and glucose levels [2–5].

The pathophysiological heterogeneity of CMVO makes its prediction challenging. Even the best available models [2–5] demonstrate limited positive predictive value (60–70%). This limitation may stem from the omission of genetic factors influencing endothelial function, microcirculation, hemostasis, and inflammation in current models.

Identifying genetic determinants of CMVO holds significant scientific novelty and could form the basis for a novel diagnostic system to predict this complication. Such a system would be clinically valuable, reducing PCI-related risks and improving patient outcomes.

Study Objective: to identify genetic determinants of coronary microvascular obstruction development during percutaneous coronary interventions.

Study Design

A matched case-control study will be conducted to examine the role of specific single nucleotide polymorphisms (SNPs) as predictors of coronary microvascular obstruction (CMVO) development. The study will enroll 80 patients meeting the inclusion/exclusion criteria who have provided written informed consent. Patients will be allocated into two groups in a 1:1 ratio:

- Group 1 (CMVO+): 40 patients with myocardial infarction (MI) who developed CMVO following PCI
- Group 2 (CMVO-, control group): 40 patients with MI who did not develop CMVO following PCI

Group allocation will be performed by the principal investigator. The groups will be matched by sex and age using the "matched pairs" method [9]. For each patient in the CMVO+ group, a corresponding patient of the same sex and similar age (±5 years) will be selected from the pool of MI patients who underwent PCI without CMVO development.

The required sample size (80 patients) was determined based on the following parameters: alpha error level – 5 %, study power – 80 %, group ratio – 1:1, minimum detectable odds ratio – 4.0, expected prevalence of the studied SNPs in the population: 12–69 % (average 50%) [10]. Among the 40 planned matched pairs, 20 pairs must be discordant (i.e., the paired patients must differ in the presence or absence of the predictor).

The study will be conducted in several stages:

1. Patient Screening
    - The principal investigator will assess inclusion/exclusion criteria.
    - Determine eligibility for study group allocation.
    - Performed in the catheterization laboratory immediately after PCI completion.
2. Informed Consent Process
    - Eligible patients will provide written informed consent for genetic testing.

- Patients will be assigned to respective study groups.
- Conducted in the catheterization laboratory.

3. Biological Sample Collection
    - Blood sampling for genetic analysis.
    - Performed in the catheterization laboratory.
4. Sample Processing and Transportation
    - Blood sample processing.
    - Transportation to the Central Research Laboratory of the Institute of Fundamental Medicine at Privolzhsky Research Medical University (Russian Ministry of Health).
5. Clinical Data Collection
    - The investigator will complete patient registry forms.
    - Includes hospitalization course and outcomes.
6. Genetic Analysis
    - Performed at the Central Research Laboratory of the Institute of Fundamental Medicine at Privolzhsky Research Medical University.
7. Data Management
    - Collected information will be entered into a unified electronic database.
8. Statistical Analysis
    - Comprehensive processing of all obtained data.

Ethical Information About the Study

The study is conducted in accordance with Good Clinical Practice (GCP) standards and the principles of the Helsinki Declaration. The study protocol has been approved by the local ethics committee. Patient information is processed anonymously. Upon inclusion in the study, each patient is assigned a serial number.
The following data are recorded in the registry of enrolled patients: serial number, last name, first name, patronymic, sex, date of birth, study group, date of inclusion in the study, medical record number, contact phone number, home address. pair number. This registry is stored at the research center. The serial number is indicated on: the

inclusion/exclusion criteria form; the patient's voluntary informed consent for study participation; blood collection tubes with patient samples; the patient registry form with hospitalization details and outcomes; the unified electronic database of study participants. All work with blood samples outside the research center and statistical processing of obtained data is performed in anonymized form.

Inclusion Criteria:

1. Signed voluntary informed consent for study participation by the patient;
2. Male or female aged 18 years or older;
3. ST-segment elevation myocardial infarction (STEMI) type I according to the Fourth Universal Definition of Myocardial Infarction criteria [11] and current clinical guidelines for this condition [12];
4. Enrollment in the study immediately after PCI with possibility of blood sampling in the catheterization laboratory after signing informed consent;
5. For "CMVO+" group: Development of CMVO as a result of PCI (CMVO is assessed according to criteria specified in the European Society of Cardiology clinical guidelines) [12];
6. For "CMVO-" group: Absence of CMVO as a result of PCI (CMVO is assessed according to criteria specified in the European Society of Cardiology clinical guidelines) [12] and matching with the paired patient from "CMVO+" group by sex and age (±5 years).

Exclusion Criteria:

1. Presentation with subacute-stage MI (>48 hours from symptom onset) or early post-infarction angina;
2. MI occurring during revascularization procedures;
3. PCI-related complications (coronary artery dissection, perforation, or acute intraprocedural thrombosis);
4. Death during PCI unrelated to CMVO development;

5. Concurrent terminal illness (unrelated to primary diagnosis) with life expectancy <1 month.

The inclusion/exclusion criteria checklist is completed by the principal investigator who signs the voluntary informed consent. The completed checklist is then attached to the patient-signed informed consent document.

Diagnostic Criteria for STEMI:

1. Symptoms of acute coronary syndrome (ACS) within 48 hours prior to hospitalization (angina or equivalent symptoms);
2. ST-segment elevation (>0.1 mV in ≥2 contiguous ECG leads) or newly developed left bundle branch block or ST-segment depression (≥0.1 mV in precordial leads V1-V4) with findings suggestive of true posterior infarction;
3. Cardiac troponin levels exceeding the 99th percentile of the upper reference limit.

Definition of CMVO and Description of Used Angiographic Scales

According to the 2017 European Society of Cardiology clinical guidelines for the management of STEMI patients [12], CMVO is defined as inadequate myocardial perfusion after successful mechanical restoration of the infarct-related artery (IRA) lumen.

Criteria of Inadequate Myocardial Perfusion (at least one required for CMVO registration):

1. IRA blood flow <3 points by TIMI flow grade [8];
2. Myocardial perfusion <2 points by Myocardial Blush Grade (MBG) [13].

Other causes of coronary artery obstruction (spasm, dissection, thromboembolism) must be confidently excluded.

Procedure for Signing the Voluntary Informed Consent

The voluntary informed consent is signed by the patient in the catheterization laboratory immediately after completion of PCI. The inclusion/exclusion criteria

checklist and the signed voluntary informed consent form constitute part of the primary study documentation. These documents are placed in the patient file, which is stored at the research center.

Protocol for Blood Sample Collection, Storage and Transportation

The study utilizes human whole peripheral blood. Blood samples are collected in the catheterization room immediately after the patient signs the voluntary informed consent form. The test tube is labeled with the patient's serial number, initials, and blood collection date/time. Sample collection, preprocessing and storage are performed according to the manufacturer's protocol for the DNA extraction kit from biological material. The following reagent kits are used in this study: "CardioGenetics Hypertension", "CardioGenetics Thrombophilia", "Folate Metabolism Genetics" (manufactured by DNA-Technology, Russia), "SNP-Express-Cardiogenetics" RV kit for detecting Lys198Asn SNP in the EDN1 gene (manufactured by Litech, Russia).

Whole peripheral blood is collected in 2.0 ml or 4.0 ml Vacuette vacuum plastic tubes containing EDTA anticoagulant at a final concentration of 2.0 mg/ml (lavender-top tubes). Sodium citrate may also be used as an alternative anticoagulant. After collection, the tube should be inverted 2-3 times to mix blood with the anticoagulant. Heparin must not be used as an anticoagulant.

Prior to transportation, samples must be stored in a medical refrigerator at 2-8°C. Collected samples must be delivered to the genetics laboratory within 24 hours of blood collection. Transportation must be performed using a specialized thermal container maintaining 2-8°C.

Completion of Patient Registry Card with Hospitalization Course and Outcomes

At the conclusion of the current hospitalization, the principal investigator completes a registry card for each patient containing information about the course and outcomes of the hospitalization. The card is anonymous, recording only the patient's serial number and initials within the study. This card forms part of the

primary documentation and is stored in the patient's file at the research center. The registry card includes: details of the performed PCI; documented risk factors for CMVO development; laboratory test results (complete blood count, biochemical blood analysis, brain natriuretic peptide, etc.); instrumental diagnostic findings (ECG, ECG monitoring, echocardiography, 6-minute walk test); complications occurring during hospitalization (ventricular fibrillation, etc.); hospitalization outcome (discharged/deceased). This information is collected solely for registry purposes to enable subsequent statistical analysis. All described laboratory and instrumental diagnostic methods are performed according to standard clinical protocols for MI patient management and their use is unrelated to the study procedures. Copies of all test results are stored anonymously in the patient file. Patient angiograms are also preserved in anonymized form at the research center.

Purpose and Methodology of Genetic Analysis

The purpose of genetic analysis is to identify SNPs serving as predictors of CMVO development during PCI in patients with MI. For the study, SNPs were selected from the "CardioGenetics Hypertension", "CardioGenetics Thrombophilia" and "Folate Metabolism Genetics" panels manufactured by "DNA-Technology" (Russia), as well as the kit for determining the Lys198Asn SNP in the EDN1 gene manufactured by "Litech" (Russia) (Appendix A). This selection is based on literature data indicating that the pathogenetic changes associated with these SNPs are similar to the mechanisms of CMVO development [1].

For the panels from "DNA-Technology", SNP detection will be performed using real-time polymerase chain reaction (PCR) with high-resolution melting curve analysis using TaqMan fluorescent probes. The method principle involves amplification (copying) of DNA fragments containing SNPs through repeated cycles of: 1) thermal DNA denaturation, 2) primer annealing to DNA sequences complementary to the studied fragment, 3) extension of polynucleotide chains from these primers using Taq polymerase.The planned kit includes a "hot start" feature to increase reaction sensitivity and specificity, achieved through the use of antibody-

blocked Taq polymerase (which prevents nonspecific primer annealing to DNA targets during initial tube heating).

The amplification mixture contains signal probes with Fam and Hex fluorescent labels for each SNP variant being analyzed, which undergo thermal melting after PCR completion. The melting temperature of these probes depends on their degree of complementarity to the studied DNA targets, which in turn affects their fluorescence level. The SNP variant is determined by analyzing the fluorescence curve. The mixture also includes a system for amplifying a fragment of human genomic DNA, which allows monitoring the DNA quantity in the tube and preventing genotyping errors.

The reagent kit includes amplification mixtures specific for each SNP. Each tube uses three fluorescent dyes, enabling simultaneous recording of results from multiple amplification reactions occurring in a single tube (determining two alleles and assessing genomic DNA quantity).

For analysis of the Lys198Asn SNP in the EDN1 gene, allele-specific PCR will be used (using the "SNP-Express-Cardiogenetics" RV reagent kit, Litech, Russia) with real-time reaction product detection (CFX96, BioRad, USA).

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Abbreviations

ACS – acute coronary syndrome

CA – coronary artery

CMVO – coronary microvascular obstruction (no-reflow phenomenon)

DNA – deoxyribonucleic acid

ECG – electrocardiogram

EchoCG – echocardiography

EDTA – ethylenediaminetetraacetic acid

IHD – ischemic heart disease

IRA – infarct-related artery

MBG – myocardial blush grade

Me – median

MI – myocardial infarction

PCI – percutaneous coronary intervention

PCR – polymerase chain reaction

[Q1; Q3] – interquartile range

SNP – single nucleotide polymorphism

STEMI – ST-segment elevation myocardial infarction

TIMI – thrombolysis in myocardial infarction (study group)

Appendix B

SNPs Planned for Investigation

Gene	SNP	Identifier	Possible genotypes and their proportion in the population (reference and alternative)
ADD1 – α-adductin	1378 G>T (Gly460Trp)	rs4961	G=0.806737; T=0.193263
AGT – angiotensinogen	704 T>C (Met235Th)	rs699	A=0.545132; G=0.454868
	521 C>T (Thr174Met)	rs4762	G=0.878133; A=0.121867
AGTR1 – angiotensin II receptor type 1	1166 A>C	rs5186	A=0.721723; C=0.278277
AGTR2 – angiotensin II receptor type 2	1675 G>A	rs1403543	G=0.478265; A=0.521735
CYP11B2 – cytochrome 11b2 – aldosterone synthase	-344 C>T	rs1799998	A=0.573264; G=0.426736
GNB3 – beta 3 subunit of G protein – guanine-binding protein	825 C>T	rs5443	C=0.668330; T=0.331670
eNOS – nitric oxide synthase	-786 T>C	rs2070744	C=0.34927; T=0.65073
	894 G>T (Glu298Asp)	rs1799983	T=0.31468; G=0.68532
EDN1 – endothelin 1	594 G>T (Lys198Asn)	rs5370	G=0.780864; T=0.219136
F2 – prothrombin	20210 G>A	rs1799963	G=0.98867; A=0.01133

Gene	**SNP**	**Identifier**	**Possible genotypes and their proportion in the population (reference and alternative)**
F5 – proaccelerin	1691 G>A (Arg506Gln)	rs6025	G=0.951; A=0.049
F7 – proconvertin	10976 G>A (Arg353Gln)	rs6046	G=0.882035; A=0.117965
F13 – fibrinase	103 G>T (Val34Leu)	rs5985	G=0.786; T=0.214
FGB – fibrinogen	-455 G>A	rs1800790	G=0.81485; A=0.18515
ITGA2-α2 – integrin	807 C>T (Phe224Phe)	rs1126643	C=0.610691; T=0.389309
ITGB3-β3 – integrin	1565 T>C (Leu33Pro)	rs5918	T=0.851958; C=0.148042
SERPINE1 (PAI-1) – serpin	-675 5G>4G	rs1799762	4G=0.48510; 5G=0.51490
MTHFR – methylenetetrahydrofolate reductase	677 C>T (Ala222Val)	rs1801133	G=0.659842; A=0.340158
	1298 A>C (Glu429Ala)	rs1801131	T=0.695830; G=0.304170
MTR – B12 dependent methionine synthase	2756 A>G (Asp919Glu)	rs1805087	A=0.808600; G=0.191400
MTRR – methionine synthase reductase	66 A>G (Ile22Met)	rs1801394	A=0.482820; G=0.517180