Study Protocol, including Statistical Analysis plan: ExINOCA part 2

Brief Title: The Impact of Exercise Training on Ischemia With Non-Obstructive Coronary Arteries (INOCA): The ExINOCA Study

Official Title: Ischemia With No Obstruction of Coronary Arteries: Underlying Mechanisms and the Impact of Exercise Training (EXINOCA)

Date of document: July 29th 2024

Unique Protocol ID: not yet assigned

Ethical application no. H-24005644 as approved by the Regional Ethical Committee of the Copenhagen Region (De Videnskabsetiske Komiteer Region Hovedstaden),

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The study is monitored by the Danish Data-protection Agency.

Study is planned to be conducted from October 2024-October 2027

FDA Regulated: No

FDA Regulated Intervention: No Sponsor: Bispebjerg-Frederiksbjerg Hospital

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Short Description

This study aims to identify mechanisms underlying Coronary microvascular dysfunction (CMD) in angina and to assess whether exercise training can improve the condition.

This study is a randomized controlled trial testing the effect of exercise training in patients with coronary microvascular dysfunction. 100 patients will be randomized 1:1 to exercise training or control. The primary outcome is coronary microvascular function, secondary outcomes include symptoms and microvascular function in cutaneous tissue, skeletal muscle, and adipose tissue.

Background

Recent evidence shows that INOCA can manifest from general microvascular disease affecting small arteries other than the coronary artery. For example, the vascular response in the finger of patients with coronary microvascular disease has been shown to be abnormal (8) and small arteries isolated from skin and adipose tissue of patients with coronary microvascular disease display increased response to the vasoconstrictor endothelin and reduced response to the endothelium dependent vasodilator acetylcholine when compared to controls (9,10). Other experiments have shown a shift in endothelial mediated vasodilation from nitric oxide to the vasodilator H_2O_2, and preliminary evidence indicates that this could be rectified through induction of the transcriptional coactivator PGC-1a (11). This is consistent with the evidence for inflammation associated with coronary microvascular disease (12).

The mechanistic findings are exceedingly interesting as they provide the first targets, which may potentially be exploited to treat patients with microvascular disease. Accordingly, there is strong preliminary data to suggest a way forward with respect to treatment of the large population – mostly women – with coronary microvascular disease. In the first part of this project two groups will be compared at baseline; a matched control group and a group with angina symptoms and reduced myocardial blood flow reserve as an indication of microvascular dysfunction; INOCA. The two groups are compared to allow for an assessment of functional and molecular mechanisms in angina patients with- versus without coronary microvascular dysfunction and to relate these aspects in both groups to a matched control group.

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We have found that INOCA is associated with development of renal and cerebral microvascular disease (13) strongly suggesting that coronary microvascular disease is a manifestation of a generalized microvascular disease. As direct investigations of microcirculatory mechanisms in the human heart are difficult, the systemic aspect of microcirculatory dysfunction provides an excellent opportunity to study the microcirculation of more accessible organs in patients with INOCA. Accordingly, in the current project we will study the microcirculation in skeletal muscle, skin and adipose tissue of patients and matched control participants to obtain insight into the mechanisms underlying INOCA.

Objective

The overarching aim of the project is to identify mechanisms underlying impaired coronary microvascular dysfunction in INOCA and to assess whether exercise training can improve the condition. The underlying premise is that microvascular dysfunction is a systemic disease and to fulfil our aim we will study multiple aspects of microvascular function in cardiac, skeletal muscle, fat and skin by different techniques. Although not part of this study registration, these studies in humans will, in parallel studies,

be combined by studies in an animal model of INOCA.

The project consists of the following aims and hypotheses.

Aim

To determine the impact of a structured exercise training program on patients with INOCA on coronary microvascular function, symptom burden, physical capacity and systemic microvascular function and related mechanisms

Hypotheses

The hypothesis of the overall project is that microvascular dysfunction is a systemic disease and that underlying mechanisms can be identified in several tissues in INOCA patients and moreover that the dysfunction can be reversed by a period of exercise training.

Our main hypothesis is that exercise training can improve myocardial microvascular dysfunction and related mechanisms in INOCA patients and thereby reduce symptom burden and improve physical capacity.

Methods

Measurements

METHODS OF ASSESSMENT	ENDPOINT	SITE
PET	Change in MBFR assessed by $[^{15}O]H_2O$-PET-scan from baseline to end of intervention (ie. $MBFR_{6end}$ -$MBFR_{Baseline}$)*	BFH
Seattle angina questionnaire/selfreported	Change in Symptom burden assessed by Seattle Angina Questionnaire at baseline and end of intervention	BFH
Oxygen uptake during exhaustive exercise	Change in VO2peak from baseline to end of intervention	NEXS/BFH
	Change in MBF assessed by [15O]H2O-PET-scan from baseline and after 6 months (ie. MBF6 months - MBFBaseline).	BFH
	Change in hMBF assessed by [15O]H2O-PET-scan from baseline and after 6 months (ie. hMBF6 months - hMBFBaseline).	BFH
Laser Speckle contrast Imaging	Skin microvascular function	BFH/UCPH
Skeletal muscle microvascular function	Brachial artery ultrasound Doppler in response to stimuli	BFH/UCPH
Arterial compliance	Calculation made from intraarterial pressure and arterial diameter measured by ultrasound doppler	BFH/UCPH
Plasma levels of markers related to vascular function	Mesoscale/ELISA	UCPH
Arterial blood pressure	Automated blood pressure at rest and during exercise	BFH/UCPH
In vitro adipose tissue microvascular function from fat biopsy**	Myography, proteomics and histology of vessels derived from fat biopsy	UCPH-BMI

Clinical chemical analysis	Plasma HbA1c, HDL and triglycerides	BFH/UCPH
Plasma levels of inflammatory markers	Mesoscale/ELISA/OLink	BFH/UCPH
In vitro adipose tissue microvascular function from fat biopsy	Myography, proteomics, and histology of vessels derived from fat biopsy	UCPH

*Change in MBFR is defined as

Myocardial Blood flow reserve $= \frac{\text{Global stress perfusion}}{\text{Global rest perfusion}}$, *ie unitless*. The primary analysis will be of comparison (intention to treat) of mean/median change in MBFR between the two groups.

**In the substudy the small arteries are derived from adipose tissue subsamples (first 20 participants in intervention group and first 20 participants from control group).

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Department of Cardiology, Bispebjerg Frederiksberg Hospital (BFH)

Department of Biomedical Science, (UCPH-BMI)

Procedures

The following examinations will be performed at baseline in Study and at baseline and follow-up in Study 2, unless otherwise indicated

PET-scan with [15O]H2O

$[^{15}O]H_2O$-PET scan is an examination routinely used in clinical practice. At Bispebjerg Frederiksberg Hospital approximately 1000 $[^{15}O]H_2O$-PET scans are performed annually.

$[^{15}O]H_2O$-PET uses water labeled with the radioactive isotope oxygen-15 (^{15}O) as the radiotracer. This radiotracer is injected into the patient's bloodstream, allowing for the reconstruction of three-dimensional images of the distribution of the radiotracer in the body.

By dynamic measurements of the concentration of the radiotracer in the myocardium, it is possible to obtain information about blood flow and tissue perfusion, thereby aiding in the diagnosis and management of coronary artery disease (17,18).

For this procedure patients will have one peripheral venous catheter. They will be given continuous adenosine 0.14 mg/kg for 6 min to provoke vasodilation.

Participants are not allowed to drink or eat coffee, tea, dark chocolate, or any other substance containing caffeine for 24 hours before their $[^{15}O]H_2O$-PET scan $[^{15}O]H_2O$-PET involves a small amount of radiation and the use of a radioactive tracer.

The healthcare professionals adhere to radiation safety protocols and regulations to ensure the patient receives the lowest possible radiation dose while still obtaining diagnostic images.

Maximum oxygen uptake

To determine maximal oxygen uptake, the participants complete a maximal incremental cycling test during which expired air is continuously analyzed. The test begins at a workload of 50 W

with a continuous increase in power of 25 W per minute until exhaustion. If expected max capacity is <100W, the protocol will be adapted. During the test heart rate and blood pressure are continuously monitored. If expected maximal exercise capacity is <75 Watt an adapted protocol will be used. The same protocol will be used before and after intervention.

Echocardiography

Echocardiographic assessment will be conducted by ultrasound doppler at baseline, guided by a standardized protocol in accordance with internationally recognized consensus guidelines. This comprehensive protocol covers various aspects of cardiac structure and function, evaluation of the heart valve system, and assessment of cardiac vasculature.

Echocardiographic outcomes will encompass a range of parameters, including but not limited to left ventricular (LV) dimensions, LV mass, LV ejection fraction (LVEF), diastolic function, and left atrial volume (LAV).

Seattle Angina Questionnaire:

The Seattle Angina Questionnaire (SAQ) is a self-administered questionnaire used in cardiac patients to quantify angina based on five scales: physical limitation scale, anginal stability scale, anginal frequency scale, treatment satisfaction scale, and the disease perception scale. The questionnaire will be in Danish. It will be answered electronically using REDCap, or by paper if the participant does not wish to do it electronically.

Skeletal muscle microcirculatory function

The procedure involves catheterization of the brachial artery and vein for blood sampling, intraarterial pressure measurement by a pressure transducer and for infusions of vasoactive compounds as described below. Catheters are placed under sterile conditions and after local anaesthesia of the skin with Lidocain.
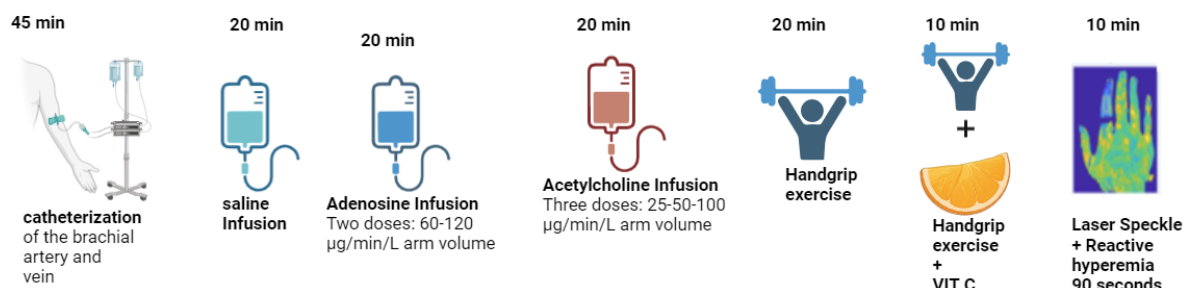
Throughout the experiments brachial artery blood flow is measured by ultrasound doppler. The procedure also involves short term (3-10 min) infusions of vasoactive compounds. Subjects will be randomly allocated to one out of two similar infusion protocols: A and B. The design for the two protocols is as follows:

Protocol A.

- Infusion of adenosine, two doses (60-120 mikrog/min/l arm volume)

- Infusion of acetylcholine in three doses (Miochol-E, Bausch & Lomb Inc.) (25-50-100 mikrog/min/l arm volume)

- Handgrip exercise at 15% of maximal capacity for 14 min, first 4 min without infusion and then with 10 min of infusion of ascorbic acid (80 mg min/l arm volume).

Illustration of Protocol A

VISIT 2a ~2½ hour:

45 min — catheterization of the brachial artery and vein

20 min — saline Infusion

20 min — Adenosine Infusion Two doses: 60-120 µg/min/L arm volume

20 min — Acetylcholine Infusion Three doses: 25-50-100 µg/min/L arm volume

20 min — Handgrip exercise

10 min — Handgrip exercise + VIT C

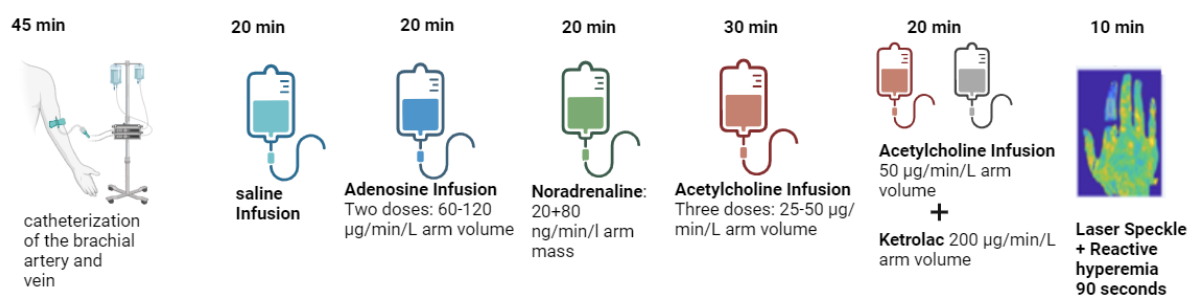10 min — Laser Speckle + Reactive hyperemia 90 seconds

Anthropometric measurements of the arm will be made to estimate arm volume for calculation of infusion dose. During the assessment of microvascular function heart rate and blood pressure are continuously monitored. After the experiment is completed, the catheters are removed and pressure is applied.

Protocol B

- Infusion of adenosine, two doses (60-120 mikrog/min/l arm volume)

- Infusion of noradrenaline two doses (20-80 ng/min/l arm volume)

- Infusion of acetylcholine in two doses (Miochol-E, Bausch & Lomb Inc.) (25-50 mikrog/min/l arm volume)

- combined infusion of 50 mikrog/min/l arm volume acetylcholine and infusion of ketorolac (Toradol; Roche) (200 mikrog/min/l arm volume) for 5 min.

Illustration of Protocol B

VISIT 2b ~2½ hour:

45 min — catheterization of the brachial artery and vein

20 min — saline Infusion

20 min — Adenosine Infusion Two doses: 60-120 µg/min/L arm volume

20 min — Noradrenaline: 20+80 ng/min/l arm mass

30 min — Acetylcholine Infusion Three doses: 25-50 µg/min/L arm volume

20 min — Acetylcholine Infusion 50 µg/min/L arm volume + Ketrolac 200 µg/min/L arm volume

10 min — Laser Speckle + Reactive hyperemia 90 seconds

Laser Speckle contrast Imaging (LSCI)

LSCI is an imaging technique that will help to visualize and quantify the blood perfusion of the skin (15).

The method will be used to quantify blood perfusion in the hand of the participant at resting baseline, after short term occlusion of the arm (reactive hyperaemia test) and during adenosine

infusion (vasodilation) conducted concomitant with the measurements described above in *Skeletal muscle microcirculatory function*.

Description of the LSCI method: Laser light will be sent to the tissue and the reflected laser light is detected as a 'Speckle' pattern. This 'Speckle pattern' is used to make a map of the blood perfusion of the skin. The LSCI imaging will be performed using an LSCI system that was constructed in collaboration with Center for Functionally Integrative Neuroscience at Aarhus University Hospital. The system consists of a 785 nm laser that is equipped with a diffuser thereby enabling large-area illumination of the skin surface. A compact CMOS camera is used to detect the reflected laser light. The camera is equipped with a polarizing filter to eliminate the surface reflected laser light and optimize the visualization of the skin perfusion. Image data are acquired using dedicated data acquisition software and post-processed in MATLAB to generate blood flow index (BFI) and relative BFI images.

Reactive hyperemia test

Occlusion by application of a tourniquet will be conducted on the upper arm for 90 seconds.

Vasodilation test

Involves a stressor-test in which low-dose adenosine is given intraarterially. The procedure is conducted concomitant with the skeletal muscle microcirculation procedure when adenosine is infused.

Adipose tissue biopsy

Biopsies will be obtained either at The Department of Nutrition, Exercise and Sports or at Bispebjerg/Frederiksberg Hospital in collaboration with University of Copenhagen.

Under local anesthesia (lidocaine 10 mg/ml with adrenalin 5 µg/ml) an adipose tissue biopsy (3x0.5x0.5 cm) will be performed in sterile environment by a medical doctor or by a trained health care professional supervised by a medical doctor.

The biopsy will be obtained from the gluteal region, where a small boat-shaped incision is made following the natural creases of the skin. The adipose tissue, along with a small skin flap, is removed (1-2 cm^3). Subsequently, the subcutaneous tissue is closed with a self-absorbable suture, and the skin is sutured (approximately 3 stitches) that should be removed after 10 days. The wound is covered with a bandage that can be removed after 24 hours, but we recommend applying a new bandage until stich removal.

The adipose biopsies are immediately transferred to ice-cold physiological buffer and transported to the laboratory at the Biomedical Institute, University of Copenhagen for analysis and storing.

From these biopsies, segments of small arteries (diameter about 200 µm) will be promptly dissected free. One segment will be mounted in a myograph for recording of isometric tension (i.e., vasoconstriction and vasodilation) and for histochemical analysis, while the other segment

will be snap-frozen for proteomic and histological analysis and the adipose tissue/perivascular adipose tissue will be used for genomic and transcriptomic analysis (see below).

The following methods will be used on the free-dissected arteries:

Myography

In the excised artery, we will evaluate the relaxation to adenosine and to acetylcholine and the dependence of the response to prostaglandins. To evaluate the role of NO and H_2O_2 we will also evaluate the relaxation to acetylcholine after inhibition of the NO-synthase with L-NAME and after metabolism of H_2O_2 with catalase. We will further evaluate the vasoconstrictor response to the neurotransmitter noradrenaline and the vasodilator response to the beta-receptor agonist isoprenaline to obtain a comprehensive evaluation of the pharmacological characteristics of the small arteries. Finally, assessment of the endothelin-1 vasoconstriction will be performed.

Analysis of microvascular segments from adipose tissue and the adipose tissue:

We will map the proteome of the small arteries. This will be done by using an arterial segment (~2 mm) obtained from the adipose tissue sample.

In brief, digested peptides from arteries will be analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS), and data analysis will be used to identify proteins and their relative abundances, which enables statistical comparison.

Significantly regulated proteins will be subjected to gene-overrepresentation analysis that can identify biological pathway associations in the data. This unbiased technology is advantageous for gaining novel understanding of the underlying biological mechanisms responsible for the dysfunction of the small arteries and arterioles in patients with microvascular disease and effect of exercise training on these. This discovery-based approach is essential hypothesis-generating research that will enable us to define the mechanisms underlying INOCA, which we will be able to test in our functional experiments. To stimulate new research and therapeutic development, we plan to make all data acquired publicly available, thereby enabling other research groups to use the data via the ProteomeXchange database. A small fraction of the arteries and perivascular fat-tissue will be sectioned for histological analysis. The adipose tissue/perivascular adipose tissue will be analyzed for mRNA and proteins by use of RNA-sequencing and proteomic analysis. This analysis will be done as part of the aim to understand microvascular changes in INOCA and how training may influence the microvasculature. The analysis will enable an assessment of how changes in mRNA and proteins are altered in relation to structure and function of the tissue. The assessment includes isolation of cell nuclei from the fat biopsies.

Oxidative stress

A part of the biopsy (mainly fat cells, connective tissue, and small arteries) will be stained for

lipid peroxidation as a measure of oxidative stress of the tissue. For this we will quantify malondialdehyde which is a general product of nonenzymatic peroxidation of polyunsaturated fatty and arachidonic acids as done by us before (14).

Medical History and Concomitant Medicine

Medical history will be registered at the baseline visit (V1) and evaluated in relation to the protocol's exclusion criteria. All medication that is necessary for the participants health and which is not in the protocol exclusion criteria may be continued during the study. At the following visits participant will be asked if they have taken any new medicine or changes in dosage since last visit. Medical History will be registered in the Medicine form in the CRF and changes will be registered in the Concomitant Medicine Form in the CRF.

Statistics

Sample size determination relates to the primary outcome myocardial blood flow reserve. Sample size calculations are based on a power of 0.80 and alpha level of 0.05. We regard 0.3 as a clinically relevant improvement in myocardial blood flow reserve. SD of repeated measurement has been 0.2-0.4 in previous studies. With a conservatively estimated SD of 0.50, alfa 0.05, power 0.8 and allowing for 15% drop-out, we will include 100 patients.

Inclusion Criteria

- Age>50
- Only for angina patients:
- Have CMD, defined as myocardial bloodflow reserve (MBFR) < 2.5 or hyperemic myocardial blood flow (hMBF) < 2.3ml/g/min
- No obstructive CAD as determined by the clinical assessment of [15O]H2O-PET
- Able and willing to provide informed consent

Exclusion Criteria

- Females of childbearing potential (defined as a premenopausal female capable of becoming pregnant). The female patient must either be postmenopausal, defined as amenorrhea for at least 1 year, or surgically sterile
- Heart failure, defined as left ventricular ejection fraction of less than 40%
- Uncontrolled hypertension defined as blood pressure above target 140/90 for all
- Uncontrolled hypertension
- Co-morbidity resulting in <1 year expected survival
- Considered by the investigator, for any reason, to be an unsuitable candidate for the study.
- Unable or unwilling to exercise, e.g. due to arthritis or injury*
- Already are regularly physically active and/or have a maximal oxygen uptake >45 ml/kg/min*

- The subject has a known allergy to either: norepinephrine, adenosine, ketorolac, and or ascorbic acid (vitamin C).

Primary Outcome Measure:

1. **Myocardial Blood Flow Reserve (MBFR)**
   - o **Measure:** Change in MBFR assessed by [15O]H2O-PET-scan from baseline to end of intervention (MBFR_end - MBFR_Baseline)

Secondary Outcome Measures:

1. **Symptom Burden**
   - o **Measure:** Change in Symptom burden assessed by Seattle Angina Questionnaire at baseline and end of intervention
2. **Exercise Capacity**
   - o **Measure:** Change in VO2peak (oxygen uptake) during exhaustive exercise
3. **Global Rest Perfusion**
   - o **Measure:** Change in MBF assessed by [15O]H2O-PET-scan
4. **Global Stress Perfusion**
   - o **Measure:** Change in hMBF assessed by [15O]H2O-PET-scan

Exploratory Outcome Measures:

6. **Vascular Function Adaptations**

   Measure: Changes in Arterial compliance ($C = \Delta V / \Delta P$) calculated from intraarterial pressure ΔP and arterial diameter (ΔD) measured by ultrasound Doppler

7. **Skin microvascular function**

   Hand microcirculatory blood flow assessed by Laser Speckle Contrast Imaging (LSCI)

8. **Plasma Markers Related to Vascular Function**

   Plasma levels of markers related to vascular function assessed by Mesoscale/ELISA

9. **Blood Pressure**

   Automated blood pressure at rest

10. **Plasma Lipids**
    Conducted through clinical chemical analysis to measure levels of HDL, and triglycerides in the blood, providing insights into lipid profiles.

11. **Plasma Levels of Inflammatory Markers**

Measured using Mesoscale/ELISA/O-Link platforms to evaluate the presence and levels of inflammatory markers, indicating systemic inflammation.

12. Proteomic and transcriptomic analyses

Changes in gene and protein expressions patterns in small arteries

Time frame for all measures are: Baseline and after the intervention (3 months)

Trial design
Study Type: Interventional Estimated Enrollment: 100 participants

Allocation: Randomized Intervention Model: Parallel Assignment

Randomized controlled trial in which 100 angina patients with reduced flow reserve (INOCA patients) are randomized 1:1 to either exercise training or control (no exer-cise training).
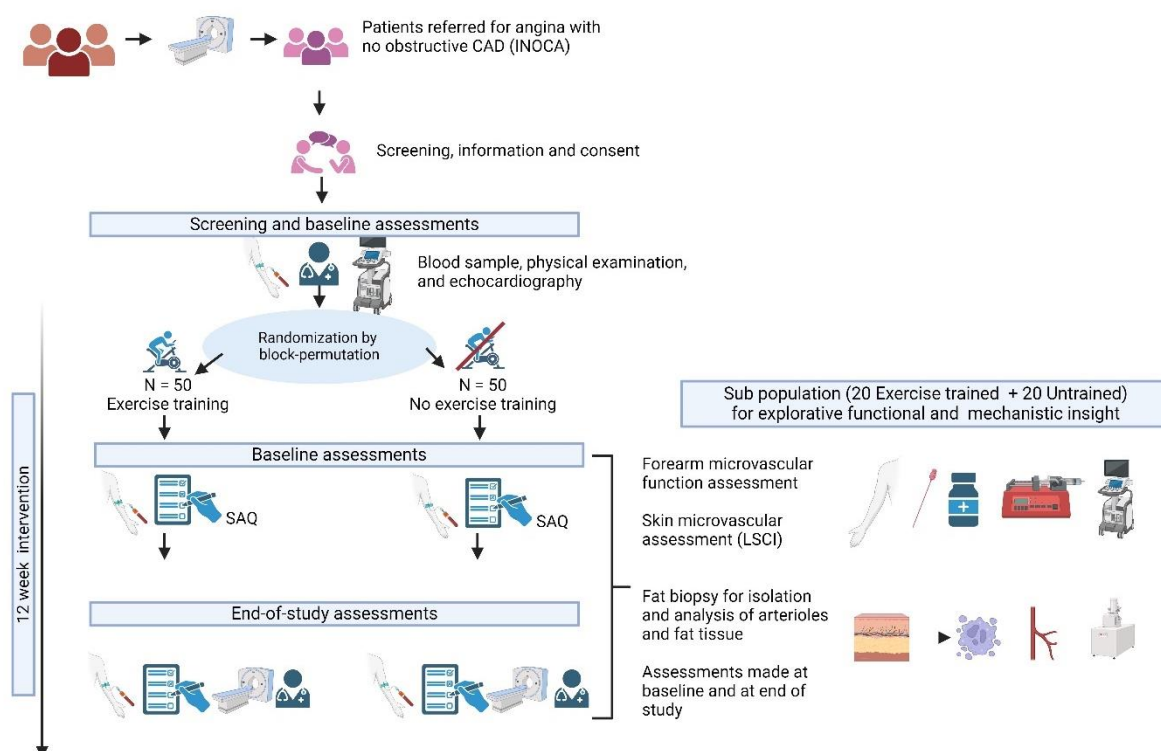

Figure 2. Study overview

Arms and Interventions

Arm	Intervention/treatment

Active Comparator: Exercise	Exercise training
The exercise intervention consists o supervised training 40-50 minutes x weekly for 12 weeks.	The training sessions are consist of cycling and as follows: ; 10 min warm-up at a light intensity, 20-35 min of cyclinș exercise in intervals at varying intensities from light (~60% of max heart rate) to more intensive (80-90% of max hear rate) and ending with 5 min of warm-down at a ligh intensity. The training intensity will be progressive during the course of the intervention period. The cycling training sessions are supervised . Home training is allowed up to once a week if participant: are able to adhere to the prescribed intensity levels. Training sessions are closely monitored to ensure effectiveness and safety. This includes heart rate monitoring perceived exertion assessment. Other Names: Physical activity Endurance training ♦
No Intervention: Control The participants who are randomizec to the no- training group will be offered exercise train-ing after the interventior is completed. Participants will be encouraged to no change their lifestyle or medicatior throughout the study period.	

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