PROTOCOL

MARC-3: Measuring Athlete's Risk of Cardiovascular events

(January 2026)

MARC-3: Measuring Athlete's Risk of Cardiovascular Events

Short title	**MARC-3**
Version	**3.0**
Date	**2026.01.20**
Project leader	**Dr. V.L. Aengevaeren (Radboudumc)** **E-mail: vincent.aengevaeren@radboudumc.nl**
Principal investigator(s) (in Dutch: hoofdonderzoeker/ uitvoerder)	**Dr. T.M.H. Eijsvogels (Radboudumc)** **E-mail: thijs.eijsvogels@radboudumc.nl** **Prof. dr. B.K. Velthuis (UMC Utrecht)** **E-mail: b.k.velthuis@umcutrecht.nl** **Dr. A. Mosterd (Meander MC)** **E-mail: a.mosterd@meandermc.nl** **Dr. J. van den Bossche (Amsterdam UMC)** **E-mail: j.vandenbossche@amsterdamumc.nl**
Sponsor (in Dutch: verrichter/opdrachtgever)	**Department of Medical Biosciences, Radboudumc** **Geert Grooteplein Zuid 30, 6525 GA, Nijmegen**
Subsidising party	**Nederlandse Hartstichting**
Independent expert(s)	**Dr. P. Damman (Radboudumc)** **E-mail: peter.damman@radboudumc.nl**

PROTOCOL SIGNATURE SHEET

Name	Signature	Date
Head of Department: ***Prof. Dr. Jolanda de Vries***		**20-01-2026**
Project leader: ***Dr. V.L. Aengevaeren***		**20-01-2026**

TABLE OF CONTENTS

LIST OF ABBREVIATIONS AND RELEVANT DEFINITIONS

AE	**Adverse Event**
CABG	**Coronary Artery Bypass Graft**
CAC	**Coronary Artery Calcification**
CAD	**Coronary Artery Disease**
CVD	**Cardiovascular disease**
CT	**Computed Tomography**
CCTA	**Coronary Computed Tomography Angiography**
DEXA	**Dual Energy X-ray Absorptiometry**
eCRF	**Electronic Case Report Form**
GCP	**Good Clinical Practice**
GDPR	**General Data Protection Regulation; in Dutch: Algemene Verordening Gegevensbescherming (AVG)**
IB	**Investigator's Brochure**
IC	**Informed Consent**
MACE	**Major Adverse Cardiovascular Event**
MARC	**Measuring Athletes' Risk of Cardiovascular Events**
PCI	**Percutaneous Coronary Intervention**
PCATa	**Pericoronary Adipose Tissue Attenuation**
Review committee	**Medical research ethics committee (MREC) or CCMO or Gezondheidsraad**
(S)AE	**(Serious) Adverse Event**
Sponsor	**The sponsor is the party that commissions the organisation or performance of the research, for example a pharmaceutical company, academic hospital, scientific organisation or investigator. A party that provides funding for a study but does not commission it is not regarded as the sponsor, but referred to as a subsidising party.**
UAVG	**Dutch Act on Implementation of the General Data Protection Regulation; in Dutch: Uitvoeringswet AVG**
Wbo	**Dutch Act on Population Screening Act; in Dutch: Wet op het bevolkingsonderzoek**

SUMMARY

Rationale: Regular endurance exercise is strongly associated with reduced cardiovascular morbidity and mortality. However, multiple imaging studies have revealed that male endurance athletes paradoxically demonstrate a higher prevalence of coronary artery calcification (CAC) and calcified plaques, compared to less active individuals. Whether this represents harmful disease progression or a more benign, stable phenotype remains unknown.

Objective: The MARC-3 study is the second follow-up of the original MARC cohort and aims to: (1) examine the association between long-term exercise characteristics and progression of coronary atherosclerosis in male amateur athletes; (2) determine the association between exercise and coronary plaque phenotypes using artificial intelligence based quantitative coronary CT angiography (AI-QCT); (3) identify biomarkers linking exercise and atherosclerosis; and (4) assess long-term clinical outcomes including all-cause mortality and major adverse cardiovascular events (MACE).

Study design: MARC-3 is a prospective, observational follow-up study (~12.5 years after baseline, ~6 years after MARC-2). All surviving participants of the original MARC cohort (n=318) will be invited for two study visits: Visit 1 at UMC Utrecht (cardiac CT, blood sampling, questionnaires) and Visit 2 at Radboudumc (cardiopulmonary exercise testing, accelerometry, DEXA, carotid ultrasound, hair cortisol, and immunophenotyping).

Study population: Middle-aged Caucasian male amateur athletes previously enrolled in the MARC cohort (≥45 years at baseline). Estimated sample size: 250–280 participants.

Intervention (if applicable): Not applicable.

Main study parameters/endpoints: The primary endpoints are CAC score, plaque prevalence, and luminal stenosis. Secondary endpoints include plaque phenotype and burden by AI-QCT, pericoronary adipose tissue attenuation (PCATa), carotid atherosclerosis, biomarkers (lipids, HbA1c, inflammatory markers, polygenic risk score, hair cortisol, immunophenotyping), and long-term outcomes (all-cause mortality and MACE).

Nature and extent of the burden and risks associated with participation, benefit and group relatedness: The study involves established, minimally invasive procedures. Radiation exposure is limited to one additional cardiac CT (~3–5 mSv), leading to a cumulative dose of ~12–15 mSv across 12 years, well within accepted research limits. Other risks include rare adverse reactions to contrast agents, minor discomfort from blood sampling, and exercise test-related risks, which are mitigated by physician supervision. The study offers participants comprehensive cardiovascular risk profiling and contributes unique insights into the “athlete’s paradox” of exercise and coronary atherosclerosis.

1. INTRODUCTION AND RATIONALE

Cardiovascular diseases (CVD) remain the leading cause of death worldwide, with approximately 21 million deaths in 2021 (1). Regular physical activity is strongly associated with reduced cardiovascular and all-cause mortality and increased life expectancy (2, 3). Exercise exerts multiple protective effects, including improved endothelial function, reduced blood pressure, favorable lipid modulation, reduced systemic inflammation, and overall improvement in cardiovascular health (4).

Despite these well-established benefits, several imaging studies have shown that middle-aged and older male endurance athletes have a higher prevalence of coronary atherosclerosis—particularly calcified plaques—compared to less active healthy controls (5-8). Coronary artery calcium (CAC) scoring with computed tomography (CT) provides a quantitative measure of calcified plaque burden and predicts future cardiovascular events (9, 10). Coronary CT angiography (CCTA) allows more detailed plaque characterization, which offers prognostic information beyond CAC scores, as calcified plaque is less frequently associated with cardiovascular events than mixed or non-calcified plaque (11).

The paradox between higher CAC in athletes and their generally superior life expectancy remains unresolved. It is unclear whether elevated CAC scores in athletes indicate an actual increase in atherosclerotic burden and cardiovascular risk, or rather a benign shift toward more stable plaque phenotypes (12). Since coronary atherosclerosis is a leading cause of exercise-related cardiovascular morbidity and mortality (13), resolving this question is essential for accurate cardiovascular risk stratification, prevention strategies, and evidence-based counselling in highly active individuals (14).

The Measuring Athlete's Risk of Cardiovascular Events (MARC) study was initiated between 2012–2014 and included 318 healthy, middle-aged Caucasian male amateur athletes. At baseline, vigorous physical activity was associated with higher CAC prevalence compared to controls, predominantly in the form of calcified plaques (5). Our follow-up study (MARC-2; 2019–2020) revealed that very vigorous exercise intensity, but not total exercise volume, was associated with accelerated CAC progression (12). However, CAC scoring cannot distinguish between beneficial plaque stabilization and harmful disease progression.

The proposed MARC-3 study represents the second follow-up (~12.5 years after baseline and ~6 years after MARC-2) and offers a unique opportunity to evaluate longitudinal changes in coronary atherosclerosis using more detailed CCTA measurements and advanced image

analysis. For the first time in this cohort, plaque composition will be quantified using an AI-based quantitative CT algorithm (AI-QCT, Cleerly Inc.). This technology enables precise measurement of total plaque volume, calcified and non-calcified plaque, low-density non-calcified plaque, luminal stenosis, and remodeling index—parameters that more accurately reflect cardiovascular risk than CAC alone (15, 16). By identifying whether high CAC in athletes represents stable calcification or progressive atherosclerotic disease, AI-QCT directly addresses the central mechanistic question underlying the so-called athlete's paradox.

In addition, coronary inflammation will be evaluated using pericoronary adipose tissue attenuation (PCATa), a validated imaging biomarker associated with vascular inflammation, plaque burden, and cardiovascular outcomes (17, 18). PCATa may provide essential insight into the role of vascular inflammation in exercise-associated atherosclerosis.

Beyond imaging, MARC-3 will integrate objectively measured exercise exposure from maximal cardiopulmonary exercise testing, wearable devices, and training data from online platforms (e.g. Strava, Garmin, Polar), thereby overcoming the limitations of previous studies that relied mainly on self-reported training history (19). Blood samples will be collected to perform biomarker analyses (e.g. lipids, inflammatory markers) and assess a polygenic risk score for coronary artery disease to investigate the role of genetic predisposition in exercise-associated atherosclerosis (20).

The findings from MARC-3 will contribute to a better understanding of whether high-volume endurance exercise accelerates or stabilizes coronary atherosclerosis, and how this subsequently impacts cardiovascular risk stratification in athletes. These insights are essential for developing and refining evidence-based cardiovascular screening and prevention measures guidelines from the American College of Cardiology (ACC), American Heart Association (AHA), and European Society of Cardiology (ESC).

2. OBJECTIVES

Primary Objective:

- To examine the association between long-term exercise characteristics and the progression of coronary atherosclerosis in male amateur athletes.

Secondary Objectives:

- To determine the association between exercise characteristics and coronary atherosclerosis characteristics and phenotypes using AI-based quantitative CCTA.
- To identify biomarkers that could link exercise training to coronary atherosclerosis characteristics.
- To assess the long-term clinical outcomes associated with coronary atherosclerosis in male athletes, including all-cause mortality and MACE.

3. STUDY DESIGN

This study is a prospective, observational cohort study and constitutes the second follow-up of the previously established MARC study. The original MARC cohort (n=318) was recruited between 2012–2014 and included healthy, middle-aged male amateur athletes. The MARC study was approved by the METC in Nieuwegein (NL38234.100.11/R-11.44M). The first follow-up (MARC-2) was conducted in 2019–2020 and approved by the Dutch Health Council (*Gezondheidsraad*) and Dutch Minister of Health, Welfare and Sport (no. 1456153-184955-PG). The present MARC-3 study aims to reassess these athletes ~12.5 years after baseline and ~6 years after the first follow-up. Participants will be invited for two study visits within a six-month period:

Visit 1 – UMC Utrecht (Radiology Department, ~2 hours)

- Informed consent;
- Screening questionnaires (health, medication use, training history, diet, stress);
- Blood pressure measurement, electrocardiogram (ECG), and venous blood sampling;
- Coronary CT scan (non-contrast CAC scan and contrast-enhanced coronary CT angiography);
- Instruction and consent for collection of digital training data via online platforms (e.g. Strava, Garmin, TrainingsPeaks).

Visit 2 – Radboudumc (Physiology Department, ~3 hours)

- Maximal cardiopulmonary exercise test with gas exchange analysis, continuous ECG, and lactate measurement ;
- DEXA scan;
- Hair sampling from the occipital region for cortisol measurement;
- Ultrasound of the carotid and femoral arteries;
- Distribution and instruction for wearing a thigh-worn accelerometer for 8 days;
- Optional: additional venous blood sampling for immunological analyses in a subset of participants stratified by coronary plaque burden (n=10 per group).

4. STUDY POPULATION

4.1 Population (base)

Participants will be reinvited from the previously established MARC cohort. The original MARC study recruited 318 Caucasian, middle-aged (≥45 years) male amateur athletes between 2012 and 2014. In the first follow-up study (MARC-2), 291 participants (92%) were included. For this second follow-up (MARC-3), all surviving members of the original cohort will be approached for participation.

4.2 Inclusion criteria

In order to be eligible to participate in MARC-3, the sole inclusion criteria is participation in the original MARC study (i.e. included between 2012–2014).

4.3 Exclusion criteria

A potential participant who meets any of the following criteria will be excluded from participation in this study:

- Inability to provide written informed consent.
- Not willing to be informed about potential incidental findings from the CT-scan
- Concurrent participation in an interventional study targeting cardiovascular health.

Additional exclusion criteria for the CCTA:

- Not willing to undergo coronary CT angiography with intravenous contrast.
- History of severe allergic reaction to iodinated contrast agents.
- Renal dysfunction (estimated GFR < 30 ml/min/1.73 m^2).

Additional exclusion criteria for participation in the exercise test:

- Presence of absolute contraindications for maximal exercise testing as defined in the Standard Operating Procedures of the Department of Medical BioSciences, Radboudumc, including but not limited to:
    - Acute myocardial infarction (3-5 days)
    - Unstable angina
    - Uncontrolled arrhythmias causing symptoms or hemodynamic compromise
    - Syncope
    - Active endocarditis
    - Acute myocarditis or pericarditis
    - Uncontrolled heart failure
    - Acute pulmonary embolus or pulmonary infarction
    - Thrombosis of lower extremities
    - Suspected dissecting aneurysm
    - Uncontrolled asthma
    - Pulmonary edema
    - Room air desaturation at rest ≤ 85% (exercise patient with supplemental 02)
    - Respiratory failure
    - Acute noncardiopulmonary disorder that may affect exercise performance or be aggravated by exercise (i.e. infection, renal failure, thyrotoxicosis)

- Mental impairment leading to inability to cooperate.

4.4 Sample size calculation

This study is a second follow-up of the previously established MARC cohort (n = 318). As such, no formal sample size calculation was performed. The primary aim is to include as many of the original participants as possible. From MARC to MARC-2, the participation rate exceeded 91%, reflecting strong cohort retention. However, due to the increasing age of the participants and known mortality, the maximum number of participants eligible for MARC-3 is 313. Based on prior participation, we estimate that 250 to 280 participants will be available and willing to participate in this follow-up study.

5. METHODS

5.1 Study parameters/endpoints

5.1.1 Main study parameter/endpoint

- CAC score
- CAC prevalence
- Plaque prevalence
- Degree of luminal stenosis

5.1.2 Secondary study parameters/endpoints

- Plaque phenotype and burden by AI-QCT
- Pericoronary adipose tissue attenuation (PCATa)
- Carotid atherosclerosis and stenosis by ultrasound (IMT in mm, % stenosis)
- Biomarkers: biochemical markers (lipid profile, HbA1c, inflammation), cortisol in hair, polygenic risk score for coronary artery disease, immunophenotyping from blood samples
- Long-term outcomes: all-cause mortality and MACE (myocardial infarction, stroke, PCI, CABG)

5.1.3 Other study parameters

- Subject characteristics (age, sex, height, weight, resting blood pressure, fat percentage, muscle mass, bone density, etc)
- Exercise characteristics (self-reported MET-hours/week, CPET VO_2max, accelerometry data, digital training data)
- Lifestyle factors (smoking, drinking, diet, sleep behaviour)
- Medical history (cardiovascular and non-cardiovascular)

- Medication use (including lipid-lowering therapy, antihypertensives, anticoagulants)

5.2 Study procedures

Participants will be invited for two study visits. Below, the specific procedures that will be performed during each visit are described in more detail.

Visit 1 (UMC Utrecht)

Blood pressure measurement, height/weight, ECG and blood draw

After a minimum of five minutes of seated rest, with the arm supported at heart level and without conversation, three blood pressure measurements will be taken. Height and weight will be recorded. A 12-lead ECG will be obtained in the supine position to assess resting cardiac rhythm and detect potential contraindications to CT imaging or medication administration. An intravenous cannula (preferably 18G) will be placed for both blood sampling and contrast infusion during the CT scan.

Laboratory tests

Approximately 30 mL of venous blood will be collected via the intravenous cannula. All samples will be processed within four hours of collection. A portion will be analyzed immediately for renal function (creatinine, eGFR) to ensure safe contrast administration. The remainder will be processed for biomarker analyses relevant to cardiovascular health and atherosclerosis, including lipid profile, high-sensitivity C-reactive protein (hs-CRP), HbA1c, fasting glucose, and electrolytes (Na, K, magnesium, phosphate, urea), 25-hydroxyvitamin D, parathyroid hormone and complete blood count with leukocyte differentiation. In addition, one serum tube will be centrifuged and aliquoted for long-term storage, two EDTA tubes will be stored as whole blood, one EDTA tube will be processed to obtain EDTA plasma, and one lithium-heparin tube will be processed for plasma storage. These extra samples will be stored at –80°C and may be used for advanced biomarker research (e.g., proteomics, metabolomics, inflammatory cytokines, genetic or epigenetic markers, and other emerging assays relevant to atherosclerosis). For genetic analyses, SNP-array genotyping and imputation will be performed to calculate a polygenic risk score (PRS) for coronary atherosclerosis (21, 22). No individual mutations or rare genetic variants will be analyzed, and participants will not receive individual PRS results.

Questionnaires

Participants will complete validated questionnaires on diet (food frequency questionnaire (23)), stress (Perceived Stress Scale) (24), lifelong exercise history (25) and (cardiovascular) health and morbidity including medical history and medication use.

Digital training data collection

Participants will be asked to provide access to their online training data from the past year (e.g. Strava, Garmin, Polar) via a secure TrainingPeaks account accessible only to the research team. Data will be exported and analyzed according to the protocol as described by Dausin et al. (26) to determine total training volume and intensity distribution.

CCTA preparation and protocol

Participants will be asked to abstain from caffeine-containing products for at least 4 hours prior to the scan. A technician will explain the procedure and position the participant feet-first in the scanner. ECG monitoring will be applied and sinus rhythm observed for one minute. A 25-second breath-hold will be practiced. If heart rate exceeds 70 bpm, intravenous metoprolol will be administered in increments until the heart rate is <60 bpm or a maximum of 20 mg is reached. Immediately prior to scanning, two puffs of sublingual nitroglycerin will be administered to dilate the coronary arteries.

CT-scan imaging protocol and analysis

All cardiac CT imaging will be performed on a spectral CT scanner at UMC Utrecht using a standardized acquisition protocol. Imaging will consist of a scout view, followed by a non-contrast prospective ECG-triggered cardiac CT for CACS and a CCTA. The scout view will cover the entire chest, followed by a non-contrast scan to detect and quantify coronary calcification using the Agatston score, as well as mass and volume scores. This non-contrast scan will also be used to define the anatomical range for the CCTA. The CCTA will be acquired prospectively at mid-diastole to minimize motion artefacts. Contrast injection will be weight-adjusted and monitored with bolus tracking in the descending aorta. When the attenuation in the region of interest reaches the predefined threshold, the scan will start after a short post-threshold delay.

Image datasets will be analyzed by experienced cardiovascular radiologists. Clinically relevant cardiac and extracardiac findings will be reported to the participant’s general

practitioner. Calcium scoring will be performed with validated software to derive Agatston, mass, and volume scores. CCTA images will be assessed segment-by-segment according to the modified American Heart Association 18-segment model. Plaque composition will be classified as calcified, mixed, or non-calcified. Atherosclerotic burden will be quantified using the segment involvement score and the segment stenosis score. Luminal stenosis will be graded as absent, minimal (1–24%), mild (25–49%), moderate (50–69%), or severe (≥70%) based on diameter measurements compared to proximal and distal reference segments. If extensive calcification prevents accurate quantification, the segment will be labelled as "calcified, stenosis unclear."

In addition to conventional assessment, all CCTA datasets will undergo quantitative plaque analysis using an FDA-cleared AI-based platform (AI-QCT, Cleerly Inc., New York). This technology provides automated and reproducible quantification of total plaque volume, calcified plaque volume, non-calcified plaque volume, and low-density non-calcified plaque volume. It also reports per-lesion and per-patient measures of luminal diameter stenosis, remodeling index, and plaque distribution across the coronary tree. Also, PCATa will be quantified from the contrast-enhanced CCTA images using validated semi-automated software (Caristo Diagnostics Ltd., Oxford). PCATa is defined as the mean attenuation (in HU) of the adipose tissue within a radial distance equal to the vessel diameter from the outer vessel wall. The right coronary artery (proximal 10–50 mm) will be used for analysis, as this location has been most strongly associated with cardiovascular outcomes in prior studies (17, 18).

<u>Visit 2 (Radboudumc)</u>

Maximal cardiopulmonary exercise test (CPET)

The CPET will be conducted on an electronically braked cycle ergometer following a standardized incremental ramp protocol to volitional exhaustion or until termination criteria are met. Continuous 12-lead ECG monitoring will be used. Breath-by-breath gas analysis (VO_2, VCO_2, VE) will be recorded, and fingertip capillary blood samples will be collected at rest, during exercise, and in recovery to measure lactate concentrations. The total duration of the CPET is around 45 minutes.

DEXA scan

Participants will undergo a whole-body DEXA scan while lying supine for approximately ten minutes to measure bone mineral density, total body fat, lean mass, and visceral adiposity. The scans will be analyzed using standardized

manufacturer software to quantify total and regional body composition parameters. Regions of interest (arms, legs, trunk) will be automatically delineated by the software and manually adjusted if necessary by a trained technician. No Vertebral Fracture Assessment (VFA) will be performed, as this does not relate to the primary research question in this population.

Hair sampling

A small hair sample (approximately 100 strands) will be cut from the posterior vertex of the scalp, as close as possible to the skin. The sample will be stored in a sealed, labelled envelope at room temperature until batch analysis.

Carotid and peripheral ultrasound

Participants will undergo B-mode ultrasound of the carotid and femoral arteries while lying in the supine position after 15 minutes of rest. Both sides will be examined to assess vessel wall thickness and luminal stenosis. The procedure is non-invasive and painless.

Accelerometry

At the end of Visit 2, a waterproof-sealed Axivity AX-3 accelerometer (Axivity Ltd, UK) will be attached to the participant's right thigh with medical-grade adhesive. The device will be worn continuously for eight days, including during bathing and swimming. After this period, the device will be returned in a prepaid envelope. Data will be used to assess characteristics of the 24-hour physical activity spectrum.

Blood sampling for immunological analyses

At Visit 2 an additional 30 mL of blood will be drawn for advanced immunological analyses, including immune cell profiling and inflammatory biomarker measurements, in collaboration with the Department of Internal Medicine, Radboudumc (Riksen group) and Department of Molecular Cell Biology and Immunology, Amsterdam UMC (Van den Bossche group).

5.3 Withdrawal of individual research participants

Participants can leave the study at any time for any reason without any consequences if they wish to do so. The investigator can decide to withdraw a participant from the study for urgent medical reasons.

5.4 Replacement of individual research participants after withdrawal.

Not applicable.

5.5 Follow-up of research participants withdrawn from treatment.

Not applicable.

5.6 Premature termination of the study

The elements of this study are relatively harmless and routinely performed, thus it is unlikely that we will have to terminate the study prematurely. Moreover, since this is an observational, diagnostic study, premature termination of the study is not expected. If for any reason participation in this study cause additional unforeseen harm to patients, the study will be terminated.

6. STATISTICAL ANALYSIS

Statistical analyses will be performed using SPSS statistics 25 or higher (IBM Corp., USA). Continuous variables will be presented as mean ± standard deviation, or median [interquartile range] when not normally distributed. Categorical variables will be expressed as numerical values and percentages. Percentages will be calculated on the number of non-missing observations. In all cases the number of missing values will be specified. All statistical tests will be two-sided and P-values less than 0.05 will be considered as statistically significant.

6.1 Primary study parameter(s)

Associations between exercise volume and intensity and changes in CAC score will be analyzed using linear mixed-effects models, incorporating all three time points (baseline and two follow-up visits). CAC values will be modeled as ln(CAC + 1) when residuals show skewness; otherwise, the original scale will be used. Models will include fixed effects for time, exercise exposure, and a time×exercise interaction to quantify differences in CAC progression across exercise levels. Participant-specific random intercepts, and where appropriate random slopes for time, will account for within-subject correlation and individual variability in baseline CAC and progression rates.

All models will be adjusted for conventional cardiovascular risk factors, including age, BMI, systolic blood pressure, smoking (pack-years), total cholesterol, family history of coronary artery disease, and use of antihypertensives, antidiabetics, and statins. Exercise exposure will be analyzed primarily as a continuous variable, with secondary analyses in tertiles to assess robustness and potential threshold effects. The assumption of linearity will be evaluated using restricted cubic splines, serving exploratory and visualization purposes rather than coefficient interpretation. Missing outcome data will not be assumed

to be random by default. We will first explore whether loss to follow-up shows any systematic patterns in relation to participant characteristics or developments during the study period. Based on these findings, we will determine whether a missing-at-random assumption is justified and apply an analytical strategy accordingly. Sensitivity analyses will be conducted to assess the potential impact of selective attrition on the results.

6.2 Secondary study parameter(s)

Plaque phenotype and burden (AI-QCT): Associations between exercise volume/intensity and plaque composition (calcified, mixed, non-calcified, low-density non-calcified) and remodelling index will be assessed using linear and logistic regression.

Pericoronary adipose tissue attenuation (PCATa): Continuous Houndsfield Units (HU) values will be analyzed in linear regression models, adjusted for confounders. Subgroup analyses will dichotomize PCATa at established cut-offs (e.g. < -70.1 HU).

Exercise characteristics (CPET VO_2max, accelerometry, digital training data): Comparisons between objective and self-reported data will be performed using Bland-Altman plots and correlation coefficients.

Carotid atherosclerosis (ultrasound): Mean intima-media thickness (IMT, mm) and percentage stenosis (%) will be analysed as continuous outcomes in relation to exercise characteristics, using linear regression. Logistic regression will be applied for presence of carotid plaque (yes/no).

Biomarkers: Associations between biochemical markers (lipid profile, HbA1c, inflammatory markers), polygenic risk scores and hair cortisol with coronary atherosclerosis will be tested using linear regression (for CAC/plaque volume) and logistic regression (for plaque presence/type). Multiplicity will be addressed using false discovery rate (FDR) correction.

Clinical outcomes (MACE and mortality): Kaplan-Meier curves will be generated to evaluate and depict unadjusted survival plots of the composite endpoint of all-cause mortality and major adverse cardiovascular events (MACE) for CAC/plaque prevalence. Cox regression models will be used to evaluate the association between baseline coronary atherosclerosis (CAC, plaque burden and phenotype) and time-to-event outcomes (mortality and MACE), expressed as hazard ratios with 95% confidence intervals.

6.3 Other study parameters

Baseline variables of all individuals will be collected to investigate and correct for potential confounders, including cardiovascular risk factors such as smoking. Baseline variables will be collected and summarized in tables.

7. ETHICAL CONSIDERATIONS

7.1 Regulation statement

The study will be conducted in accordance with the principles of the Declaration of Helsinki (latest version). This study falls under the scope of the Dutch Population Screening Act (Wet op het bevolkingsonderzoek, Wbo) due to the use of ionising radiation in a screening context involving a healthy population. Therefore, a separate assessment and permit application will be submitted to and reviewed by the Dutch Health Council (Gezondheidsraad), in accordance with the legal requirements for Wbo-licensed studies.

7.2 Recruitment and consent

One of the investigators (or authorized delegate) will recruit the subjects, explain the study procedures and ask the participants for their consent.
Subjects will be recruited via e-mail and/or telephone to contact original MARC participants. Interested individuals are invited to respond via email and then receive additional information on the study (‘Proefpersoneninformatie’). They will be given at least one week to consider their decision regarding study participation. If they agree to participate, a screening telephone call will be made, and the study visit day will be planned. The informed consent will be signed at the start of the first visit. The patient information letter and informed consent form are attached as a separate document.

7.3 Benefits and risks assessment, group relatedness

Participants will undergo a contrast-enhanced coronary CT scan, maximal cardiopulmonary exercise test, venous blood sampling, and several non-invasive assessments. These procedures carry minimal risk.

<u>Risk of ionising radiation</u>

Participants in this study have previously undergone two contrast-enhanced coronary CT scans as part of the MARC and MARC-2 studies, conducted approximately 6 and 12.5 years ago. The radiation dose in MARC-1 was on average 3.9 mSv (27). Although this

constitutes repeated exposure to ionising radiation, the total cumulative radiation dose from three serial cardiac CT scans (estimated 9–15 mSv over 12 years) remains well within internationally accepted research exposure levels. According to the ICRP and European Commission guidelines, cumulative doses up to 50 mSv may be acceptable for research purposes in healthy adults >50 years old, provided the risk–benefit ratio is favourable and as-low-as-reasonable principles are respected (28, 29). The use of low-dose protocols and spectral CT technology further minimises radiation exposure. Nonetheless, this cumulative exposure is clearly communicated to participants during the informed consent process.

Other risks associated with cardiac CT-scan:

- Adverse reaction to the contrast medium (nausea, skin hives in 1 to 3% of participants, fatal adverse reactions in 0.001%) (30).
- Discomfort, such as headache or light-headedness, due to nitroglycerine or bètablocker administration.
- Contrast media-induced renal insufficiency (contrast nephropathy). The occurrence of contrast nephropathy is directly related to renal function; by selecting participants with a GFR higher than 30 mL/min contrast nephropathy is unlikely to occur (31).
- Incidental findings in the scan field may lead to additional diagnostic tests with extra costs and risks not covered by this study. Incidental findings will be reported to the general practitioner of the participant. Common incidental findings include pulmonary nodules, pulmonary embolism and liver abnormalities. All patients with relevant coronary artery disease are discussed with a cardiologist. Management and treatment decisions are left to the discretion of the cardiologist. As a general rule, participants with a CAC score of 100-400 AU without obstructive coronary artery disease will receive lifestyle advice and may be recommended to initiate treatment with statins. Participants with a CAC score ≥400 AU or coronary artery stenosis ≥50% will be offered a consultation with a cardiologist to discuss management options. Other relevant cardiac findings will be discussed with the cardiologist. The participant's general practitioner will be informed of these recommendations to ensure appropriate follow-up, treatment initiation, or referral if indicated. If incidental extra-cardiac findings, such as lung or liver lesions, are considered to be of clinical importance, recommendations of further testing, follow-up or referral to another specialist will be made by the radiologist.
- For the management of small pulmonary nodules incidentally detected with cardiac CT the Fleischner criteria will be used and in all cases of incidental findings the general practitioner will be informed as well (32).

- False-positive results may lead to additional diagnostic tests with extra costs and risks not covered by this study.
- A venous cannula (with blood draw) may cause a small haemorrhage (5% of cases), which can be uncomfortable but harmless.

Risks associated with DEXA scan

The DEXA scan involves a very low dose of ionizing radiation. In rare cases, evident vertebral fractures or abnormal bone densities may be visible. In such instances, the scan will be reviewed by a radiologist. If the finding is considered clinically relevant, follow-up evaluation through the participant's general practitioner will be recommended.

Risks associated with exercise test

The risk of adverse events is minimal. A physician will be present during testing. The test will be terminated upon reaching termination criteria or on participant request.

Potential benefits for participants

Participants will receive a comprehensive cardiovascular risk assessment, including coronary artery imaging. Results will be shared with the participant only if they have provided consent for this. If relevant, evidence-based advice on lifestyle or medical treatment will be provided. Abnormal findings (e.g. hypertension, hyperlipidaemia, coronary atherosclerosis) will be reported to the general practitioner for further management.

Group relatedness

This study focuses specifically on asymptomatic, middle-aged male athletes to investigate the long-term effects of endurance exercise on coronary atherosclerosis. The research question cannot be addressed using another population group.

Risk–benefit balance

The study involves limited risk and burden. The procedures are non-invasive or minimally invasive, and the potential for indirect health benefit is substantial. Therefore, the overall risk–benefit ratio is considered favourable.

7.4 Incentives

There will be no compensation for study subjects besides compensation for travel expenses for both study visits. However, after the end of the study, study participants will

receive a report of their personal study results. We expect that our target study population (enthusiastic athletes) will appreciate this extra information on their cardiac health.

8. ADMINISTRATIVE ASPECTS, MONITORING AND PUBLICATION

8.1 Handling and storage of data and documents

All study data will be handled in compliance with the GDPR and the UAVG. Participant confidentiality will be strictly maintained throughout the study.

Each participant will be assigned a unique study code (i.e. MARC001, MARC002). Study data will be stored under this code and will not contain directly identifying personal information. The key to the coding system (code list) will be stored separately in a secured and access-restricted location at the coordinating site (Radboudumc), and will only be accessible to designated study personnel.

Study data will be entered and managed using an eCRF in Castor EDC (Castor EDC, Ciwit B.V., The Netherlands), a secure and validated data capture system. Only authorised users will be granted access to Castor, and all data handling will be logged and audited according to GCP standards.

Electronic data (including imaging data, exercise test results, and digital training data) will be stored on secure, access-controlled servers of the participating institutions. Paper-based source documents, if applicable, will be stored in locked cabinets in restricted-access research areas.

Biological samples will be collected in tubes labelled with the participant's unique study code and a code for the specific timepoint. Samples will be stored in a temperature-monitored freezer at the study site or transferred to an institutional biobank for long-term storage.

All study-related data and materials (including the eCRF, coded data, and biological samples) will be retained for 15 years after study completion, in accordance with applicable laws and institutional policies. Data and samples may be used for future research questions related to the aims of the study, i.e. coronary atherosclerosis and related cardiovascular risks. If analyses are proposed that fall outside the direct scope of the current study protocol, an application will be submitted for medical ethical review.

Data transfer between participating sites will take place using secure, encrypted methods, and only in pseudonymised form. Data Transfer Agreements (DTAs) are in place or will be established with collaborating partners.
Participants will be informed of their rights concerning access to, correction, or deletion of their personal data, as detailed in the patient information letter and informed consent form.

For several secondary objectives within the MARC-3 project, collaboration with international partners is required to perform advanced analyses on CCTA. Only pseudonymised imaging data will be shared, containing no directly identifiable personal information. Data exchange will occur exclusively under approved Data Transfer Agreements (DTAs), reviewed by the legal and privacy officers of the participating institutions. Collaborations include Caristo Diagnostics Ltd. (Oxford, United Kingdom) for analysis of pericoronary adipose tissue attenuation (PCATa), Cleerly Inc. (New York, United States) for AI-based quantification of plaque volume and composition, and Philips Healthcare (Best, the Netherlands) for research aimed at improving spectral CT data reconstruction and image processing algorithms. Data will be transferred via secure, encrypted institutional channels. These measures ensure that all data handling fully complies with the GDPR and the UAVG.

8.2 Amendments

Amendments are changes made to the research after a favourable opinion by the review committee has been given. All amendments will be notified to the review committee that gave a favourable opinion. Non-substantial amendments will not be notified to the review committee, but will be recorded and filed by the sponsor.

8.3 Temporary halt and (prematurely) end of study report

The investigator/sponsor will notify the review committee of the end of the study within a period of 8 weeks. The end of the study is defined as the patient's last visit.
The sponsor will notify the review committee immediately of a temporary halt of the study, including the reason of such an action.
In case the study is ended prematurely, the sponsor will notify the review committee within 15 days, including the reasons for the premature termination.
Within one year after the end of the study, the investigator/sponsor will submit a final study report with the results of the study, including any publications/abstracts of the study, to the review committee.

8.4 Public disclosure and publication policy

The intention is to publish the results of this study in a peer reviewed scientific journal. All results, both positive and negative, will be published without restrictions. Authorship and manuscript composition will reflect joint cooperation between multiple investigators and sites. Authorship will be established prior to writing of the manuscript. No individual publications will be allowed prior to the completion of the final report for this study and as agreed in writing by the PI. All data will be reported anonymously.

9. REFERENCES

1. Lindstrom M, DeCleene N, Dorsey H, Fuster V, Johnson CO, LeGrand KE, et al. Global Burden of Cardiovascular Diseases and Risks Collaboration, 1990-2021. Journal of the American College of Cardiology. 2022;80(25):2372-425.
2. Shiroma EJ, Lee IM. Physical activity and cardiovascular health: lessons learned from epidemiological studies across age, gender, and race/ethnicity. Circulation. 2010;122(7):743-52.
3. Lear SA, Hu W, Rangarajan S, Gasevic D, Leong D, Iqbal R, et al. The effect of physical activity on mortality and cardiovascular disease in 130 000 people from 17 high-income, middle-income, and low-income countries: the PURE study. Lancet. 2017;390(10113):2643-54.
4. Green DJ, Hopman MT, Padilla J, Laughlin MH, Thijssen DH. Vascular Adaptation to Exercise in Humans: Role of Hemodynamic Stimuli. Physiol Rev. 2017;97(2):495-528.
5. Aengevaeren VL, Mosterd A, Braber TL, Prakken NHJ, Doevendans PA, Grobbee DE, et al. Relationship Between Lifelong Exercise Volume and Coronary Atherosclerosis in Athletes. Circulation. 2017;136(2):138-48.
6. Merghani A, Maestrini V, Rosmini S, Cox AT, Dhutia H, Bastiaenan R, et al. Prevalence of Subclinical Coronary Artery Disease in Masters Endurance Athletes With a Low Atherosclerotic Risk Profile. Circulation. 2017;136(2):126-37.
7. Möhlenkamp S, Lehmann N, Breuckmann F, Bröcker-Preuss M, Nassenstein K, Halle M, et al. Running: the risk of coronary events : Prevalence and prognostic relevance of coronary atherosclerosis in marathon runners. Eur Heart J. 2008;29(15):1903-10.
8. De Bosscher R, Dausin C, Claus P, Bogaert J, Dymarkowski S, Goetschalckx K, et al. Lifelong endurance exercise and its relation with coronary atherosclerosis. Eur Heart J. 2023;44(26):2388-99.
9. Silverman MG, Blaha MJ, Krumholz HM, Budoff MJ, Blankstein R, Sibley CT, et al. Impact of coronary artery calcium on coronary heart disease events in individuals at the extremes of traditional risk factor burden: the Multi-Ethnic Study of Atherosclerosis. Eur Heart J. 2014;35(33):2232-41.
10. Budoff MJ, Young R, Burke G, Jeffrey Carr J, Detrano RC, Folsom AR, et al. Ten-year association of coronary artery calcium with atherosclerotic cardiovascular disease (ASCVD) events: the multi-ethnic study of atherosclerosis (MESA). Eur Heart J. 2018;39(25):2401-8.
11. Hou ZH, Lu B, Gao Y, Jiang SL, Wang Y, Li W, et al. Prognostic value of coronary CT angiography and calcium score for major adverse cardiac events in outpatients. JACC Cardiovasc Imaging. 2012;5(10):990-9.
12. Aengevaeren VL, Mosterd A, Sharma S, Prakken NHJ, Möhlenkamp S, Thompson PD, et al. Exercise and Coronary Atherosclerosis: Observations, Explanations, Relevance, and Clinical Management. Circulation. 2020;141(16):1338-50.
13. Kim JH, Rim AJ, Miller JT, Jackson M, Patel N, Rajesh S, et al. Cardiac Arrest During Long-Distance Running Races. Jama. 2025;333(19):1699-707.
14. Claessen G, Eijsvogels TMH, Albert CM, Baggish AL, Levine BD, Marijon E, et al. Coronary atherosclerosis in athletes: emerging concepts and preventive strategies. Eur Heart J. 2025;46(10):890-903.
15. Nurmohamed NS, Bom MJ, Jukema RA, de Groot RJ, Driessen RS, van Diemen PA, et al. AI-Guided Quantitative Plaque Staging Predicts Long-Term Cardiovascular Outcomes in Patients at Risk for Atherosclerotic CVD. JACC Cardiovasc Imaging. 2024;17(3):269-80.
16. van Assen M, von Knebel Doeberitz P, Quyyumi AA, De Cecco CN. Artificial intelligence for advanced analysis of coronary plaque. Eur Heart J Suppl. 2023;25(Suppl C):C112-c7.
17. Oikonomou EK, Marwan M, Desai MY, Mancio J, Alashi A, Hutt Centeno E, et al. Non-invasive detection of coronary inflammation using computed tomography and prediction of residual cardiovascular risk (the CRISP CT study): a post-hoc analysis of prospective outcome data. Lancet. 2018;392(10151):929-39.
18. Tzolos E, Williams MC, McElhinney P, Lin A, Grodecki K, Flores Tomasino G, et al. Pericoronary Adipose Tissue Attenuation, Low-Attenuation Plaque Burden, and 5-Year Risk of Myocardial Infarction. JACC Cardiovasc Imaging. 2022;15(6):1078-88.

19. Dausin C, Tironi RM, Cornelissen V, Hespel P, Willems R, Haykowsky M, et al. Your Heart Can't See What Sneakers You Are Wearing: Exercise Training Load in Endurance Athletes Is Inadequately Quantified in Sports Cardiology. Canadian Journal of Cardiology. 2025;41(3):354-63.
20. Klarin D, Natarajan P. Clinical utility of polygenic risk scores for coronary artery disease. Nat Rev Cardiol. 2022;19(5):291-301.
21. Khera AV, Chaffin M, Aragam KG, Haas ME, Roselli C, Choi SH, et al. Genome-wide polygenic scores for common diseases identify individuals with risk equivalent to monogenic mutations. Nat Genet. 2018;50(9):1219-24.
22. Patel AP, Wang M, Ruan Y, Koyama S, Clarke SL, Yang X, et al. A multi-ancestry polygenic risk score improves risk prediction for coronary artery disease. Nat Med. 2023;29(7):1793-803.
23. Ocké MC, Bueno-de-Mesquita HB, Pols MA, Smit HA, van Staveren WA, Kromhout D. The Dutch EPIC food frequency questionnaire. II. Relative validity and reproducibility for nutrients. Int J Epidemiol. 1997;26 Suppl 1:S49-58.
24. Cohen S, Kamarck T, Mermelstein R. A global measure of perceived stress. J Health Soc Behav. 1983;24(4):385-96.
25. Friedenreich CM, Courneya KS, Bryant HE. The lifetime total physical activity questionnaire: development and reliability. Med Sci Sports Exerc. 1998;30(2):266-74.
26. Dausin C, Ruiz-Carmona S, De Bosscher R, Janssens K, Herbots L, Heidbuchel H, et al. Semiautomatic training load determination in endurance athletes. Journal for the Measurement of Physical Behaviour. 2023;6(3):193-201.
27. Braber TL, Mosterd A, Prakken NH, Rienks R, Nathoe HM, Mali WP, et al. Occult coronary artery disease in middle-aged sportsmen with a low cardiovascular risk score: The Measuring Athlete's Risk of Cardiovascular Events (MARC) study. European Journal of Preventive Cardiology. 2020;23(15):1677-84.
28. The 2007 Recommendations of the International Commission on Radiological Protection. ICRP publication 103. Ann ICRP. 2007;37(2-4):1-332.
29. European Commission. Directorate-General for Environment NS, Civil P. Radiation protection 99 : guidance on medical exposures in medical and biomedical research. Luxembourg: Office for Official Publications of the European Communities; 1998.
30. Cohan RH, Dunnick NR. Intravascular contrast media: adverse reactions. AJR Am J Roentgenol. 1987;149(4):665-70.
31. Stacul F, van der Molen AJ, Reimer P, Webb JA, Thomsen HS, Morcos SK, et al. Contrast induced nephropathy: updated ESUR Contrast Media Safety Committee guidelines. Eur Radiol. 2011;21(12):2527-41.
32. MacMahon H, Naidich DP, Goo JM, Lee KS, Leung ANC, Mayo JR, et al. Guidelines for Management of Incidental Pulmonary Nodules Detected on CT Images: From the Fleischner Society 2017. Radiology. 2017;284(1):228-43.