TREAT study

Improving the interpretation of **TR**oponin concentrations following **E**xercise **A**nd **T**heir clinical significance

(January 2023)

TREAT study: Improving the interpretation of TRoponin concentrations following Exercise And Their clinical significance

Protocol ID	NL79864.091.22
Short title	TREAT study
Version	5.0
Date	January 4th, 2023
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Pharmacy	Not applicable

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LIST OF ABBREVIATIONS AND RELEVANT DEFINITIONS

ABR	**General Assessment and Registration form (ABR form), the application form that is required for submission to the accredited Ethics Committee; in Dutch: Algemeen Beoordelings- en Registratieformulier (ABR-formulier)**
AE	**Adverse Event**
AR	**Adverse Reaction**
CACS	**Coronary Artery Calcium Score**
CCMO	**Central Committee on Research Involving Human Subjects; in Dutch: Centrale Commissie Mensgebonden Onderzoek**
CV	**Curriculum Vitae**
CVD	**Cardiovascular Diseases**
CT	**Computed Tomography**
FFR-CT	**Fractional Flow Reserve derived from Computed Tomography**
CCTA	**Coronary Computed Tomography Angiography**
cMyC	**Cardiac Myosin Binding Protein C**
eGFR	**estimated Glomerular Filtration Rate in ml/min**
EU	**European Union**
GCP	**Good Clinical Practice**
GDPR	**General Data Protection Regulation; in Dutch: Algemene Verordening Gegevensbescherming (AVG)**
hs-cTnT	**High-sensitivity Cardiac Troponin T**
hs-cTnI	**High-sensitivity Cardiac Troponin I**
IC	**Informed Consent**
MACE	**Major Adverse Cardiac Event**
METC	**Medical research ethics committee (MREC); in Dutch: medisch-ethische toetsingscommissie (METC)**
(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.**
SUSAR	**Suspected Unexpected Serious Adverse Reaction**
UAVG	**Dutch Act on Implementation of the General Data Protection Regulation; in Dutch: Uitvoeringswet AVG**
WMO	**Medical Research Involving Human Subjects Act; in Dutch: Wet Medisch-wetenschappelijk Onderzoek met Mensen**

SUMMARY

Rationale: Cardiac troponins (cTn) have a key role in the diagnosis of a myocardial injury[1, 2]. Intriguingly, exercise produces transient elevations of cTn concentrations[3], mimicking the cTn kinetics of a myocardial infarction[4]. The clinical relevance of exercise-induced elevations remains unclear. We recently showed that the magnitude of post-exercise cTn concentrations is associated with an increased risk for mortality and major adverse cardiovascular events in long-distance walkers[5]. Furthermore, exercise-induced cTn responses were different in recreational cyclists with occult obstructive coronary artery disease compared to healthy controls[6, 7]. Hence, post-exercise elevations of cTn concentrations could represent: 1) an acute coronary event, 2) (sub)clinical myocardial injury, or 3) a physiological response. The interpretation of cTn concentrations following exercise is, therefore, challenging and causes clinical confusion[8]. Therefore, more insight into physiological *versus* pathological post-exercise cTn concentrations is needed.

Objectives: Primary aim: To establish reference values for exercise-induced elevations of cTn concentrations following walking, cycling and running exercise.

Secondary aim: Assess the prevalence of (sub)clinical coronary artery disease in individuals with high *versus* low post-exercise cTn concentrations.

Tertiary aim: To determine the association between post-exercise cTn concentrations and major adverse cardiovascular events and mortality during long-term follow-up.

Study design: Observational cohort study.

Study population: 1500 participants of mass-participation exercise events, stratified for type of exercise (walking *versus* running *versus* cycling; n=500 each).

Intervention (if applicable): Not applicable.

Main study parameters/endpoints: Baseline and post-exercise concentrations of high-sensitivity cTn I (hs-cTnI) and T (hs-cTnT) will be established for our primary aim. Subsequently, the prevalence and magnitude of coronary artery calcification (i.e. Agatston score) and atherosclerotic plaque characteristics (density, phenotype (calcified/partially calcified/non-calcified), degree of stenosis, CT-derived fractional flow reserve), will be assessed in a subgroup of n=100 individuals with high *versus* n=50 with low post-exercise hs-cTnI concentrations. Finally, we will assess the incidence of major adverse cardiovascular events yearly and (cardiovascular and all-cause) mortality at 5-, 10-, 15-, and 20-years of follow-up.

Nature and extent of the burden and risks associated with participation, benefit and group relatedness:

Overall, the risks of this study are low, and all efforts will be made to further minimize these risks. Visits 1 to 3 include a venous blood draw at baseline (V1), within 6 hours post-exercise

(V2) and after 24 – 48 hours post-exercise (V3). All blood draws (3x30mL) are performed by an experienced researcher/nurse/physician, but a hematoma may occur in ± 5% of the participants. This will typically disappear within 2 weeks and is not associated with any (functional) limitations. Participants may experience transient pallor, dizziness, weakness or sweating following the blood draw of 30 ml per time point (90 ml in total). These symptoms generally disappear rapidly.

A subgroup of 150 athletes will be invited for visit 4 to determine the presence of (sub)clinical coronary artery disease using a contrast enhanced coronary CT angiography. The occurrence of contrast nephropathy is extremely rare in athletes with an estimated GFR >30 ml/min[9]. Mean radiation dose is estimated at 4.5 mSv, while a recent study observed no evidence of DNA damage in patients undergoing CT angiography with <7.5 mSv of radiation dose[10]. Incidental findings in the scan field may lead to additional diagnostic tests with extra costs and risks not covered by this study. Incidental findings (i.e. pulmonary nodules, pulmonary embolism and liver abnormalities) will be reported to the general practitioner of the participant. For subjects with heart rates >60 bpm, metoprolol will be administered iv. to lower heart rate and all subjects will receive 2 puffs of nitro-glycerine (0,8mg) sublingually directly before scanning to dilate the coronary arteries in concordance with clinical routine protocol. This may temporarily lower blood pressure.

1. INTRODUCTION AND RATIONALE

Cardiac troponin (cTn) has a key role in the diagnosis of a myocardial infarction next to clinical symptoms and/or imaging findings. Due to the unique cardiac-specific isoforms[11], cardiac troponin T or I (cTnT/cTnI) assessment is routinely used to differentiate myocardial infarction from other causes of chest-pain in patients presenting at the emergency department[1].

Intriguingly, exercise produces transient elevations of cTn concentrations[3], mimicking the cTn kinetics of a myocardial infarction[4]. Recent studies showed that the magnitude of exercise-induced cTn elevations, and especially post-exercise troponin concentrations above the 99th percentile, are predictive of adverse cardiovascular outcomes[5]. High post-exercise cTn concentrations may therefore be indicative of subclinical cardiovascular disease, such as coronary atherosclerosis.

Exercise-induced elevations in cTn concentrations are commonly observed in amateur athletes[12], also in healthy individuals, and are not directly related to cardiac symptoms. Post-exercise elevations of cTn concentrations in physically active individuals could thus represent: 1) an acute coronary event, 2) (sub)clinical myocardial injury, or 3) a physiological response. Therefore, the interpretation of cTn concentrations following exercise is challenging and can cause clinical confusion when an athlete presents to the emergency department with atypical chest pain and an elevated cTn concentration following exercise[8]. Hence, there is need for reference values for post-exercise cTn concentrations to aid in estimating the probability of either an acute coronary syndrome or a physiological cTn elevation following exercise. Therefore, our first aim is to collect real-life field data among a heterogeneous (age, sex, health status) group of middle-aged and older individuals participating in different types of mass-participation exercise events (i.e. walking, cycling, running) to establish reference values for exercise-induced cTn elevations in different sports.

Although exercise-induced cTn elevations are typically not associated with cardiac symptoms, higher concentrations of post-exercise cTn may represent myocardial injury due to underlying, subclinical, cardiac pathology. This is supported by a higher incidence of post-exercise cTn elevations in participants with CVD, but also the association between post-exercise cTn concentrations and MACE *versus* mortality[5]. Exposure of individuals to bouts of exercise can unmask myocardial vulnerability[13], which might remain unnoticed under resting conditions. Thus, exercise-induced cTn elevations could be a novel marker of cardiovascular risk. Indeed, a small Norwegian pilot study reported higher post-exercise cTnI concentrations in cyclists with obstructive coronary artery disease (n=9) compared to healthy peers (n=109)[6]. This is important as coronary atherosclerosis is more prevalent in amateur athletes compared to inactive controls[14]. 19% of asymptomatic, middle-aged male amateur athletes without abnormalities on sports medical examination, had substantial coronary atherosclerosis (defined as coronary artery calcification score (CACS) ≥100 and/or a ≥50% stenosis)[15] and

the prevalence was dependent on their lifelong exercise volumes[16]. Hence, early identification of athletes with coronary atherosclerosis is warranted, and a (delayed) elevation of post-exercise cTn concentrations may be indicative of such subclinical disease. The secondary aim of our study is, therefore, to compare the prevalence of subclinical coronary artery disease in a subgroup of participants free from known CVD (myocardial infarction, stroke, heart failure, peripheral vascular disease) with high *versus* low post-exercise cTn concentrations, matched for age and sex.

The predictive capacity of post-exercise cTn concentrations for long-term cardiovascular outcomes have only been assessed in a single study[5]. An editorial[17] and American Heart Association press release[18] accompanying our work[5] highlighted the need to conduct large-scale follow-up studies, to assess whether these findings can be extrapolated to other types of exercise, such as long-distance running or cycling. Therefore, our tertiary aim is to assess the incidence of major adverse cardiovascular events and mortality during long-term follow-up.

2. OBJECTIVES

Primary Objective:

To establish reference values for exercise-induced elevations of cTn concentrations following walking, cycling and running.

> ***Hypothesis***: We hypothesize that the exercise-induced cTn release is different following walking, cycling and running exercise. Therefore, we will establish reference values for post-exercise cTn concentrations across each of these sport types.

Secondary Objective:

To assess the prevalence of (sub)clinical coronary artery disease in individuals with high *versus* low post-exercise cTn concentrations.

> ***Hypothesis***: We hypothesize that athletes with the highest post-exercise cTn concentrations have a higher prevalence of coronary atherosclerosis compared to athletes matched for sex and age with the lowest post-exercise cTn concentrations.

Tertiary Objective:

To assess the incidence of major adverse cardiovascular events and mortality between athletes with post-exercise cTn concentrations above and below the upper reference limit of normal (i.e. 99th percentile).

> ***Hypothesis:*** We hypothesize that post-exercise cTn concentrations beyond the 99th percentile are associated with an increased risk for MACE and mortality during follow-up.

3. STUDY DESIGN

The TREAT study is a prospective cohort study consisting of three phases:

Phase 1) Assessment of cTn concentrations to establish reference values

Baseline and post-exercise cTn concentrations will be assessed in the full cohort of n=1500 middle-aged and older participants of mass participation exercise events, stratified for walking, cycling and running exercise. As exercise duration and exercise intensity are known predictors for the magnitude of exercise-induced elevations in cTn concentrations[19-21], we will focus on long distance events only (i.e. walking ≥30 km, cycling ≥100 km, running ≥15 km). Within each type of sport, we aim to balance our inclusion rates for sex (men/women) and age (40-49 / 50-59 / 60-69).

All participants will be invited for two blood draws:

- pre-exercise (i.e. baseline; ≤5 days before the start of the mass-participation event)
- <6 hours post-exercise

And are invited for an optional 3rd blood draw:

- 24-48 hours post-exercise

Participants will be recruited via the organizer's website, newsletter, social media platforms, as well as via (social) media channels of the Radboudumc and our research group. Only athletes that already registered for participation in the mass-participation exercise event can take part in this study. Upon expression of interest to take part in the TREAT study, participants will receive a written explanation of the study ("Proefpersoneninformatie"), whereafter participants will have at least 1 week to consider study participation. If a participant chooses to participate, written informed consent will be obtained at their first visit. An overview of the study design is shown in **Figure 1**.

Phase 2) Assessment of (sub)clinical coronary artery disease

The secondary aim of this study is to assess the presence of occult coronary artery disease in individuals with high *versus* low post-exercise cTnI concentrations. For this exploratory study, a subgroup of participants free from known cardiovascular diseases will be invited for an additional study visit at the Radboudumc. Specifically, we will select n=100 eligible participants with the highest post-exercise cTnI concentrations, as well as an age-, sex-, and sport-type matched control group of n=50 participants with low cTnI concentrations post-exercise. So, n=100 participants with a high and n=50 participants with a low post-exercise troponin

concentration will be invited for this additional study visit (**Figure 1**). We strive to include participants evenly from all three sport disciplines.

Phase 3) Incidence of long-term outcomes

The tertiary study endpoint will be a composite of all-cause mortality and major adverse cardiovascular events (MACE). In a previous study by our group 62 of 725 (9%) of walkers experienced an end point after a median follow-up of 43 months; 29 died and 33 had major adverse cardiovascular events[5]. We will assess the incidence of major adverse cardiovascular events yearly and (cardiovascular and all-cause) mortality at 5-, 10-, 15-, and 20-years of follow-up. MACE will be collected from annual questionnaires sent to participants and will be defined as myocardial infarction, stroke, heart failure, revascularization (both acute and elective) or sudden cardiac arrest during follow-up. Using the questionnaires, we will also evaluate medication use to ensure it matches the reported diagnoses. Survival status of study participants (i.e. mortality data) will be retrieved from the Dutch population register.

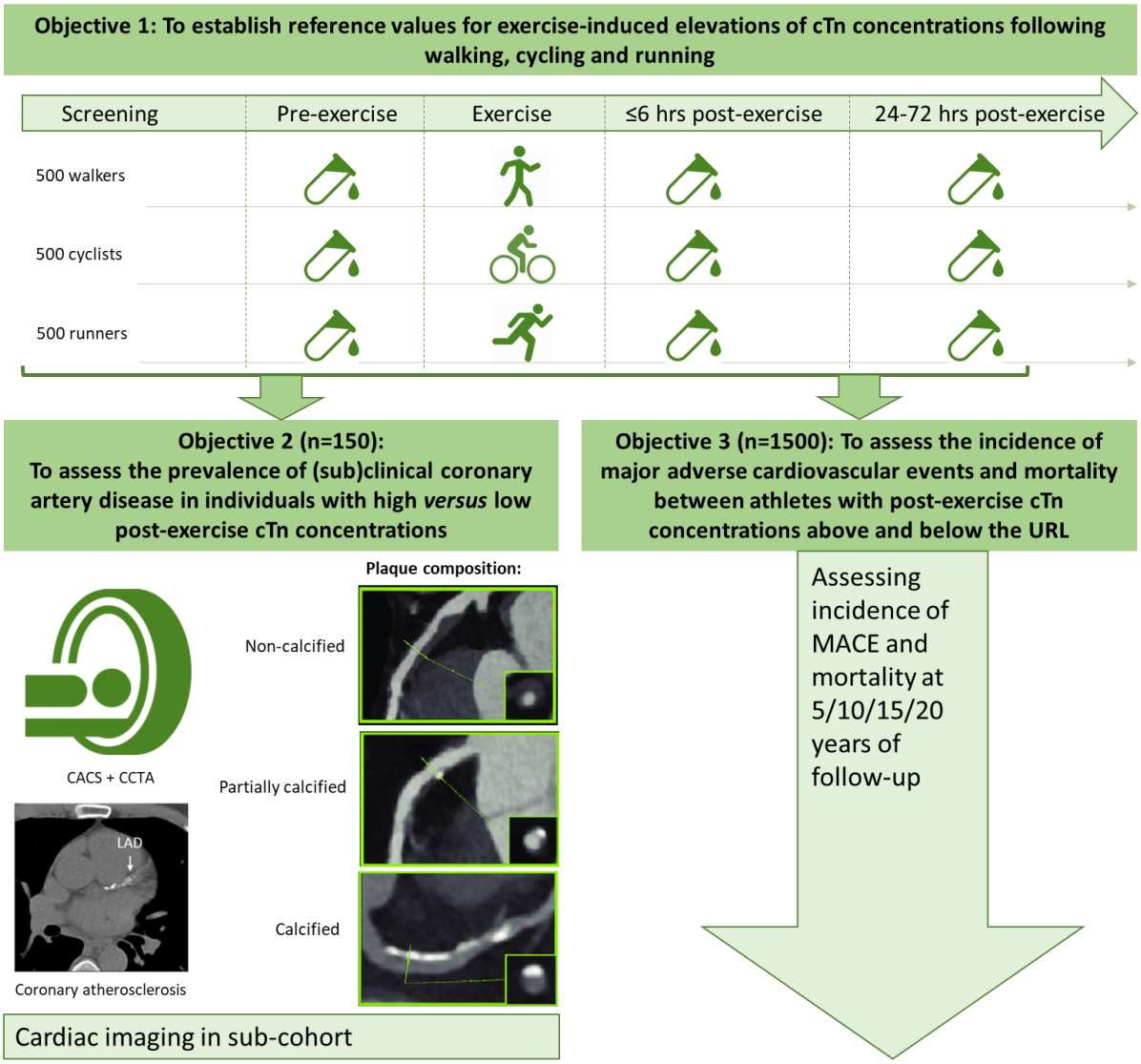

Figure 1. Summary of study design. Plaque composition images derived from Kolossváry *et al.*[22] cTn: cardiac troponin; hrs: hours; CACS: coronary artery calcium score; CCTA: coronary computed tomography angiography; URL: upper reference limit of normal (i.e. 99th percentile); MACE: major adverse cardiovascular events.

4. STUDY POPULATION

4.1 Population (base)

1500 Athletes will be recruited at mass-participation long-distance exercise events and stratified for type of sport (e.g. walking, cycling, running). Recruitment will take place via official websites/newsletters of the associated exercise events and social media channels.

Our research has extensive experience performing studies in mass-participation exercise events, such as the Four Days Marches, Seven Hills Run, Eindhoven Marathon, Amsterdam Marathon, Maastricht Marathon in which we successfully included hundreds of participants. Therefore, we expect no difficulty to reach our target number of included participants.

4.2 Inclusion criteria

In order to be eligible to participate in this study, a subject must meet all of the following criteria:

- Participant of a mass-participation exercise event with a:
  - Walking distance ≥20 km
  - Cycling distance ≥100 km
  - Running distance ≥15 km
- Age: ≥ 40 and <70 years old
- Able to understand and perform study related procedures

For Phase 2 of the study (i.e. assessment of (sub)clinical coronary artery disease), the following additional criteria are present:

- Free from (known) cardiovascular diseases

4.3 Exclusion criteria

A potential subject who meets any of the following criteria will be excluded from participation in Phase 2 of the study:

- Renal transplantation in the past
- Contrast nephropathy in the past
- eGFR < 30 ml/min
- Atrial fibrillation (heart rhythm disorder)
- Previous allergic reaction to iodine contrast
- Participation in other studies involving radiation
- Not willing to be informed about potential incidental findings from the CT-scan

4.4 Sample size calculation

The primary aim of this study is to establish reference values for post-exercise cTn concentrations in participants of mass-participation exercise events. There are no specific sample size calculations available for such studies, but a sufficient number of observations is needed to determine the 99th percentile upper reference limit in the whole cohort, as well as specific subgroups (i.e. sport type / sex / age). For this purpose, we aim to include 500 participants per type of sport. This is a common sample size for establishing reference values within the field of clinical chemistry[23, 24] and allows us to explore sex and age specific values beyond the impact of sport type, even when there is some loss-to-follow-up, drop-out of participants and/or missing values. This sample size also follows the recommendations by the Clinical and Laboratory Standards Institute (CLSI), as mentioned in their guideline[25].

Our secondary objective (to determine the prevalence and magnitude of coronary atherosclerosis between participants with high *versus* low post-exercise cTn concentrations) is explorative, so no sample size calculation was conducted. We aim to compare the prevalence and magnitude of coronary atherosclerosis between 100 high *versus* 50 low cTn responders, which are stratified for sport type.

Our third objective is also explorative. In a previous study by our group 62 of 725 (9%) of walkers experienced an end point; 29 died and 33 had MACE[5]. However, these walkers had a median age of 61 years. As many of our participants are younger, and thus have a lower probability of MACE and (cardiovascular and all-cause) mortality, we expect a lower event rate in our study population. Therefore, we will assess the incidence of MACE and mortality at 5-, 10-, 15-, and 20-years of follow-up.

5. TREATMENT OF SUBJECTS

Not applicable.

6. INVESTIGATIONAL PRODUCT

Not applicable.

7. NON-INVESTIGATIONAL PRODUCT

Not applicable.

8. METHODS

8.1 Study parameters/endpoints

8.1.1 Main study parameter/endpoint

- Baseline and post-exercise concentrations of high sensitive cTnT and cTnI (objective 1)

8.1.2 Secondary study parameters/endpoints (if applicable)

- Coronary artery calcification score, expressed in Agatston score (objective 2) and specified by coronary vessel (LM, LAD, RCA en RCX)
- Plaque characteristics (calcified/non-calcified/partially calcified) and degree of coronary artery stenosis (objective 2) according to CAD-RADS classification system
- On-site CT-derived Fractional Flow Reserve per coronary artery (i.e. left main (LM), left anterior descending (LAD), left circumflex (LCX), right coronary artery (RCA) (objective 2)
- Incidence of mortality (cardiovascular and all-cause) (objective 3)
- Incidence of major adverse cardiovascular events (myocardial infarction, stroke, heart failure, revascularization (both acute and elective) or sudden cardiac arrest during follow-up) (objective 3)

8.1.3 Other study parameters (if applicable)

- Subject characteristics (age, sex, height, weight, resting blood pressure, fat percentage, muscle mass, etc)
- Lifestyle factors (smoking, drinking, sleep behaviour)
- Cardiovascular health characteristics
- Exercise training status and physical activity characteristics (current and historic training frequency, distance, intensity)
- Other (cardiac) biomarkers: creatinine, albumin, cardiac myosin binding protein C (cMyC), future biomarkers.
- Exercise duration
- Exercise intensity (heart rate during exercise)
- Power output during exercise (in subcohort)

8.2 Randomisation, blinding and treatment allocation

Not applicable.

8.3 Study procedures

All procedures are extra for this study and are not part of medical treatment. No diagnostic procedures or treatment will be postponed for this study.

Recruitment and informed consent

Recruitment will take place via official websites/newsletters of the associated exercise events and social media channels. Interested individuals are invited to respond via e-mail and will then receive participant information on the study ('Proefpersoneninformatie'). They will be given at least 1 week to consider their decision regarding study participation. If they agree to participate, a screening e-mail will be sent, in which we screen for inclusion criteria. Participants will be asked to refrain from vigorous exercise in the 48 hours before study visit 1. In addition, if participants agree to visit us a third time (for the optional blood draw where recovery blood samples will be obtained), they will be asked to refrain from vigorous exercise in the time between the finish of the sports event and visit 3. Low to moderate intensity exercise up to 1 hour (e.g. walking the dog) is still allowable. During study visit 1, a member of the research team will go over the participant information and can answer any remaining questions. If the participant is interested in participation, we will obtain informed consent at the beginning of visit 1.

Visit 1: Pre-exercise blood draw (10 minutes) and baseline measurements

Following informed consent, height, body mass and resting blood pressure will be assessed. Subsequently, a pre-exercise blood sample (30 ml) will be drawn from an antecubital vein by an experienced researcher, nurse or physician. This blood draw will be performed ≤5 days before the start of the mass-participation event. Blood samples will be centrifuged according to the manufacturer's instructions and aliquots will be stored at -80°C. After analysis the collected blood samples will be stored in the freezer of the study location or the Biobank for maximum 20 years in case additional analyses are warranted.

Following visit 1, participants will receive an email with the request to complete online questionnaires about their health and training status.

Exercise (is NOT part of our study protocol)

Participants will voluntarily participate in a mass-participation exercise event (i.e. walking, cycling or running). Participation in this exercise event is voluntary and independent from

participation in this study (i.e. participants would also take part in the exercise event if they did not participate in our study). Therefore, we do not consider the performed exercise an intervention or part of our study protocol. Hence, we consider our study design to be observational. Information about exercise characteristics, such as heart rate and power output, will be collected during exercise using commercially available devices in order to quantify the exercise exposure.

Visit 2: Acute post-exercise blood draw (10 minutes)

This blood draw (30 ml from an antecubital vein) will be performed within 6 hours after exercise cessation.

Visit 3 (optional): Delayed post-exercise blood draw (10 minutes)

This blood draw (30 ml from an antecubital vein) will be performed between 24 and 48 hours after exercise cessation. Visit 3 is optional and this blood draw will only be performed if a participant agrees to visit 3 (where recovery blood samples will be obtained).

Visit 4: Cardiac CT-scan (60 minutes)

A subgroup of 150 athletes (free from CVD and stratified for sport type) with high post-exercise cTnI concentrations and age- and sex- matched controls with low acute post-exercise cTnI concentrations, will be scheduled for coronary CT-angiography (CCTA) (including calcium scoring and CT angiography imaging) on a state-of-the-art CT scanner, with a total on-table examination time of approximately 15 minutes. This procedure is described in more detail below. The CT scans will be performed at the Department of Radiology of the Radboudumc by trained cardiac CT technicians. Creatinine levels will be assessed in baseline (pre-exercise) blood samples to determine their glomerular filtration rate (GFR). GFR will be determined at maximum of 3 months before the CT-scan.

Participant Preparation

The participant preparation will proceed according to the following protocol:

- Participant is asked to abstain from caffeine (e.g. coffee, tea, coca cola, energy drinks like Red Bull) for 12 hours prior to the CT examination.
- Participant will be asked to empty their bladder, shortly before scanning.
- Participant is positioned in the scanner.
- Participant is connected to the ECG monitor – sinus rhythm is monitored for 1 minute.
- Participant practices a 25 second breath-hold.
- Technician inserts an IV tube according to clinical routine protocol

- Participants who have a heart rate above 60 beats per minute (bpm) will be administered intravenous beta-blockers (minimum of 5 mg Metoprolol). Participants will continue to receive beta-blockers until their heart rates are below 60 bpm, or 10 mg metoprolol has been administered. As athletes usually have low heart rates (e.g. a mean heart rate of 57±10 bpm in a recent study in 55 older athletes with a mean age of 50±6.6 years)[26], it is expected that administration of metoprolol will not be necessary for the majority of participants. A decrease in blood pressure may occur as a side-effect after administration of metoprolol.
- Participants will receive two puffs of Nitroglycerine sublingually (0,8mg) to dilate the coronary arteries, directly prior to CT scanning. A decrease in blood pressure may occur as a side-effect.

Imaging protocol

The imaging will consist of a scout view, followed by CACS and coronary CT-angiography.

Scout view and CACS

The first scan will include an initial scout view of the entire chest, that will be followed by a prospective ECG-triggered calcium scoring to detect and quantify the amount of calcification in the coronary arteries.

Contrast injection & bolus tracking

Following the calcium scoring examination, the contrast injection will be set up. The localizer for bolus tracking will be placed in the aorta. The contrast volume will be calculated individually depending on participant weight. The contrast injection will be started. The contrast tracker will be used to monitor the infusion of the contrast agent into the circulatory system. When the image intensity within the region of interest (ROI) reaches the threshold, the acquisition will automatically start after a 4-6 second post-threshold delay.

The CCTA examination is a prospectively ECG triggered study performed at the diastolic portion (70-99%) of the cardiac cycle.

If despite intravenous betablockers administration, heart rate remains high, padding (systolic and diastolic) imaging could be used.

Image Analysis

General

The cardiac CT data will be analyzed by the site investigators. The radiology site investigator is responsible for reading the dataset for relevant extracardiac and cardiac findings (such as pulmonary nodules, pulmonary embolism, mammary nodules, liver abnormalities, 3-vessel

disease, significant stenosis of the proximal LAD or left main stenosis >50%). Incidental findings will be reported to the general practitioner of the participant. If a person does not wish to be informed about incidental findings, this person cannot participate in this study.

Calcium Scoring

An established analysis program will be used to determine the amount of coronary calcium using the Agatston score as well as density and volume scores[27, 28].

Coronary CT-angiography (CCTA)

The CCTA will be evaluated by the investigators as follows

- Load the CCTA dataset in a workstation and create a semi-automatic curved MPR along the vessel centerline of each coronary artery.
- Evaluate the CCTA in terms of contrast opacification, assessability, stenoses and plaques.
- Evaluate the overall coronary artery contrast opacification using a 5-point scale (1=non-diagnostic, 2=limited diagnostic, 3=acceptable, 4=good, 5=excellent).
- The diagram of the Society of Cardiovascular Computed Tomography (SCCT) guideline will be used to indicate where the stenoses are located[29].
- The CCTA will be scored following the CAD-RADS methodology[30]. Lesion type (if present) will be indicated as follows: non-calcified, partially calcified or calcified.

On-site FFR derived from CT (FFR-CT)

Because CCTA only provides anatomical delineation, we will also perform CT-derived fractional flow reserve (FFR-CT) to determine the functional significance of stenosis. FFR-CT involves computing the FFR from a CT data set which can be acquired in addition to the procedure of the standard coronary CT. FFR-CT will be performed according to the clinical Radboudumc protocols. For FFR-CT it is crucial that heart rate is ≤60 bpm to have diagnostic FFR-CT images. If we are not able to slow heart rate to ≤60 bpm, we will not perform the FFR-CT. In that case, we will only perform the cardiac CT-scan with CACS and the CCTA.

Radiation Dose

The total estimated radiation dose to which participants will be exposed will be 4.5 mSv on average (based on historical data from CT angiography performed in the Radboudumc).

Follow-up

We will assess the incidence of major adverse cardiovascular events yearly and (cardiovascular and all-cause) mortality at 5-, 10-, 15-, and 20-years of follow-up. MACE will

be collected from annual questionnaires sent to participants and will be defined as myocardial infarction, stroke, heart failure, revascularization (both acute and elective) or sudden cardiac arrest during follow-up. Using the questionnaires, we will also evaluate medication use to ensure it matched the reported diagnoses. Survival status of study participants (i.e. mortality data) will be retrieved from the Dutch population register.

Flowchart of study design

An overview of the TREAT study protocol is presented in Figure 2.

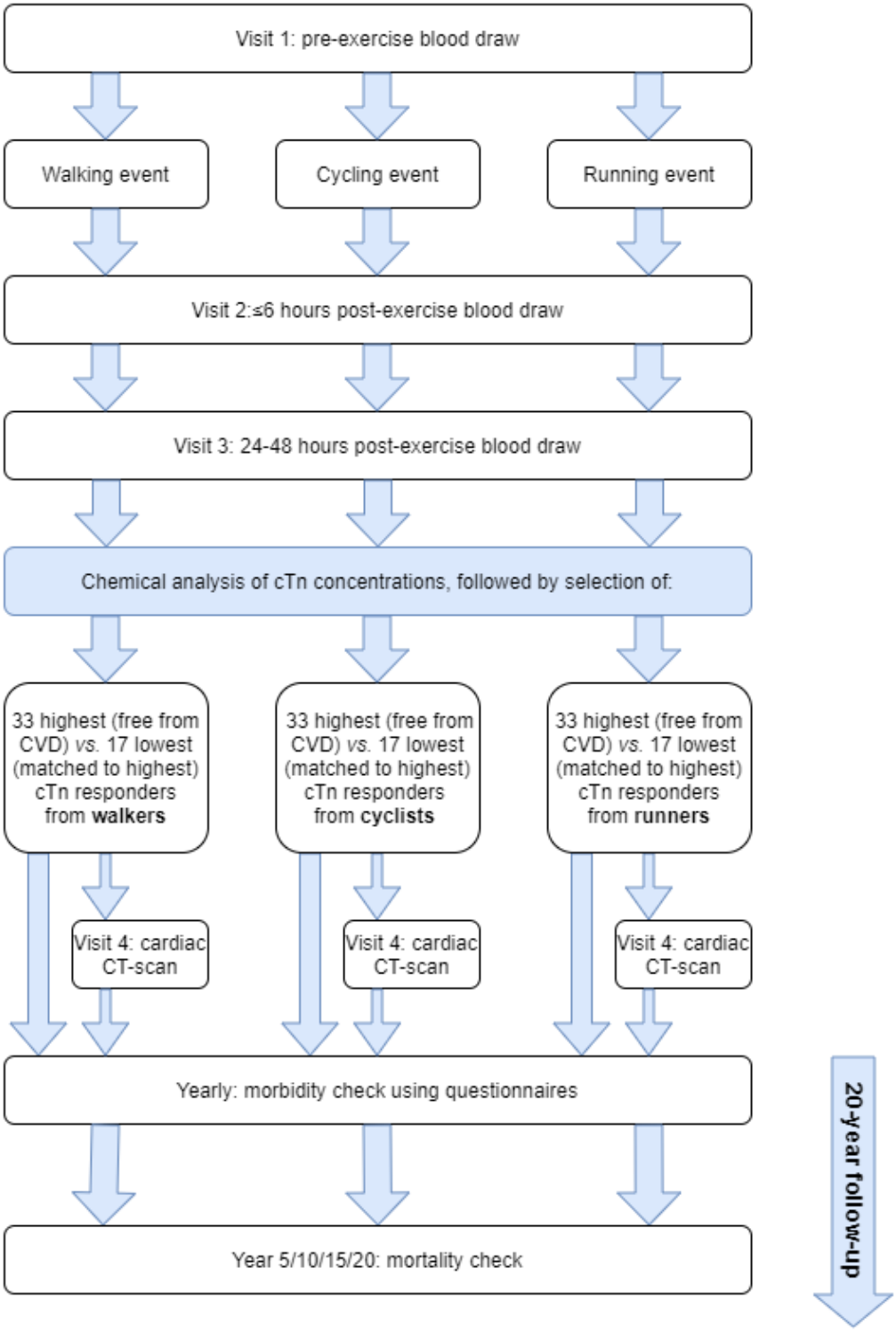

Figure 2. Flowchart of study design

8.4 Withdrawal of individual subjects

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

8.4.1 Specific criteria for withdrawal (if applicable)

Not applicable.

8.5 Replacement of individual subjects after withdrawal

Subjects who withdraw before CT scanning will be replaced by reserve subjects from a back-up list. Subjects who withdraw after CT scanning or subjects with a non-diagnostic CT-scan (which only rarely happens), will not be replaced.

8.6 Follow-up of subjects withdrawn from treatment

Not applicable.

8.7 Premature termination of the study

Adverse reaction to the contrast medium in the form of nausea, skin hives are estimated to occur in 1 to 3% of participants and are likely to occur during our study[2, 32]. Fatal adverse reactions are estimated to occur in <0.001% of participants. Considering our sample size, this is not expected to occur. Therefore, we have no reason to prematurely terminate the study.

9. SAFETY REPORTING

9.1 Temporary halt for reasons of subject safety

In accordance to section 10, subsection 4, of the WMO, the sponsor will suspend the study if there is sufficient ground that continuation of the study will jeopardise subject health or safety. The sponsor will notify the accredited METC without undue delay of a temporary halt including the reason for such an action. The study will be suspended pending a further positive decision by the accredited METC. The investigator will take care that all subjects are kept informed.

9.2 AEs, SAEs and SUSARs

9.2.1 Adverse events (AEs)

Adverse events are defined as any undesirable experience occurring to a subject during the study, whether or not considered related to trial procedure. All adverse events reported spontaneously by the subject or observed by the investigator or his staff will be recorded.

9.2.2 Serious adverse events (SAEs)

A serious adverse event is any untoward medical occurrence or effect that

- results in death;
- is life threatening (at the time of the event);
- requires hospitalisation or prolongation of existing inpatients' hospitalisation;
- results in persistent or significant disability or incapacity;
- is a congenital anomaly or birth defect; or
- any other important medical event that did not result in any of the outcomes listed above due to medical or surgical intervention but could have been based upon appropriate judgement by the investigator.

An elective hospital admission will not be considered as a serious adverse event.

SAE may be caused by the CT coronary angiography as this visit encompasses the use of a contrast medium. Adverse reaction to the contrast medium (nausea, skin hives in 1 to 3% of participants, fatal adverse reactions in 0.001%) and contrast medium-induced renal insufficiency may occur, which will be a reason for hospitalization. CT will be performed in the hospital and therefore acute matters will be handled immediately by notifying the emergency department, which is next to the scanning room.

The sponsor will report the SAEs through the web portal *ToetsingOnline* to the accredited METC that approved the protocol, within 7 days of first knowledge for SAEs that result in death or are life threatening followed by a period of maximum of 8 days to complete the initial preliminary report. All other SAEs will be reported within a period of maximum 15 days after the sponsor has first knowledge of the serious adverse events.

9.2.3 Suspected unexpected serious adverse reactions (SUSARs)

Not applicable.

9.3 Annual safety report

Not applicable.

9.4 Follow-up of adverse events

All AEs will be followed until they have abated, or until a stable situation has been reached. Depending on the event, follow up may require additional tests or medical procedures as indicated, and/or referral to the general physician or a medical specialist. SAEs need to be reported till end of study within the Netherlands, as defined in the protocol.

9.5 Data Safety Monitoring Board (DSMB)

Not applicable.

10. 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.

10.1 Primary study parameter(s)

cTnT and cTnI concentrations will be expressed in ng/L. Descriptive statistics will be applied to determine reference values. Upper reference values will be defined at the 95th percentile and 99th percentile upper reference limit (URL), including sex and sport specific values (following recent recommendations by Ichihara et al.[23]). We will analyse the results using multiple regression analysis to determine factors that influence the test values in the reference population. Non-parametric analyses will be performed to determine 99th percentile upper reference limits of hs-cTnT and hs-cTnI, stratified by sex and age. Uncorrected and outlier-adjusted 99th percentiles will be calculated according to both Dixon's and Tukey's outlier detection methods. To verify that results are robust and independent of the chosen outlier detection method (Dixon vs. Tukey), sensitivity analysis will be performed to compare results from both outlier detection methods.

10.2 Secondary study parameter(s)

Normality of all variables will be tested using Shapiro-Wilk tests and visual inspection for skewness and kurtosis. Homogeneity of variance between groups will be tested using Levene's tests. When comparing two groups of participants (e.g. high *versus* low hs-cTn responders) conditional logistic regression will be used. When comparing three or more groups of participants (e.g. cyclists *versus* runners *versus* walkers), one-way ANOVA (when normally distributed) or Kruskal–Wallis tests (when not normally distributed) will be used. Categorical variables will be compared with the Chi-square test or, in case expected count for any cell is below 5, with Fisher's exact tests.

Because the high *versus* low troponin responders will be matched (if possible at individual level and otherwise at group level) and included in a 2:1 ratio, these are paired data that will thus be analysed using paired analytical methods. To determine the prevalence of coronary atherosclerosis between participants with high *versus* low post-exercise cTn concentrations, conditional logistic regression will be used. Lumen stenosis is also a dichotomous variable with a cut-off point for percentage lumen stenosis (>50%) and will also be compared between both

groups using conditional logistic regression. To compare the magnitude of coronary atherosclerosis between participants with high *versus* low post-exercise cTn concentrations, we will look into plaque characteristics and CAC score. Plaque characteristics will be described as a categorical variable (non-calcified/mixed/calcified) and will be analysed using conditional multinomial logistic regression. CAC score will be calculated using the Agatston scoring protocol[33] and expressed in Agatston units (AU). To compare CAC scores between participants with high *versus* low post-exercise cTn, ANOVA with a blocking factor will be used. Participants will first (before CT-scanning) be divided into (relatively) homogeneous groups regarding sex and age which constitute the blocks for this analysis. Doing this, we aim to minimize the variance among units within blocks (i.e. within the matched pairs) relative to the variance among blocks (i.e. between the matched pairs).

Univariate regression analyses will be used to study the association between post-exercise cTn concentrations and CAC scores.

Creatinine concentrations will not be statistically analysed because they are only for screening purposes (to prevent contrast nephropathy). These will only be used to determine individuals' GFR. We will only select participants with a GFR higher than 30 ml/min, because in these individuals contrast nephropathy is unlikely to occur.

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 troponine concentrations above and below a certain threshold (threshold to be determined). Univariate regression analyses will be used to study the relation between exercise-induced troponin and prevalence of MACE. Cox-proportional hazards regression analyses will be performed to calculate unadjusted and adjusted hazard ratios for the incidence of MACE and mortality. Moreover, Kaplan-Meier curves and Cox proportional-hazards models will be generated to investigate the association between delta (post-exercise minus baseline) cTn concentrations and MACE and mortality. Binary logistic regression will be used to calculate unadjusted and multivariable adjusted odds ratios for the association between participant- and exercise characteristics and post-exercise cTn concentrations above the URL. First, univariable logistic regression analysis of variables will be used to select potential predictors for multivariable analysis. Variables that show to be associated ($p<.10$) with post-exercise cTn concentrations above the URL will subsequently be included in a multivariable backward logistic regression analysis to gain insight into which variables could independently predict post-exercise cTn concentrations >URL.

10.3 Other study parameters

Subject characteristics (age, smoking, other cardiovascular risk factors, training status) will be analysed as previously mentioned. Other study parameters, such as exercise characteristics, will be analysed as previously mentioned.

10.4 Interim analysis (if applicable)

Not applicable.

11. ETHICAL CONSIDERATIONS

11.1 Regulation statement

This study will be conducted according to the principles of the Declaration of Helsinki (World Medical Association, 2013, Brazil) and in accordance with the Medical Research Involving Human Subjects Act (WMO).

11.2 Recruitment and consent

Recruitment will take place via official websites/newsletters of the associated exercise events and social media channels. Interested individuals are invited to respond via email and will then receive participant information on the study ('Proefpersoneninformatie'). They will be given at least 1 week to consider their decision regarding study participation. If they agree to participate, a screening e-mail will be sent, in which we screen for exclusion criteria and possible contra-indications. Participants will be asked to refrain from vigorous exercise in the 48 hours before study visit 1. Low to moderate intensity exercise up to 1 hour (e.g. walking the dog) is still allowable. During study visit 1, a member of the research team will go over the participant information and can answer any questions. If the participant is interested in participation, informed consent will be obtained at the beginning of visit 1.

11.3 Objection by minors or incapacitated subjects (if applicable)

Not applicable.

11.4 Benefits and risks assessment, group relatedness

<u>Possible inconveniences for the participant</u>

All venous blood draws (3x 30ml) are performed by an experienced researcher, nurse or physician. Nevertheless, a hematoma may occur in ± 5% of the participants. This will typically disappear within 2 weeks and is not associated with any (functional) limitations. Participants may experience transient pallor, dizziness, weakness or sweating following the blood draw of 30 ml per time point (90 ml in total). These symptoms generally disappear rapidly.

A selection of participants (n=150) will undergo cardiac CT-scans. This radiological examination is generally considered safe, but not entirely without risks (next paragraph)[34, 35]. Participants may be anxious when undergoing this examination. Participants will receive extensive written and oral information about the radiological procedures prior to examination. All questions about the examinations will be answered. The examinations will be terminated on participant's request.

Possible risks for the participant

The following are potential risks of cardiac CT:

- adverse reaction to the intravenous contrast media (nausea, skin hives in 1 to 3% of participants, fatal adverse reactions in 1 in 100,000 participants)[34].
- 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[9].
- Radiation exposure may lead to DNA damage and subsequent side effects. The total radiation dose to which participants will be exposed during CT scanning of the heart is anticipated to be approximately 4.5 mSv on average. A recent study observed no evidence of DNA damage in patients undergoing CT angiography with <7.5 mSv of radiation dose[10].
- Incidental findings will be reported to the general practitioner of the participant. If a person does not wish to be informed about incidental findings, this person cannot participate in this study. Common incidental findings include conditions such as pulmonary nodules, pulmonary embolism, mammary nodules, liver abnormalities, 3-vessel disease, significant stenosis of the proximal LAD. (False-positive) Incidental findings in the scan field which may lead to additional diagnostic tests with extra costs and risks not covered by this study.

These risks are the same risks that all participants have when they undergo CT of the chest with contrast infusion; a standard examination performed routinely in all radiology departments.

Possible advantages for the participant

Participants undergoing a cardiac CT scan will be provided with a state-of-the-art evaluation of the presence or absence of coronary artery disease. The results of the investigations will be shared with the participant. If necessary, e.g. in case of a (very) high coronary artery calcium score, we will inform the participant's general practitioner. The results of the hs-cTn analysis and the FFR-CT protocol will generally not be shared with the participant and his/her general practitioner because (for the time being) the clinical relevance of these results is currently unclear and primarily collected for research purposes.

11.5 Compensation for injury

The sponsor/investigator has a liability insurance which is in accordance with article 7 of the WMO.

The sponsor (also) has an insurance which is in accordance with the legal requirements in the Netherlands (Article 7 WMO). This insurance provides cover for damage to research subjects through injury or death caused by the study.
1. € 650.000,-- (i.e. six hundred and fifty thousand Euro) for death or injury for each subject who participates in the Research;
2. € 5.000.000,-- (i.e. five million Euro) for death or injury for all subjects who participate in the Research;
3. € 7.500.000,-- (i.e. seven million five hundred thousand Euro) for the total damage incurred by the organization for all damage disclosed by scientific research for the Sponsor as 'verrichter' in the meaning of said Act in each year of insurance coverage.
The insurance applies to the damage that becomes apparent during the study or within 4 years after the end of the study.

11.6 Incentives (if applicable)

Participants will undergo CT coronary angiography and will receive the results of this study, and therefore will receive extra information on their cardiovascular health status. Other than financial compensation for travel expenses for visit 4, no compensation will be available for the participants.

12. ADMINISTRATIVE ASPECTS, MONITORING AND PUBLICATION

12.1 Handling and storage of data and documents

Data will be handled confidentially. After informed consent, each participant will be given a unique code consisting of letters (the code name of the study) and a number (e.g. TREAT001). The key linking to the patient identity will be stored in a secured file and only the involved investigators will have access to this key. Personal data will be handled in accordance with the EU General Data Protection Regulation and the Dutch Act on Implementation of the General Data Protection Regulation. (in Dutch: Uitvoeringswet AVG, UAVG). The research data will be stored for 15 years after finalization of the project. All blood samples will be collected in tubes identified by the unique subject code and a code for the specific timepoint (date of study visit, consecutive timepoints pre- and post-exercise). Collected samples will be stored in the -80°C freezer of the study location for maximum 20 years in case additional analyses are warranted. The data required for the trail will be collected and stored in the electronic Case Report Form (eCRF) using Castor (Castor EDC 2019.1.15 or further, Ciwit B.V., The Netherlands) and afterwards exported to SPSS statistics 25 or further (IBM Corp., USA) for the analyses. Only the involved investigators will have unrestricted access to all pseudonymized data. Study related correspondence, signed Informed Consent forms, and source documents are to be maintained by the study site and archived in a locked cabinet for a minimum of 15 years after the end of this study. Source data will be entered at worksheets in the eCRF and afterwards exported to SPSS statistics 25 or further (IBM Corp., USA) for the analyses.

12.2 Monitoring and Quality Assurance

Written procedures have been established by the PI for monitoring clinical investigations, to assure the quality of the study and to assure that each person involved in the monitoring process carries out his or her duties. Standardized written procedures, sufficiently detailed to cover the general aspects of clinical investigations, will be used as a basic monitoring plan and will be supplemented by more specific or additional procedures, as required by the clinical investigation. Periodic monitoring visits will be conducted with adequate frequency to ensure that the PI’s obligations are being fulfilled and that the facilities continue to be acceptable. Monitoring will be carried out by a BROK certified internal monitor of the Radboud university medical center. The stored data in the CRFs, all informed consents, SAE reports, the trial master file and storage of blood samples will be monitored.

12.3 Amendments

Amendments are changes made to the research after a favourable opinion by the accredited METC has been given. All amendments will be notified to the METC that gave a favourable opinion.

12.4 Annual progress report

The sponsor/investigator will submit a summary of the progress of the trial to the accredited METC once a year. Information will be provided on the date of inclusion of the first subject, numbers of subjects included and numbers of subjects that have completed the trial, serious adverse events/ serious adverse reactions, other problems, and amendments.

12.5 Temporary halt and (prematurely) end of study report

The investigator/sponsor will notify the accredited METC of the end of the study within a period of 8 weeks. The end of the study is defined as the last patient's last visit. The sponsor will notify the METC 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 accredited METC 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 accredited METC.

12.6 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 among involved investigators. 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. This study will be registered in a WHO recognized primary registry by the investigator to safeguard an open documentation policy and a future scientific publication.

13. STRUCTURED RISK ANALYSIS

Not applicable.

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