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The effects of Menaquinone-7 supplementation in patients with aortic valves calcification: study protocol for a randomized controlled trial

Acronym: The Aortic Valve DECalcification (AVADEC) trial

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1. Abstract

Introduction

Aortic stenosis is a common heart valve disease and due to the growing elderly population the prevalence is increasing. The disease is progressive with increasing calcification of the valve cusps. A few attempts with medical preventive treatment have failed, thus presently the only effective treatment of aortic stenosis is surgery. This study will examine the effect of menaquinone-7 (MK-7) supplementation on progression of aortic valve calcification (AVC). We hypothesize that MK-7 supplementation will slow down the calcification process.

Method and analysis

In this multicenter and double-blinded placebo-controlled study, 400 men aged 65-74 years with severe AVC are randomized (1:1) to treatment with MK-7 (720 µg/day) supplemented by the recommended daily dose of vitamin D (25 µg/day) or placebo treatment (no active treatment) for two years. Exclusion criteria are treatment with vitamin K antagonist or coagulation disorders. To evaluate AVC score, a non-contrast CT-scan is performed at baseline and repeated after 12 and 24 months of follow-up. Primary outcome is difference in AVC score from baseline to follow-up at two years. Intention-to-treat principle is used for all analyses.

Ethics and dissemination

There are no reported adverse effects associated with the use of MK-7. The protocol is approved by the Regional Scientific Ethical Committee for Southern Denmark (S-20170059) and the Data Protection Agency (17/19010). It is conducted in accordance with the Declaration of Helsinki. Positive as well as negative findings will be reported.

Trial registration number

ClinicalTrials.gov Identifier: NCT03243890.

Strengths and limitations of this study

- The study is the first to investigate the effect of menaquinone-7 supplementation on progression of aortic valve calcification.
- Strengths include the stratified randomization, double-blind placebo-controlled design and being a multi-centre trial.
- A clinical relevant dose of Menaquinone-7 supplementation is unknown, and accordingly the chosen dose might be insufficient.
- A confirmatory trial with clinical outcomes is needed, if progression of aortic valve calcification.is decreased by menaquinone-7 supplementation.

2. Introduction

In the ongoing Danish Cardiovascular Screening (DANCAVAS) trial, we are randomizing (1:2) 45,000 Danish men aged 65-74 years to a screening examination comprising a non-contrast CT scan.[1] The purpose is to investigate whether an advanced cardiovascular screening will prevent death and cardiovascular events. In a supplementary PhD study, we have been studying aortic valve calcification (AVC). With prevalence ranging from 2 to 7%, aortic valve stenosis is the most common heart valve disease in the western world,[2;3] and combined with the rapidly growing elderly population it is likely that the prevalence will increase further in the future. Central in the pathogenesis is pro-osteogenic factors entailing active bone formation in the valve cusps and this drive disease progression.[4;5] To halt aortic stenosis progression preventive medical treatments with statins and bisphosphonates have been explored in randomized clinical trials, but with discouraging results. Thus at present medical treatment is not an option.[6]

In DANCAVAS, we have made an interesting observation. Patients on vitamin K antagonist (VKA) treatment had a significant increased AVC score (median 32 versus 11, p=0.004). Adjusting for age, smoking, hypertension and cardiovascular disease, this was confirmed in binominal negative (IRR 1.70, 95%CI: 1.25-2.31) and logistic regression (OR 1.66, 95%CI 1.19-2.30) (unpublished results). Thus, in DANCAVAS, patients on VKA seem to have increased aortic valve calcifications.

Vitamin K and the calcification process

Calcification is a slowly progressive process and caused by an imbalance between the mechanisms that promotes and inhibits the deposition of calcium in the vascular wall, and the vitamin K-dependent proteins play an essential role in this inhibition. The most familiar of the K vitamins are phylloquinone (VK1), as this is essential in activation of several coagulation factors, but menaquinone (MK) is another very important vitamin K species. MK is deemed necessary for γ-carboxylation of proteins involved inhibition of arterial calcification, i.e. matrix-Gla proteins (MGP).[7-10] Without these activated proteins, the balance of cellular calcium uptake and the mineralization process in bone and blood vessels is impaired. Additionally, clinical studies suggest that MK preserves bone structure.[11]

The inhibiting process of the vitamin K-dependent proteins was originally showed by Luo et al. in 1997.[12] In a mice model they described MGP to be an important inhibitor of calcification of arteries. In other animal studies, the inhibition of the vitamin K-dependent proteins by VKA resulted in arterial and soft tissue calcification.[13-16] These observations are in agreement with our findings from the DANCAVAS trial, and other human studies have also shown that long-term use of VKA is associated with both increased coronary- and extra-coronary vascular calcification.[17-20] Furthermore, in Japanese, the use of VKA was associated to exacerbate the risk of degenerative aortic valve disease.[21] Finally, low circulating MGP and an impaired carboxylation at its tissue site of expression is associated with the development and progression of cardiovascular disease.[22]

Since VKA seems to induce vascular calcification, MK intake may be beneficial to reduce these calcifications. No recommendations of MK are available, however we know that the daily intake in the Western world is not sufficient to meet the request for a complete activation of MGP.

Observational studies in healthy elderly have shown an inverse relationship between MK-4 intake and coronary artery calcification (CAC),[23] and VK1 did slow the progression of CAC after 3 years of follow-up.[24] Furthermore, VK1 and MK-7 decreased arterial stiffness and improved elastic properties of the carotid artery.[25;26] Dalmeijer et al. performed a randomized, double blind, placebo controlled trial to investigate the effect of MK-7 supplementation (180 µg/day, 360 µg/day or placebo) and found a dose-dependent decrease of uncarboxylated MGP concentrations.[27] Two subsequent studies in haemodialysis patients found an almost linear dose–response decrease of uncarboxylated MGP without an upper limit, with doses ranging between 360 µg/day and 1080 µg trice weekly.[28;29] In a supplementary study, MK-7 was well tolerated and did not cause a hypercoagulable state.[30] Finally, there is no documented toxicity for VK1 or MK-4, and MK-7, and the WHO has set no upper tolerance level for vitamin K intake.[31]

2.1. Hypothesis

In a randomized setup we test the hypothesis that supplementation with MK-7 (720 µg per day) and vitamin D (25 µg/day) in comparison to placebo will half the progression of further aortic valve calcification in patients with severe valve calcification, but without aortic valve stenosis.

3. Methods

3.1. Trial design

The study is a double-blind, randomized, placebo-controlled study.

3.2. Participants

In DANCAVAS we are performing echocardiography in all participants with an AVC score above the 90% percentile (AVC score above 300).[32] Patients with an AVC score above 300, but without aortic valve stenosis are eligible patients in AVADEC.

Exclusion criteria are:

- Prior heart valve surgery
- Known significant aortic valve disease (peak velocity ≥3.0 m/s)
- History of venous thrombosis including pulmonary embolism
- Coagulation disorders
- VKA use
- Disorders of calcium and phosphate metabolism
- A life-expectancy < 5 years

The study takes place at Odense University Hospital, and the hospitals in Svendborg, Vejle and Silkeborg, Denmark, from 2018 to 2020.

3.3. Intervention

In AVADEC, half of the patients are randomized to supplementation with MK-7 (720 µg/day) including the recommended daily dose of vitamin D (25 µg/day) and the other half to placebo treatment (no active treatment). Treatment of both groups will last for at least 24 months. During

this time participants will visit our research unit five times, at 6-month intervals (Figure 1). To evaluate AVC score, we will perform a non-contrast CT-scan at baseline and after 12 and 24 months of follow-up.

3.4. Outcome

The *primary endpoint* is the change in AVC score from baseline to two years. The natural history of the aortic valve calcification is not adequately understood, and accordingly the changes are analyzed in two prespecified patient subgroups (AVC score 300-599 and ≥600, respectively).

Secondary endpoints are:

- Change in calcifications in the coronaries, carotid, aortic, renal, iliac and femoral arteries by non-contrast CT.
- Change in coronary and carotid plaque composition by contrast CT
- Change in aortic valve area by transthoracic echocardiography.
- Change in bone density as quantitative CT of the columna lumbalis and hip region.
- Change in MGP and osteocalcin with different phosphorylation (p and dp) and carboxylation forms (c and uc).
- Quality of life.

Safety endpoints are:

- Death
- Cardiovascular events (myocardial infarction, coronary revascularization, stroke, heart valve surgery, significant aortic disease (including dissection, ruptur and surgery) and significant peripheral artery disease (including thromboembolisms and surgery))
- Progressive aortic valve disease (more than 50% increase in AVC score)
- Venous thromboembolism including pulmonary embolism
- Bleeding (including intracranial bleeding and hemorrhage associated with a drop in hemoglobin of ≥ 2mmol/l)
- Low-energy or spontaneous fracture
- Cancer, including solid and hematologic
- Significant deterioration in laboratory measurements (calcium, magnesium, albumin, phosphate, alkaline phosphatase, bone specific alkaline phosphatase, Parathyroid Hormone, vitamin D or Prothrombin time-International normalized ratio (PT-INR)).

3.5 Sample size

We are planning a study of a continuous response variable from independent control and experimental subjects with 1 control per experimental subject. The mean annual AVC progression is unknown, but based on data from the DANCAVAS study we estimate the progression to be 32 units in two years. Likewise we estimate the standard deviation to be 59 units. We expect that the treatment will reduce the AVC progression by half, and if this is true (a AVC progression at 16 and 32 among experimental and control subjects, respectively), we will need to study 200 experimental subjects and 200 control subjects to be able to reject the null hypothesis that the population means

of the experimental and control groups are equal with probability (power) 0.8. The Type I error probability associated with this test of this null hypothesis is 0.05.
The sample size is based on two years of treatment, but the Steering Committee may decide to continue the study beyond the planned trial end, if they believe the primary end point may be reached after one additional year of treatment.

3.6 Stratified randomization

Subjects will be randomized 1:1 after stratification for site (Odense University Hospital, Svendborg Hospital, Vejle Hospital or Silkeborg Hospital), and AVC score (300-599 or ≥600). Each site will be provided with sequentially numbered, opaque and sealed envelopes containing randomly generated treatment allocations. Two types of envelopes are provided; 1) AVC 300-599; and 2) AVC ≥600.

3.7 Blinding

The randomization-list is available to the data and safety monitoring board, but patients, nurses, physicians and other data collectors are kept blinded to the allocation during the study. The placebo is matched to the study drug for taste, color, and size.

3.8 Statistical methods

We will use the intention-to-treat principle for all analyses. The primary endpoint (change in AVC score) will be presented as continuous variables. Additional, the changes are analyzed in two pre-specified patient subgroups (AVC score 300-599 and ≥600, respectively). Secondary endpoints include 1) change in calcifications in the coronaries, carotid, aortic, renal, iliac and femoral arteries; 2) change in coronary and carotid plaque composition by contrast CT; 3) change in aortic valve area by transthoracic echocardiography and 4) change in bone-density, -geometry and -microstructure as quantitative CT of the columna lumbalis and hip region. We use an analysis of covariance (ANCOVA) for the primary and for secondary endpoints as well as potential harms and this is supplemented by a repeated measures analysis.

4. Organization

The study is a part of the DANCAVAS trial, and conducted in collaboration with Centre for Individualized Medicine in Arterial Diseases (CIMA). The DANCAVAS secretariat at OUH will identify eligible patients in the DANCAVAS database, and an invitation is send by mail to these patients. If a patient is interested, he is invited to the local site to discuss the trial with a study nurse. If he is willing to participate in the study, informed consent is obtained, and he is randomly assigned to the MK-7 or placebo group. Nurses, radiographers. biomedical technicians and a PhD student are responsible for the treatment and examinations. During the study, only the independent data and safety monitoring board will have access to the complete database including the randomization-list. The data registration is performed via REDCap (Research Electronic Data Capture) with logging and secure storage directly on a server under Odense Patient data Explorative Network (OPEN), Region of Southern Denmark.

The Executive Committee, consisting Professor Jes Lindholt (JL, Department of Cardiothoracic and Vascular Surgery, OUH), MD Niels Erik Frandsen (NEF) and associate professor Axel Diederichsen (AD, Department of Cardiology, OUH) conceived and designed the study, and will handle the decisions regarding the overall organization including administration, budget and use of the database.
The Steering Committee will consist of the members of the executive committee, and Jordi Dahl (JD, Department of Cardiology, OUH), Professor Lars Melholt Rasmussen (LMR, Department of Clinical Biochemistry and Pharmacology) both OUH, and two from each screening site. All practical issues concerning the treatment and data sampling will be handled by the steering committee.
The data and safety monitoring board (DSMB) consists of the following experts: Professor of Cardiology Hans Mickley (HM, OUH), Professor of Cardiology and Clinical Epidemiology Christian Torp-Pedersen (Aalborg University) and Professor of Cardiology Lars Køber (Rigshospitalet), who all have large have experience with clinical randomized trials.

5. Publication

Project results reporting the primary endpoint will be published in peer reviewed international journals. The order of the authors will be PhD student (to be appointed), JL, JD, LF, two from each screening site, LMR, NEF and AD. Positive as well as negative findings will be reported.

6. Feasibility

By December 2017 more than 10000 participants have been included in DANCAVAS, and 800 of these are eligible to participate in AVADEC. Thus we are able to identify enough participants. AD and JL are PI's of the main study, DANCAVAS. In addition, several experts assist with AVADEC: JD is an expert in aortic stenosis, LMR is an expert in biochemistry, while PhD Lars Folkestad (LF) has undertaken several studies in bone-density, -geometry and -microstructure. In addition, local cardiologist from Vejle, Svendborg and Silkeborg will be responsible for securing local practical feasibility of the project at the specific screening sites.

7. Safety and Ethics

Pure natural MK-7 is used in the study. A daily dose at 720 µg MK-7 has not been examined on patients with aortic stenosis, but in a Belgian dose-finding study using 360, 720 or 1080 µg of MK-7 thrice weekly for 8 weeks in chronic haemodialysis patients no severe adverse effects were observed.[29] Presently the Belgian group are performing a randomized trial exploring the efficacy of 2000 µg MK-7 thrice weekly.[33] MK-7 is well tolerated and does not cause a hypercoagulable state.[30] There are no reported adverse effects associated with the use of MK-7.[31]

Each patient has three CT scans during the study. Epidemiological studies do suggest that radiation exposure is associated with a slightly increased risk of cancer. The best studied cohort is the Japanese atomic bomb survivor cohort. In a group exposed to a mean radiation dose of 29 mSv, an

excess of solid cancers – corresponding to an excess relative risk of 2% – were observed.[34] No large studies involving medically exposed adult cohorts are available, but a linear no-threshold model has been considered. Thus, there may be no minimal radiation dose for an increased cancer risk, and the risk increases linearly with the radiation dose. The average dose of one non-contrast CT scan is 3 mSv. Two additionally contrast CT scans are performed (baseline and 24 months) with an average dose of 3 mSv each, thus at average the participants in AVADEC will receive 15 mSv. For comparison, the annual background radiation dose in Denmark is 3 mSv, and the average annual limit for radiation workers is 20 mSv.[35]

An independent DSMB is established to perform ongoing safety surveillance. None of the DSMB-members are directly or indirectly involved in the coordination, execution or analysis of the study. The following is assessed: 1) death, myocardial infarction, coronary revascularization, stroke, heart valve surgery and venous thromboembolism, 2) progressive aortic valve disease, and 3) laboratory measurements (Calcium, Magnesium, Albumin, Phosphate and alkaline phosphatase, bone specific alkaline phosphatase, Parathyroid Hormone and vitamin D or Prothrombin time-International normalized ratio (PT-INR)). If there is are a reason for concern, the DSMB can advise to interrupt the study for further analysis, and the study can be terminated prematurely if the number of severe adverse events is significantly higher in the treatment group versus the placebo group. This will be discussed in a meeting with the investigators and DSMB. The investigator will inform the subjects in case of interruption or termination of the study.

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 or in case of demonstrable poor adherence to the study medication. This is assessed by interview and pill-count. If subjects are required to take VKA during the course of the study they will be withdrawn.

The protocol is approved by the Regional Scientific Ethical Committee for Southern Denmark (S-20170059) and the Data Protection Agency (17/19010). It is conducted in accordance with the Declaration of Helsinki. According to Danish legislation vitamin K is a dietary supplement, and accordingly license from the Danish Medicines Agency is not needed. Written informed consent is obtained from each participant. The study is registered at clinicaltrials.gov: NCT03243890.

8. Discussion

Aortic stenosis is a common heart valve disease and due to the growing elderly population the prevalence is increasing. The disease is progressive with increasing calcification of the valve cusps. A few attempts with medical preventive treatment have failed, thus presently the only effective treatment of aortic stenosis is surgery. This study will examine the effect of MK-7 supplementation on progression of AVC in a randomized, placebo-controlled study. We hypothesize that MK-7 supplementation will slow down the progression of valves calcification. If positive effects are shown a new treatment options may be available to prevent progression of aortic valve calcification. The result of this study will be expected at in 2021.

9. Applied tests during the study

9.1. Medical interview

At baseline, all relevant data are supplied from the DANCAVAS trial (e.g. AVC score, medical history and lifestyle factors).[1] At every visit, an interview is conducted and evaluating the following: incident cardiovascular disease, dyspnea, chest pain and quality of life (EurQol 5D).

9.2. Laboratory Assessment

Blood samples are obtained at every visit. Routine parameters include:

- Circulating MGP species with different phosphorylation (p and dp) and carboxylation forms (c and uc) are measured using a sandwich enzyme-linked immunosorbent assay (ELISA) based on monoclonal antibodies.
- Creatinine (eGFR), Natrium, Potassium, Calcium, Magnesium, Albumin, Phosphate, alkaline phosphatase, bone specific alkaline phosphatase, Parathyroid Hormone, Vitamin D and INR.

As a part of the study, a biobank at baseline and after 24 months will be organized. 40 mL of blood form each of the participants are centrifuged, labeled, and stored at -80°C until serial testing.

9.3. Multi-Slice Computed Tomography Scans

CT scans will be performed using a high end CT- scanner like dual-source CT-scanner (Somatom Definition Flash, Siemens Medical Solutions, Forchheim, Germany). To assess the AVC scores the following CT settings are used: Gantry rotation time 0.28 s, 3.0 mm collimation, acquisition 128 x 0.6 mm, 120 kV tube voltage, 90 mAs tube current, and a prospectively electrocardiographic (ECG) -triggered scan (gating at 65%-75% of the R-R interval if the heart rate were <75 or at 250-400ms after the QRS-complex if heart rate were >75). Calculation of the AVC scores is performed off-line by summing-up all spots of calcifications in the aortic valve area. AVC is defined as calcification below the ostia of the coronaries in the aortic sinus Valsalva, within the valve leaflet, or in the aortic annulus.[36] The CAC score is assessed as previously described.[32] To assess the calcifications in the carotid, aortic, renal, iliac and femoral arteries a CT scanning proximal from the mandibular bone and distally to the proximal third of the femur are performed with the following settings: Spiral scan with a pitch of 3.2 (Flash), 100 kV tube voltage, 90 mAs, collimation of 128 x 0.6mm, Safire 3 and slice thickness 5 mm. The calcifications scores are measured using the Agatston method.

To examine vessel plaques in the coronaries and carotid arteries, an ordinary contrast CT will be performed. The scanning protocol depends on the local CT scanner. Typically 80–100 mL of contrast agent are injected into an antecubital vein at a rate of 6.0 mL/s followed by 60 mL intravenous saline (6.0 mL/s) using a dual-head power injector. A prospectively gated high pitch spiral “flash” protocol will be used in patients with a stable heart rate <60 beats per minute (bpm). In patients with a stable heart rate between 60 and 90 bpm, intravenously β-blocker is typically injected until the heart rate is appropriate, and a prospectively gated axial “adaptive sequence” protocol is used. In patients with a heart rate > 90 bpm or in case of an irregular heart rhythm, a retrospectively gated “helical” protocol with dose modulation will be used. Data acquisition parameters are 2*128*0.6 mm slice collimation, a gantry rotation time of 280 ms and a tube voltage of 100 or 120 kV depending on patients’ height and weight. The coronary artery tree will be

analyzed for the presence and severity of CAD, according to the classification of the American Heart Association 16-segment model. Coronary plaques are defined as visible structures within or adjacent to the coronary artery lumen, which can be clearly distinguished from the vessel lumen and the surrounding pericardial tissue. Quantification of coronary plaque components is done via semi-automated analysis. Scans are analyzed an experienced cardiologist.

9.4 Echocardiographic measurements

A comprehensive transthoracic echocardiography is performed at baseline and hereafter annually. Left ventricle (LV) volume and ejection fraction (EF) are estimated. LV longitudinal function is assessed using global strain analysis. LV remodelling is assessed by relative wall thickness and LV mass using the Devereaux formula. LV filling pressure is estimated from assessment of mitral inflow and assessment of diastolic motion of the mitral plane using tissue Doppler imaging. Left atrial size is assessed using biplane planimetry, and longitudinal left atrial strain is estimated using 2D speckle tracking. Aortic valve area is estimated by quantitative Doppler ultrasound using the continuity equation. LV outflow tract time-velocity integral is measures with pulsed-wave Doppler by placing the sample volume just below the region of flow convergence. Peak flow velocity across the valve is determined in the window with the highest velocity. AS severity is graded according to current guidelines (*secondary endpoints*).[37]

9.5. Bone mineral-density, -geometry and -microstructure

Using the images obtained from the multi-slice computed tomography the lumbar spine and hip can be evaluated. The currently available software from Mindways® allows for volumetric bone mineral-density of the trabecular compartment in the spine, thus making it possible to calculate T- and Z-scores for volumetric bone mineral density in the spine. At the hip both cortical, trabecular and total hip volumetric bone mineral density can be evaluated for the femoral neck, the trochanter region and femoral shaft. Again both T- and Z-scores can be calculated. The software also offers evaluation of the total bone area at the spine, and hip.

10. Figure legends

Figure 1.
Timeline and applied tests, for details please see "Applied tests during the study".

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12. Footnotes

12.1. Contributors

AD, JL, NEF and LMR conceived and designed the study. AD wrote the study protocol and is the principal investigator while JL, NEF and LMR are coinvestigators. MF is the clinical research fellow responsible for the running of the clinical trial. JD and JEM are responsible for the echocardiography protocol. Grazina Urbonaviciene (Silkeborg), Søren Warberg Becker (Silkeborg), Jess Lambrechtsen (Svendborg), Kenneth Egstrup (Svendborg), Flemming Hald (Vejle) and Martin Busk (Vejle), are site specific coinvestigators. LF is involved in the bone-related substudy. HM is representative for the DSMB. All authors have contributed to the revision of the manuscript.

12.2. Funding statement

The trial is supported by the Danish Heart Foundation [grant number 17-R116-A7569-22071], Region Syddanmarks Research council [grant number 17/15638] and the Novo Nordisk Foundation [grant number NNF17OC0029076].

The study-tablets, including placebo, are provided for free of charge by Kappa Bioscience A/S, Silurveien 2B, 0380 Oslo and Orkla Care, Industrigrenen 10, 2635 Ishøj. The companies are not involved in the execution of the study or analysis of the data.

12.3. Competing interests statement

None declared.

12.4. Ethics approval

The protocol is approved by the Regional Scientific Ethical Committee for Southern Denmark.

12.5. Data sharing statement:

Positive as well as negative findings will be reported via conference presentations and peer-reviewed publications. All the data will be available upon request.

Month	Baseline	3	6	9	12	15	18	21	24	(27)	(30)
Info and consent	X										
Interview	X		X		X		X		X		(X)
Web based survey		X		X		X		X		(X)	
Biochemical measurements	X		X		X		X		X		(X)
Non-contrast CT	X				X				X		(X)
Contrast CT	X								X		
Echocardiography	X				X				X		(X)

Timeline and applied tests, for details please see "Applied tests during the study".

180x34mm (96 x 96 DPI)

SPIRIT 2013 Checklist: Recommended items to address in a clinical trial protocol and related documents*

Section/item	Item No	Description	Addressed on page number
Administrative information			
Title	1	Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym	1
Trial registration	2a	Trial identifier and registry name. If not yet registered, name of intended registry	2
	2b	All items from the World Health Organization Trial Registration Data Set	
Protocol version	3	Date and version identifier	1
Funding	4	Sources and types of financial, material, and other support	15
Roles and responsibilities	5a	Names, affiliations, and roles of protocol contributors	15
	5b	Name and contact information for the trial sponsor	15
	5c	Role of study sponsor and funders, if any, in study design; collection, management, analysis, and interpretation of data; writing of the report; and the decision to submit the report for publication, including whether they will have ultimate authority over any of these activities	15
	5d	Composition, roles, and responsibilities of the coordinating centre, steering committee, endpoint adjudication committee, data management team, and other individuals or groups overseeing the trial, if applicable (see Item 21a for data monitoring committee)	7, 8, __

Introduction			
Background and rationale	6a	Description of research question and justification for undertaking the trial, including summary of relevant studies (published and unpublished) examining benefits and harms for each intervention	4,5
	6b	Explanation for choice of comparators	
Objectives	7	Specific objectives or hypotheses	5
Trial design	8	Description of trial design including type of trial (eg, parallel group, crossover, factorial, single group), allocation ratio, and framework (eg, superiority, equivalence, noninferiority, exploratory)	5
Methods: Participants, interventions, and outcomes			
Study setting	9	Description of study settings (eg, community clinic, academic hospital) and list of countries where data will be collected. Reference to where list of study sites can be obtained	5
Eligibility criteria	10	Inclusion and exclusion criteria for participants. If applicable, eligibility criteria for study centres and individuals who will perform the interventions (eg, surgeons, psychotherapists)	5
Interventions	11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered	5,6
	11b	Criteria for discontinuing or modifying allocated interventions for a given trial participant (eg, drug dose change in response to harms, participant request, or improving/worsening disease)	6
	11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return, laboratory tests)	6
	11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial	
Outcomes	12	Primary, secondary, and other outcomes, including the specific measurement variable (eg, systolic blood pressure), analysis metric (eg, change from baseline, final value, time to event), method of aggregation (eg, median, proportion), and time point for each outcome. Explanation of the clinical relevance of chosen efficacy and harm outcomes is strongly recommended	6
Participant timeline	13	Time schedule of enrolment, interventions (including any run-ins and washouts), assessments, and visits for participants. A schematic diagram is highly recommended (see Figure)	6

Sample size	14	Estimated number of participants needed to achieve study objectives and how it was determined, including clinical and statistical assumptions supporting any sample size calculations	___7_____
Recruitment	15	Strategies for achieving adequate participant enrolment to reach target sample size	___8_____
Methods: Assignment of interventions (for controlled trials)			
Allocation:			
Sequence generation	16a	Method of generating the allocation sequence (eg, computer-generated random numbers), and list of any factors for stratification. To reduce predictability of a random sequence, details of any planned restriction (eg, blocking) should be provided in a separate document that is unavailable to those who enrol participants or assign interventions	___7_____
Allocation concealment mechanism	16b	Mechanism of implementing the allocation sequence (eg, central telephone; sequentially numbered, opaque, sealed envelopes), describing any steps to conceal the sequence until interventions are assigned	___7_____
Implementation	16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions	___7_____
Blinding (masking)	17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how	___7_____
	17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial	_____
Methods: Data collection, management, and analysis			
Data collection methods	18a	Plans for assessment and collection of outcome, baseline, and other trial data, including any related processes to promote data quality (eg, duplicate measurements, training of assessors) and a description of study instruments (eg, questionnaires, laboratory tests) along with their reliability and validity, if known. Reference to where data collection forms can be found, if not in the protocol	___7,8_____
	18b	Plans to promote participant retention and complete follow-up, including list of any outcome data to be collected for participants who discontinue or deviate from intervention protocols	_____

Data management	19	Plans for data entry, coding, security, and storage, including any related processes to promote data quality (eg, double data entry; range checks for data values). Reference to where details of data management procedures can be found, if not in the protocol	___8_____
Statistical methods	20a	Statistical methods for analysing primary and secondary outcomes. Reference to where other details of the statistical analysis plan can be found, if not in the protocol	___7_____
	20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)	___7_____
	20c	Definition of analysis population relating to protocol non-adherence (eg, as randomised analysis), and any statistical methods to handle missing data (eg, multiple imputation)	___7_____
Methods: Monitoring			
Data monitoring	21a	Composition of data monitoring committee (DMC); summary of its role and reporting structure; statement of whether it is independent from the sponsor and competing interests; and reference to where further details about its charter can be found, if not in the protocol. Alternatively, an explanation of why a DMC is not needed	___8, 9_____
	21b	Description of any interim analyses and stopping guidelines, including who will have access to these interim results and make the final decision to terminate the trial	___7,9_____
Harms	22	Plans for collecting, assessing, reporting, and managing solicited and spontaneously reported adverse events and other unintended effects of trial interventions or trial conduct	___6, 8, 9____
Auditing	23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor	_____
Ethics and dissemination			
Research ethics approval	24	Plans for seeking research ethics committee/institutional review board (REC/IRB) approval	___9_____
Protocol amendments	25	Plans for communicating important protocol modifications (eg, changes to eligibility criteria, outcomes, analyses) to relevant parties (eg, investigators, REC/IRBs, trial participants, trial registries, journals, regulators)	_____

Consent or assent	26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32)	___7,8_____
	26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable	_____
Confidentiality	27	How personal information about potential and enrolled participants will be collected, shared, and maintained in order to protect confidentiality before, during, and after the trial	_____
Declaration of interests	28	Financial and other competing interests for principal investigators for the overall trial and each study site	___15_____
Access to data	29	Statement of who will have access to the final trial dataset, and disclosure of contractual agreements that limit such access for investigators	___8_____
Ancillary and post-trial care	30	Provisions, if any, for ancillary and post-trial care, and for compensation to those who suffer harm from trial participation	_____
Dissemination policy	31a	Plans for investigators and sponsor to communicate trial results to participants, healthcare professionals, the public, and other relevant groups (eg, via publication, reporting in results databases, or other data sharing arrangements), including any publication restrictions	___8_____
	31b	Authorship eligibility guidelines and any intended use of professional writers	___8_____
	31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code	_____
Appendices			
Informed consent materials	32	Model consent form and other related documentation given to participants and authorised surrogates	_____
Biological specimens	33	Plans for collection, laboratory evaluation, and storage of biological specimens for genetic or molecular analysis in the current trial and for future use in ancillary studies, if applicable	___10_____

*It is strongly recommended that this checklist be read in conjunction with the SPIRIT 2013 Explanation & Elaboration for important clarification on the items. Amendments to the protocol should be tracked and dated. The SPIRIT checklist is copyrighted by the SPIRIT Group under the Creative Commons "Attribution-NonCommercial-NoDerivs 3.0 Unported" license.

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The effects of Menaquinone-7 supplementation in patients with aortic valves calcification: study protocol for a randomized controlled trial

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Secondary Subject Heading:	Pharmacology and therapeutics, Radiology and imaging, Diabetes and endocrinology
Keywords:	menaquinone-7, aortic valve calcification, Valvular heart disease < CARDIOLOGY, Calcium & bone < DIABETES & ENDOCRINOLOGY, Cardiovascular imaging < RADIOLOGY & IMAGING, randomized clinical trial

The effects of Menaquinone-7 supplementation in patients with aortic valves calcification: study protocol for a randomized controlled trial

Acronym: The Aortic Valve DECalcification (AVADEC) trial

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1. Abstract

Introduction

Aortic stenosis is a common heart valve disease and due to the growing elderly population the prevalence is increasing. The disease is progressive with increasing calcification of the valve cusps. A few attempts with medical preventive treatment have failed, thus presently the only effective treatment of aortic stenosis is surgery. This study will examine the effect of menaquinone-7 (MK-7) supplementation on progression of aortic valve calcification (AVC). We hypothesize that MK-7 supplementation will slow down the calcification process.

Method and analysis

In this multicenter and double-blinded placebo-controlled study, 400 men aged 65-74 years with substantial AVC are randomized (1:1) to treatment with MK-7 (720 µg/day) supplemented by the recommended daily dose of vitamin D (25 µg/day) or placebo treatment (no active treatment) for two years. Exclusion criteria are treatment with vitamin K antagonist or coagulation disorders. To evaluate AVC score, a non-contrast CT-scan is performed at baseline and repeated after 12 and 24 months of follow-up. Primary outcome is difference in AVC score from baseline to follow-up at two years. Intention-to-treat principle is used for all analyses.

Ethics and dissemination

There are no reported adverse effects associated with the use of MK-7. The protocol is approved by the Regional Scientific Ethical Committee for Southern Denmark (S-20170059) and the Data Protection Agency (17/19010). It is conducted in accordance with the Declaration of Helsinki. Positive as well as negative findings will be reported.

Trial registration number

ClinicalTrials.gov Identifier: NCT03243890.

Strengths and limitations of this study

- The study is the first to investigate the effect of menaquinone-7 supplementation on progression of aortic valve calcification.
- Strengths include the stratified randomization, double-blind placebo-controlled design and being a multi-center trial.
- A clinical relevant dose of Menaquinone-7 supplementation is unknown, and accordingly the chosen dose might be insufficient.
- A confirmatory trial with clinical outcomes is needed, if progression of aortic valve calcification.is decreased by menaquinone-7 supplementation.

2. Introduction

In the ongoing Danish Cardiovascular Screening (DANCAVAS) trial, we are randomizing (1:2) 45,000 Danish men aged 65-74 years to a screening examination comprising a non-contrast CT scan.[1] The purpose is to investigate whether an advanced cardiovascular screening will prevent death and cardiovascular events. In a supplementary PhD study, we have been studying aortic valve calcification (AVC). With prevalence ranging from 2 to 7%, aortic valve stenosis is the most common heart valve disease in the western world,[2;3] and combined with the rapidly growing elderly population it is likely that the prevalence will increase further in the future. Central in the pathogenesis is pro-osteogenic factors entailing active bone formation in the valve cusps and this drive disease progression.[4;5] To halt aortic stenosis progression preventive medical treatments with statins and bisphosphonates have been explored in randomized clinical trials, but with discouraging results. Thus at present medical treatment is not an option.[6]

In DANCAVAS, we have made an interesting observation. Patients on vitamin K antagonist (VKA) treatment had a significant increased AVC score (median 32 versus 11, p=0.004). Adjusting for age, smoking, hypertension and cardiovascular disease, this was confirmed in binominal negative (IRR 1.70, 95% CI: 1.25-2.31) and logistic regression (OR 1.66, 95% CI 1.19-2.30) (unpublished results). Thus, in DANCAVAS, patients on VKA seem to have increased aortic valve calcifications.

Vitamin K and the calcification process

Calcification is a slowly progressive process and caused by an imbalance between the mechanisms that promotes and inhibits the deposition of calcium in the vascular wall, and the vitamin K-dependent proteins play an essential role in this inhibition. The most familiar of the K vitamins are phylloquinone (VK1), as this is essential in activation of several coagulation factors, but menaquinone (MK) is another very important vitamin K species. MK is deemed necessary for γ-carboxylation of proteins involved inhibition of arterial calcification, i.e. matrix-Gla proteins (MGP).[7-10] Without these activated proteins, the balance of cellular calcium uptake and the mineralization process in bone and blood vessels is impaired. Additionally, clinical studies suggest that MK preserves bone structure.[11]

The inhibiting process of the vitamin K-dependent proteins was originally showed by Luo et al. in 1997.[12] In a mice model they described MGP to be an important inhibitor of calcification of arteries. In other animal studies, the inhibition of the vitamin K-dependent proteins by VKA resulted in arterial and soft tissue calcification.[13-16] These observations are in agreement with our findings from the DANCAVAS trial, and other human studies have also shown that long-term use of VKA is associated with both increased coronary- and extra-coronary vascular calcification.[17-20] Furthermore, in Japanese, the use of VKA was associated to exacerbate the risk of degenerative aortic valve disease.[21] Finally, low circulating MGP and an impaired carboxylation at its tissue site of expression is associated with the development and progression of cardiovascular disease.[22]

Since VKA seems to induce vascular calcification, MK intake may be beneficial to reduce these calcifications. No recommendations of MK are available; however, we know that the daily intake in the Western world is not sufficient to meet the request for a complete activation of MGP.

Observational studies in healthy elderly have shown an inverse relationship between MK-4 intake and coronary artery calcification (CAC),[23] and VK1 did slow the progression of CAC after 3 years of follow-up.[24] Furthermore, VK1 and MK-7 decreased arterial stiffness and improved elastic properties of the carotid artery.[25;26] Dalmeijer et al. performed a randomized, double blind, placebo controlled trial to investigate the effect of MK-7 supplementation (180 µg/day, 360 µg/day or placebo) and found a dose-dependent decrease of uncarboxylated MGP concentrations.[27] Two subsequent studies in haemodialysis patients found an almost linear dose–response decrease of uncarboxylated MGP without an upper limit, with doses ranging between 360 µg/day and 1080 µg trice weekly.[28;29] In a supplementary study, MK-7 was well tolerated and did not cause a hypercoagulable state.[30] Finally, there is no documented toxicity for VK1 or MK-4, and MK-7, and the WHO has set no upper tolerance level for vitamin K intake.[31]

2.1. Hypothesis

In a randomized setup we test the hypothesis that supplementation with MK-7 (720 µg per day) and vitamin D (25 µg/day) in comparison to placebo will half the progression of further aortic valve calcification in patients with substantial valve calcification, but without aortic valve stenosis.

3. Methods

3.1. Trial design

The study is a double-blind, randomized, placebo-controlled study.

3.2. Participants

In DANCAVAS we are performing echocardiography in all participants with an AVC score above the 90% percentile (AVC score above 300).[32] Patients with an AVC score above 300, but without aortic valve stenosis are eligible patients in AVADEC.

Exclusion criteria are:

- Prior heart valve surgery
- Known significant aortic valve disease (peak velocity ≥3.0 m/s)
- History of venous thrombosis including pulmonary embolism
- Coagulation disorders
- VKA use
- Disorders of calcium and phosphate metabolism
- A life-expectancy < 5 years

The study takes place at Odense University Hospital, and the hospitals in Svendborg, Vejle and Silkeborg, Denmark, from 2018 to 2020.

3.3. Intervention

In AVADEC, half of the patients are randomized to supplementation with MK-7 (720 µg/day) including the recommended daily dose of vitamin D (25 µg/day) and the other half to placebo treatment (no active treatment). Treatment of both groups will last for at least 24 months. During

this time participants will visit our research unit five times, at 6-month intervals (Figure 1). To evaluate AVC score, we will perform a non-contrast CT-scan at baseline and after 12 and 24 months of follow-up.

3.4. Outcome

The *primary endpoint* is the change in AVC score from baseline to two years. The natural history of the aortic valve calcification is not adequately understood, and accordingly the changes are analyzed in two pre-specified patient subgroups (AVC score 300-599 and ≥600, respectively).

Secondary endpoints are:

- Change in calcifications in the coronaries, carotid, aortic, renal, iliac and femoral arteries by non-contrast CT.
- Change in coronary and carotid plaque composition by contrast CT
- Change in aortic valve area by transthoracic echocardiography.
- Change in bone density as quantitative CT of the columna lumbalis and hip region.
- Change in MGP and osteocalcin with different phosphorylation (p and dp) and carboxylation forms (c and uc).
- Quality of life.

Safety endpoints are:

- Death
- Cardiovascular events (myocardial infarction, coronary revascularization, stroke, heart valve surgery, significant aortic disease (including dissection, ruptur and surgery) and significant peripheral artery disease (including thromboembolisms and surgery))
- Progressive aortic valve disease (more than 50% increase in AVC score)
- Venous thromboembolism including pulmonary embolism
- Bleeding (including intracranial bleeding and hemorrhage associated with a drop in hemoglobin of ≥ 2mmol/l)
- Low-energy or spontaneous fracture
- Cancer, including solid and hematologic
- Significant deterioration in laboratory measurements (calcium, magnesium, albumin, phosphate, alkaline phosphatase, bone specific alkaline phosphatase, Parathyroid Hormone, vitamin D or Prothrombin time-International normalized ratio (PT-INR)).

3.5 Sample size

We are planning a study of a continuous response variable from independent control and experimental subjects with 1 control per experimental subject. The mean annual AVC progression is unknown, but based on data from the DANCAVAS study we estimate the progression to be 100 units in two years with a joint standard deviation 67 units. We expect that the treatment will reduce the AVC progression by 20% (i.e. to 80), leading to the inclusion of 177 experimental subjects and 177 control subjects to be able to reject the null hypothesis that the population means of the experimental and control groups are equal with probability (power) 0.8. The Type I error

probability associated with this test of this null hypothesis is 0.05. Accordingly, 354 subjects are needed, but in order to account for drop-out 400 patients will be included.
Interim analysis: The sample size is based on two years of treatment. One member of the The data and safety monitoring board (HM) and a statistician (OG) will evaluate the available primary endpoint one-year data of approximately 100 patients around 01 July 2019 in order to assess whether the treatment period should be prolonged by six months. The number of patients to be included in this study is unaffected by the decision to prolong treatment by six months or not.

3.6 Stratified randomization

Subjects will be randomized 1:1 after stratification for site (Odense University Hospital, Svendborg Hospital, Vejle Hospital or Silkeborg Hospital), and AVC score (300-599 or ≥600). Each site will be provided with sequentially numbered, opaque and sealed envelopes containing randomly generated treatment allocations. Two types of envelopes are provided; 1) AVC 300-599; and 2) AVC ≥600.

3.7 Blinding

The randomization-list is available to the data and safety monitoring board, but patients, nurses, physicians and other data collectors are kept blinded to the allocation during the study. The placebo is matched to the study drug for taste, color, and size.

3.8 Statistical methods

We will use the intention-to-treat principle for all analyses. The primary endpoint (change in AVC score) will be presented as continuous variables. Additionally, the changes are analyzed in two pre-specified patient subgroups (AVC score 300-599 and ≥600, respectively). Primary hypothesis testing will be done hierarchically to maintain a closed testing procedure: only if the overall treatment effect is statistically significant, testing in AVC strata will be performed with confirmatory intent, otherwise solely for explorative reasons. Secondary endpoints include 1) change in calcifications in the coronaries, carotid, aortic, renal, iliac and femoral arteries; 2) change in coronary and carotid plaque composition by contrast CT; 3) change in aortic valve area by transthoracic echocardiography and 4) change in bone-density, -geometry and -microstructure as quantitative CT of the columna lumbalis and hip region.
We use general linear models (employing group, time point, and group x time point interaction) for the primary and for secondary endpoints as well as potential harms. Missing data will be treated as such; supplementary sensitivity analyses making use of imputed values under the missing at random assumption will be conducted for the primary analysis if more than 5% of expected data points will be missing.

4. Organization

The study is a part of the DANCAVAS trial, and conducted in collaboration with Centre for Individualized Medicine in Arterial Diseases (CIMA). The DANCAVAS secretariat at OUH will identify eligible patients in the DANCAVAS database, and an invitation is send by mail to these patients. If a patient is interested, he is invited to the local site to discuss the trial with a study nurse.

If he is willing to participate in the study, informed consent is obtained, and he is randomly assigned to the MK-7 or placebo group. Nurses, radiographers. biomedical technicians and a PhD student are responsible for the treatment and examinations. During the study, only the independent data and safety monitoring board will have access to the complete database including the randomization-list. The data registration is performed via REDCap (Research Electronic Data Capture) with logging and secure storage directly on a server under Odense Patient data Explorative Network (OPEN), Region of Southern Denmark.

The Executive Committee, consisting Professor Jes Lindholt (JL, Department of Cardiothoracic and Vascular Surgery, OUH), MD Niels Erik Frandsen (NEF) and associate professor Axel Diederichsen (AD, Department of Cardiology, OUH) conceived and designed the study, and will handle the decisions regarding the overall organization including administration, budget and use of the database.

The Steering Committee will consist of the members of the executive committee, and Jordi Dahl (JD, Department of Cardiology, OUH), Professor Lars Melholt Rasmussen (LMR, Department of Clinical Biochemistry and Pharmacology) both OUH, and two from each screening site. All practical issues concerning the treatment and data sampling will be handled by the steering committee.

The data and safety monitoring board (DSMB) consists of the following experts: Professor of Cardiology Hans Mickley (HM, OUH), Professor of Cardiology and Clinical Epidemiology Christian Torp-Pedersen (Aalborg University) and Professor of Cardiology Lars Køber (Rigshospitalet), who all have large have experience with clinical randomized trials.

5. Publication

Project results reporting the primary endpoint will be published in peer reviewed international journals. The order of the authors will be PhD student (to be appointed), JL, JD, LF, two from each screening site, LMR, NEF and AD. Positive as well as negative findings will be reported.

6. Feasibility

By December 2017 more than 10000 participants have been included in DANCAVAS, and 800 of these are eligible to participate in AVADEC. Thus we are able to identify enough participants. AD and JL are PI's of the main study, DANCAVAS. In addition, several experts assist with AVADEC: JD is an expert in aortic stenosis, LMR is an expert in biochemistry, while PhD Lars Folkestad (LF) has undertaken several studies in bone-density, -geometry and -microstructure. In addition, local cardiologist from Vejle, Svendborg and Silkeborg will be responsible for securing local practical feasibility of the project at the specific screening sites.

7. Safety and Ethics

Pure natural MK-7 is used in the study. A daily dose at 720 µg MK-7 has not been examined on patients with aortic stenosis, but in a Belgian dose-finding study using 360, 720 or 1080 µg of MK-7 thrice weekly for 8 weeks in chronic haemodialysis patients no severe adverse effects were

observed.[29] Presently the Belgian group are performing a randomized trial exploring the efficacy of 2000 µg MK-7 thrice weekly.[33] MK-7 is well tolerated and does not cause a hypercoagulable state.[30] There are no reported adverse effects associated with the use of MK-7.[31]

Each patient has three CT scans during the study. Epidemiological studies do suggest that radiation exposure is associated with a slightly increased risk of cancer. The best studied cohort is the Japanese atomic bomb survivor cohort. In a group exposed to a mean radiation dose of 29 mSv, an excess of solid cancers – corresponding to an excess relative risk of 2% – were observed.[34] No large studies involving medically exposed adult cohorts are available, but a linear no-threshold model has been considered. Thus, there may be no minimal radiation dose for an increased cancer risk, and the risk increases linearly with the radiation dose. The average dose of one non-contrast CT scan is 3 mSv. Two additionally contrast CT scans are performed (baseline and 24 months) with an average dose of 3 mSv each, thus at average the participants in AVADEC will receive 15 mSv. For comparison, the annual background radiation dose in Denmark is 3 mSv, and the average annual limit for radiation workers is 20 mSv.[35]

An independent DSMB is established to perform ongoing safety surveillance. None of the DSMB-members are directly or indirectly involved in the coordination, execution or analysis of the study. The following is assessed: 1) death, myocardial infarction, coronary revascularization, stroke, heart valve surgery and venous thromboembolism, 2) progressive aortic valve disease, and 3) laboratory measurements (Calcium, Magnesium, Albumin, Phosphate and alkaline phosphatase, bone specific alkaline phosphatase, Parathyroid Hormone and vitamin D or Prothrombin time-International normalized ratio (PT-INR)). If there is are a reason for concern, the DSMB can advise to interrupt the study for further analysis, and the study can be terminated prematurely if the number of severe adverse events is significantly higher in the treatment group versus the placebo group. This will be discussed in a meeting with the investigators and DSMB. The investigator will inform the subjects in case of interruption or termination of the study.

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 or in case of demonstrable poor adherence to the study medication. This is assessed by interview and pill-count. If subjects are required to take VKA during the course of the study they will be withdrawn.

The protocol is approved by the Regional Scientific Ethical Committee for Southern Denmark (S-20170059) and the Data Protection Agency (17/19010). It is conducted in accordance with the Declaration of Helsinki. According to Danish legislation vitamin K is a dietary supplement, and accordingly license from the Danish Medicines Agency is not needed. Written informed consent is obtained from each participant. The study is registered at clinicaltrials.gov: NCT03243890.

8. Discussion

Aortic stenosis is a common heart valve disease and due to the growing elderly population the prevalence is increasing. The disease is progressive with increasing calcification of the valve cusps.

A few attempts with medical preventive treatment have failed, thus presently the only effective treatment of aortic stenosis is surgery. This study will examine the effect of MK-7 supplementation on progression of AVC in a randomized, placebo-controlled study. We hypothesize that MK-7 supplementation will slow down the progression of valves calcification. If positive effects are shown a new treatment options may be available to prevent progression of aortic valve calcification. The result of this study will be expected at in 2021.

9. Applied tests during the study

9.1. Medical interview

At baseline, all relevant data are supplied from the DANCAVAS trial (e.g. AVC score, medical history and lifestyle factors).[1] At every visit, an interview is conducted and evaluating the following: incident cardiovascular disease, dyspnea, chest pain and quality of life (EurQol 5D).

9.2. Laboratory Assessment

Blood samples are obtained at every visit. Routine parameters include:

- Circulating MGP species with different phosphorylation (p and dp) and carboxylation forms (c and uc) are measured using a sandwich enzyme-linked immunosorbent assay (ELISA) based on monoclonal antibodies.
- Creatinine (eGFR), Natrium, Potassium, Calcium, Magnesium, Albumin, Phosphate, alkaline phosphatase, bone specific alkaline phosphatase, Parathyroid Hormone, Vitamin D and INR.

As a part of the study, a biobank at baseline and after 24 months will be organized. 40 mL of blood form each of the participants are centrifuged, labeled, and stored at -80°C until serial testing.

9.3. Multi-Slice Computed Tomography Scans

CT scans will be performed using a high end CT- scanner like dual-source CT-scanner (Somatom Definition Flash, Siemens Medical Solutions, Forchheim, Germany). To assess the AVC scores the following CT settings are used: Gantry rotation time 0.28 s, 3.0 mm collimation, acquisition 128 x 0.6 mm, 120 kV tube voltage, 90 mAs tube current, and a prospectively electrocardiographic (ECG) -triggered scan (gating at 65%-75% of the R-R interval if the heart rate were <75 or at 250-400ms after the QRS-complex if heart rate were >75). Calculation of the AVC scores is performed off-line by summing-up all spots of calcifications in the aortic valve area. AVC is defined as calcification below the ostia of the coronaries in the aortic sinus Valsalva, within the valve leaflet, or in the aortic annulus.[36] The CAC score is assessed as previously described.[32] To assess the calcifications in the carotid, aortic, renal, iliac and femoral arteries a CT scanning proximal from the mandibular bone and distally to the proximal third of the femur are performed with the following settings: Spiral scan with a pitch of 3.2 (Flash), 100 kV tube voltage, 90 mAs, collimation of 128 x 0.6mm, Safire 3 and slice thickness 5 mm. The calcifications scores are measured using the Agatston method.

To examine vessel plaques in the coronaries and carotid arteries, an ordinary contrast CT will be performed. The scanning protocol depends on the local CT scanner. Typically 80–100 mL of contrast agent are injected into an antecubital vein at a rate of 6.0 mL/s followed by 60 mL

intravenous saline (6.0 mL/s) using a dual-head power injector. A prospectively gated high pitch spiral "flash" protocol will be used in patients with a stable heart rate <60 beats per minute (bpm). In patients with a stable heart rate between 60 and 90 bpm, intravenously β-blocker is typically injected until the heart rate is appropriate, and a prospectively gated axial "adaptive sequence" protocol is used. In patients with a heart rate > 90 bpm or in case of an irregular heart rhythm, a retrospectively gated "helical" protocol with dose modulation will be used. Data acquisition parameters are 2*128*0.6 mm slice collimation, a gantry rotation time of 280 ms and a tube voltage of 100 or 120 kV depending on patients' height and weight. The coronary artery tree will be analyzed for the presence and severity of CAD, according to the classification of the American Heart Association 16-segment model. Coronary plaques are defined as visible structures within or adjacent to the coronary artery lumen, which can be clearly distinguished from the vessel lumen and the surrounding pericardial tissue. Quantification of coronary plaque components is done via semi-automated analysis. Scans are analyzed an experienced cardiologist.

9.4 Echocardiographic measurements

A comprehensive transthoracic echocardiography is performed at baseline and hereafter annually. Left ventricle (LV) volume and ejection fraction (EF) are estimated. LV longitudinal function is assessed using global strain analysis. LV remodelling is assessed by relative wall thickness and LV mass using the Devereaux formula. LV filling pressure is estimated from assessment of mitral inflow and assessment of diastolic motion of the mitral plane using tissue Doppler imaging. Left atrial size is assessed using biplane planimetry, and longitudinal left atrial strain is estimated using 2D speckle tracking. Aortic valve area is estimated by quantitative Doppler ultrasound using the continuity equation. LV outflow tract time-velocity integral is measures with pulsed-wave Doppler by placing the sample volume just below the region of flow convergence. Peak flow velocity across the valve is determined in the window with the highest velocity. AS severity is graded according to current guidelines (*secondary endpoints*).[37]

9.5. Bone mineral-density, -geometry and -microstructure

Using the images obtained from the multi-slice computed tomography the lumbar spine and hip can be evaluated. The currently available software from Mindways® allows for volumetric bone mineral-density of the trabecular compartment in the spine, thus making it possible to calculate T- and Z-scores for volumetric bone mineral density in the spine. At the hip both cortical, trabecular and total hip volumetric bone mineral density can be evaluated for the femoral neck, the trochanter region and femoral shaft. Again both T- and Z-scores can be calculated. The software also offers evaluation of the total bone area at the spine, and hip.

10. Figure legends

Figure 1.
Timeline and applied tests, for details please see "Applied tests during the study".

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12. Footnotes

12.1. Contributors

AD, JL, NEF and LMR conceived and designed the study. AD wrote the study protocol and is the principal investigator while JL, NEF and LMR are coinvestigators. MF is the clinical research fellow responsible for the running of the clinical trial. Kristian Øvrehus is responsible for the Cardiac-CT contrast protocol. JD and Jacob E Møller are responsible for the echocardiography protocol. Grazina Urbonaviciene (Silkeborg), Søren Warberg Becker (Silkeborg), Jess Lambrechtsen (Svendborg), Søren Auscher (Svendborg), Flemming Hald (Vejle) and Martin Busk (Vejle), are site specific coinvestigators. LF is involved in the bone-related substudy. Oke Gerke is responsible for statistics. HM is representative for the DSMB. All authors have contributed to the revision of the manuscript.

12.2. Patient and Public Involvement

Patients and public were not involved in the design of study, but as members of the Regional Scientific Ethical Committee for Southern Denmark the public have approved the written participant information.

12.3. Funding statement

The trial is supported by the Danish Heart Foundation [grant number 17-R116-A7569-22071], Region of Southern Denmark's Research council [grant number 17/15638] and the Novo Nordisk Foundation [grant number NNF17OC0029076].

The study-tablets, including placebo, are provided for free of charge by Kappa Bioscience A/S, Silurveien 2B, 0380 Oslo and Orkla Care, Industrigrenen 10, 2635 Ishøj. The companies are not involved in the execution of the study or analysis of the data.

12.4. Competing interests statement

None declared.

12.5. Ethics approval

The protocol is approved by the Regional Scientific Ethical Committee for Southern Denmark.

12.6. Data sharing statement:

Positive as well as negative findings will be reported via conference presentations and peer-reviewed publications. All the data will be available upon request.

Month	0	3	6	9	12	15	18	21	24	(27)	(30)
Informed consent	X										
Medical interview	X		X		X		X		X		(X)
Web-based survey		X		X		X		X		(X)	
Biochemical measurements	X		X		X		X		X		(X)
Non-contrast CT	X				X				X		(X)
Contrast CT	X								X		
Echocardiography	X				X				X		(X)

Timeline and applied tests, for details please see "Applied tests during the study".

154x75mm (300 x 300 DPI)

SPIRIT
STANDARD PROTOCOL ITEMS: RECOMMENDATIONS FOR INTERVENTIONAL TRIALS

SPIRIT 2013 Checklist: Recommended items to address in a clinical trial protocol and related documents*

Section/item	Item No	Description	Addressed on page number
Administrative information			
Title	1	Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym	1
Trial registration	2a	Trial identifier and registry name. If not yet registered, name of intended registry	2
	2b	All items from the World Health Organization Trial Registration Data Set	
Protocol version	3	Date and version identifier	1
Funding	4	Sources and types of financial, material, and other support	15
Roles and responsibilities	5a	Names, affiliations, and roles of protocol contributors	15
	5b	Name and contact information for the trial sponsor	15
	5c	Role of study sponsor and funders, if any, in study design; collection, management, analysis, and interpretation of data; writing of the report; and the decision to submit the report for publication, including whether they will have ultimate authority over any of these activities	15
	5d	Composition, roles, and responsibilities of the coordinating centre, steering committee, endpoint adjudication committee, data management team, and other individuals or groups overseeing the trial, if applicable (see Item 21a for data monitoring committee)	7, 8,

Introduction

Background and rationale	6a	Description of research question and justification for undertaking the trial, including summary of relevant studies (published and unpublished) examining benefits and harms for each intervention	4,5
	6b	Explanation for choice of comparators	
Objectives	7	Specific objectives or hypotheses	5
Trial design	8	Description of trial design including type of trial (eg, parallel group, crossover, factorial, single group), allocation ratio, and framework (eg, superiority, equivalence, noninferiority, exploratory)	5

Methods: Participants, interventions, and outcomes

Study setting	9	Description of study settings (eg, community clinic, academic hospital) and list of countries where data will be collected. Reference to where list of study sites can be obtained	5
Eligibility criteria	10	Inclusion and exclusion criteria for participants. If applicable, eligibility criteria for study centres and individuals who will perform the interventions (eg, surgeons, psychotherapists)	5
Interventions	11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered	5,6
	11b	Criteria for discontinuing or modifying allocated interventions for a given trial participant (eg, drug dose change in response to harms, participant request, or improving/worsening disease)	6
	11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return, laboratory tests)	6
	11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial	
Outcomes	12	Primary, secondary, and other outcomes, including the specific measurement variable (eg, systolic blood pressure), analysis metric (eg, change from baseline, final value, time to event), method of aggregation (eg, median, proportion), and time point for each outcome. Explanation of the clinical relevance of chosen efficacy and harm outcomes is strongly recommended	6
Participant timeline	13	Time schedule of enrolment, interventions (including any run-ins and washouts), assessments, and visits for participants. A schematic diagram is highly recommended (see Figure)	6

Sample size	14	Estimated number of participants needed to achieve study objectives and how it was determined, including clinical and statistical assumptions supporting any sample size calculations	___7_____
Recruitment	15	Strategies for achieving adequate participant enrolment to reach target sample size	___8_____
Methods: Assignment of interventions (for controlled trials)			
Allocation:			
Sequence generation	16a	Method of generating the allocation sequence (eg, computer-generated random numbers), and list of any factors for stratification. To reduce predictability of a random sequence, details of any planned restriction (eg, blocking) should be provided in a separate document that is unavailable to those who enrol participants or assign interventions	___7_____
Allocation concealment mechanism	16b	Mechanism of implementing the allocation sequence (eg, central telephone; sequentially numbered, opaque, sealed envelopes), describing any steps to conceal the sequence until interventions are assigned	___7_____
Implementation	16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions	___7_____
Blinding (masking)	17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how	___7_____
	17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial	_____
Methods: Data collection, management, and analysis			
Data collection methods	18a	Plans for assessment and collection of outcome, baseline, and other trial data, including any related processes to promote data quality (eg, duplicate measurements, training of assessors) and a description of study instruments (eg, questionnaires, laboratory tests) along with their reliability and validity, if known. Reference to where data collection forms can be found, if not in the protocol	___7,8_____
	18b	Plans to promote participant retention and complete follow-up, including list of any outcome data to be collected for participants who discontinue or deviate from intervention protocols	_____

Data management	19	Plans for data entry, coding, security, and storage, including any related processes to promote data quality (eg, double data entry; range checks for data values). Reference to where details of data management procedures can be found, if not in the protocol	___8_____
Statistical methods	20a	Statistical methods for analysing primary and secondary outcomes. Reference to where other details of the statistical analysis plan can be found, if not in the protocol	___7_____
	20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)	___7_____
	20c	Definition of analysis population relating to protocol non-adherence (eg, as randomised analysis), and any statistical methods to handle missing data (eg, multiple imputation)	___7_____
Methods: Monitoring			
Data monitoring	21a	Composition of data monitoring committee (DMC); summary of its role and reporting structure; statement of whether it is independent from the sponsor and competing interests; and reference to where further details about its charter can be found, if not in the protocol. Alternatively, an explanation of why a DMC is not needed	___8, 9_____
	21b	Description of any interim analyses and stopping guidelines, including who will have access to these interim results and make the final decision to terminate the trial	___7,9_____
Harms	22	Plans for collecting, assessing, reporting, and managing solicited and spontaneously reported adverse events and other unintended effects of trial interventions or trial conduct	___6, 8, 9____
Auditing	23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor	_____
Ethics and dissemination			
Research ethics approval	24	Plans for seeking research ethics committee/institutional review board (REC/IRB) approval	___9_____
Protocol amendments	25	Plans for communicating important protocol modifications (eg, changes to eligibility criteria, outcomes, analyses) to relevant parties (eg, investigators, REC/IRBs, trial participants, trial registries, journals, regulators)	_____

Consent or assent	26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32)	___7,8_____
	26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable	_____
Confidentiality	27	How personal information about potential and enrolled participants will be collected, shared, and maintained in order to protect confidentiality before, during, and after the trial	_____
Declaration of interests	28	Financial and other competing interests for principal investigators for the overall trial and each study site	___15_____
Access to data	29	Statement of who will have access to the final trial dataset, and disclosure of contractual agreements that limit such access for investigators	___8_____
Ancillary and post-trial care	30	Provisions, if any, for ancillary and post-trial care, and for compensation to those who suffer harm from trial participation	_____
Dissemination policy	31a	Plans for investigators and sponsor to communicate trial results to participants, healthcare professionals, the public, and other relevant groups (eg, via publication, reporting in results databases, or other data sharing arrangements), including any publication restrictions	___8_____
	31b	Authorship eligibility guidelines and any intended use of professional writers	___8_____
	31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code	_____
Appendices			
Informed consent materials	32	Model consent form and other related documentation given to participants and authorised surrogates	_____
Biological specimens	33	Plans for collection, laboratory evaluation, and storage of biological specimens for genetic or molecular analysis in the current trial and for future use in ancillary studies, if applicable	___10_____

*It is strongly recommended that this checklist be read in conjunction with the SPIRIT 2013 Explanation & Elaboration for important clarification on the items. Amendments to the protocol should be tracked and dated. The SPIRIT checklist is copyrighted by the SPIRIT Group under the Creative Commons "Attribution-NonCommercial-NoDerivs 3.0 Unported" license.

BMJ Open

The effects of Menaquinone-7 supplementation in patients with aortic valves calcification: study protocol for a randomized controlled trial

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Primary Subject Heading:	Cardiovascular medicine
Secondary Subject Heading:	Pharmacology and therapeutics, Radiology and imaging, Diabetes and endocrinology
Keywords:	menaquinone-7, aortic valve calcification, Valvular heart disease < CARDIOLOGY, Calcium & bone < DIABETES & ENDOCRINOLOGY, Cardiovascular imaging < RADIOLOGY & IMAGING, randomized clinical trial

The effects of Menaquinone-7 supplementation in patients with aortic valves calcification: study protocol for a randomized controlled trial

Acronym: The Aortic Valve DECalcification (AVADEC) trial

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1. Abstract

Introduction

Aortic stenosis is a common heart valve disease and due to the growing elderly population the prevalence is increasing. The disease is progressive with increasing calcification of the valve cusps. A few attempts with medical preventive treatment have failed, thus presently the only effective treatment of aortic stenosis is surgery. This study will examine the effect of menaquinone-7 (MK-7) supplementation on progression of aortic valve calcification (AVC). We hypothesize that MK-7 supplementation will slow down the calcification process.

Method and analysis

In this multicenter and double-blinded placebo-controlled study, 400 men aged 65-74 years with substantial AVC are randomized (1:1) to treatment with MK-7 (720 µg/day) supplemented by the recommended daily dose of vitamin D (25 µg/day) or placebo treatment (no active treatment) for two years. Exclusion criteria are treatment with vitamin K antagonist or coagulation disorders. To evaluate AVC score, a non-contrast CT-scan is performed at baseline and repeated after 12 and 24 months of follow-up. Primary outcome is difference in AVC score from baseline to follow-up at two years. Intention-to-treat principle is used for all analyses.

Ethics and dissemination

There are no reported adverse effects associated with the use of MK-7. The protocol is approved by the Regional Scientific Ethical Committee for Southern Denmark (S-20170059) and the Data Protection Agency (17/19010). It is conducted in accordance with the Declaration of Helsinki. Positive as well as negative findings will be reported.

Trial registration number

ClinicalTrials.gov Identifier: NCT03243890.

Strengths and limitations of this study

- The study is the first to investigate the effect of menaquinone-7 supplementation on progression of aortic valve calcification.
- Strengths include the stratified randomization, double-blind placebo-controlled design and being a multi-center trial.
- A clinical relevant dose of Menaquinone-7 supplementation is unknown, and accordingly the chosen dose might be insufficient.
- A confirmatory trial with clinical outcomes is needed, if progression of aortic valve calcification.is decreased by menaquinone-7 supplementation.

2. Introduction

In the ongoing Danish Cardiovascular Screening (DANCAVAS) trial, we are randomizing (1:2) 45,000 Danish men aged 65-74 years to a screening examination comprising a non-contrast CT scan.[1] The purpose is to investigate whether an advanced cardiovascular screening will prevent death and cardiovascular events. In a supplementary PhD study, we have been studying aortic valve calcification (AVC). With prevalence ranging from 2 to 7%, aortic valve stenosis is the most common heart valve disease in the western world,[2;3] and combined with the rapidly growing elderly population it is likely that the prevalence will increase further in the future. Central in the pathogenesis is pro-osteogenic factors entailing active bone formation in the valve cusps and this drive disease progression.[4;5] To halt aortic stenosis progression preventive medical treatments with statins and bisphosphonates have been explored in randomized clinical trials, but with discouraging results. Thus at present medical treatment is not an option.[6]

In DANCAVAS, we have made an interesting observation. Patients on vitamin K antagonist (VKA) treatment had a significant increased AVC score (median 32 versus 11, p=0.004). Adjusting for age, smoking, hypertension and cardiovascular disease, this was confirmed in binominal negative (IRR 1.70, 95% CI: 1.25-2.31) and logistic regression (OR 1.66, 95% CI 1.19-2.30) (Axel Diederichsen, DANCAVAS). Thus, in DANCAVAS, patients on VKA seem to have increased aortic valve calcifications.

Vitamin K and the calcification process

Calcification is a slowly progressive process and caused by an imbalance between the mechanisms that promotes and inhibits the deposition of calcium in the vascular wall, and the vitamin K-dependent proteins play an essential role in this inhibition. The most familiar of the K vitamins are phylloquinone (VK1), as this is essential in activation of several coagulation factors, but menaquinone (MK) is another very important vitamin K species. MK is deemed necessary for γ-carboxylation of proteins involved inhibition of arterial calcification, i.e. matrix-Gla proteins (MGP).[7-10] Without these activated proteins, the balance of cellular calcium uptake and the mineralization process in bone and blood vessels is impaired. Additionally, clinical studies suggest that MK preserves bone structure.[11]

The inhibiting process of the vitamin K-dependent proteins was originally showed by Luo et al. in 1997.[12] In a mice model they described MGP to be an important inhibitor of calcification of arteries. In other animal studies, the inhibition of the vitamin K-dependent proteins by VKA resulted in arterial and soft tissue calcification.[13-16] These observations are in agreement with our findings from the DANCAVAS trial, and other human studies have also shown that long-term use of VKA is associated with both increased coronary- and extra-coronary vascular calcification.[17-20] Furthermore, in Japanese, the use of VKA was associated to exacerbate the risk of degenerative aortic valve disease.[21] Finally, low circulating MGP and an impaired carboxylation at its tissue site of expression is associated with the development and progression of cardiovascular disease.[22]

Since VKA seems to induce vascular calcification, MK intake may be beneficial to reduce these calcifications. No recommendations of MK are available; however, we know that the daily intake in

the Western world is not sufficient to meet the request for a complete activation of MGP. Observational studies in healthy elderly have shown an inverse relationship between MK-4 intake and coronary artery calcification (CAC),[23] and VK1 did slow the progression of CAC after 3 years of follow-up.[24] Furthermore, VK1 and MK-7 decreased arterial stiffness and improved elastic properties of the carotid artery.[25;26] Dalmeijer et al. performed a randomized, double blind, placebo controlled trial to investigate the effect of MK-7 supplementation (180 µg/day, 360 µg/day or placebo) and found a dose-dependent decrease of uncarboxylated MGP concentrations.[27] Two subsequent studies in haemodialysis patients found an almost linear dose–response decrease of uncarboxylated MGP without an upper limit, with doses ranging between 360 µg/day and 1080 µg trice weekly.[28;29] In a supplementary study, MK-7 was well tolerated and did not cause a hypercoagulable state.[30] Finally, there is no documented toxicity for VK1 or MK-4, and MK-7, and the WHO has set no upper tolerance level for vitamin K intake.[31]

2.1. Hypothesis

In a randomized setup we test the hypothesis that supplementation with MK-7 (720 µg per day) and vitamin D (25 µg/day) in comparison to placebo will half the progression of further aortic valve calcification in patients with substantial valve calcification, but without aortic valve stenosis.

3. Methods

3.1. Trial design

The study is a double-blind, randomized, placebo-controlled study.

3.2. Participants

In DANCAVAS we are performing echocardiography in all participants with an AVC score above the 90% percentile (AVC score above 300).[32] Patients with an AVC score above 300, but without aortic valve stenosis are eligible patients in AVADEC.

Exclusion criteria are:

- Prior heart valve surgery
- Known significant aortic valve disease (peak velocity ≥3.0 m/s)
- History of venous thrombosis including pulmonary embolism
- Coagulation disorders
- VKA use
- Disorders of calcium and phosphate metabolism
- A life-expectancy < 5 years

The study takes place at Odense University Hospital, and the hospitals in Svendborg, Vejle and Silkeborg, Denmark, from 2018 to 2020.

3.3. Intervention

In AVADEC, half of the patients are randomized to supplementation with MK-7 (720 µg/day) including the recommended daily dose of vitamin D (25 µg/day) and the other half to placebo

treatment (no active treatment). Treatment of both groups will last for at least 24 months. During this time participants will visit our research unit five times, at 6-month intervals (Figure 1). To evaluate AVC score, we will perform a non-contrast CT-scan at baseline and after 12 and 24 months of follow-up.

3.4. Outcome

The *primary endpoint* is the change in AVC score from baseline to two years. The natural history of the aortic valve calcification is not adequately understood, and accordingly the changes are analyzed in two pre-specified patient subgroups (AVC score 300-599 and ≥600, respectively).

Secondary endpoints are:

- Change in calcifications in the coronaries, carotid, aortic, renal, iliac and femoral arteries by non-contrast CT.
- Change in coronary and carotid plaque composition by contrast CT
- Change in aortic valve area by transthoracic echocardiography.
- Change in bone density as quantitative CT of the columna lumbalis and hip region.
- Change in MGP and osteocalcin with different phosphorylation (p and dp) and carboxylation forms (c and uc).
- Quality of life.

Safety endpoints are:

- Death
- Cardiovascular events (myocardial infarction, coronary revascularization, stroke, heart valve surgery, significant aortic disease (including dissection, ruptur and surgery) and significant peripheral artery disease (including thromboembolisms and surgery))
- Progressive aortic valve disease (more than 50% increase in AVC score)
- Venous thromboembolism including pulmonary embolism
- Bleeding (including intracranial bleeding and hemorrhage associated with a drop in hemoglobin of ≥ 2mmol/l)
- Low-energy or spontaneous fracture
- Cancer, including solid and hematologic
- Significant deterioration in laboratory measurements (calcium, magnesium, albumin, phosphate, alkaline phosphatase, bone specific alkaline phosphatase, Parathyroid Hormone, vitamin D or Prothrombin time-International normalized ratio (PT-INR)).

3.5 Sample size

We are planning a study of a continuous response variable from independent control and experimental subjects with 1 control per experimental subject. The mean annual AVC progression is unknown, but based on data from 37 subjects of the DANCAVAS study we estimate the progression to be 100 units in two years with a joint standard deviation 67 units. We expect that the treatment will reduce the AVC progression by 20% (i.e. to 80), leading to the inclusion of 177 experimental subjects and 177 control subjects to be able to reject the null hypothesis that the

population means of the experimental and control groups are equal with probability (power) 0.8. The Type I error probability associated with this test of this null hypothesis is 0.05. Accordingly, 354 subjects are needed, but in order to account for drop-out 400 patients will be included.
Interim analysis: The sample size is based on two years of treatment. One member of the The data and safety monitoring board (HM) and a statistician (OG) will evaluate the available primary endpoint one-year data of approximately 100 patients around 01 July 2019 in order to assess whether the treatment period should be prolonged by six months. The number of patients to be included in this study is unaffected by the decision to prolong treatment by six months or not.

3.6 Stratified randomization

Subjects will be randomized 1:1 after stratification for site (Odense University Hospital, Svendborg Hospital, Vejle Hospital or Silkeborg Hospital), and AVC score (300-599 or ≥600). Each site will be provided with sequentially numbered, opaque and sealed envelopes containing randomly generated treatment allocations. Two types of envelopes are provided; 1) AVC 300-599; and 2) AVC ≥600.

3.7 Blinding

The randomization-list is available to the data and safety monitoring board, but patients, nurses, physicians and other data collectors are kept blinded to the allocation during the study. The placebo is matched to the study drug for taste, color, and size.

3.8 Statistical methods

We will use the intention-to-treat principle for all analyses. The primary endpoint (change in AVC score) will be presented as continuous variable. Additionally, the changes are analyzed in two pre-specified patient subgroups (AVC score 300-599 and ≥600, respectively). Primary hypothesis testing will be done hierarchically to maintain a closed testing procedure: only if the overall treatment effect is statistically significant, testing in AVC strata will be performed with confirmatory intent, otherwise solely for explorative reasons. Secondary endpoints include 1) change in calcifications in the coronaries, carotid, aortic, renal, iliac and femoral arteries; 2) change in coronary and carotid plaque composition by contrast CT; 3) change in aortic valve area by transthoracic echocardiography and 4) change in bone-density, -geometry and -microstructure as quantitative CT of the columna lumbalis and hip region.
We use general linear models (employing group, time point, and group x time point interaction) for the primary and for secondary endpoints as well as potential harms. Missing data will be treated as such; supplementary sensitivity analyses making use of imputed values under the missing at random assumption will be conducted for the primary analysis if more than 5% of expected data points will be missing.

3.9. Patient and Public Involvement

Patients and public were not involved in the design of study, but as members of the Regional Scientific Ethical Committee for Southern Denmark the public have approved the written participant information.

4. Organization

The study is a part of the DANCAVAS trial, and conducted in collaboration with Centre for Individualized Medicine in Arterial Diseases (CIMA). The DANCAVAS secretariat at OUH will identify eligible patients in the DANCAVAS database, and an invitation is send by mail to these patients. If a patient is interested, he is invited to the local site to discuss the trial with a study nurse. If he is willing to participate in the study, informed consent is obtained, and he is randomly assigned to the MK-7 or placebo group. Nurses, radiographers. biomedical technicians and a PhD student are responsible for the treatment and examinations. During the study, only the independent data and safety monitoring board will have access to the complete database including the randomization-list. The data registration is performed via REDCap (Research Electronic Data Capture) with logging and secure storage directly on a server under Odense Patient data Explorative Network (OPEN), Region of Southern Denmark.

The Executive Committee, consisting Professor Jes Lindholt (JL, Department of Cardiothoracic and Vascular Surgery, OUH), MD Niels Erik Frandsen (NEF) and associate professor Axel Diederichsen (AD, Department of Cardiology, OUH) conceived and designed the study, and will handle the decisions regarding the overall organization including administration, budget and use of the database.

The Steering Committee will consist of the members of the executive committee, and Jordi Dahl (JD, Department of Cardiology, OUH), Professor Lars Melholt Rasmussen (LMR, Department of Clinical Biochemistry and Pharmacology) both OUH, and two from each screening site. All practical issues concerning the treatment and data sampling will be handled by the steering committee.

The data and safety monitoring board (DSMB) consists of the following experts: Professor of Cardiology Hans Mickley (HM, OUH), Professor of Cardiology and Clinical Epidemiology Christian Torp-Pedersen (Aalborg University) and Professor of Cardiology Lars Køber (Rigshospitalet), who all have large have experience with clinical randomized trials.

5. Publication

Project results reporting the primary endpoint will be published in peer reviewed international journals. The order of the authors will be PhD student (to be appointed), JL, JD, LF, two from each screening site, LMR, NEF and AD. Positive as well as negative findings will be reported.

6. Feasibility

By December 2017 more than 10000 participants have been included in DANCAVAS, and 800 of these are eligible to participate in AVADEC. Thus we are able to identify enough participants. AD and JL are PI's of the main study, DANCAVAS. In addition, several experts assist with AVADEC: JD is an expert in aortic stenosis, LMR is an expert in biochemistry, while PhD Lars Folkestad (LF) has undertaken several studies in bone-density, -geometry and -microstructure. In addition, local cardiologist from Vejle, Svendborg and Silkeborg will be responsible for securing local practical feasibility of the project at the specific screening sites.

7. Safety and Ethics

Pure natural MK-7 is used in the study. A daily dose at 720 µg MK-7 has not been examined on patients with aortic stenosis, but in a Belgian dose-finding study using 360, 720 or 1080 µg of MK-7 thrice weekly for 8 weeks in chronic haemodialysis patients no severe adverse effects were observed.[29] Presently the Belgian group are performing a randomized trial exploring the efficacy of 2000 µg MK-7 thrice weekly.[33] MK-7 is well tolerated and does not cause a hypercoagulable state.[30] There are no reported adverse effects associated with the use of MK-7.[31]

Each patient has three CT scans during the study. Epidemiological studies do suggest that radiation exposure is associated with a slightly increased risk of cancer. The best studied cohort is the Japanese atomic bomb survivor cohort. In a group exposed to a mean radiation dose of 29 mSv, an excess of solid cancers – corresponding to an excess relative risk of 2% – were observed.[34] No large studies involving medically exposed adult cohorts are available, but a linear no-threshold model has been considered. Thus, there may be no minimal radiation dose for an increased cancer risk, and the risk increases linearly with the radiation dose. The average dose of one non-contrast CT scan is 3 mSv. Two additionally contrast CT scans are performed (baseline and 24 months) with an average dose of 3 mSv each, thus at average the participants in AVADEC will receive 15 mSv. For comparison, the annual background radiation dose in Denmark is 3 mSv, and the average annual limit for radiation workers is 20 mSv.[35]

An independent DSMB is established to perform ongoing safety surveillance. None of the DSMB-members are directly or indirectly involved in the coordination, execution or analysis of the study. The following is assessed: 1) death, myocardial infarction, coronary revascularization, stroke, heart valve surgery and venous thromboembolism, 2) progressive aortic valve disease, and 3) laboratory measurements (Calcium, Magnesium, Albumin, Phosphate and alkaline phosphatase, bone specific alkaline phosphatase, Parathyroid Hormone and vitamin D or Prothrombin time-International normalized ratio (PT-INR)). If there is are a reason for concern, the DSMB can advise to interrupt the study for further analysis, and the study can be terminated prematurely if the number of severe adverse events is significantly higher in the treatment group versus the placebo group. This will be discussed in a meeting with the investigators and DSMB. The investigator will inform the subjects in case of interruption or termination of the study.

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 or in case of demonstrable poor adherence to the study medication. This is assessed by interview and pill-count. If subjects are required to take VKA during the course of the study they will be withdrawn.

The protocol is approved by the Regional Scientific Ethical Committee for Southern Denmark (S-20170059) and the Data Protection Agency (17/19010). It is conducted in accordance with the Declaration of Helsinki. According to Danish legislation vitamin K is a dietary supplement, and accordingly license from the Danish Medicines Agency is not needed. Written informed consent is obtained from each participant. The study is registered at clinicaltrials.gov: NCT03243890.

8. Discussion

Aortic stenosis is a common heart valve disease and due to the growing elderly population the prevalence is increasing. The disease is progressive with increasing calcification of the valve cusps. A few attempts with medical preventive treatment have failed, thus presently the only effective treatment of aortic stenosis is surgery. This study will examine the effect of MK-7 supplementation on progression of AVC in a randomized, placebo-controlled study. We hypothesize that MK-7 supplementation will slow down the progression of valves calcification. If positive effects are shown a new treatment options may be available to prevent progression of aortic valve calcification. The result of this study will be expected in 2021.

9. Applied tests during the study

9.1. Medical interview

At baseline, all relevant data are supplied from the DANCAVAS trial (e.g. AVC score, medical history and lifestyle factors).[1] At every visit, an interview is conducted and evaluating the following: incident cardiovascular disease, dyspnea, chest pain and quality of life (EurQol 5D).

9.2. Laboratory Assessment

Blood samples are obtained at every visit. Routine parameters include:

- Circulating MGP species with different phosphorylation (p and dp) and carboxylation forms (c and uc) are measured using a sandwich enzyme-linked immunosorbent assay (ELISA) based on monoclonal antibodies.
- Creatinine (eGFR), Natrium, Potassium, Calcium, Magnesium, Albumin, Phosphate, alkaline phosphatase, bone specific alkaline phosphatase, Parathyroid Hormone, Vitamin D and INR.

As a part of the study, a biobank at baseline and after 24 months will be organized. 40 mL of blood form each of the participants are centrifuged, labeled, and stored at -80°C until serial testing.

9.3. Multi-Slice Computed Tomography Scans

CT scans will be performed using a high end CT- scanner like dual-source CT-scanner (Somatom Definition Flash, Siemens Medical Solutions, Forchheim, Germany). To assess the AVC scores the following CT settings are used: Gantry rotation time 0.28 s, 3.0 mm collimation, acquisition 128 x 0.6 mm, 120 kV tube voltage, 90 mAs tube current, and a prospectively electrocardiographic (ECG) -triggered scan (gating at 65%-75% of the R-R interval if the heart rate were <75 or at 250-400ms after the QRS-complex if heart rate were >75). Calculation of the AVC scores is performed off-line by summing-up all spots of calcifications in the aortic valve area. AVC is defined as calcification below the ostia of the coronaries in the aortic sinus Valsalva, within the valve leaflet, or in the aortic annulus.[36] The CAC score is assessed as previously described.[32] To assess the calcifications in the carotid, aortic, renal, iliac and femoral arteries a CT scanning proximal from the mandibular bone and distally to the proximal third of the femur are performed with the following settings: Spiral scan with a pitch of 3.2 (Flash), 100 kV tube voltage, 90 mAs, collimation of 128 x 0.6mm, Safire 3 and slice thickness 5 mm. The calcifications scores are measured using the Agatston method.

To examine vessel plaques in the coronaries, an ordinary contrast CT will be performed. CT scanners with a minimum of 64 detector rows will be used. The scanning protocol depends on the local CT scanner and the patient heart rate. In patients with a stable heart rate above 60 beats per minute, orally or intravenously β-blocker are administered until the heart rate is appropriate (if possible below 60), and a prospectively gated protocol is used. In patients with a heart rate > 70 bpm despite β-blocker pretreatment a retrospectively gated scan with dose modulation will be performed. In case of an irregular heart rhythm, a prospectively scan 250-400ms after the QRS-complex is performed. Additionally, sublingual nitrates are administered prior to the scan. 50-80 mL of contrast agent are injected into an antecubital vein at a rate of 6.0 mL/s followed by 60 mL intravenous saline (6.0 mL/s) using a dual-head power injector. Data acquisition parameters depends on the local CT scanner, but slice collimation will be below 0.6mm, gantry rotation time as fast as possible and a tube voltage of 100 or 120 kV depending on patients' weight. The coronary artery tree will be analyzed for the presence and severity of CAD, according to the classification of the American Heart Association 16-segment model. Coronary plaques are defined as visible structures within or adjacent to the coronary artery lumen, which can be clearly distinguished from the vessel lumen and the surrounding pericardial tissue. All coronary segments ≥2 mm in diameter with plaque will be analyzed using a semi-automated software. Scans are analyzed an experienced cardiologist.

9.4 Echocardiographic measurements

A comprehensive transthoracic echocardiography is performed at baseline and hereafter annually. Left ventricle (LV) volume and ejection fraction (EF) are estimated. LV longitudinal function is assessed using global strain analysis. LV remodelling is assessed by relative wall thickness and LV mass using the Devereaux formula. LV filling pressure is estimated from assessment of mitral inflow and assessment of diastolic motion of the mitral plane using tissue Doppler imaging. Left atrial size is assessed using biplane planimetry, and longitudinal left atrial strain is estimated using 2D speckle tracking. Aortic valve area is estimated by quantitative Doppler ultrasound using the continuity equation. LV outflow tract time-velocity integral is measures with pulsed-wave Doppler by placing the sample volume just below the region of flow convergence. Peak flow velocity across the valve is determined in the window with the highest velocity. AS severity is graded according to current guidelines (*secondary endpoints*).[37]

9.5. Bone mineral-density, -geometry and -microstructure

Using the images obtained from the multi-slice computed tomography the lumbar spine and hip can be evaluated. The currently available software from Mindways® allows for volumetric bone mineral-density of the trabecular compartment in the spine, thus making it possible to calculate T- and Z-scores for volumetric bone mineral density in the spine. At the hip both cortical, trabecular and total hip volumetric bone mineral density can be evaluated for the femoral neck, the trochanter region and femoral shaft. Again both T- and Z-scores can be calculated. The software also offers evaluation of the total bone area at the spine, and hip.

10. Figure legends

Figure 1.
Timeline and applied tests, for details please see "Applied tests during the study".

11. Reference List

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12. Footnotes

12.1. Contributors

AD, JL, NEF and LMR conceived and designed the study. AD wrote the study protocol and is the principal investigator while JL, NEF and LMR are coinvestigators. MF is the clinical research fellow responsible for the running of the clinical trial. Kristian Øvrehus is responsible for the Cardiac-CT contrast protocol. JD and Jacob E Møller are responsible for the echocardiography protocol. Grazina Urbonaviciene (Silkeborg), Søren Warberg Becker (Silkeborg), Jess Lambrechtsen (Svendborg), Søren Auscher (Svendborg), Susanne Hosbond (Vejle) and Dilek Hunerel Alan (Vejle), are site specific coinvestigators. LF is involved in the bone-related substudy. Oke Gerke is responsible for statistics. HM is representative for the DSMB. All authors have contributed to the revision of the manuscript.

12.2. Funding statement

The trial is supported by the Danish Heart Foundation [grant number 17-R116-A7569-22071], Region of Southern Denmark's Research council [grant number 17/15638] and the Novo Nordisk Foundation [grant number NNF17OC0029076].

The study-tablets, including placebo, are provided for free of charge by Kappa Bioscience A/S, Silurveien 2B, 0380 Oslo and Orkla Care, Industrigrenen 10, 2635 Ishøj. The companies are not involved in the execution of the study or analysis of the data.

12.3. Competing interests statement

None declared.

12.4. Ethics approval

The protocol is approved by the Regional Scientific Ethical Committee for Southern Denmark.

12.5. Data sharing statement:

Positive as well as negative findings will be reported via conference presentations and peer-reviewed publications. All the data will be available upon request.

Month	0	3	6	9	12	15	18	21	24	(27)	(30)
Informed consent	X										
Medical interview	X		X		X		X		X		(X)
Web-based survey		X		X		X		X		(X)	
Biochemical measurements	X		X		X		X		X		(X)
Non-contrast CT	X				X				X		(X)
Contrast CT	X								X		
Echocardiography	X				X				X		(X)

Timeline and applied tests, for details please see "Applied tests during the study".

154x75mm (300 x 300 DPI)

SPIRIT
STANDARD PROTOCOL ITEMS: RECOMMENDATIONS FOR INTERVENTIONAL TRIALS

SPIRIT 2013 Checklist: Recommended items to address in a clinical trial protocol and related documents*

Section/item	Item No	Description	Addressed on page number
Administrative information			
Title	1	Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym	1
Trial registration	2a	Trial identifier and registry name. If not yet registered, name of intended registry	2
	2b	All items from the World Health Organization Trial Registration Data Set	
Protocol version	3	Date and version identifier	1
Funding	4	Sources and types of financial, material, and other support	15
Roles and responsibilities	5a	Names, affiliations, and roles of protocol contributors	15
	5b	Name and contact information for the trial sponsor	15
	5c	Role of study sponsor and funders, if any, in study design; collection, management, analysis, and interpretation of data; writing of the report; and the decision to submit the report for publication, including whether they will have ultimate authority over any of these activities	15
	5d	Composition, roles, and responsibilities of the coordinating centre, steering committee, endpoint adjudication committee, data management team, and other individuals or groups overseeing the trial, if applicable (see Item 21a for data monitoring committee)	7, 8,

Introduction

Background and rationale	6a	Description of research question and justification for undertaking the trial, including summary of relevant studies (published and unpublished) examining benefits and harms for each intervention	4,5
	6b	Explanation for choice of comparators	
Objectives	7	Specific objectives or hypotheses	5
Trial design	8	Description of trial design including type of trial (eg, parallel group, crossover, factorial, single group), allocation ratio, and framework (eg, superiority, equivalence, noninferiority, exploratory)	5

Methods: Participants, interventions, and outcomes

Study setting	9	Description of study settings (eg, community clinic, academic hospital) and list of countries where data will be collected. Reference to where list of study sites can be obtained	5
Eligibility criteria	10	Inclusion and exclusion criteria for participants. If applicable, eligibility criteria for study centres and individuals who will perform the interventions (eg, surgeons, psychotherapists)	5
Interventions	11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered	5,6
	11b	Criteria for discontinuing or modifying allocated interventions for a given trial participant (eg, drug dose change in response to harms, participant request, or improving/worsening disease)	6
	11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return, laboratory tests)	6
	11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial	
Outcomes	12	Primary, secondary, and other outcomes, including the specific measurement variable (eg, systolic blood pressure), analysis metric (eg, change from baseline, final value, time to event), method of aggregation (eg, median, proportion), and time point for each outcome. Explanation of the clinical relevance of chosen efficacy and harm outcomes is strongly recommended	6
Participant timeline	13	Time schedule of enrolment, interventions (including any run-ins and washouts), assessments, and visits for participants. A schematic diagram is highly recommended (see Figure)	6

Sample size	14	Estimated number of participants needed to achieve study objectives and how it was determined, including clinical and statistical assumptions supporting any sample size calculations	___7_____
Recruitment	15	Strategies for achieving adequate participant enrolment to reach target sample size	___8_____
Methods: Assignment of interventions (for controlled trials)			
Allocation:			
Sequence generation	16a	Method of generating the allocation sequence (eg, computer-generated random numbers), and list of any factors for stratification. To reduce predictability of a random sequence, details of any planned restriction (eg, blocking) should be provided in a separate document that is unavailable to those who enrol participants or assign interventions	___7_____
Allocation concealment mechanism	16b	Mechanism of implementing the allocation sequence (eg, central telephone; sequentially numbered, opaque, sealed envelopes), describing any steps to conceal the sequence until interventions are assigned	___7_____
Implementation	16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions	___7_____
Blinding (masking)	17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how	___7_____
	17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial	_____
Methods: Data collection, management, and analysis			
Data collection methods	18a	Plans for assessment and collection of outcome, baseline, and other trial data, including any related processes to promote data quality (eg, duplicate measurements, training of assessors) and a description of study instruments (eg, questionnaires, laboratory tests) along with their reliability and validity, if known. Reference to where data collection forms can be found, if not in the protocol	___7,8_____
	18b	Plans to promote participant retention and complete follow-up, including list of any outcome data to be collected for participants who discontinue or deviate from intervention protocols	_____

Data management	19	Plans for data entry, coding, security, and storage, including any related processes to promote data quality (eg, double data entry; range checks for data values). Reference to where details of data management procedures can be found, if not in the protocol	___8_____
Statistical methods	20a	Statistical methods for analysing primary and secondary outcomes. Reference to where other details of the statistical analysis plan can be found, if not in the protocol	___7_____
	20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)	___7_____
	20c	Definition of analysis population relating to protocol non-adherence (eg, as randomised analysis), and any statistical methods to handle missing data (eg, multiple imputation)	___7_____
Methods: Monitoring			
Data monitoring	21a	Composition of data monitoring committee (DMC); summary of its role and reporting structure; statement of whether it is independent from the sponsor and competing interests; and reference to where further details about its charter can be found, if not in the protocol. Alternatively, an explanation of why a DMC is not needed	___8, 9_____
	21b	Description of any interim analyses and stopping guidelines, including who will have access to these interim results and make the final decision to terminate the trial	___7,9_____
Harms	22	Plans for collecting, assessing, reporting, and managing solicited and spontaneously reported adverse events and other unintended effects of trial interventions or trial conduct	___6, 8, 9____
Auditing	23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor	_____
Ethics and dissemination			
Research ethics approval	24	Plans for seeking research ethics committee/institutional review board (REC/IRB) approval	___9_____
Protocol amendments	25	Plans for communicating important protocol modifications (eg, changes to eligibility criteria, outcomes, analyses) to relevant parties (eg, investigators, REC/IRBs, trial participants, trial registries, journals, regulators)	_____

Consent or assent	26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32)	___7,8_____
	26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable	_____
Confidentiality	27	How personal information about potential and enrolled participants will be collected, shared, and maintained in order to protect confidentiality before, during, and after the trial	_____
Declaration of interests	28	Financial and other competing interests for principal investigators for the overall trial and each study site	___15_____
Access to data	29	Statement of who will have access to the final trial dataset, and disclosure of contractual agreements that limit such access for investigators	___8_____
Ancillary and post-trial care	30	Provisions, if any, for ancillary and post-trial care, and for compensation to those who suffer harm from trial participation	_____
Dissemination policy	31a	Plans for investigators and sponsor to communicate trial results to participants, healthcare professionals, the public, and other relevant groups (eg, via publication, reporting in results databases, or other data sharing arrangements), including any publication restrictions	___8_____
	31b	Authorship eligibility guidelines and any intended use of professional writers	___8_____
	31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code	_____
Appendices			
Informed consent materials	32	Model consent form and other related documentation given to participants and authorised surrogates	_____
Biological specimens	33	Plans for collection, laboratory evaluation, and storage of biological specimens for genetic or molecular analysis in the current trial and for future use in ancillary studies, if applicable	___10_____

*It is strongly recommended that this checklist be read in conjunction with the SPIRIT 2013 Explanation & Elaboration for important clarification on the items. Amendments to the protocol should be tracked and dated. The SPIRIT checklist is copyrighted by the SPIRIT Group under the Creative Commons "Attribution-NonCommercial-NoDerivs 3.0 Unported" license.