This supplement contains the following items:

1. Original protocol, final protocol, summary of changes.

2. Original analysis plan, final statistical analysis plan, summary of changes.
Original Protocol
VIDEO PROTOCOL

Vitamin D Effect on Osteoarthritis study

Scientific title
Does vitamin D supplementation prevent progression of knee osteoarthritis?
A randomised controlled trial

Sponsor Institution
Menzies Research Institute & Monash University

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1. Background

Osteoarthritis (OA) is characterized by gradual loss of articular cartilage and changes of other joint structures (i.e., subchondral bone and meniscus) leading, eventually, to total joint replacement. It is the most common joint disorder in the world and in Western populations is one of the most frequent causes of pain, loss of function and disability in adults. Approximately 25% of people 55 years of age or older have had knee pain on most days in a month in the past year. Of these about half have radiographic knee OA and are considered to have symptomatic OA. The recognition of arthritis as a National Health Priority Area and the establishment of the “Bone and Joint Decade 2000 – 2010” organization highlight the importance of arthritis and musculoskeletal disorders. There is clearly an urgent need for research that investigates innovative and cost-effective approaches to slow the progression of OA.

Vitamin D deficiency [defined as serum level of 25-hydroxy-vitamin D(25-(OH)D) < 50 nmol/l] is very common in older people. High rates of vitamin D deficiency have been reported in all sectors of the community especially in Tasmania and Victoria where this study will be conducted.

Pathophysiological processes of vitamin D in normal and OA joints

Vitamin D may have direct effects on chondrocytes in osteoarthritic cartilage. Vitamin D receptors (VDR) have been demonstrated in human articular chondrocytes (HAC) of osteoarthritic cartilage, especially the superficial zone. VDR expression by HAC is often associated with sites where matrix metalloproteinases (MMPs) expression is prevalent, and 1α,25(OH)2D3 contributes to the regulation of MMP and PGE2 production by HAC in osteoarthritic cartilage. Thus, articular cartilage, including cartilage affected by OA, seems to be sensitive to the effects of vitamin D.

Vitamin D may also exert an effect on OA through bone. During bone growth, vitamin D regulates the transition from growth plate cartilage to bone. Chronic vitamin D inadequacy in adults has adverse effects on calcium metabolism, osteoblast activity, matrix ossification, and bone density, resulting in increased bone turnover and enhanced bone loss. Thus vitamin D deficiency could impair the ability of bone to respond optimally to pathophysiological processes in OA, and predispose to disease progression.

Muscle weakness is another possible mediator of effects of vitamin D on OA. Some studies have demonstrated that low 25-(OH)D (calcidiol) levels are associated with reduced muscle function. As reviewed by Bischoff et al, it is postulated that the beneficial effect of vitamin D on muscle strength is mediated by 1,25(OH)2D binding to a vitamin D–specific nuclear receptor in muscle tissue leading to de novo protein synthesis, muscle cell growth, and improved muscle function.

In summary, there is potential for vitamin D supplementation to have beneficial effects on OA through its direct action on cartilage, and/or its effects on bone and muscle health.

Evidence from observational studies

Cross-sectional data

An early study reported that in women aged between 27 to 79 years, low serum 25-(OH)D levels were associated with prevalent knee osteophytes but this association became non-significant after adjustment for BMI and age. In this study, serum 25-OHD levels were not associated with joint space narrowing. However, recent studies reported at the 2008 American College of Rheumatology Annual Meeting suggested that serum 25-(OH)D levels were associated with OA. Kinjo reported that in the Third National Health and Nutrition
Examination Survey (NHANES III), women (but not men) with knee OA who used chronic analgesics were more likely to have low 25-(OH)D levels than subjects with asymptomatic OA. Similar findings were observed in back pain, suggesting vitamin D may be an important pain modulator in some groups or that reverse causation applies whereby subjects with pain have less sun exposure as a result. Chaganti reported that in the community-dwelling ambulatory elderly men, those with low serum 25-(OH)D levels were twice as likely to have prevalent radiographic hip OA.

Radiographic assessment of OA is two dimensional in nature, lacks sensitivity to change and is highly susceptible to measurement error through factors such as joint position. Magnetic resonance imaging (MRI) has more recently been used to study OA. Standard techniques such as fat-saturated T1-weighted spoiled gradient echo and T2-weighted proton density-weighted fast spin echo sequences, have been utilized to directly assess knee structural alterations such as cartilage volume, cartilage defects, subchondral bone changes and meniscal lesions. This has increased our understanding of early joint changes. In the Tasmania Older Adult Cohort (TASOAC) study (NHMRC, 302204), in cross-sectional analysis, serum 25-(OH)D level was significantly and positively associated with knee cartilage volume in older men and women. This was a continuous association with no evidence to support a threshold. Furthermore, 25-(OH)D insufficiency was weakly and positively associated with medial (yes vs no: β=29 mm², P=0.12) and lateral tibial bone area (yes vs no: β=21 mm², P=0.07) in women in adjusted analysis. Because decreased cartilage volume and increased bone area play important roles in the aetiology of knee OA, these results suggest vitamin D insufficiency appears detrimental to joint health.

**Longitudinal data**

Data from the Framingham OA cohort study suggests that vitamin D insufficiency contributes to the progression and possibly development of OA. In this study, the risk of progression of OA in older patients with knee OA was three times higher in the middle and lowest tertiles for both intake and serum levels of vitamin D as compared with patients in the highest tertiles. Low serum levels of vitamin D also predicted loss of cartilage, as assessed by loss of joint space (OR: 2.3, 95% CI: 0.9, 5.5), as well as osteophyte growth (OR: 3.1, 95% CI: 1.3, 7.5). Two studies at the 2008 American College of Rheumatology Annual Scientific Meeting provided further evidence suggesting vitamin D levels are important for OA. Javaid reported from the Multicentre Osteoarthritis (MOST) Study, that there were no significant associations between low serum vitamin D status and incident radiographic knee OA; however, 25-(OH)D as measured by quartiles was predictive of incident radiographic knee OA, with the lowest risk in the middle quartiles. This quadratic association remained significant (P=0.007) after adjusting for vitamin D supplement use and baseline season. Lo reported that in those with symptomatic knee OA, a high baseline vitamin D level was associated with a lower odds of increase in the medial: lateral tibial BMD ratio (M:L BMD) and higher odds of decrease in M:L BMD ratio over 1 year. High M:L BMD may be related to increased medial tibiofemoral OA.

The only longitudinal MRI data is from our TASOAC study and this strongly supports a role of vitamin D in OA. We reported that higher baseline serum levels of 25-(OH)D predicted reduced loss of cartilage volume over 2 years, and increases in vitamin D levels were associated with a further protective association. These suggest vitamin D is important in maintaining knee cartilage health in older adults.

**VIDEO study**
VIDEO study will be the first randomised controlled trial to explore the effects of the vitamin D supplementation on knee structural changes (cartilage and bone) utilizing pioneering MRI techniques and limb muscle strength assessment in OA patients. It has been supported by National Health & Medical Research Council (NHMRC ID 605501).

Sub-studies

In this study, we will also explore the vitamin D supplementation on bone microarchitecture and density at the distal radius and distal tibia assessed by three-dimensional high-resolution peripheral quantitative computed tomography (HR-pQCT)\(^ {14}\). Although effects of vitamin D on bone mineral density assessed by dual energy X-ray absorptiometry (DXA) have widely reported\(^ {15}\), the effects of vitamin D supplementation on bone microarchitecture have not been reported.

Furthermore, we will determine the effect of vitamin D supplementation on blood pressure (clinic, ambulatory, upper arm and central measures) and arterial stiffness (aortic pulse wave velocity). Vitamin D deficiency is associated with increased ischaemic and non-ischaemic cardiovascular disease\(^ {16}\). While these associations are thought to occur independently from traditional risk factors for cardiovascular disease, several lines of evidence suggest that vitamin D deficiency may influence blood pressure via mechanisms including activation of the renin–angiotensin–aldosterone system (RAAS)\(^ {17-19}\). A recent systematic review reported that there is accumulating evidence to support the hypothesis that vitamin D deficiency contributes to hypertension\(^ {20}\). This study also concluded that randomised, placebo-controlled trials are “greatly needed to clarify and definitively prove the effect of vitamin D on blood pressure”\(^ {20}\). VIDEO study aims to be the first to assess this. Central and ambulatory blood pressure will be the main outcomes because central blood pressure is the actual pressure load experienced by the heart (and other organs such as the kidneys and brain) rather than the pressure at the upper arm\(^ {21}\), and ambulatory blood pressure is regarded as the “gold standard” technique because it correlates with target organ damage and provides more accurate information on daily (including night time) blood pressure fluctuations\(^ {22}\).

These 2 sub-studies will be performed with collaboration of Prof Ego Seeman from University of Melbourne and Dr James Sharman from Menzies, and are self-funded.

A third sub-study (also self-funded) is to examine the effects of vitamin D supplementation on the function of deep lumbo-pelvic stabilising muscles. The protective deep lumbo-pelvic stabilising muscles [including, but not exclusively, transversus abdominus (TrAb) and lumbar multifidus (LM); also known as “core muscles”] become dysfunctional shortly after the onset of low back pain, and that ongoing muscle dysfunction is associated with persistent low back pain\(^ {23,24}\). Muscle weakness can be a sign of vitamin D deficiency\(^ {25}\), and therefore, vitamin D supplementation may have beneficial effects on functionally important core muscles.

2. Aims

- To determine whether vitamin D supplementation reduces the loss of knee cartilage volume (~2% per year less loss than placebo group), as assessed by MRI, over a 2-year period in patients with symptomatic knee OA.
- To determine whether vitamin D supplementation significantly prevents the progression of knee cartilage defects and enlargement of tibial bone area over a 2-year period in patients with knee OA.
- To determine whether vitamin D supplementation significantly reduces loss of limb muscle strength over a 2-year period in patients with knee OA.
To determine whether vitamin D supplementation significantly increase bone microarchitecture and density at the distal radius and distal tibia in patients with knee OA.

To determine whether vitamin D supplementation significantly reduce blood pressure (both upper arm and central) and aortic pulse wave velocity in older people.

To determine whether vitamin D supplementation improves measures of deep lumbo-pelvic stabilising muscle function, particularly transversus abdominus and lumbar multifidus.

3. Methods

3.1 Study design

VIDEO is a randomised, placebo-controlled double-blind clinical trial.

We will recruit 400 subjects with symptomatic knee OA, 200 patients in Southern Tasmania and 200 in Melbourne, by using a combined strategy, including collaboration with general practitioners, specialist rheumatologists, and orthopaedic surgeons, as well as advertising through local media. This is the approach we have taken to successfully recruit participants for large community-based trials across a range of musculoskeletal conditions.

3.2 Inclusion criteria:

1) Age 50-79 years old;
2) Men and women with symptomatic knee OA for at least 6 months with a pain visual analogue scale (VAS) of at least 20 mm;
3) Meet the America College of Rheumatology (ACR) criteria for symptomatic knee OA assessed by a rheumatologist;
4) Have an ACR functional class rating of I, II and III;
5) Have relatively good health (0-2 according to the investigator’s global assessment of disease status on a 5-point Likert scale, range 0 [very well] to 4 [very poor]); and
6) Have serum vitamin D level of >12.5 nmol/L and <60 nmol/L.
7) Is able to read, speak and understand English, capable of understanding the study requirements and willing to co-operate with the study instructions.

3.3 Exclusion criteria:

1) Patients with severe radiographic knee OA (grade 3 according to Altman’s atlas);
2) Patients with severe knee pain (on standing more than 80 mm on a 100-mm VAS);
3) Any contra-indication to having an MRI.
4) Patients with rheumatoid arthritis, psorotic arthritis, lupus, or cancer;
5) Patients with severe cardiac or renal function impairment
6) Patients with hypersensitivity to vitamin D;
7) Patients with any condition possibly affecting oral drug absorption (eg, gastrectomy or clinically significant diabetic gastroenteropathy);
8) having significant trauma to the knees including arthroscopy or significant injury to ligaments or menisci of the knee within 1 year preceding the study;
9) having anticipated need for knee or hip surgery in the next 2 years;
10) having taken Vitamin D supplements in last 30 days;
11) having taken an investigational drug in last 30 days.

4. Study medications and allocation
Participants in the intervention arm will receive 50,000 IU (1.25 mg) compounding vitamin D3 (cholecalciferol, Nationwide Compounding Pharmacy, Melbourne) capsules given once monthly \(^{29,30}\), and the control arm will receive an identical inert placebo (provided by the same company). Allocation of participants will be based on computer generated random numbers. Allocation concealment will be ensured by the use of identical inert placebo, and the use of a central automated allocation procedure, with security in place to ensure allocation data cannot be accessed or influenced by any person. Thus the randomized controlled trial (RCT) will be double blind. Monthly treatment at this dose will achieve serum vitamin D levels above 60 nmol/l in all compliant subjects \(^{29}\), and will have less cost and better compliance than daily treatment. Toxicity is extremely unlikely with this dose. All participants will be provided recommended standard of care.

5. Scheduled visits and measurements

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If the participant withdraws after a minimum of 6 months of treatment, he/she will be requested to have a second knee MRI (HR-pQCT or DXA) scan.

5.1 MRI assessment of structural changes

This will be assessed in the right knee. Knees will be imaged in the sagittal plane on a 1.5-T whole body magnetic resonance unit with use of a commercial transmit-receive extremity coil. Fat-saturated T1-weighted spoiled gradient echo and T2-weighted proton density-weighted fast spin echo sequences will be used.

**Cartilage volume:** The volumes of individual cartilage plates (medial tibial, lateral tibial and patella) are isolated from the total volume by manually drawing disarticulation contours around the cartilage boundaries on a section by section basis. These data are then resampled by means of bilinear and cubic interpolation (area of 312 and 312 µm and 1.5 mm thickness, continuous sections) for the final 3D rendering.

**Cartilage defects assessment:** The cartilage defects (0-4) will be graded at medial tibial and femoral, lateral tibial and femoral, and patellar sites: grade 0, normal cartilage; grade 1, focal blistering and intracartilaginous low-signal intensity area with an intact surface and bottom; grade 2, irregularities on the surface or bottom and loss of thickness of less than 50%; grade 3, deep ulceration with loss of thickness of more than 50%; grade 4, full-thickness chondral wear with exposure of subchondral bone.

**Knee tibial plateau bone area:** The area of medial and lateral tibial plateau bone will be measured manually on the three reformatted images closest to tibial cartilage. An average of these three areas will be used as an estimate of the tibial plateau bone area.

**Subchondral bone marrow lesions:** will be assessed on the T2-weighted MR images and defined as discrete areas of increased signal adjacent to the subcortical bone at the lateral, medial femur and/or tibia. Each bone marrow lesion will be scored on the basis of lesion size, e.g., a lesion was scored as grade 1 if it was only present on 1 slice, grade 2 if present on 2 consecutive slices, or grade 3 if present on >3 consecutive slices.

**Meniscal tear assessment:** The menisci will be assessed in the sagittal view and be confirmed in the coronal and axial views as previously described. In brief, the presence or absence of a tear was based on the presence of a signal, which was line shaped, brighter than the dark meniscus, and reached the surface of the meniscus at both ends within 6 defined regions (anterior horn, body, and posterior horn at both medial and lateral tibiofemoral compartments).

**Meniscal extrusion assessment:** The extent of meniscal extrusion on the medial or lateral edges of the tibial femoral joint space for the anterior, body, and posterior horns of the...
menisci will be graded (CIA19) with scores of 0 = no extrusion, 1 = partial meniscal extrusion, and 2 = complete meniscal extrusion with no contact with the joint space.

5.2 Lower limb muscle strength
Lower limb muscle strength will be measured by dynamometry at the lower limb (involving both legs simultaneously). The muscles measured with this technique are mainly the quadriceps and hip flexors. The devices will be calibrated by suspending known weights at regular intervals.

5.3 Knee pain
Knee pain, stiffness and function later will be assessed by the Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC), a self-administered questionnaire. Knee pain will be also assessed using visual analogue scale (VAS).

5.4 Measurements of bone microarchitecture and density
Bone macro-, micro-architecture and volumetric BMD (vBMD) were measured at the distal radius and distal tibia using three-dimensional high-resolution peripheral quantitative computed tomography (HR-pQCT, Xtreme CT, Scanco Medical AG, Bassersdorf, Switzerland). This system uses a two-dimensional detector array in combination with a 0.08-mm point-focus x-ray tube. This enables the simultaneous acquisition of a stack of parallel CT slices with a resolution (voxel size) of 82 μm. The following settings were used: effective energy of 60 kVp, x-ray tube current of 95 mA, and matrix size of 1536 x 1536. During measurement, the non-dominant arm or leg of the patient was immobilized in an anatomically formed carbon fibre shell. The reference line was manually placed at the middle point of the endplate of the radius and tibia on the scout view. As a default, the first CT slice commenced at a certain distance from the reference line, i.e., 9.5 mm at radius, 22.5 mm at tibia, respectively. One hundred and ten slices were obtained at each skeletal site over a 9.02 mm distance. The effective dose was less than 3 μSv per measurement with a measurement time of 2.8 min.

Analyses use a threshold-based algorithm to separate the volume of interest into the cortical and trabecular regions. Attenuation data are converted to equivalent hydroxyapatite (HA) densities. The vBMD (mgHA/cm³) in the trabecular region (vBMD trab), cortical region (vBMD cort) and the combined total vBMD (vBMD tot) were computed as the average mineral density within the trabecular, cortical and entire volume of interest respectively. Trabecular bone volume / tissue volume (BV/TV,%) was determined by dividing the apparent trabecular bone density by 1200 mgHA/cm³ which represents fully mineralised bone [i.e. BV/TV(%) = 100 x (vBMD trab/1200)].

Trabecular number (TbN) was defined as the mean inverse distance between the ridges (the centre points of trabeculae). Briefly, the three-dimensional ridges were extracted from the original three-dimensional gray-scale images. The distances of the ridges were then directly assessed by the newly developed methods derived from distance transformation. This is not affected by the structure of trabeculae whether it is plate- or rod-like. Trabecular thickness (TbTh, mm) and separation (TbSp, mm) were calculated from BV/TV and TbN using histomorphometry methods [i.e., TbTh = (BV/TV) /TbN and TbSp = (1 – BV/TV)/TbN]. Cortical thickness (CTh) was calculated by dividing the mean cortical volume by the outer bone surface.

5.5 Bone mineral density and fat mass
Bone mass was measured using dual-energy X-ray absorptiometry (DXA) at the right total hip and spine at baseline and follow up. The instrument used was a Hologic Delphi densitometer on array setting (Hologic, Inc., Waltham, MA). The software program was not
altered during the study timeframe. Bone mass was examined as areal BMD (g/cm²), which is calculated by dividing the bone mineral content (BMC) by the area measured.

Fat mass and lean mass (both in kg) were also measured using DXA.

5.6 Blood pressure and arterial stiffness
Clinic upper arm blood pressure will be read twice after 5 minutes seated rest using a validated semi-automated device (Omron HEM-907). Clinic central blood pressure will be read twice after 5 minutes seated rest using radial applanation tonometry (SphygmoCor 8.1, AtCor Medical). 24 hour ambulatory blood pressure monitoring (TM2430, A&D mercury) and aortic pulse wave velocity (SphygmoCor 8.1, AtCor Medical) will be performed in a subgroup of patients at the Hobart site. This method derives an estimate of aortic pulse wave velocity, which is the “gold standard” clinical arterial stiffness measure known to independently correlate with mortality.

5.7 Serum vitamin D and other measurements
Serum samples were treated initially with acetone to rapidly extract 25-hydroxyvitamin D [25-(OH)D] and other hydroxylated metabolites. 25-(OH)D was then assayed utilising a Liquid Phase radioimmunoassay (Immunodiagnostic Systems Ltd, Boldon, Tyne & Wear, UK). Serum calcium, phosphate and renal function will be assessed using routine biochemical methods. Serum will be stored for other measures.

5.8 Radiographic osteoarthritis
This will be assessed by a standing semiflexed AP radiograph of right knee as per the Altman atlas. X-rays will also be scored for osteophytes and joint space narrowing on a four point scale (0-3) utilising two simultaneous observers and the OARSI atlas.

5.9 Physical activity
Physical activity will primarily be assessed by pedometry at baseline and two years. An Omron pedometer which measures vertical displacement (steps per day) will be utilised. Each subject will be instructed prior to use and a diary will be kept. The pedometer will be worn for seven consecutive days on two occasions. Up to seven days is required to accurately assess habitual physical activity. We will also measure physical activity by the International Physical Activity Questionnaire (IPAQ) short version.

5.10 Anthropometry
Height was measured to the nearest 0.1 cm (with shoes, socks, and headgear removed) using a stadiometer. Weight was measured to the nearest 0.1 kg (with shoes, socks, and bulky clothing removed) using a single pair of electronic scales (Delta Model 707; Seca, Hamburg, Germany) that were calibrated using a known weight at the beginning of each clinic. Waist and hip circumference were measured using a tape measure. Skinfold at triceps, subscapular, biceps, Iliac crest, supraspinale and midabdominal sits will be measured using Slim Guide skinfold callipers.

5.11 Questionnaires
Depression (using Patients Health Questionnaire-9, PHQ-9), cigarette smoking status (currently smoking, level of smoking, ever smoked, when gave up), previous knee injury and occupation, low back pain, foot pain and quality of life will be assessed by questionnaires.

5.12 Real-time dynamic ultrasound of the core musculature (transversus abdominus, internal obliques, multifidus) using a fully featured ‘big box’ diagnostic ultrasound machine will be used to measure thickness of transversus abdominus and lumbar multifidus muscles at rest and during contractions. These measures have a high degree of reliability (ICC >0.90 across a range of studies). We will do this a baseline, 12 and 24 months.
5.13 Safety
Adverse events will be recorded during the study period. Intensity and relationship with the study medication will be investigated.

6. Statistics and sample size
Paired t-test and logistic regression will be used to compare changes in cartilage volume, cartilage defects, bone marrow lesions, and meniscal abnormalities in univariable and multivariable modelling (adjusted by confounding factors) between groups. Both intention to treat analysis and per protocol will be utilized. Per protocol will be defined as achieving a vitamin D level >60 nmol/l at month 3.

Based on sample size calculations, 200 patients in each arm (allowing for a 20% dropout over the trial) will be sufficient to detect the specified differences (2.2% for medial tibial cartilage loss, 0.9% for increase in medial tibial bone area, 15% for increase in cartilage defects) between the placebo and vitamin D arms with at least 80% power for each outcome.

7. Termination criteria
As the aim of the study is to assess ultimate outcome following randomisation to one of the treatment arms, participants will be strongly encouraged to attend the study. However, it is recognised that situations will arise that will necessitate withdrawal of a participant from the study. Such situations include a participant’s refusal to continue, moved to overseas or interstate, deceased, institutionalised, or physical unable, etc.

In general there will be no specific withdrawal criterion relating to adverse effects of drug therapy as the analysis will be on an intention-to-treat basis. If a participant withdraws or is removed from the study for any reason, the reason for and date of the discontinuation and date of the last dose of study medication should be recorded in the appropriate section of the form.

8. Product liability
VIDEO study is not sponsored by a pharmaceutical company. We will obtain compounding vitamin D3 (cholecalciferol) 1.25 mg capsule and placebo from Nationwide Compounding Pharmacy in Melbourne. This is an unregistered (S4) product in Australia but it can be obtained under the Special Access Scheme (SAS). It has been available for use through some hospitals (including the Royal Hobart Hospital) for the treatment of vitamin D deficiency.

9. Report of the project
VIDEO study research team will provide a statement to the Tasmania Health & Medical Human Ethics Committee and Monash University Human Research Ethics Committee annually or as required. When the research is completed, published papers and abstracts will inform the Ethics Committees of the outcome.

10. Ethic conduct of the trial
This study will be conducted in accordance this protocol, ICH GCP Note for Guidance on Good Clinical Practice (CPMP/ICH/135/95) Annotated with TGA comments and NHMRC National Statement on Ethical Conduct in Human Research 2007 and in keeping with local regulations.

11. Informed consent
11.1 Consent Form
Before obtaining consent from each participant, he/she must be informed of the objectives, benefits, risks and requirements of the study, as well as the nature of the test medication. An information sheet should be given to every participant prior to screening and randomisation. Participants will be giving their own consent after having read all content of the information sheet and consent form. Participant and investigator each retain a copy of the signed consent form.

11.2 Obtaining Consent

The Investigator, or a person designated by the Investigator, and under the Investigator's responsibility, should fully inform the participant of all pertinent aspects of the VIDEO study including the written participant information sheet. All participants should be informed to the fullest extent possible about the study, in language and terms they are able to understand. Participants are expected to giving their own consents by reading content in the information sheet and consent form and giving their signatures by themselves. In those who might have vision impairment, a research officer may read out content and ensure a thoroughly understanding of all content before asking for signature.

- Prior to a subject’s participation in the study, the written Informed Consent Form should be signed, name filled in and personally dated by the participant or by the participant’s legally acceptable representative, and by the person who conducted the informed consent discussion.
- A copy of the signed and dated written Informed Consent Form will be provided to the participant. The original consent is to be stored in the participant’s individual file, held by the investigator. A second copy may be filed in the participant’s file at the general practice.
- The Participant information Sheet and Consent Form used for obtaining the participant’s informed consent must be the current version that has been reviewed and approved by the appropriate Ethics Committee.

12. References

Final Protocol

(Published)
Vitamin D supplementation in the management of knee osteoarthritis: study protocol for a randomized controlled trial

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Abstract

Background: Osteoarthritis (OA) is a common health issue worldwide in the aging population who are also commonly deficient in vitamin D. Our previous study suggested that higher serum 25-(OH)D levels were associated with reduced knee cartilage loss, implying that vitamin D supplementation may prevent the progression of knee OA. The aim of the VItamin D Effects on OA (VIDEO) study is to compare, over a 2-year period, the effects of vitamin D supplementation versus placebo on knee structural changes, knee pain, and lower limb muscle strength in patients with symptomatic knee OA.

Methods/design: Randomised, placebo-controlled, and double-blind clinical trial aiming to recruit 400 subjects (200 from Tasmania and 200 from Victoria) with both symptomatic knee OA and vitamin D deficiency (serum [25-(OH)D] level of >12.5 nmol/liter and <60 nmol/liter). Participants will be randomly allocated to vitamin D supplementation (50,000 IU compounded vitamin D3 capsule monthly) or identical inert placebo group for 2 years. The primary endpoint is loss of knee cartilage volume measured by magnetic resonance imaging (MRI) and Western Ontario and McMaster Universities Index of OA (WOMAC) knee pain score. The secondary endpoints will be other knee structural changes, and lower limb muscle strength. Several other outcome measures including core muscle images and central blood pressure will be recorded. Linear and logistic regression will be used to compare changes between groups using univariable and multivariable modeling analyses. Both intention to treat and per protocol analyses will be utilized.

Discussion: The trial is designed to test if vitamin D supplementation will reduce loss of knee cartilage volume, prevent the progression of other knee structural abnormalities, reduce knee pain and strengthen lower limb muscle strength, thus modify disease progression in knee OA.

Trial registration: ClinicalTrials.gov identifier: NCT01176344; Australian New Zealand Clinical Trials Registry: ACTRN12610000495022

Keywords: Vitamin D, Osteoarthritis, Magnetic resonance imaging
Background

Osteoarthritis (OA) is the most common form of arthritis in the world and one of the most common chronic conditions managed in Australian general practice [1,2]. It is characterized by the gradual loss of articular cartilage and changes to other joint structures eventually leading to total joint replacement. Currently there is no cure for OA, and the development of innovative and cost-effective approaches to prevent the development and progression of OA is urgent and important.

Vitamin D comprises a group of fat-soluble secosteroids encompassing two major molecules, vitamin D2 and vitamin D3. Vitamin D is circulated to the liver where it is converted to the prohormone calcidiol, or 25-hydroxy-vitamin D (25-(OH)D), which is the best indicator of vitamin D status [3-5]. Vitamin D deficiency is very common in older people. It is estimated that 20 to 100% of elderly men and women in North America and Europe are vitamin D deficient (mostly defined as a serum level of 25-(OH)D < 50 nmol/liter)[6,7]. High rates of vitamin D deficiency have also been reported in all sectors of the community of Australia, especially in Tasmania and Victoria where this study will be conducted [8-10].

It has been widely recognized that OA is a disease affecting the whole joint, including cartilage, bone and muscle. Through targeting these joint tissues vitamin D supplementation may modify disease progression in OA. Vitamin D receptors (VDRs) are found in human articular chondrocytes [11], and 1α-25(OH)2D3 regulates the expression of metalloproteinase (MMP) and prostaglandin E2 (PGE2) in chondrocytes via VDRs [11]. Vitamin D could enhance the ability of bone to respond optimally to pathophysiological processes in OA, thus prevent disease progression [12,13]. Furthermore, 1,25(OH)2D leads to de novo protein synthesis, muscle cell growth, and improved muscle function, and thus has a beneficial effect on muscle strength [14].

Epidemiological studies have provided preliminary evidence supporting the potential use of vitamin D for the treatment of OA. Lower serum levels of 25-(OH)D were associated with greater knee pain [15] and higher prevalence of radiographic OA [16], and predicted incidence of knee pain [17], progression/incidence of radiographic OA [18,19], and loss of joint space, as well as osteophyte growth [18]. Magnetic resonance imaging (MRI) has been utilized to directly assess knee structural alterations such as cartilage volume, cartilage defects, subchondral bone changes and meniscal lesions. Using MRI, we reported that, in cross-sectional analysis, serum 25-(OH)D level was significantly and positively associated with knee cartilage volume in older men and women, and vitamin D insufficiency was positively associated with medial and lateral tibial bone area in women. Longitudinally, higher baseline serum levels of 25-(OH)D predicted reduced loss of cartilage volume over 2 years, and increases in vitamin D levels were associated with further protective association [15,16]. Furthermore, serum levels of 25-(OH)D were also associated with increased leg muscle strength and quality, and thus may be important for the maintenance of muscle function [20].

Based on this experimental and epidemiological evidence, we have initiated a randomized, placebo-controlled trial (Vitamin D Effect on Osteoarthritis, VIDEO study) to determine if vitamin D supplementation can reduce loss of knee cartilage volume, prevent the progression of other knee structural abnormalities and strengthen lower limb muscle strength, and thus modify disease progression in knee OA. The effects of vitamin D supplementation on the progression of knee pain will also be determined.

In a sub-study, we will examine the effects of vitamin D supplementation on the function of deep lumbo-pelvic stabilising muscles. The protective deep lumbo-pelvic stabilising muscles, which include (but not exclusively) the transversus abdominus (TrAb) and lumbar multifidus and which are known as core muscles, become dysfunctional shortly after the onset of low back pain, and that ongoing muscle dysfunction is associated with persistent low back pain [18,21]. Muscle weakness can be a sign of vitamin D deficiency [19] and therefore, vitamin D supplementation may have beneficial effects on functionally important core muscles.

Furthermore, we will determine the effect of vitamin D supplementation on blood pressure (clinical, ambulatory, upper arm and central measures) and arterial stiffness (aortic pulse wave velocity). Several lines of evidence suggest that vitamin D deficiency may influence blood pressure via mechanisms including activation of the renin-angiotensin-aldosterone system (RAAS) [22-24]. Central and ambulatory blood pressure will be the main outcomes because central blood pressure is the actual pressure load experienced by the heart (and other organs such as the kidneys and brain) rather than the pressure at the upper arm [25], and ambulatory blood pressure is regarded as the gold standard technique because it correlates with target organ damage and provides more accurate information on daily (including night time) blood pressure fluctuations [26].

Methods/design

Study design

VIDEO is a randomized, placebo-controlled double-blind clinical trial. Four hundred subjects (200 from Tasmania and 200 from Victoria) with symptomatic knee OA and serum 25-(OH)D > 12.5 nmol/liter and < 60 nmol/liter will be recruited and randomly allocated to either the
treatment or placebo control group. Recruitment methods will include advertisements through the local media and community groups as well as liaisons with general practitioners, specialist rheumatologists, and orthopedic surgeons. Ethics approval has been received from The Tasmania Health and Human Medical Research Ethics Committee (reference number H1040) and Monash University Human Research Ethics Committee (reference number CF10/1182 - 2010000616). Informed written consent will be obtained from all participants.

Inclusion criteria
The inclusion criteria were as follows: age 50 to 79 years; symptomatic knee OA for at least 6 months with a pain at least 20 mm on a 100 mm visual analogue scale (VAS); American College of Rheumatology (ACR) criteria for symptomatic knee OA assessed by a rheumatologist [27]; ACR functional class rating of I, II and III [28]; relatively good health, with a score of 0 to 2 on a 5-point Likert scale (with a range of 0 indicating very good health to 4 indicating very poor health), according to the investigators global assessment of disease status; serum 25-(OHD)D > 12.5 nmol/liter and < 60 nmol/liter; able to read, speak and understand English, capable of understanding the study requirements and willing to cooperate with the study instructions.

Exclusion criteria
Exclusion criteria were as follows: severe radiographic knee OA, grade 3 according to Altman’s atlas [29]; severe knee pain on standing (more than 80 mm on 100-mm VAS); any contraindication to having MRI; rheumatoid or psoriatic arthritis, lupus or cancer; severe cardiac or renal impairment; hypersensitivity to vitamin D; any condition possibly affecting oral drug absorption (for example, gastrectomy or malabsorption syndromes); significant trauma to knees, including arthroscopy or significant injury to ligaments or menisci of the knee within one preceding the study; anticipated need for knee or hip surgery within the next 2 years; history of taking vitamin D supplements within the previous 30 days; history of taking an investigational drug within the previous 30 days.

Randomization
Participants in each site will be randomly assigned to the intervention arm or placebo arm in a ratio of 1:1 and the randomization will be double-blind. Allocation of participants will be based on computer-generated random numbers. Allocation concealment will be ensured by the use of an identical inert placebo, and a central automated allocation procedure, with security in place to ensure allocation data cannot be accessed or influenced by any person.

Intervention
Participants in the intervention arm will take one capsule per month of 50,000 IU (1.25 mg) of a vitamin D3 compound (cholecalciferol), purchased from Nationwide Compounding Pharmacy, Melbourne, Australia[30], and patients in the control arm will receive an identical inert placebo provided by the same company. Patients are required to record their medication information in personal diaries and a reminder will be given each month. All participants will receive the recommended standard of care. The duration of the study is 2 years.

Quality assurance
To ensure that this trial will be of a high standard and delivered in accordance with the trial protocol, all research staff will be provided with a standard protocol and case report form, and will be trained to competently administer items as per protocol. The investigators, research assistants and outcome assessors are different people. Protocols will not be altered during the study timeframe.

Outcome measures
The co-primary efficacy endpoints of the study will be MRI assessment of volume changes in knee cartilage from baseline to month 24, as well as the Western Ontario and McMaster Universities Index of OA (WOMAC) score [31] (Table 1). The secondary endpoints will be other knee structural changes (cartilage defects, tibial plateau bone area, and bone marrow lesions, meniscal tear and extrusion) from baseline to month 24, and lower limb muscle strength at months 3, 6, 12 and 24 (Table 1).

MRI assessment of knee structural changes
Knees will be imaged in the sagittal plane on a 1.5-T whole body MRI unit using a commercial transmit-receive extremity coil. Fat-saturated T1-weighted spoiled gradient echo (GRE) and T2-weighted/proton density-weighted fast spin echo (FSE) sequences will be used. The images will be assessed by two readers blinded to the treatment according to the methods described in our previous publications [15,32]. Cartilage volume: the volumes of individual cartilage plates (medial tibial, lateral tibial and patella) are isolated from the total volume by manually drawing disarticulation contours around the cartilage boundaries on a section-by-section basis. Sagittal images will be obtained at a partition thickness of 1.5 mm and an in-plane resolution of 0.31 x 0.31 mm (512 x 512 pixels), then resampled by means of bilinear and cubic interpolation (area of 312 μm x 312 μm and multiplied by 1.5 mm thickness, continuous sections) for the final 3D rendering. Particular cartilage volume was then determined by
Table 1 Timetable and measures to be made

<table>
<thead>
<tr>
<th>Measure</th>
<th>Screening</th>
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<th>3</th>
<th>6</th>
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</tr>
</tbody>
</table>

Participants who withdraw within one year will be asked to have MRI at month 12; patients who withdraw after one year will be asked to have MRI straight away. MRI: magnetic resonance imaging; WOMAC: Western Ontario McMaster Universities Osteoarthritis Index; 25-(OH)D: 25-hydroxy-vitamin D; FFQ: food frequency questionnaire.

summing all the pertinent voxels within the resultant binary volume.

Cartilage defects assessment: the cartilage defects (0 to 4) will be graded at medial tibial and femoral, lateral tibial and femoral, and patellar sites: grade 0, normal cartilage; grade 1, focal blistering and intracartilaginous low-signal intensity area with an intact surface and bottom; grade 2, irregularities on the surface or bottom and loss of thickness of less than 50%; grade 3, deep ulceration with loss of thickness of more than 50%; grade 4, full-thickness chondral wear with exposure of subchondral bone.

Knee tibial plateau bone area: the area of the medial and lateral tibial plateau bone will be measured manually on the three reformatted images closest to the tibial cartilage. An average of these three areas will be used as an estimate of the tibial plateau bone area.

Subchondral bone marrow lesions: This will be assessed on the T2-weighted MRI and defined as discrete areas of increased signal adjacent to the subcortical bone at the lateral, medial femur and/or tibia. Each bone marrow lesion will be scored on the basis of lesion size, for example, a lesion is scored as grade 1 if it occupies < 25% of the region; grade 2 if it occupies 25% to 50% of the region; or grade 3 if it occupies > 50% of the region.

Meniscal tear assessment: the menisci will be assessed in the sagittal view and confirmed in the coronal and axial views as previously described [33]. In brief, the presence or absence of a tear is based on the presence of a signal, which is line shaped, brighter than the dark meniscus, and reaches the surface of the meniscus at both ends within six defined regions (anterior horn, body, and posterior horn at both medial and lateral tibiofemoral compartments).

Meniscal extrusion assessment: the extent of meniscal extrusion on the medial or lateral edges of the tibial femoral joint space for the anterior, body, and posterior horns of the menisci will be graded, where a score of 0 = no extrusion, 1 = partial meniscal extrusion, and 2 = complete meniscal extrusion with no contact with the joint space.

Lower limb muscle strength
This will be assessed by dynamometry (TTM Muscle Meter, Tokyo, Japan) at the lower limb (involving both legs simultaneously). The muscles measured with this technique are mainly the quadriceps and hip flexors. The device will be calibrated by suspending known weights at regular intervals.

WOMAC
Knee pain will be assessed by both WOMAC pain subscale (walking on a flat surface, going up/down stairs, at night in the bed, sitting/lying and standing upright) and a 100 mm VAS.

Other measurements
Core musculature measure: Core muscle images will be taken at baseline and 12 months. Images of the core muscles (TrArb, internal oblique muscles and LM) are taken with real-time dynamic ultrasound using a fully featured big box diagnostic ultrasound machine (Phillips HDI 5000, Bothell, WA, US) with a hand held 7.5 mHz linear array transducer. Images are taken of right and left sides, both at rest and during contraction (drawing in of abdomen) using previously published protocols [34,35].
These measures have a high degree of reliability with an interclass correlation coefficient (ICC) > 0.90 across a range of studies [36].

Upper arm blood pressure, central blood pressure and aortic stiffness: Clinical upper arm blood pressure will be measured twice after 5 minutes seated rest using a validated device (Omron HEM-907, Kyoto, Japan). Seated clinical central blood pressure will be recorded (immediately after upper arm blood pressure) using radiacl application tonometry (SphygmoCor 8.1, AtCor Medical, Sydney, Australia). Aortic stiffness will be measured by electrocardiogram-gated, sequential carotid to femoral pulse wave velocity as per expert consensus [37].

Physical activity: Physical activity will primarily be assessed using a pedometer (SW 200 Digi-Walker, Yamax Corporation, Tokyo, Japan), which measures vertical displacement (steps per day). The pedometer will be worn for seven consecutive days on two occasions (baseline and 2 years) as up to seven days is required to accurately assess habitual physical activity [38]. We will also measure physical activity using the International Physical Activity Questionnaire (IPAQ) short version [39].

Body fat: Body fat will be assessed using bioelectrical impedance analysis (BIA) (BIA analyser, Quantum II, RJL Systems, Michigan, USA). Fat-free mass, % fat-free mass, fat mass and % fat mass will be assessed [40].

Hand grip strength: Hand grip strength will be assessed to the nearest kg in both the right and left hand using a hydraulic hand dynamometer (Saehan Corporation, Masan, Korea). Both hands will be alternately measured in triplicate.

Radiographic OA: this will be assessed at baseline by a standing semiflexed anterior-posterior (AP) radiograph as per the Altman atlas [29]. Radiographs will also be assessed simultaneously by two observers using the Osteoarthritis Research Society International (OARSI) atlas to score osteophytes and joint space narrowing on a four-point scale (0 to 3).

Laboratory measurements: serum 25-(OH)D will be assayed at month 0, 3 and 24, utilizing a Liquid Phase radioimmunoassay (Immunodiagnostics Systems Ltd, Boldon, Tyne & Wear, UK). Serum calcium, phosphate and renal function will be assessed at month 0 and 3 using routine biochemical methods.

Anthropometrics and other questionnaires: Height will be measured to the nearest 0.1 cm (with shoes removed) using a stadiometer (Leicester Height Measure, Invicta Plastics Ltd, Leicester, UK). Weight will be measured to the nearest 0.1 kg (with shoes and bulky clothing removed) using electronic scales (Heine S-7307, Heine, New Hampshire, USA). Waist and hip measurements will be assessed using a tape measure to the nearest 0.1 cm (Figure Finder Tape Measure, Novel Products Inc, Illinois, USA). Sun exposure, employment status and occupation, depression, smoking status, previous knee injury, dietary intake, low back and foot pain and quality of life will be assessed by questionnaires.

Safety assessments
Spontaneously reported adverse events will be recorded throughout the study. Intensity and relationship with the study medication will be ascribed.

Sample size
All sample size calculations assume α = 0.05 and β = 0.20 and are performed based upon formulae provided by Cohen [41]. Table 2 describes the sample size (each arm) needed to detect the specified differences between the placebo and vitamin D arms with at least 80% power for each outcome.

Previous studies, including our own, suggest that OA patients have a loss of cartilage volume of 4 to 5% per year at different joint sites, respectively [42]. Vitamin D supplementation in doses ranging from 400 to 800 IU/d increased the serum level of 25-(OH)D by 27 nmol/liter per year in 7,964 men and women from five studies [43]. We estimate from our published data [15] that this change will lead to absolute reduction in loss of cartilage volume by 2.2% at the medial tibial site after vitamin D supplementation. The sample size that is needed to detect this difference is calculated (Table 2).

We have shown that male OA patients have an increase in medial tibial bone area of 1.6 ± 2.8% per year [44], and an incidence of knee cartilage defects of 80% over 2 years [45]. There are no data known to the investigators about the associations between change in vitamin D and change in bone area or cartilage defects. However, healthy subjects have been shown to have an increase in tibial bone area of 0.7% per year (CD et al., unpublished), and an incidence of knee cartilage defects of 65% over 2 years in older people [46]. Assuming that changes in cartilage defects and bone area in OA patients will be suppressed by vitamin D supplementation to the levels in the healthy subjects, the sample size needed to detect these differences is given in Table 2.

Table 2 Sample size calculation

<table>
<thead>
<tr>
<th>Outcome</th>
<th>Mean (SD)</th>
<th>Detectable difference</th>
<th>Calculated sample size (per arm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Loss of volume of medial tibial cartilage</td>
<td>4.5% ± 6.5%</td>
<td>2.16%</td>
<td>143</td>
</tr>
<tr>
<td>Increase in medial tibial bone area</td>
<td>1.6% ± 2.8%</td>
<td>0.9%</td>
<td>153</td>
</tr>
<tr>
<td>Incidence of knee cartilage defects</td>
<td>80%</td>
<td>15%</td>
<td>136</td>
</tr>
</tbody>
</table>
Therefore, 200 patients in each arm (allowing for a 20% dropout over the trial) will be sufficient to detect the differences between treatment groups.

**Analysis plan**

Statistical primary comparisons for total and subscale WOMAC scores will be made using a repeated measures mixed model with terms for treatment, month, center and the corresponding baseline values as the covariates. The independent $t$-tests will be used to compare changes between groups in quantitative data from baseline to the end of follow-up. Linear regression (annual changes in cartilage volume, cartilage defects, bone area and muscle strength as the dependent variables, and treatment as the independent variable) and logistic regression (development/progression of bone marrow lesions and meniscal abnormalities as the dependent variables, treatment as the independent variable) analyses will be applied in univariate and multivariate modeling adjusted for age, sex, body mass index, baseline 25-\((\text{OH})\text{D}\) and other disease status.

In secondary analysis of loss in cartilage volume, the minimal clinically important differences (MCID) in cartilage volume will be calculated [47] and logistic regression will be used to determine the association between cartilage loss ($>\text{MCID}$ vs. $<\text{MCID}$) and treatment before and after adjustment for the covariates described above.

Both intention to treat and per protocol analyses will be utilized. Per protocol will be defined as achieving a 25-\((\text{OH})\text{D}\) level $>60$ nmol/liter at month 3. The last observation carried forward method will be used in the analysis of all outcomes among patients who made at least one follow-up visit but did not complete the whole study.

**Data integrity and management**

All data obtained will be kept strictly confidential and will be stored electronically on a database with secured and restricted access. Data transfer will be encrypted and any information capable of identifying individuals will be removed.

**Withdrawal**

If a participant withdraws or is removed from the study, the reason and date of discontinuation will be recorded. Any participant who withdraws within year 1 will be asked to have MRI at the end of year 1; participants withdrawing after year 1 will be asked to have MRI on leaving the study.

**Monitoring**

The trial will be overseen and monitored by a project manager. The project manager will visit each site to examine trial procedures to ensure data quality and compliance with the trial protocol.

**Discussion**

We have proposed this protocol to determine if vitamin D supplementation can slow disease progression in patients with knee OA. Vitamin D may have beneficial effects for the treatment of OA, although there are currently no recommended guidelines for this approach [48]. Hence, well-designed randomized controlled trials are required to test if vitamin D has disease-modifying and pain-relieving effects. Such studies also need an appropriate follow-up period to capture joint structural changes using objective measurements over the course of OA, and this has been incorporated into the design of the VIDEO study.

As suggested by the 2011 Endocrine Society Clinical Practice Guideline, all adults aged 50 to 70 years, and those over 70 years old require at least 600 and 800 IU/d of vitamin D respectively, to maximize bone health and muscle function. To raise blood levels of 25-\((\text{OH})\text{D}\) above 75 nmol/liter (the lowest sufficient threshold) requires at least 1500 to 2000 IU/d of supplemental vitamin D [52]. Thus, our study design provides a dose of 50,000 IU monthly to achieve serum 25-\((\text{OH})\text{D}\) levels above 60 nmol/liter in all compliant subjects [53]. This method will be less costly and will be more convenient than daily treatment. Toxicity is extremely unlikely with this dose.

Two sub-studies will simultaneously be included in this trial. Firstly, we will examine the effects of vitamin D on the function of the deep lumbo-pelvic stabilizing muscles. Besides implications for low back pain, in healthy people core muscles have also been implicated in varying aspects of physical function. The lateral abdominal muscles are theorized to control movement and provide stability to the trunk for functional activities and this is supported in a number of studies [54]. Vitamin D supplementation may have beneficial effects on...
functionally important core muscles. Secondly, we will determine the effect of vitamin D supplementation on blood pressure and aortic stiffness. A recent systematic review suggested that there is accumulating evidence to support the hypothesis that vitamin D deficiency contributes to hypertension, and randomized controlled trials (RCTs) are greatly needed to clarify and to definitively prove the effect of vitamin D on blood pressure [55]. The VIDEO study aims to be the first to assess this.

In summary, knee OA is a major, but poorly understood, public health problem. Vitamin D deficiency may play a role in the progression of OA, and based on our novel preliminary data, the VIDEO study has been designed to determine whether intervening with vitamin D supplementation can in fact slow the progression of this disease and relieve knee pain. If correcting vitamin D deficiency can reduce rates of cartilage loss to lower levels as seen in older people without OA, it will significantly prolong the time it takes to reach end-stage OA eventually necessitating joint replacement. This suggests great potential for substantial cost savings through reductions in joint replacement surgery, as well as potential for great improvements in the quality of life for people with OA. The success of this study will provide scientific evidence for using a cost-effective and innovative approach to addressing this clinically significant problem and will lend itself to an easy public health intervention.

**Trial status**

Upon submission, VIDEO study is in the process of patient recruitment.

**Abbreviations**


**Competing interests**

The authors declare that they have no competing interests.

**Authors’ contributions**

CD and GJ conceived the study, CD, GJ, FC, TW, AW, JS, KN and YC participated in its design and coordination, and performed the research. YC, GJ, KN and CD drafted the manuscript. All authors revised the manuscript and gave final approval of the version to be submitted.

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**References**

Summary of changes

Primary outcomes

Original: Loss of knee cartilage volume from baseline to month 24.

Final: Addition of change in knee pain assessed using the Western Ontario and McMaster Universities Index of OA (WOMAC) score.

MRI assessment of subchondral bone marrow lesions (BMLs)

Original: BMLs were scored as following:

- **Grade 1**: BMLs were present on 1 slice.
- **Grade 2**: BMLs were present on 2 consecutive slices.
- **Grade 3**: BMLs were present on >3 consecutive slices.

Final: BMLs were scored using a Modified Whole Organ MR Scoring (WORMS) system:

- **Grade 1**: BMLs occupy <25% of the region.
- **Grade 2**: BMLs occupy 25% to 50% of the region.
- **Grade 3**: BMLs occupy >50% of the region.

Statistical analysis plan

No change.