

Study protocol: A randomised, double blinded, placebo-controlled clinical trial testing the effects of a vitamin D-enriched mushroom supplement on cognitive performance and mood in healthy elderly adults

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Abstract

Background: Cross-sectional evidence suggests a positive relationship between vitamin D status and cognitive performance and mood; however, interventional clinical evidence is lacking. Vitamin D deficiency is prevalent in the elderly. If justified, supplementation offers a potentially cost-effective approach for maintaining cognition-dependent quality of life in aging populations. Exposure to UV light elevates the vitamin D content of mushrooms, which represent a novel and convenient source of dietary vitamin D (D₂). Here we present the protocol for a study to determine whether increasing vitamin D status improves cognitive function, mood and depressive symptoms in healthy older subjects.

Methods: The study is a double blind, placebo-controlled clinical trial with 400 healthy male and female subjects aged 60–90 years old. Subjects pre-screened for confounders of cognition but not vitamin D status, are randomised across four groups, receiving daily supplement treatments in parallel over six months; either: vitamin D₂-enriched mushroom solids, vitamin D₃ alone, standard mushroom solids, or placebo. Primary endpoints are: changes in serum 25-OH-D₂ and 25-OH-D₃ metabolites and general cognitive performance. Secondary endpoints include mood and depressive symptoms. Data analysis will adjust for covariate measures. Blood samples taken at the three clinic visits (baseline, 5 weeks and 6 months) will be stored.

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Introduction

Vitamin D, or calciferol, is a steroid hormone comprised of a group of fat-soluble seco-sterols. The two major forms are vitamin D₂ (ergocalciferol) and vitamin D₃ (cholecalciferol), which require activation *in vivo* by a series of enzyme-controlled steps in different tissues. Vitamin D₂ is primarily produced by ultraviolet (UV) irradiation of ergosterol, a sterol

found in fungi and plants [1]. Vitamin D₃ is synthesised through the action of UV-B radiation on 7-dehydrocholesterol in the skin and, since most adults are unlikely to obtain more than 5–10% of their vitamin D requirement from dietary sources, is generally considered to be the main determinant of vitamin D status in Australia [2]. Dietary sources of vitamin D₃ include oily fish, egg yolks and meat, while vitamin D₂ can be sourced from UV-treated

mushrooms, for which availability in the marketplace is growing. In Australia, the synthetic form of vitamin D used in supplements and food fortification is predominantly vitamin D3.

The conversion of vitamin D2 and vitamin D3 to the biologically active form, calcitriol (1,25-dihydroxyvitamin D), involves two enzymatic hydroxylation reactions in the liver and kidneys, respectively. The precursor of calcitriol, 25-hydroxyvitamin D (25-OH-D), is the major circulating form of vitamin D and serum levels of this metabolite are measured as an indicator of vitamin D status in the body [3].

Vitamin D directly and indirectly regulates more than 200 genes and exerts multiple bioactive roles including as a hormone, anti-inflammatory agent and regulator of cell growth. All tissues and cells contain a receptor for vitamin D that recognises the active form: 1, 25-hydroxyvitamin D [4]. In particular, 1 α -hydroxylase, the enzyme responsible for producing the bioactive form of vitamin D, and the 1,25-dihydroxy vitamin D3 receptor, are both found throughout the human brain [5].

Current recommendations for vitamin D adequacy are associated with requirements for skeletal maintenance. Calcium and vitamin D are essential nutrients for regulating calcium absorption and bone mineral density. The recommended daily allowances (RDA) for vitamin D, as defined for bone health, are 600 IU (15 μ g)/day up to 70 years of age, and 800 IU/day over 70 years of age [6]. These values are supported by the Endocrine Society [7]. Consensus of the Australian and New Zealand Bone and Mineral Society, Endocrine Society of Australia, Osteoporosis Australia and others have defined adequate vitamin D status for mineral homeostasis, bone health and muscle function as serum levels of 25-OH-D \geq 50 nM at the end of winter [8]. On the other hand, the Endocrine Society defines adequacy as being above 75 nM, insufficiency in the range of 52.5–72.5 nM and deficiency below 50 nM [6, 7]. For prevention of other diseases including cardiovascular disease, hypertension, colon and breast cancer, and multiple sclerosis, target levels of 25-OH-D may need to be higher, in the range of 75–100 nM; however, the evidence to support this remains limited [9]. Similarly, interventional evidence of vitamin D

adequacy for maintaining cognitive health is lacking [10]. Of concern is that vitamin D deficiency is widespread among people who are at risk of certain diseases, particularly osteoporosis, hyperparathyroidism, cancer, type 2 diabetes and auto-immune and cardiovascular diseases. Vitamin D deficiency is itself a risk factor for multiple chronic diseases [11]. It is estimated that 31% of all adults in Australia have vitamin D deficiency, and that it is even more prevalent in those aged over 60 years [12].

In recent years, several studies have identified associations between low vitamin D levels and cognitive decline, Alzheimer's disease (AD) and dementia, particularly in elderly populations. A recent prospective study reported that older adults with 25-OH-D levels \geq 50 nM had substantially decreased risk of all-cause dementia and AD, compared to 25-OH-D levels \leq 50 nM [10]. However, systematic reviews containing meta-analyses of like cross-sectional studies [13–18] have concluded that the consistent association between low serum 25-OH-D levels and diminished cognitive function should not be interpreted as a causal relationship, rather that well designed, randomised controlled trials are required to confirm any relationship between vitamin D and cognition. A recent systematic review of effects of vitamin D supplementation on depressive symptoms suggested that a moderate, statistically significant benefit was evident for those with clinical depression, but it was not significant in non-depressed individuals [19]. As for the putative benefits of vitamin D for cognition, more conclusive clinical evidence is required.

The bioavailability of vitamin D2 from UV-B irradiated mushrooms, and mushrooms' ability to raise and maintain 25-OH-D levels similar to those achieved by supplements, has been confirmed through several small clinical trials dosing at 400 IU to 2000 IU/day, from durations of 4 weeks to 3 months [20–23]. Furthermore, several clinical trials of healthy adults have shown that supplementation with vitamin D2 induced downward adjustment of the level of 25-OH-D3, proportional to the increase in 24-OH-D2, with no overall change in total vitamin D status [22, 24]. Superior effects on cognitive performance were demonstrated in mice treated with vitamin D2 mushrooms, when compared with mice having equivalent serum levels of 25-OH-D3, in both wild

type (healthy) and AD transgenic phenotypes [25]. The present study aims to investigate whether these findings are replicable in healthy humans. Furthermore, the presence of other bioactive factors in mushrooms including: polysaccharides, protein, amino acids, ergothioneine and minerals, particularly for regulation of inflammation [26], may also contribute to the hypothesized benefits of vitamin D2 mushrooms on cognition.

The aim of the present clinical trial is to determine whether increasing vitamin D status by consumption of either vitamin D2-enriched mushrooms or vitamin D3 alone will have an effect on cognitive function, mood and depressive symptoms, compared to placebo. We hypothesise that taking the daily requirement of vitamin D2 via mushroom solids for six months will have a greater effect on cognitive function, mood and depressive symptoms than the same daily dose of vitamin D3. We report the trial design and methodology that will be used herein.

Methods

Study design and location

The study is a randomised, double blind, placebo-controlled clinical trial with four parallel treatment arms. Healthy participants aged 60–90 years old will be recruited from the community by advertisement. Following screening, 600 participants will be enrolled in the study and block-randomised into four treatment groups using a computer-based randomisation model. The four study arms will be equally weighted with regards to age and gender. The clinical intervention will be conducted between April and October when the potential for environmental vitamin D exposure is lowest for Australians living in southern latitudes. For logistical reasons, the study will be conducted over the same period in two successive years, each involving half of the total participant pool ($n = \sim 300 \times 2$).

For the duration of the six-month intervention period, participants will ingest two capsules daily, containing one of the following: vitamin D2-enriched mushroom (100 mg mushroom solids, 300 IU of vitamin D2); vitamin D3 (300 IU), standard mushroom (100 mg control mushroom solids), or placebo (carrier alone). The capsules will be sealed in bottles containing 180

capsules per bottle, together with a desiccant pouch, and stored at 4°C for the duration of the study, either at the Commonwealth Scientific and Industrial Research Organisation (CSIRO), Canberra, Australia, or by participants. Participants will attend the CSIRO Clinical Research Unit at the South Australian Health and Medical Research Institute (SAMHRI) at baseline, five-week and six-month time points. At these visits, participants will undergo cognitive testing, complete questionnaires and donate blood. At the five-week visit, a saliva sample will be collected for DNA analysis. A summary of the biometric and biochemical measures used for the study is shown in Table 1. Compliance will be measured by counting the number of returned capsules at the end of the study and by participant interviews during the scheduled clinic visits.

CSIRO's Food and Nutritional Sciences (CFNS) Human Research Ethics Committee (HREC) has approved the study protocol (reference: F&N-HREC-13/05). All participants will provide informed written consent. All clinical procedures will be conducted in accordance with the National Health and Medical Research Council (NHMRC)'s National Statement on Ethical Conduct in Research Involving Humans (2007) and good clinical practice guidelines. The trial has been registered with the Australian and New Zealand Clinical Trials Registry: ACTRN12613000891729.

Participants and recruitment

Study participants will be aged 60 years and over because this age group is at the highest risk of cognitive decline with associated changes to mood and depressive symptoms [27]. In addition, vitamin D deficiency tends to be more prevalent in this group [12]. In the target study population, > 30% are expected to be vitamin D deficient and > 95% without detectable levels of vitamin D2 metabolite (unpublished data). Ethical issues preclude the possibility to pre-screen and select participants with low vitamin D status because of obligation to treat all people whereas the study design will only permit 2/4 groups to receive vitamin D.

Table 1. Summary of biometric and biochemical measures and testing schedule for each year of the study

Outcome measures and covariates	Testing time point (week)			
	~ -12	0	5	24
Standard medical questionnaire	X			
Height	X			
Weight	X	X	X	X
Blood pressure	X			
OSA50 questionnaire	X			
MMSE	X			
CESD	X	X	X	X
MBA20	X			
Vitamin B12	X			
TSH	X			
HbA1c	X			
Medications, supplement use, adverse effects and health changes	X	X	X	X
Vitamin D		X	X	X
Cognitive assessment battery		X	X	X
Questionnaires (various)		X	X	X
DNA genotyping (vitamin D, B-group vitamins, APOE4, inflammatory cytokines)			X	

Participants will be recruited using social media, newspaper advertisements, advertisements at public libraries, television news interest stories, South Australia electoral roll data, collaboration with the Council of the Aging (COTA; www.cota.org.au) and existing participant databases within CSIRO.

Inclusion criteria

Healthy males and females aged 60–90 years of age; fluent in the English language (for valid completion of

the cognitive test battery); not taking any form of vitamin D supplementation for at least three months prior to the study, or any additional supplementation during the study.

Exclusion criteria

Exclusion assessment for the following criteria will be self-reported and include: those already taking vitamin D supplements; an inability to swallow tablets; physical inability to attend the SAHMRI clinic; or inability to follow basic cognitive testing instructions. Persons possessing any of the following confounding factors affecting cognitive status will also be excluded: diagnosis of intellectual disability, dementia, or neurological disorder including but not limited to cerebral vascular disease; previous head injury, stroke, or coronary artery bypass or neurosurgical procedure; history of smoking, alcohol or drug abuse; metabolic disease including diabetes; untreated asthma; shift workers; people who habitually sleep < 6 hours per night; untreated obstructive sleep apnoea (as determined by a medical doctor); untreated depression (unless taking SSRI medication and stabilised for > 6 months); abnormal thyroid function; abnormal vitamin B12 status; history of psychosis and/or taking anti-psychotic medication; or history of epilepsy and/or taking anti-epileptic medication. Persons possessing any of the following confounding factors affecting vitamin D status will further be ineligible to participate in the study: kidney disease or kidney function impairment, and/or a gastro-intestinal condition that interferes with nutrient absorption; sensitivity to or intolerance of consuming mushrooms.

Screening

Screening will occur up to three months prior to the beginning of each year of the intervention. Participants will be asked to complete a standard medical questionnaire, The Centre for Epidemiological Studies-Depression Scale (CES-D) questionnaire, and the obstructive sleep apnoea (OSA) 50 questionnaire, to determine their eligibility. Participants will present to the CSIRO Clinical Research Unit for height, weight and blood pressure measures, basic cognitive impairment testing using

the mini-mental state examination (MMSE) test [28], and to donate a blood sample for the determination of HBA1c, thyroid stimulation hormone (TSH), basic biochemistry using the MBA20 suite of tests, and vitamin B12 status. Those scoring > 16 on the CES-D and/or < 24 on the MMSE will be ineligible for the study. An officiating medical doctor will identify any persons at risk of untreated OSA; such persons will be ineligible for the study and referred to their general practitioner for further tests and diagnosis.

Adverse events

The following protocol for documenting and reporting serious adverse events (SAE), whether causally related to the treatment or not, was approved by the CSIRO HREC:

- Study participant reports SAE to a staff member
- Details of the SAE are documented by that staff member on the participant's case report form (CRF), while the participant is present.
- Medical Officer is immediately notified of all SAEs (by scanning and emailing, or showing him/her the CRF) for his/her assessment of the study participant.
- CRF is also taken to the Trial Manager to advise them of the SAE. The SAE must be documented in the study's master data file on the same day. Follow up of the SAE is the responsibility of the Trial Coordinator for that study, or as instructed by the Medical Officer until the SAE is resolved.
- SAE must be reported to the Chair of the CSIRO HREC within 24 hours; the Trial Manager will email a scanned copy of the completed SAE form directly from the CRF to the CSIRO HREC Secretary (fnshrec@csiro.au).
- Information about any AE is sent to the Trial Manager at the end of a study, but information about any SAE is sent to the Medical Officer as they occur, and to the HREC secretary.

Power and statistical analysis

Power analyses were based on the Multivariate Repeated Measures Anova (MANOVA) procedure and conducted using G*Power v3.1.7 (Heinrich Heine

University, Düsseldorf, Germany). Recruitment of 100 participants per group is calculated to yield power of 95% to detect a small overall interaction Time × Intervention effect size of $f = 0.15$. Accounting for a high drop-out rate of 25%, there will remain 80% power to detect an interaction effect size of $f = 0.15$. In the event of significant main or interaction effects, then post hoc pair-wise comparisons will be performed. With $n = 100$ per group, there will be 80% power to detect an effect size of 0.4 (Cohen's d) between any two groups. Allowing for attrition (~25%) there will remain 80% power to detect a medium effect size of ~0.45 (Cohen's d).

A biostatistician will perform statistical analysis of trial data. Repeated Measures Analysis of Variance and/or Linear Mixed Models will be used to assess the effect of the treatments within the intervention. Significant interaction effects will be investigated post hoc and adjusted for multiple comparison using the Bonferroni correction, which permits adjustments for multiple covariates.

Preparation and composition of vitamin D-enriched and control mushrooms

Mushrooms (*Agaricus bisporus*, Button variety) will be sanitised using established protocols for fresh vegetables so as to meet Australian food standards for human consumption with respect to chemical residues and microbiological safety. Freeze-drying enriches the solids approximately 10-fold compared with fresh mushrooms; after powder grinding, the product contained < 5% moisture and water activity of < 0.065. Permitted levels of non-pathogenic microorganisms for sanitised fresh vegetables were highly stable in dried solids form under refrigerated storage throughout the trial.

The vitamin D₂ loading of mushrooms is achieved using UV light to drive the conversion of ergosterol to vitamin D₂ in dried mushroom powder. After sanitising and rinsing equipment with de-ionised water, UV treatment is conducted using a light-proof box assembled with an array of six parallel UV-B tubes (Phillips, PRP TL 40 W, NSW, Australia), as previously described [29]. Batches of mushroom solids in ground powder form are exposed to UV-B light, before vacuum sealing and storage at 4°C,

pending encapsulation. Typical proximate and mineral composition of the vitamin D2-enriched mushroom solids has been reported previously [25]. The vitamin D2 concentration of the UV-treated mushroom solids was 100 mg/kg, measured by liquid chromatography–mass spectrometry (LC-MS), as described previously [25].

Dried mushroom solids, either vitamin D2-enriched or standard, vitamin D3 and placebo will be formulated with suitable excipients and sealed in ‘Vegi-cap’ capsules made from plant cellulose..

Outcome measures

Primary outcome measures include: changes in vitamin D2, vitamin D3 and total vitamin D status and change in cognitive performance as measured by the CSIRO Cognitive Assessment Battery (C-CAB). Secondary outcome measures include mood, measured by a series of validated questionnaires detailed in Table 2. In addition to primary and secondary outcomes, a range of additional measures, described below, will be collected for potential inclusion as covariates during data analysis.

Vitamin D status

Vitamin D status (25-OH-D level in serum) may be elevated by vitamin D supplementation but understanding of the independent and interacting effects of age, gender, baseline status, type of vitamin D and dosing efficacy is limited. The recommended daily intake (RDI) of vitamin D of 600 IU is reported to elevate deficiency (<75 nM 25-OH-D) in both younger and older adults to sufficiency (≥75 nM) over 4–6 months of supplementation [30]. For example, in an Irish study, supplementing healthy young adults with 600 IU vitamin D3 per day, for eight weeks over winter months, caused a significant elevation of vitamin D status from 48 nM to 87 nM [31].

Table 2. Questionnaire measures summarised by task description and time point of collection

Questionnaire	Description	Time point (week)			
		~12	0	5	24
Mini Mental State Examination	Screening tool used to identify dementia or cognitive impairment	X			
Centre for Epidemiology Studies Depression Scale	Screening tool used to identify clinical depression	X			
Positive and Negative Affect Schedule	Twenty items measuring current positive and negative Affect states	X	X	X	X
Depression, Anxiety and Stress Scale (DASS) 21	Twenty-one items measuring depression, anxiety and stress levels during preceding week	X	X	X	X
General Happiness Questionnaire	Four items measuring overall happiness	X	X	X	X
Sun Exposure Questionnaire	Quantitative measure of time spent outdoors and skin exposed for preceding seven days	X	X	X	X
Prospective and Retrospective Memory Questionnaire	Sixteen-item self-evaluation of memory deficits		X		
Computer Use Questionnaire	Four items measuring use of computers	X			
Brain Training Questionnaire	Five items measuring participation in brain training tasks		X		

In the Netherlands, supplementing elderly adults with 600 IU/day vitamin D achieved elevation from deficiency to sufficiency for 37% of the participants after four months, with a group mean of 70 nM [32]. High (2.5–80 × RDI) daily, weekly or monthly doses of vitamin D in elderly subjects produced proportional increases in vitamin D status depending on duration of intervention [30]. However, the reverse J-shaped relationship between vitamin D status and all-cause mortality [33] suggests that there may be risks associated, not only with vitamin D deficiency, but also with significantly elevated storage vitamin D. The current study therefore adopted the RDI of vitamin D intake (600 IU), with the expectation that the greatest supplementation effect would be seen in those with lower baseline vitamin D status. However, capacity to utilise oral vitamin D could be adjusted for by participant genotyping with respect to regulation of vitamin D metabolism.

Cognitive performance

The C-CAB will be used to measure cognitive performance across a range of distinct cognitive abilities (Table 3). Abilities measured are those considered most susceptible to change in response to normal aging or other intervention, and are commonly included in batteries designed to measure the impact of nutraceuticals on cognitive performance [34-37]. To ensure sufficient sensitivity to detect any subtle changes caused by the intervention, all tasks emphasise the need to respond quickly, with participant response times measured at the millisecond scale.

The C-CAB is a fully automated assessment battery that guides participants through tasks in an identical sequence across study time points. This procedure is employed by similar assessment batteries used in this field, including the computerised Cognitive Drug Research (CDR) assessment system [38] and the Swinburne University Computerised Cognitive Assessment Battery (SUCCAB) [36]. Up to four participants are supervised by a trained assessor. At the start of the automated battery at session 1, participants undertake response key training in order to familiarise themselves with the responses required throughout the battery.

At subsequent sessions, participants choose whether or not to complete this component. For each subsequent cognitive task, participants receive a comprehensive set of standardised task instructions, as well as a series of practice trials, where practicable, to ensure they understand task requirements. Trained assessors are available to answer queries and assist participants at all times.

The C-CAB takes approximately 1 hour and 15 minutes to complete on each occasion. The battery consists of 16 individual tasks. Each task is designed to measure at least one distinct cognitive construct, and each construct is measured by at least two tasks, except for Long-term Retrieval, which is measured by only one task at each time point. The tasks comprising the C-CAB and the cognitive abilities that they measure are outlined in Table 3.

Mood and depressive symptoms

Several standardised questionnaires will be used to assess changes in mood and depressive symptoms during the study. At each session, participants will complete a paper-based questionnaire before they commence the C-CAB. An overview of the measures administered and their corresponding time points is provided in Table 2.

Covariate measures

Many factors are positively and negatively correlated with cognitive function and mood status, and rate of change during aging. Longitudinal changes strongly implicate relationships between early life conditions and health outcomes, including mental health, in later life in low and middle income countries [39]. In general, cognitive capacity declines with aging but the rate of decline may be modulated by genetic and environmental factors. The interactions of these factors, which are most likely different for each individual, may make identification of the relative importance of a specific factor difficult. Nevertheless, apart from age, there are several factors with an established relationship to cognitive capacity and performance status, including: education (typically correlated with income and socio-economic status), *APOE* genotype, cardiovascular health (typically

correlated with BMI and chronic inflammation), exercise and dietary intake patterns [40]. Relevant baseline biometric data for trial participants will be included as covariates when correlated with primary and secondary outcome measures (potential confounding variables).

Table 3. Cognitive measures, domains characterised and method of scoring

Task	Description	Measure	Cognitive factor/ability				
Symbol digit	Quickly code target symbols with numbers using a code table	Number correct (<i>N</i>) [#]	Processing speed	Symbol memory scanning	Decide quickly whether a target symbol was shown in a preceding symbol series	Response time (<i>ms</i>) Number correct (<i>N</i>)	Speed of memory Scanning Quality of memory
Number comparisons	Compare two number strings and determine as quickly as possible whether they are the same or different	Number correct (<i>N</i>)	Processing speed	Letter memory task 1	Memorise five letters and decide on subsequent trials whether the displayed letter matches a memorised letter	Response time (<i>ms</i>) Number correct (<i>N</i>)	Verbal working memory Quality of memory
Finding letters	Decide whether presented words contain the letter A, or not, as quickly as possible	Number correct (<i>N</i>)	Processing speed	Letter memory task 2	Memorise eight letters and decide on subsequent trials whether the displayed letter matches a memorised letter	Response time (<i>ms</i>) Number correct (<i>N</i>)	Verbal working memory Quality of memory
Two-choice reaction time	Respond as fast as possible to left or right on-screen arrows	Response time (<i>ms</i>)	Reaction time Attention	Spatial memory task 1	Memorise a picture consisting of open and closed doors and compare subsequent pictures as the same or different	Response time (<i>ms</i>) Number correct (<i>N</i>)	Spatial working memory Quality of memory
Four-choice reaction time	Respond as fast as possible to left, right, up or down on-screen arrows	Response time (<i>ms</i>)	Reaction time Attention	Spatial memory task 2	Memorise a picture consisting of open and closed doors and compare subsequent pictures as the same or different	Response time (<i>ms</i>) Number correct (<i>N</i>)	Spatial working memory Quality of memory
Odd man out – lights	Quickly determine whether the odd-light-out is located to the left or right of the stimulus	Response time (<i>ms</i>) Number correct (<i>N</i>)	Speed of reasoning	Word recognition – delayed	Decide whether words shown match words learnt approximately 20 minutes earlier	Response time (<i>ms</i>) Number correct (<i>N</i>)	Short-term recognition Memory Quality of memory
Odd man out - shapes	Quickly determine whether the odd-shape-out is located to the left or right of the stimulus	Response time (<i>ms</i>) Number correct (<i>N</i>)	Speed of reasoning	Face recognition – delayed	Decide whether faces shown match faces learnt approximately 20 minutes earlier	Response time (<i>ms</i>) Number correct (<i>N</i>)	Short-term recognition Memory Quality of Memory
Number memory scanning	Decide quickly whether a target number was shown in a preceding number series	Response time (<i>ms</i>) Number correct (<i>N</i>)	Speed of memory Scanning Quality of memory	Word endings	Free recall of words ending with a specific letter set such as ‘ate’.	Number correct (<i>N</i>)	Long-term retrieval

[#]*N* = Raw number of items; **ms* = millisecond

Genotyping for vitamin D metabolism

Study participants will be genotyped for three genes with a proven relationship to vitamin D status: *DHCR7*, *CYP2R1* and *GC*. *DHCR7* encodes 7-dehydrocholesterol reductase, which converts 7-dehydrocholesterol to cholesterol, regulates its conversion to vitamin D, and its subsequent absorption. *DHCR7* occurs in three genotypes (TT, GT, GG), are associated with normal, moderate and high risk of vitamin D deficiency, respectively. *GC* encodes a vitamin D-binding protein that is required for circulatory transport. *GC* occurs in three genotypes [AA, AC, CC), which are associated with normal, high or higher risk of vitamin D deficiency, respectively. Association between *GC* genotype and change in vitamin D status is different for vitamin D3 versus D2 supplementation [41]. Therefore, genotype associations with vitamin D2 status may not necessarily correlate with patterns for vitamin D3, but may account for observations of differences in elevation of vitamin D status by equivalent supplementation doses of vitamin D2 versus D3 [1]. *CYP2R1* encodes a vitamin D 25-hydroxylase; it is the kidney enzyme responsible for the first step in the conversion of 25-hydroxy-vitamin D towards its active form, 25-OH-vitamin D. *CYP2R1* occurs in three genotypes (AA, AG, GG), which are associated with normal, moderate or high risk of vitamin D deficiency, respectively. These genetic markers will be used as covariates to determine capacity for vitamin D status to be elevated by respective supplement treatments and comparisons between changes in status for vitamin D2 versus D3.

Genotyping for regulation of inflammation

Chronic disease conditions associated with chronic inflammation, such as metabolic [42], cardiovascular and kidney diseases [43], are also associated with vitamin D deficiency. Vitamin D receptor and 1-alpha-hydroxylase functions are expressed by many immune system cells and support a direct role for these cells in both the translation of vitamin D to its active form [44], and to signalling and amplification of innate and adaptive immune system responses.

Regulation of inflammation in diseases of aging is important, therefore participants' genotypic

characteristics – with respect to 2 important signalling cytokines, IL-6 and TNF- α will be assessed. Specific genotypes of these cytokines are known to regulate circulating levels and therefore the status of chronic 'baseline' inflammation. For the *IL-6* gene, three genotypes (CC, CG, GG) are associated with normal, normal and elevated risk of higher circulating levels of IL-6, respectively [45]. Although the C allele is not associated with higher IL-6, it may be associated with higher levels of C-reactive protein, which is a delayed response inflammatory mediator. For the *TNFA* gene, three genotypes (GG, AG, AA) are associated with normal, elevated and elevated risk of higher circulating levels of TNF- α , respectively [46]. These genotypes will be used to account for magnitude of benefit or the opposite, to cognition and mood measures, from vitamin D supplementation.

Genotyping for APOE

The *APOE* gene consists of three major allelic variants: ϵ 2, ϵ 3 and ϵ 4. The APOE- ϵ 4 allele is a known risk factor for cognitive decline and late onset AD, despite its relatively low allelic frequency (~15%) [47, 48]. The rare ϵ 2 allele (~7%) is thought to be protective against AD [49]. It has been reported that memory status and serum vitamin D concentrations may be modifiable by *APOE* genotype [50]. Carriers of two APOE- ϵ 4 alleles appear to possess higher memory function with higher vitamin D status than those with zero or one APOE- ϵ 4 allele [50]. These data are from a single study and more evidence is required to determine any true relationship between cognition, vitamin D status and *APOE* genotype.

Genotyping for folate and co-factors

B-group vitamins including B2 (riboflavin), B6, B9 (folate) and B12 (cobalamin) are essential for critical cellular processes such as red blood cell production, DNA repair and replication. Impaired function owing to altered genetic regulation or deficiency of the B-group vitamins predisposes to amplification of age-related and chronic diseases [51]. In particular, B12 is an essential cofactor for methionine synthase to convert homocysteine to methionine, the precursor of the methyl donor S-adenosylmethionine [52]. When

B12 is deficient, homocysteine accumulates in cells and in the blood [52-54]. Higher levels of plasma homocysteine are associated with increased risk of cardiovascular and neurodegenerative diseases, including mild cognitive impairment and AD [53, 54]. Furthermore, the high affinity vitamin D receptor acts as a ligand-activated transcription factor of the *cystathionine β -synthase* gene, suggesting a role for vitamin D in homocysteine metabolism [55].

Recently, in an epidemiological study of 14,630 asymptomatic adults aged ≥ 18 years, an inverse relationship between plasma homocysteine and vitamin D status was reported for those with low baseline vitamin D [56], inferring a potential benefit for regulation of homocysteine by vitamin D supplementation. In addition, cognitive decline in people aged ≥ 65 years over a 10 year period was independently associated with B12 and homocysteine status [57]. A prospective study of 107 community-dwelling volunteers aged 61–87 years without cognitive impairment at enrolment, showed that decrease in brain volume was greater among those with lower serum vitamin B12 and higher plasma homocysteine levels at baseline [58]. A cross-sectional analysis of vitamin D status in 387 participants from the EPOCH study observed that 44% were deficient in vitamin D (< 75 nM), and there was an inverse correlation between homocysteine status and cognitive performance [59]. Genetic markers of the regulation of folate and its cofactors will be used to account for relationships between changes in vitamin D status and cognitive function. Blood samples will be taken and stored for later analysis of B12 and homocysteine to verify significant observations.

Sunlight exposure

The contribution of environmental vitamin D to changes in participants' vitamin D status will be accounted for in several ways. Firstly, the placebo treatment group will permit evaluation of changes in vitamin D status occurring over the study period (winter seasons of 2014 and 2015), which are independent of oral vitamin D intake (subjects are asked to avoid vitamin D supplementation during the study). Based on findings of changes in vitamin D status for a similar participant pool in the EPOCH

study [37], it is expected that vitamin D status will remain stable for men, but for women in the placebo group may decline over winter months [59]. Secondly, the participants will complete a seven-day recall questionnaire at each of the three clinic visits, from which habitual opportunity for exposure to sunlight will be quantified, including natural skin pigment. The usefulness of the seven-day recall measure in predicting vitamin D status was demonstrated in a study of healthy Italian adults, in which individuals' sun exposure scores in summer months correlated with sun exposure scores in winter months [60]. However, when relative contributions of oral and environmental intakes of vitamin D are evaluated, it appears that oral forms of vitamin D, either D2 or D3, suppressed utilisation of environmental D3 [24, 61], presumably because fewer bioconversion steps are required for the absorption of oral vitamin D versus 7-dehydrocholesterol. Therefore, oral and environmental intakes of vitamin D are not additive, and may reflect individuals' bio-utilisation efficiencies owing to health and genetic factors. Questionnaire data will be used to derive an index of environmental vitamin D intake and assess its contribution to observed changes in vitamin D status between study groups.

Discussion

Receptors for the pleiotropic hormone vitamin D are present on over 37 cell types and are involved in the regulation of more than 200 genes [62]. The prevalence of vitamin D deficiency is concerning, especially the higher incidences seen in at-risk sub-populations such as those living south of 35° latitude, pregnant women or those who wear a veiled habit and people who otherwise have low exposure to sunlight [12]. Some of these factors can converge for the elderly, particularly in association with declining cognitive function. Epidemiological studies consistently report positive associations between vitamin D status and cognitive performance, and also mood [17], but causal evidence is lacking. The scientific literature calls for well-designed, adequately powered intervention studies to inform understanding of the importance of vitamin D status as a risk factor and treatment biomarker for managing cognitive decline in the elderly [15].

The protocol for the clinical study presented here addresses this need and describes a controlled design comparing vitamin D2 and D3. The study also addresses that, in addition to the usual supplemented form of vitamin D (D3), vitamin D2-enriched mushrooms offer an unprecedented and convenient source of vitamin D2 in a matrix of additional bioactive compounds. These bioactives include polysaccharides, proteins, amino acids, ergothioneine and minerals; which are also associated with a wide range of health benefits [26]. It is possible that this micronutrient cocktail might provide synergistic benefits in combination with vitamin D2, thus the study design permits the effect of vitamin D2 mushrooms to be resolved from standard mushrooms.

The double-blind intervention study will target healthy, elderly adults (60–90 years), receiving one of four treatments including placebo. A comprehensive battery of cognitive performance and mood measures will be undertaken at baseline, five-week and six-month time points. The study will quantify a broad range of co-variates of vitamin D status (e.g., sunlight exposure, metabolic genotypes of vitamin D, APOE4 and inflammation) and cognition (age, gender, ethnicity, physical activity, education, income, sample date c.f. season, body mass index, smoking, alcohol consumption, cardiovascular conditions, diabetes, homocysteine, unmanaged depression, medications for depression).

Notwithstanding the multi-causal and gradual nature of cognitive decline in aging, this study is designed to test effects of supplementation with two types of vitamin D, and appropriate controls, to ascertain whether any correlation exists between changes in vitamin D metabolite status and changes in cognition and depressive symptoms. By focusing on a ‘healthy’ elderly population and avoiding pathological confounders of cognition and mood, the study has maximal potential to detect whether or not elevation of vitamin D status is specifically correlated with measurable effects on cognition and mood. The study is limited by the inherent physiological heterogeneity of decline in the elderly, but data will be adjusted for a set of biometric, biochemical and genetic covariates in order to isolate vitamin D-specific effects. The trial duration is of adequate length to alter vitamin D status and it is not known if this will translate to measurable

effects on cognition and mood within the same timeframe.

The results of this trial will be the first to report on the effects of vitamin D2 in its natural mushroom matrix, compared with synthetic vitamin D3 and controls, on cognition, mood and depressive symptoms. The results will be relevant and generalisable to healthy elderly populations, according to the study inclusion criteria. If successful, the data may justify the potential benefits of vitamin D supplementation and possibly demonstrate specific benefits of vitamin D2 sourced from mushroom, or synergistic effects of mushroom vitamin D2 taken with other components of mushroom, for promoting cognitive health in the aging population.

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