Mushrooms, Vitamin D and Cognition – Human Studies

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Summary

This project aimed to increase understanding of the role of vitamin D in cognition and mood, and in particular, to undertake a clinical study investigating the effects of vitamin D2 in mushrooms which represent a convenient dietary source of vitamin D and also contain a range of other bioactive micronutrients.

Both vitamin D status and cognitive function decline with ageing but the dependence of the relationship is associative in nature, lacking direct causal evidence. It is necessary to determine if in fact the high prevalence of vitamin D deficiency in elderly people reflects poor nutrition and lower vitamin D uptake as a result of declining cognition (ie, reverse causality) and not the opposite.

The international consensus is that randomised clinical trials are needed to test for causal effects of vitamin D on regulating cognition and mood. These studies need to examine vitamin D supplementation for *preventing* cognitive decline in cognitively healthy elderly people and also as a potential therapy for slowing cognitive decline when cognitive impairment is present. Our focus was on vitamin D2 mushroom efficacy for either cognitive improvement or preventing decline in healthy people, representing a challenging but worthwhile aim in preventative health.

In work package 2 of the project, we showed positive relationships between vitamin D status and selected brain functions for a cognitively-healthy older study group (65-90 years). This supported a link between vitamin D and healthy brain function and justified progressing to a clinical intervention study comparing efficacy of vitamin D mushroom supplementation on cognition and mood in a similar study group.

We conducted a randomized, placebo-controlled clinical study (RCT) to investigate if vitamin D2-enriched mushrooms, compared with vitamin D3 supplementation, standard mushrooms and placebo, improved cognition in a cohort of healthy older people. Additional measures included effects of vitamin D intake on measures of mood and depression and Amyloid Precursor Protein (APP). APP is the precursor for amyloid beta peptide, the primary component of amyloid plaques found in the brains of AD patients. When comparing effects of treatments in the RCT, the results suggested no notable effects of vitamin D supplementation for improving mood but evidence for some benefits of vitamin D2-enhanced mushrooms for improving cognitive function in particular genetically-defined sub-groups.

In sub-groups defined by genotypes relating to vitamin D bioavailability, inflammation and Alzheimer's disease (AD) risk, significant improvements were observed for some cognitive domains, namely, Recognition Speed, Reaction Time, Memory Scanning and Spatial Working Memory. However, these

effects were generally related to micronutrients in mushrooms and not to the vitamin D *per se*. The results, although insufficiently powered to substantiate at this time, supported a potential benefit for regular intake of mushrooms for improving cognition in people who were at-risk of cognitive decline and other age-related conditions.

Amyloid Precursor Protein (APP) is proposed to function as signaling molecule of the innate immune system. Based on the known regulation by vitamin D of innate immunity and inflammation, a relationship between vitamin D intake and expression of APP was also investigated in this RCT. The results suggested that there may be a possible link of APP circulating in blood, APP expression in the brain and outcomes for cognition. Furthermore, correlation between vitamin D status and APP may reflect that vitamin D can influence APP and the importance of vitamin D intake for managing the dysregulation of brain APP in AD. Further clinical studies are required to substantiate these observations.

In conclusion, mushroom supplementation may provide a small benefit for some cognition domains in some older at-risk groups, and vitamin D in mushrooms may assist in regulation of APP expression with possible consequences for managing AD risk. Further clinical studies in at-risk groups are required to substantiate these findings and provide the support needed to enable functional claims.

Keywords

Vitamin D, vitamin D2, vitamin D3, mushroom, cognition, mood, clinical study, supplement, inflammation, amyloid precursor protein, vitamin D metabolism, dietary vitamin D, Alzheimer's disease, ageing

Introduction

Vitamin D, also known as calciferol, refers to a family of fat-soluble seco-sterols hormones. The two major forms are vitamin D2 (ergocalciferol) and vitamin D3 (cholecalciferol) which are both functionally inactive and converted to active forms through hepatic and renal metabolism and also by specific types of cells. Vitamin D2 is primarily produced by UV-mediated transformation of ergosterol, a plant sterol found in plants and fungi. Vitamin D3, is synthesized through the action of ultraviolet B (UVB) radiation on 7-dehydrocholesterol in the skin and is considered to be the main determinant of vitamin D status in Australia, as most adults are unlikely to obtain more than 5-10% of their vitamin D requirement from dietary sources. Dietary sources of vitamin D3 include oily fish, egg yolks and meat while a source of vitamin D2 is UV-treated mushrooms. Vitamin D3 and vitamin D2 are synthesized commercially, however vitamin D3 is generally the major supplemental form available in Australia.

The conversion of vitamin D2 and vitamin D3 to biologically active forms involves two enzymatic hydroxylation reactions (**Figure 1**). The first takes place in the liver, mediated by the 25-hydroxylase (most likely cytochrome P450 2R1 [CYP2R1]) which forms 25-hydroxyvitamin D (25-OH-D). The second reaction takes place in the kidney, mediated by 1α-hydroxylase (CYP27B1), which converts 25-OH-D to the biologically active hormone, calcitriol (1,25-dihydroxyvitamin D). The 1α-hydroxylase gene is also expressed in several extra-renal tissues, but its contribution to calcitriol formation in these tissues is unknown. 25-OH-D, the precursor of calcitriol, is the major circulating form of vitamin D and levels of this metabolite in blood are measured as an indicator of vitamin D status in the body.

Calcium and vitamin D are two essential nutrients necessary for bone health. In recent years, levels of vitamin D intake and circulating 25-OH-D needed for health has been reviewed. In Australia, the consensus view of the Australian and New Zealand Bone and Mineral Society, Endocrine Society of Australia and Osteoporosis Australia, defines adequate levels of vitamin D for mineral homeostasis, bone health and muscle function at a cut-off of $\geq$50 nM 25-OH-D (at the end of winter) and severe deficiency at <12.5 nM. However, the target range of >50-62.5 nM 25-OH-D serum concentration was nominated for prevention of adverse musculoskeletal outcomes, including falls and fractures. For 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 is still limited.

While the role of vitamin D in skeletal health is well established, vitamin D deficiency is also linked to a range of non-skeletal conditions such as cardiovascular disease, cancer, stroke, diabetes, cognitive impairment and dementia. Several cohort studies have identified associations between low vitamin D levels and cognitive decline, Alzheimer's disease (AD) and dementia. Systematic reviews of a number

of cross-sectional and observational studies consistently report a relationship between low vitamin D status and poor cognitive performance and dementia risk. In support, meta-analysis of 5 cross-sectional and 2 longitudinal studies also reported an increased risk of cognitive impairment in those with low versus normal vitamin D. Vitamin D deficiency is also a risk factor in mood and depressive symptoms. However, there is strong agreement that evidence of causality is currently lacking and the need for large, well-designed, randomised controlled trials to determine whether vitamin D supplementation is effective at preventing or treating cognitive decline, AD and dementia.

This project addressed the opportunity for convenient provision of dietary vitamin D from UV-enhanced mushroom and the need for substantiation of efficacy of vitamin D *per se* for improving cognition and also mood, in a high quality clinical intervention study. The aim was to explore the relationship between vitamin D intake (as either vitamin D2-enhanced mushroom solids or vitamin D3) and cognition, mood and depressive symptoms in healthy, older people. In addition, relationship of vitamin D and expression of Amyloid Precursor Protein was measured as a secondary endpoint. The research was planned around the following 3 work packages:

P1. Review of human clinical trials using systematic review protocols and critical analysis of the levels of evidence relating to Vitamin D status and cognitive health.

P2. Cross-sectional analysis of Vitamin D status (to be measured in stored bloods) and cognition and mood measures assessed in healthy older subjects enrolled in the completed 'EPOCH' study.

P3. Conduct a randomised placebo-controlled double-blind dietary intervention study with 4 treatments to determine the effect on primary outcome measures of change in cognition, mood and depressive symptoms and the secondary measure of Amyloid Precursor Protein expression, following vitamin D supplementation supplied by either vitamin D2-enhanced mushrooms (D2M) or vitamin D3 (D3) supplement versus controls of standard mushroom (CM) and placebo (P).

Methodology, outputs, outcomes, evaluation and discussion are reported for each work package.

Methodology

P1. Review of human clinical trials (Appendix 1)

Topic and search strategy

The following 'review topic' was defined as the focus of the literature review: "**Is there a relationship between serum Vitamin D levels and cognitive status?**". The literature search strategy targeted both published and unpublished English language studies from 1990 to present (2012). A three-step search strategy was utilised comprising an initial limited search of MEDLINE followed by analysis of the text words contained in the title and abstract and index terms. A second search using all identified keywords, MESH and index terms, was then undertaken across all included databases. Thirdly, the reference list of all identified reports and articles was searched for additional studies. The initial search terms were as follows: Vitamin D [Title/Abstract] Filters: Publication date from 1990/01/01; Humans AND (alzheimer OR alzheimer's OR memory OR brain OR neuroprotect* OR cognition OR cognitive OR dementia[Title/Abstract]) Filters: Publication date from 1990/01/01; Humans. The list of databases searched and types of records gathered, including unpublished but complete, clinical trials, and included in an EndNote database, are provided in Appendix 1.

Inclusion and exclusion criteria: participants and intervention types

The review included males and females of all age groups and excluded the following: subjects with confirmed dementia or cognitive impairment due to physical injury or other disease state; subjects with confirmed chronic kidney disease or depression (where stated); animal studies. Studies were included that reported the following outcomes: serum 25OHD concentrations, cognitive status or diagnosed AD or dementia due to ageing as primary or secondary outcomes. Changes in vitamin D status following from combination anti-AD/dementia drugs and from vitamin D2 versus vitamin D3 supplementation were included and cognitive test measures included: Mini Mental State Examination (MMSE), Trail Making Test A and B, Episodic memory score, or other measures of cognitive performance.

P2. Cross-sectional analysis of vitamin D (Appendix 2)

Study Design

The cohort used for this cross-sectional study was recruited for the 18 month intervention study of the effects of omega-3 long-chain fatty acids on changes in cognitive functioning in older adults (EPOCH Study) as previously described. The EPOCH Study baseline dataset including clinical, biochemical, cognitive and mood measures, was correlated against the concentration of vitamin D metabolite (25-OH-D2 and 25-OH-D3) taken from analysis of stored plasma samples.

Briefly, the study included 391 participants (46.3% male) of 65 to 90 years of age who were fluent in the English language and who did not suffer from mood disorders nor suspected cognitive impairment (MMSE score of >24). The study design was based on cognitive measures as primary outcomes with mood and biochemical measures treated as secondary outcomes. Self-reported supplementation usage indicated that 33/391 were taking vitamin D regularly either alone or in a multivitamin formulation.

Clinical and biochemical measures and genotyping

Participant demographics and health status were assessed by self-report using standardised questionnaires. Information collated included: sex, age, working occupation, years of education, income, smoking history, diagnosed medical conditions and prescribed medications, alcohol consumption and physical activity, and also baseline anthropometric measures. Dietary intakes of energy, alcohol and vitamin D were assessed using the Victorian Cancer Council food frequency questionnaire (FFQ). Blood collection, analytical procedures for biochemical measures and APOE genotyping included: plasma homocysteine, hemoglobin A1c, low density lipoprotein (LDL) cholesterol and C-reactive protein.

Cognitive and mood measures

The cognition assessment battery was designed to be sensitive to subtle nutrition-mediated effects, to age-related changes in cognitive capacity and to be predictive of cognitive impairment and dementia. The following cognitive domains were assessed by two or more tests: Perceptual Speed, Simple and Choice-Reaction Time, Speed of Memory-Scanning, Reasoning Speed, Inhibition, Psychomotor Speed, Reasoning, Working memory, Short-term Memory and Retrieval Fluency. Mood symptoms were measured using a self-report questionnaire, including the Positive and Negative Affect Schedule and depression symptoms using the Centre for Epidemiology Studies Depression Scale (CES-D).

Serum vitamin D metabolite analysis

Blood sera of participants of the EPOCH Study collected at baseline (N=388) were subjected to analysis of vitamin D metabolites following approval from the CSIRO HREC. Baseline samples were collected over a 3 month period from 14/01/08 to 15/04/08 (with collection day factored into statistical analysis), and stored at -80°C without thawing. Levels of 25-hydroxy vitamin D2 and 25-hydroxy vitamin D3 were measured in blood sera using high throughput liquid chromatography tandem mass spectroscopy (LC-MSMS).

P3. Randomised placebo-controlled, double-blind clinical study (Appendices 3, 4, 5)

Study design

The protocol for the current randomised controlled trial has been published (Appendix 3). Briefly, this study is a randomised, double-blind, placebo controlled, 4-armed, parallel groups clinical trial. Participants were screened prior to enrolment into the study to ensure compliance with the eligibility criteria and then block-randomised into one of the four study groups matched for age and gender. The intervention period was 6 months and performed in 2 cohorts over 2 years (winter months of 2014 and 2015) at the CSIRO Human Nutrition Clinic at the South Australia Health and Medical Research Institute in Adelaide, Australia. The study was approved by the CSIRO Food and Nutrition Human Research Ethics Committee (F&N-HREC-13/05) and the trial was registered with the Australian and New Zealand Clinical Trials Registry (ACTRN: 12613000891729).

Healthy participants were recruited based on the following eligibility criteria: male or female aged 60-90 years old, fluent in the English language, not consuming Vitamin D supplements, able to swallow tablets, physically able to attend the CSIRO clinic and use a computer and pen and paper, able to consume mushrooms and healthy cognitive status. From 925 initial respondents, after screening and withdrawals, 436 were randomised into treatment groups and 361 completed the study and their data was analysed (approx.. 90 per group).

The four study arms contained the following experimental dosages delivered daily over 6 months via 2 capsules per day: 1) vitamin D2-enriched mushroom (200 mg mushroom solids, 600 IU of vitamin D2); 2) vitamin D3 (600 IU of vitamin D3); 3) standard mushroom (200 mg control mushroom solids); and 4) placebo (carrier alone). During the intervention phase, participants attended the CSIRO clinic at 3 time points: baseline, 6 weeks and 6 months. At each clinic visit participants completed cognitive assessment tasks, mood and wellbeing questionnaires and volunteered blood samples for later analysis. At 6 weeks, participants provided an additional saliva sample for DNA genotyping analysis.

Capsules

Vitamin D2-enriched (D2M), standard mushroom solids (CM), vitamin D3 (D3) or placebo (P) capsules were manufactured with a shelf life of 2 years under storage at 4°C. Vitamin D content of capsules was analysed at the end of year 1 and year 2 to verify stability. Capsule containers were color-coded and the contents blinded to clinical staff and during primary effects analysis.

Analysis of Vitamin D metabolite and serum biomarkers

Vitamin D metabolites, 25-OH-D2 and 25-OH-D3 were analysed at baseline and 6 months follow-up using high throughput liquid chromatography tandem mass spectroscopy (LC-MSMS).

Cognition, mood and depressive symptoms

Cognition was assessed using the CSIRO Cognitive Assessment Battery (C-CAB) at each of the 3 clinic visits. The C-CAB was designed to maximise sensitivity to changes associated with normal ageing or impact of nutritional interventions cognitive performance. Standardised questionnaires were used to assess mood and depressive symptoms at each of the 3 clinic visits.

Genotyping for APOE, inflammation, vitamin D and folate metabolism

Participants were characterized for a number of genotypes related to AD risk, inflammation, vitamin D and folate metabolism. These data were used to stratify participants for main effects analysis in comparison with the total cohort.

Analysis of APP mRNA expression

Blood samples for APP gene expression were collected, stored and analysed by RT-qPCR assays of paired samples that were set up in duplicate to measure APP mRNA expression. TATA box binding protein (TBP) and succinate dehydrogenase complex, subunit A, SDHA) were chosen as internal reference genes and an anchor sample used to correct for inter-plate variation. Real-time RT-qPCR threshold cycle (Ct) values were calculated and reported using the $2^{\Delta\Delta Ct}$ method, representing the fold-difference in cDNA concentration for APP compared to the anchor sample. Data from clinic Visit 3 $2^{\Delta\Delta Ct}$ value was subtracted from the clinic Visit 1 $2^{\Delta\Delta Ct}$ value to give the relative fold-change in APP mRNA expression between samples and were standardized to the mean of the V1 and V3 result to correct data skewness. Negative and positive changes in relative fold-change signify increasing and decreasing expression of APP, respectively, over 6 months of the study intervention.

Statistical Analysis

All statistical analysis was conducted using the statistical software program R (Version 3.0.0, [5]). Data outliers were reduced to the next highest/lowest score within the main body of the distribution before calculating descriptive statistics (means, SD, 95% confidence intervals) to provide an overview of the relative distribution of variables within the sample and treatment arms. For analysis of primary effects, Linear Mixed Effects (LME) models were conducted for all mood and cognition variables independently and specified to include: 1) time-wise changes in mood and cognition measures obtained at V1, V2 and V3; 2) treatment effects to determine whether there were overall differences between the treatment groups independent of time; and 3) time $\times$ treatment effects to determine differences between treatments in either mood or cognition over time. A significance level of $p \leq 0.05$ was adopted for overall model effects and in the event of a significant effect, contrasts were examined for the relevant effect to aid interpretation of the result. Statistical analysis of APP expression data is described in Appendix 5.

Outputs

P1. Review of human clinical trials (Appendix 1)

The review targeted the highest level of evidence including systematic reviews, meta-analyses, randomised clinical trials and other human clinical studies. Where such high levels of evidence were not available, human population studies were included. Included studies were classified by I-III levels of evidence, using the National Health and Medical Research Council (NHMRC) criteria (NHMRC, 2000). From initial capture of 283 records and 97 clinical trials, a total of 14 publications and 11 clinical trials were included in the final meta-analysis and review comprising 4 systematic reviews, 1 clinical review, 1 RCT and 7 cohort studies met the selection criteria. Included studies were grouped and adapted in tabular PICO Format (Population, Intervention, Comparison, Outcome) before analysis as Level I, II and III evidence.

Level I evidence (4 systematic reviews and 1 clinical review)

Studies assessed observational, cross-sectional and small randomised control trial studies of vitamin D with a primary outcome related to cognition (until August 2010). Meta-analyses from the systematic reviews showed association between low vitamin D levels and diminished cognitive function but causality was not proven, and all studies concluded the need for clinical intervention trials to confirm the apparent relationship between vitamin D and cognition. In studies focused on older adults where vitamin D deficiency was prevalent, this raised the possibility of results reflecting reverse causality whereby individuals with cognitive decline are more likely to have poor nutrition and spend less time outdoors.

Level II Evidence (2 randomised controls)

The two randomised control trials attempted to assess the effect of supplements on cognition. Both studies had limitations either due to low participant numbers and short duration. A long-term study over 8 years with more than 4,000 participants, provided good assessment of cognition (Rossom et al., 2012), but lacked vitamin D status analysis. Neither trial permitted conclusions of effect differences that could be related to differences in vitamin D status.

Level 3 Evidence: Cohort studies (8 cohort studies)

The eight cohort studies assessed varied in quality. There were two longitudinal studies of elderly populations of 4 and 6 years duration, both reporting outcomes associating lower vitamin D levels with cognitive decline. Three cross-sectional studies varied in size from 288 – 463 participants, and two had primary outcomes not related to either vitamin D status or cognition. The third study associated vitamin D deficiency with moderately severe to severe dementia, but detail with respect to study design and confounding factors were missing from the paper. A further cross-sectional study of

1765 adolescents found no association of vitamin D levels and cognitive function. A small clinical cohort of 44 assessed the effect of a vitamin D3 supplement on cognition in an elderly group and found an improvement in cognitive performance in the supplemented group compared to a control group over 16 months, however this study lacked detail with respect to confounding factors and aspects of study design. A further small clinical cohort from the same author assessed the effects of vitamin D alone and in combination with memantine on cognition in patients with Alzheimer's disease. This study found improvements in MMSE scores over six months for patients taking vitamin D in combination with memantine but not with vitamin D alone, however supplementation doses were not available for this newly reported study.

P2. Cross-sectional analysis of vitamin D (Appendix 2)

Summary statistics of data

The cohort of 387 older individuals was 54% female and ranged from 65 to 92 years of age with a median of 72 years. Median, mean and ranges of clinical characteristics and other indices of health status were summarised. Each cognition score reflected a combined index (factor score derived from CFA [6], of related tasks designed to assess distinct cognitive domains. Median, mean and ranges of cognition and mood measures demonstrated the strong discriminatory capacity of this test battery.

Serum levels of vitamin D metabolites: total 25-OH-D, 25-OH-D3 and 25-OH-D2, were measured in 387 EPOCH Study participants. Based on a cut-off of 75 nM total 25-OH-D levels, 44.4% were classified as deficient. The mean 25-OH-D levels of the subgroup reporting regular intake of vitamin D supplements (N=32, 24 females, 8 males) was not significantly different to the mean for the total cohort (p=0.39). The metabolite 25-OH-D2 was detected in only 8/387 people (2.1%) and although above limits of analytical detection (5 nM), 25-OH-D2 levels were generally low, ranging from 5.1 to 9.9 nM, compared with 25-OH-D3, which ranged from 20.5 to 161 nM. The results indicate generally low 'exposure' to vitamin D2, reflecting a combination of low natural abundance and low intakes of the 'natural' dietary form of vitamin D.

Total 25-OH-D correlations with clinical and biochemical measures

Vitamin D metabolite levels were significantly positively correlated with gender and alcohol consumption and negatively with BMI. The negative correlation with BMI applied to females, males, APOE4(-) and APOE4(+) sub-groups but was uncorrelated to vitamin D sufficiency status. Additional significant relationships were present for females only. For example, 25-OH-D levels declined over the 3 month sampling period for females but not for males whereas 25-OH-D was positively correlated with total energy consumption in males only. Other correlations are discussed in the published manuscript (Appendix 2).

Total 25-OH-D correlations with cognition and mood measures

Total 25-OH-D levels were significantly correlated with 1/10 cognition measures (reasoning speed factor score) in the total and vitamin D sufficient groups. Moreover, 2/5 mood measures were correlated with 25-OH-D in the total cohort (general positive affect and attentiveness). Correlations further varied depending on vitamin D sufficiency status, gender and APOE4 genotype. 25-OH-D was significantly correlated with perceptual speed for females and APOE4(+) subgroups. There was also a positive correlation between reasoning speed and 25-OH-D in those who were vitamin D sufficient or of APOE4 (-) genotype. For mood measures, general positive affect and attentiveness score were significantly correlated with 25-OH-D in the total cohort. However, subgroup analysis showed the relationship was only present for male and APOE4(-) sub-groups.

Fully-adjusted regression analysis

Relationships of cognition and mood measures to vitamin D status after adjusting for those variables significantly correlated with either vitamin D status or cognition/mood measures, were collated, as well as other variables known to impact on these measures in general. Detailed analysis of significant relationships were described (Appendix 2).

P3. Randomised placebo-controlled, double-blind clinical study (Appendices 3, 4, 5)

Capsule composition and stability

Vitamin D-enhanced mushroom and standard mushrooms were prepared from the same batch of mushrooms so non-vitamin D composition was equivalent between these capsule types. Capsules were certified with a 2 year shelf life for refrigerated storage and stability of vitamin D2 and D3 over the duration of the study verified.

Participant biometric profiles for treatment groups

Means of a range of biometric and biochemical measures at baseline demonstrated generally effective randomisation across the parameters, gender, age and BMI. Analysis of sun exposure by recall at each clinic visit was used to compare equivalence of the 2014 and 2015 cohorts.

Effects of treatments on vitamin D metabolite status

For ethical reasons, vitamin D metabolite analysis was not conducted during screening and therefore not factored into randomisation. However, baseline means of total 25-OH-D were similar between treatment groups (p=0.06), as were the proportions of sufficient in total 25-OH-D between groups (p=0.11). Treatments produced a lowering of vitamin D status in 3/4 groups and a significant decline in the proportion of people in the sufficient range of vitamin D status (>75 nM) after 6 months.

However, differences in vitamin D status changes between specific sub-groups and the total cohort suggested that there were other factors regulating vitamin D status independently of treatment-based vitamin D intake.

Effects of treatments on mood measures

Main effects modelling was conducted for the total cohort and for sub-groups based on gender, baseline vitamin D status and its change relative to 'low', 'mid' and 'high' baseline levels between V1 and V3. In addition to stratification based on gender, vitamin D status and responses, participants were characterised for multiple genotypes relevant to vitamin D and folate metabolism, inflammation and AD risk, permitting these factors to also be considered in main effects modelling, by examining effects within specified sub-groups.

Means and confidence intervals for all treatment groups across time points indicated there was no significant time $\times$ treatment interaction effects for any of the mood variables for the total cohort. However, there were significant time effects, independent of treatment, for the following 3 measures: Stress, Negative Affect and Happiness, with improvement observed in each case.

Effects of treatments on cognition

Means and confidence intervals of cognition measures for all treatment groups and time points are presented for the total cohort and specific sub-groups. For the total cohort, there were no treatment $\times$ time interaction effects but there was a significant increase in performance over time for all cognitive speed variables. Increases tended to occur primarily between baseline and week 6. Improvement was also evident for 2/3 memory measures for the total cohort. In contrast with the total cohort, there was a significant time, treatment and interaction effects for selected genetic sub-groups. In each case, benefit was associated with the standard mushroom and not vitamin D.

Temporal trends in APP expression

Mean and median expression levels of APP at V1 and V3 were similar to each other ($p>0.05$) and mean delta APP was close to zero (0.025). The data was checked for normality with APP-V1, APP-V3 and delta APP all found to be significantly non-normal ($p<0.001$) and highly skewed requiring logarithm transformation for further analysis.

The data was analysed as the total cohort and also after stratification by magnitude of the delta APP into sub-sets either: unchanging or 'normal' (25-75% quartile, N=176), decreasing (<25% quartile, N=90) or increasing (>25% quartile, N=90). For the total cohort, APP-V1 was negatively correlated with MMSE at screening ($p<0.05$) that did not persist at V3. In the total cohort, APP-V1 was not correlated with any baseline measures of cognition, noting a time difference of 3-6 weeks between

MMSE measured at screening and baseline measurements. Standardised delta APP was also significantly correlated with change in 25-OH-D (V3-V1). For the mid-range quartiles (25-75%) of delta APP representing unchanging (putative 'normal') levels of APP expression, APP at both V1 and V3 was positively correlated with recognition speed at V1 (p<0.05) also suggesting a relationship between APP expression status and this cognitive measure, that was present at V1 and V3 because APP was unchanging in this sub-group. However, for those in the lowest quartile of delta APP (APP-V3 < APP-V1), suggesting tendency for decreasing APP between V1 and V3, there were no correlations with any cognitive measures (p<0.05). For those in the highest quartile of delta APP (APP-V3 > APP-V1), suggesting tendency for increasing APP between V1 and V3, the coefficients for positive correlation with age and negative correlation with MMSE were significant (p<0.05). APP-V1 and APP-V3 were each positively correlated with age and negatively with MMSE at screening. The positive correlation between delta APP and delta vitamin D status seen in the total cohort was also significant and the coefficient strengthened for this sub-group, suggesting particular importance of the relationship for this sub-group.

Outcomes

P1. Review of human clinical trials (Appendix 1)

The review assessed the current evidence available on the association between Vitamin D and cognitive status. Although this review has retrieved four current systematic reviews and associated meta-analyses, two randomised clinical trials and seven cohort studies the conclusiveness of the evidence was undermined by the possibility of reverse causality where high prevalence of vitamin D deficiency in elderly people might reflect poor nutrition and lower vitamin D3 uptake as a result of compromised cognition, not the reverse.

P2. Cross-sectional analysis of vitamin D (Appendix 2)

The cross-sectional study tested the hypothesis that measures of cognition and mood in cognitively healthy, community-dwelling older people are related to vitamin D status, measured as total 25-OH-D. Importantly, the analyses showed that some associations are present between 25-OH-D and some cognition and mood measures, even after rigorous adjustments for confounding variables. However, a number of the clinical and biochemical measures also correlated with vitamin D status. Thus, the possibility that cognition and mood could be either directly or indirectly influenced by vitamin D status cannot be excluded based on the present analyses, as represented in **Figure 2**. The data is discussed by considering both direct and indirect pathways of regulation of cognition and mood involving vitamin D.

Fully-adjusted models of 25-OH-D and cognition measures were significant for reasoning speed (All), speed of memory scanning (vitamin D deficient) and perceptual speed (females, Table 6). APOE4 status did not influence these cognitive measures. These observations are supported by other reports of positive correlations with vitamin D status for executive reasoning but lack of significant correlation with memory performance. The positive associations of 25-OH-D with reasoning speed may reflect benefits for cognitive domains associated with subcortical function and a vasculo-protective effect of vitamin D.

Vitamin D and mood

Vitamin D status has been previously inversely correlated with risk of depression across male and female study populations and also in older men. Furthermore, supplementation of overweight and obese individuals with vitamin D has been shown to improve depressive symptoms. The current study did not detect any significant correlation between 25-OH-D and depression *per se* in either men or women but it did detect significant relationships for general positive affect and attentiveness in the total sample, whilst these constructs as well as general negative effect related to 25-OH-D in some subgroups. In the present study, the correlation of vitamin D status with negative affect was only applicable to those vitamin D sufficient and not significant for the total cohort.

Vitamin D status – regulation by sunlight (link with blood sampling date) and intake

Vitamin D can be obtained via sunlight-mediated, dietary and supplement sources which have been collectively modelled in order to define guidelines for overall intake that elicits sufficiency of the circulating active metabolite, 25-OH-D. However, sufficiency of 25-OH-D, based on an adequacy cut-off of 50 nM 25-OH-D, is mostly defined in terms of requirements for skeletal health and not yet for non-skeletal health indices such as cognition, which requires further evidence. To meet requirements for health throughout life to prevent non-skeletal, longer latency diseases, a revised target level of 75 nM 25-OH-D has been nominated and if accepted will affect intake guidelines.

The 25-OH-D2 status of this cohort, reflecting exclusively dietary sources of vitamin D (from plants and fungi), ranged from 5.1 to 9.9 nM, or approximately 9% of mean total 25-OH-D. The results demonstrate that vitamin D2 accounts for a low proportion of vitamin D intake in the present as well as other cohorts, and therefore that vitamin D status mostly reflected environmental and dietary sources of vitamin D3.

Vitamin D status and gender

The significantly lower mean level of 25-OH-D observed in South Australian women versus men is supported by similar trends towards lower vitamin D dietary intakes for European women compared with European men. If not due to differences in intake patterns, decline of 25-OH-D status for

women and not for men over the sampling period may infer a relatively higher dependence on environmental sources of vitamin D by women and also reflect biochemical differences in vitamin D metabolism between females and males.

Vitamin D status and Body Mass Index (BMI)

The mean BMI and vitamin D cohort characteristics of the current study population compare well with those of the female and male subsets of previous studies. Vitamin D deficiency is significantly prevalent in overweight and obese individuals. In the current cohort, 25-OH-D declined rapidly with increasing BMI in older subjects (aged>73, $p<0.001$), and in particular for older females ($p<0.001$), supporting that BMI differences between males and females may be attributed to loss of bone mineral density that is known to accelerate for females post menopause.

P3. Randomised placebo-controlled, double-blind clinical study (Appendices 3, 4, 5)

Main Effects

Levels of total 25-OH-D were either maintained (D3) or depleted ($p<0.05$) by D2M, SM or P treatments. For the total cohort, there were significant ($p<0.01$) time-wise improvements for all treatments for 7/8 cognitive measures and 3/6 mood measures, but there were no time ×treatment interaction effects. However, in some sub-groups relating to vitamin D bioavailability and selected genotypes, significant time × treatment interaction effects were observed for some cognitive domains (Recognition Speed, Reaction Time, Memory Scanning and Spatial Working Memory). These effects, however, generally appeared related to micronutrients in CM and not vitamin D in either D2M or D3 treatments.

The results from this study do not support a strong benefit of vitamin D supplementation for mood or cognition in healthy older people. However, there is some evidence that particular at-risk sub-groups could benefit from supplementation of mushroom micronutrients and potentially, vitamin D, to support cognitive function. Considering that observed improvements in mood and cognition were accompanied by either unchanging or declining vitamin D status, the results also suggest that the 25-OH-D metabolite may provide an index of recent history of vitamin D supply but is not well correlated with the distribution of vitamin D to cells and tissues relevant to mood or cognitive function.

Amyloid Precursor Protein

Amyloid Precursor Protein (APP) is proposed to function as signaling molecule of the innate immune system. Based on the known regulation by vitamin D of innate immunity and inflammation, a relationship between vitamin D intake and expression of APP, was also investigated. Longitudinal changes in peripheral APP expression determined at baseline (V1) and 6 months follow-up (V3) are

reported here. APP at V1 was negatively correlated with MMSE at screening (p<0.05) and delta APP with change in vitamin D status. Delta APP expression was stratified into sub-groups displaying unchanging (N=176), negative (N=90) and positive (N=90) changes. Positive delta APP expression correlated inversely with MMSE-screening (p<0.05), positively with age (p<0.05) and also positively with change in vitamin D. The results support a possible link of peripheral APP expression with brain APP expression and outcomes for cognition. Furthermore, for those with increasing APP expression, correlation between vitamin D status and APP expression may reflect a role of vitamin D in regulation of APP expression and explain the consistently reported associative relationship between vitamin D deficiency and cognitive decline in AD.

Evaluation and Discussion

P1. Review of human clinical trials (Appendix 1)

All systematic reviews concluded the need for well-designed randomised controlled trials to determine whether there is any clinically relevant relationship between vitamin D and cognitive status and to establish causality. Furthermore, the review also confirmed that the forthcoming trial on vitamin D-enriched mushrooms and cognition currently appears to be the only registered study to address the effects of vitamin D2 either alone or in a mushroom matrix on cognition and mood in a healthy older cohort, in order to minimise the possibility of reverse causality.

P2. Cross-sectional analysis of vitamin D (Appendix 2)

Vitamin D is an important vitamin for skeletal health but its non-skeletal functions, particularly its role in preventing chronic diseases and decline in brain function, are of increasing interest. This cross-sectional analysis identified relationships between 25-OH-D status and a number of factors that appeared to regulate vitamin D metabolism including gender, body mass index, alcohol consumption and APOE4 genotype. The results could be interpreted as reflecting that vitamin D metabolism is differentially regulated in older females versus older males with different risk factors for vitamin D adequacy status and deficiency. Fully-adjusted models revealed that vitamin D status was significantly positively correlated with one measure of cognition (reasoning speed) and 2 measures of mood (general positive affect and attentiveness). Vitamin D-deficient and female sub-groups displayed additional significant associations between vitamin D status and speed of memory scanning and perpetual speed, respectively, whereas relationships with general positive affect and attentiveness were strongest in male or APOE4(-) subgroups relative to the total sample. Gender differences in the models further supported that vitamin D influenced brain functions of older males and females differently. In general, benefits of APOE4(+) status, which provides for more efficient absorption of lipid-soluble vitamin D, was not evident in this older cohort, and the results suggested that cognition measures were independent of APOE4 status but vitamin D adequacy positively

affected selected mood measures, particularly in males. The positive cross-sectional relationships between vitamin D status and brain functions observed within this cognitively healthy cohort lend some support to the hypothesis that vitamin D has an important protective role for brain health in ageing.

P3. Randomised placebo-controlled, double-blind clinical study (Appendices 3, 4, 5)

This is the first clinical evaluation of effects of supplementation of vitamin D2-enhanced or standard mushrooms on cognition and mood in a healthy older cohort. The 4 treatment arms compared vitamin D2 mushroom (D2M), vitamin D3 (D3), standard mushroom (CM) and placebo (P). As such, 3/4 treatments contained potentially beneficial vitamin D or mushroom micronutrients including amino acids, ergothioniene and minerals (or both). Under the RCT conditions and intervention doses tested, the results showed significant timewise improvements across all treatment groups for multiple mood and cognitive domains, which varied between sub-groups defined by vitamin D metabolism and genotype. However, the study did not detect advantage of any particular treatment, including vitamin D, for either mood or cognition in the **total cohort**. For mood measures, treatment-specific effects for happiness favoured the Placebo. In contrast to this, for cognitive measures, treatment effects in selected sub-groups favoured the effects of mushroom micronutrients and not vitamin D.

Importantly, the main effect trends of improvement over time were accompanied by either depleting (P, D2M, CM treatments) or maintained levels of total 25-OH-D (D3 treatment), and highlight that 25-OH-D may provide an index of recent history of vitamin D supply but may not usefully inform about vitamin D mobilisation, activation or turn-over, nor the distribution of vitamin D to cells and tissues competing for its hormonal regulatory functions. Nevertheless, the results are relevant and generalizable to healthy, older populations and provide a basis for further investigation of the potential benefits of vitamin D versus mushroom micronutrients during ageing.

The study also demonstrated a relationship between vitamin D status and APP expression, suggesting interaction of vitamin D and APP signaling pathways in the periphery, and potentially in the brain. In this context, the supply of vitamin D in the form of mushrooms can provide a convenient dietary source for maintaining adequacy of this important nutritional hormone.

Recommendations

This research program culminated in a high quality randomized clinical study seeking to demonstrate a causal relationship between vitamin D mushroom intake and increased vitamin D status and improvement in cognition and mood in healthy, older people. The interpretations of the results lead to several hypotheses that require further clinical substantiation research. The following recommendations for further research are proposed:

1. Measure levels of RNA expression of the enzymes responsible for conversion of storage to active vitamin D and turnover of active vitamin D, respectively. It is necessary to show that vitamin D intake influenced changes in active vitamin D (1,25-OH-D) and that changes in 25-OH-D metabolite were not predictive of changes in active vitamin D. These results may have important implications for understanding the relationship of daily incremental dosing of vitamin D for conversion to active vitamin D and by implication, usefulness of vitamin D-enriched mushrooms for dietary supply of vitamin D.

The interpretations proposed that particular at-risk sub-groups of healthy people are more likely to benefit from supplementation of vitamin D or mushroom micronutrients also requires further clinical substantiation in the nominated sub-groups, with the following potential opportunities, dependent on outcomes of further clinical studies:

2. If incremental daily dosing of vitamin D at the RDI (versus alternative regimens such as bolus dosing) is preferential for maximizing levels of *active* vitamin D, this understanding has direct relevance to the opportunity for vitamin D-enhanced mushroom present in a year-round, convenient supply of dietary vitamin D. This can be achieved by promoting use of vitamin D-enhanced mushroom in both supplement, fresh or other food-related forms for delivery of the daily requirement of vitamin D for older people, and potentially other at-risk populations. It is likely that the supply of vitamin D (ie, dose, intake cycles) would require optimization for people with particular baseline status and genetic predispositions in order to overcome metabolic 'bottlenecks' in conversions to active vitamin D.
3. The clinical study revealed cohort sub-groups who responded to either vitamin D2 (one cognitive outcome) or mushroom micronutrients (several cognitive outcomes) affecting particular cognitive domains and who might be expected to benefit from supplementation. In particular, those with (1) anomalies in 25-OH-D status and (2) specific genotypes regulating AD risk, vitamin D bioavailability and inflammation risk factors, were identified.
4. The link demonstrated in this study of a relationship of APP expression with 25-OH-D may implicate the importance of vitamin D adequacy for supporting healthy regulatory functions of APP in the body and managing APP-related cognitive decline in AD. In this context, vitamin D adequacy can be promoted by regular dietary intake of vitamin D-enriched mushrooms.

Scientific Refereed Publications

Journal articles:

Barnes, M. B., Danthiir, V., Noakes, M., Macaulay, S. L., Zajac I. and Bennett, L. (2016) Cross-sectional relationships of serum 25-OH-D metabolite of vitamin D with cognition and mood in a cognitively-healthy older cohort**.** *Current Psychopharmacology*, **5**. P*ublished ahead of print.*

Zajac, I., Cavuoto, P., Danthiir, V., Wittert, G. A., Krause, D., Lawson, L., Noakes, M., Weaver, J. and Bennett, L. 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: study protocol. Accepted in: *Healthy Aging Research*.

Zajac, I. and Bennett, L. Development of an automated and reliable cognitive assessment battery for measuring and monitoring change in clinical trials. Submitted to: *Aging, Neuropsychology and Cognition.*

Intellectual Property/Commercialisation

The patentability or potential IP value (pending additional confirmatory clinical studies) of the following outcomes from the clinical study require expert evaluation:

1. Relationship of vitamin D and mushroom micronutrient intake dose to improvements in cognition for older people in general and for specific sub-sets of people with respect to vitamin D metabolism and genotype.
2. Efficacy of vitamin D supply and stimulation of active vitamin D, specifically as vitamin D2 present in the mushroom matrix, as per the content of vitamin D2-enhanced mushroom capsules used in this clinical study. The results may suggest that daily intake of vitamin D at doses of the recommended daily requirement may produce different (superior?) effects regarding conversion to active vitamin D, versus alternative regimens of dosing (eg, bolus).
3. Advantages of vitamin D2 versus D3 for supply of vitamin D to people with elevated bodymass index, other biometric or genotype sub-groups, with differentiated tendency for tissue uptake, circulation and conversion to active forms of vitamin D.

Figures

Figure 1. Vitamin D metabolism.

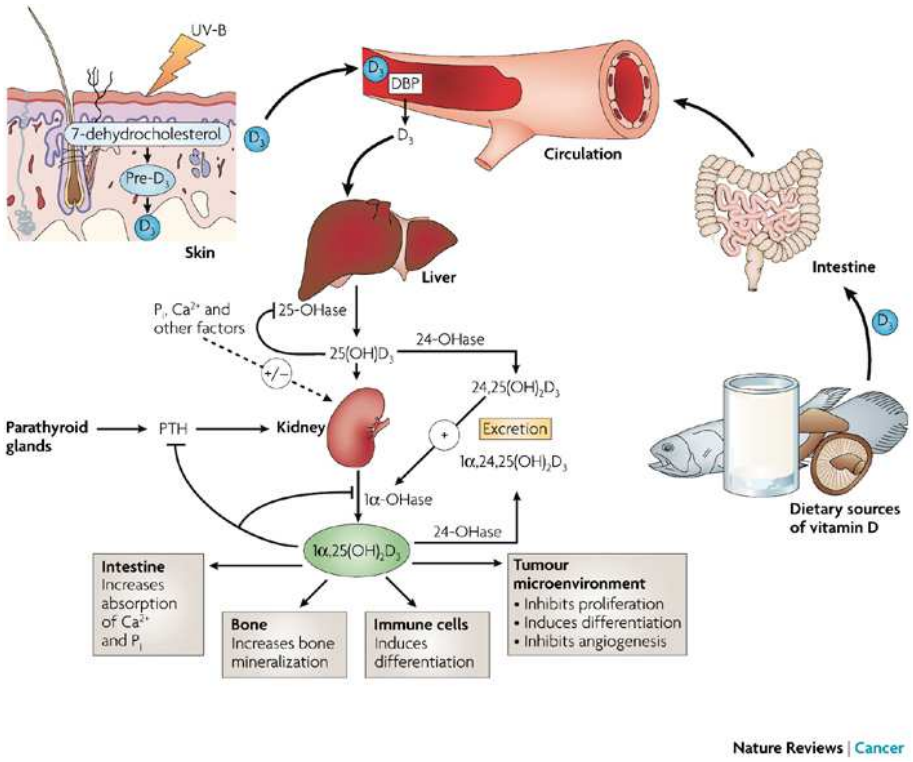
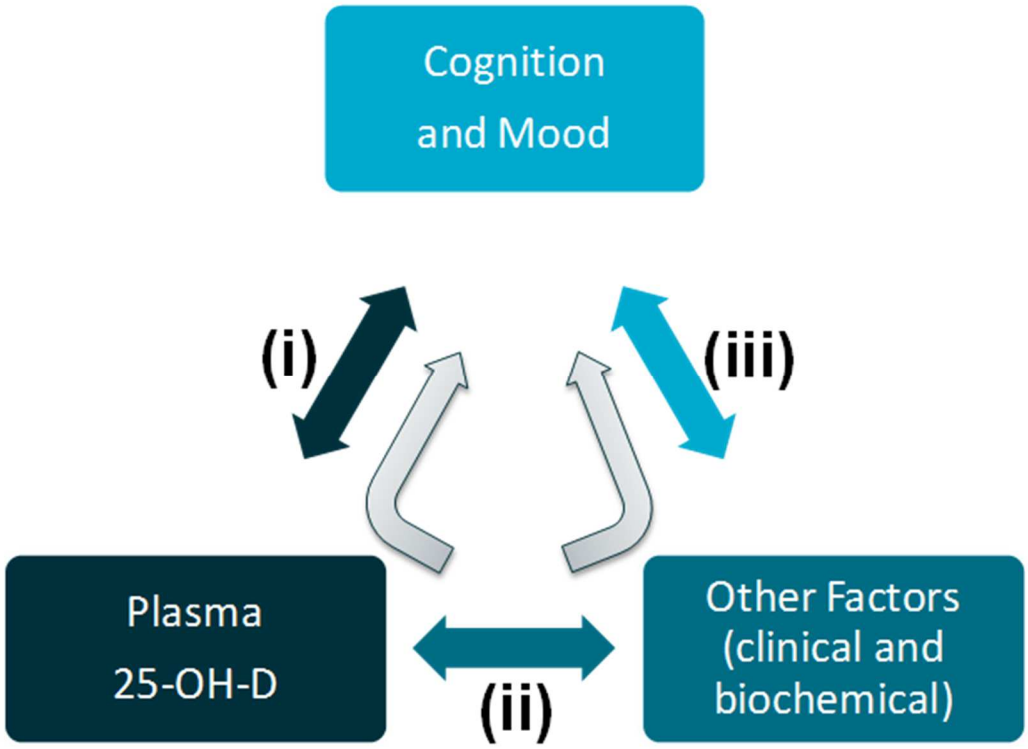


Figure 2. Relationships of vitamin D and other factors to cognition and mood.



Appendices and References

The research outcomes from this project have been fully described in the following appendices which also include details of all references.

No.	Description
1	**Report:** Review of vitamin D and cognitive status: evaluation of the evidence (Milestone 102)
2	**Manuscript:** Cross-Sectional Relationships of Serum 25-OH-D Metabolite of Vitamin D with Cognition and Mood in a Cognitively-Healthy Older Cohort. (Milestone 103)
3	**Manuscript:** 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 older adults. (Milestone 190)
4	**Draft manuscript:** 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 older adults: main effect outcomes. (Milestone 190)
5	**Draft manuscript:** Longitudinal changes in expression of amyloid precursor protein in a healthy older cohort and relationships with vitamin D status. (Milestone 190)