

HUMAN AND CLINICAL NUTRITION

Effect of supplementation with vitamin D₂-enhanced mushrooms on vitamin D status in healthy adults

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Abstract

Vitamin D deficiency is emerging worldwide and many studies now suggest its role in the development of several chronic diseases. Due to the low level of vitamin D naturally occurring in food there is a need for supplementation and use of vitamin D-enhanced products. The aim of the present study was to determine if daily consumption of vitamin D₂-enhanced mushrooms increased vitamin D status in free-living healthy adults or affected markers of the metabolic syndrome. A total of ninety volunteers (aged 40–65 years) were randomly assigned to one of two 4-week studies: mushroom study (15 µg vitamin D₂ or placebo mushroom powder) and capsule study (15 µg vitamin D₃ or placebo capsules). Consumption of vitamin D₂-enhanced mushrooms increased serum 25-hydroxyvitamin D₂ (25(OH)D₂) by 128 % from baseline (3.9 (SD 1.9) nmol/l; $P < 0.05$). Serum 25(OH)D₃ increased significantly in the vitamin D₃ capsule group (a 55 % increase from a baseline of 44.0 (SD 17.1) nmol/l; $P < 0.05$). Vitamin D status (25(OH)D) was affected only in the vitamin D₃ group. Plasminogen activator inhibitor-1 was lowered by vitamin D₂ intake. Vitamin D₂ from enhanced mushrooms was bioavailable and increased serum 25(OH)D₂ concentration with no significant effect on 25(OH)D₃ or total 25(OH)D.

Key words: Food enhancement: Metabolic syndrome: Mushrooms: Vitamin D status

In recent years, a large body of evidence has emerged to support the role of vitamin D in a broad range of biological roles other than Ca absorption and bone metabolism. Though the evidence is less robust, vitamin D deficiency is now implicated in a range of diseases including psoriasis, multiple sclerosis, inflammatory bowel disease, type 1 and 2 diabetes, hypertension, CVD, the metabolic syndrome (MetS) and various cancers⁽¹⁾. In recent years, studies have documented that hypovitaminosis D may be a risk factor for the development of the MetS^(2,3). There is an inverse relationship between serum 25-hydroxyvitamin D (25(OH)D) levels and several MetS markers, such as fasting plasma glucose, blood pressure, waist circumference, TAG

and markers of systemic inflammation^(4–6). However, intervention studies have yielded contradictory results: some studies have confirmed the effect of vitamin D intake on insulin sensitivity, lipid profile and inflammation^(7–9), while others have not^(10–12). Discrepancy in the intervention results may be due to the range of populations studied, the length, frequency and the level of vitamin D supplementation used.

Vitamin D insufficiency, defined as 25(OH)D concentration below 50 nmol/l, is prevalent in nearly half of the population^(13–15) and less than a quarter of adults reach levels considered sufficient (i.e. above 75 nmol/l)⁽¹⁶⁾. Even during the summer months most individuals do not reach satisfactory

Abbreviations: 25(OH)D, 25-hydroxyvitamin D; hsCRP, high-sensitivity C-reactive protein; MetS, metabolic syndrome; PAI-1, plasminogen activator inhibitor-1; PTH, parathyroid hormone.

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25(OH)D levels⁽¹⁷⁾. Recent work has demonstrated that daily intakes between 7.9 and 42.8 µg of vitamin D are required to maintain adequate vitamin D levels in the elderly⁽¹⁸⁾. Furthermore, the US Institute of Medicine has recently increased the RDA for vitamin D to 15 µg/d (600 IU)⁽¹⁹⁾. Since natural dietary sources are a poor provider of vitamin D with values typically ranging from 0.1 to 16.1 µg/100 g for whole milk and oil-rich fish, respectively⁽²⁰⁾, the dietary intake of vitamin D is significantly below 15 µg/d in most European countries, with the highest median daily intakes of about 10 µg in the Nordic countries⁽²¹⁾. In Ireland, 72 % of men and 78 % of women had average daily vitamin D intakes of less than 5 µg/d, and over 90 % less than 10 µg/d⁽²²⁾.

In recent years, in an attempt to combat low dietary intakes of vitamin D, attention has turned to vitamin D-enhanced foods⁽²³⁾. In the USA, enhanced foods have been found to contribute approximately 80 % of total vitamin D intake⁽²⁴⁾. A recent systematic review evaluated the efficacy of vitamin D fortification and concluded that from each 1 µg of vitamin D ingested daily a 1.2 nmol/l increase in 25(OH)D is obtained, highlighting the potential effectiveness of the approach in the prevention of vitamin D deficiency⁽²⁵⁾. However, vitamin D enhancement is not straightforward and much debate exists around whether enhancement should be performed with vitamin D₃ or vitamin D₂^(26,27), and at what level vitamin D should be added to foods^(28,29). To date, the majority of the randomised controlled trials that have investigated the effect of intake of vitamin D-enhanced milk, yogurt or cheese at the level of 10–100 µg/d recorded an improvement in vitamin D status^(30–33). In recent years, alternatives such as fortified orange juice have been introduced and research has demonstrated that daily consumption of juice enhanced with 25 µg of vitamin D₂ or D₃ for 3 months resulted in improved 25(OH)D levels^(34,35). More recently, the use of UVB radiation as an effective method for increasing the concentration of vitamin D₂ in mushrooms has emerged⁽³⁶⁾. Mushrooms are low in energy (120–150 kJ per 100 g), a source of protein (approximately 30 % DM) and carbohydrates (47 %), as well as B vitamins and trace elements (Na, K, P, Mg, Ca and S), with eminent flavour properties^(37,38). The bioavailability of vitamin D₂ from wild-grown⁽³⁹⁾, as well as from UV-treated mushrooms^(40,41) has been confirmed in human subjects.

The objective of the present study was to expand our knowledge on the impact of consumption of such vitamin D₂-enhanced mushrooms. With this in mind the primary objective was to examine the impact of daily consumption of 15 µg of vitamin D₂-enhanced mushrooms for 4 weeks on vitamin D status in comparison with the effect of the same level of intake of vitamin D₃ from capsules. The secondary objective of the study was to examine the impact of increased vitamin D₂ and D₃ intake on markers of the MetS.

Experimental methods

Study participants and design

The present study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving

human subjects/patients were approved by the Human Research Ethics Committee in University College Dublin. Written informed consent was obtained from all subjects.

Healthy Caucasian men and women living in Dublin and its surroundings (latitude 53.3° N) between the age of 40 and 65 years were recruited by advertisement in local and college newspapers, flyers, posters, university-based emails, website and radio advertising. The participants were initially screened and, if the inclusion criteria were met, the participants were asked to sign a consent form. Participants were excluded if they had a BMI below 18 or above 32 kg/m², used medication or hormone replacement therapy (with the exception of the contraceptive pill), had a chronic or infectious disease, were pregnant or lactating, took part in any other dietary intervention study or had allergy to mushrooms. Abstention from taking any vitamin D supplements for 4 weeks preceding the study and during the intervention was imposed. The intervention was carried out between February and March 2011. Anthropometric measurements were performed during the screening visit. Body weight and height were measured in duplicate to the second decimal place and BMI was calculated: BMI = weight (kg)/height² (m²). Blood pressure was measured and heart rate recorded using an Omron M6 Comfort digital automatic blood pressure monitor (Omron Healthcare Europe) in the subject's steady state. Power calculations were performed based on published results for 25(OH)D levels following consumption of vitamin D₂ mushrooms and no supplementation⁽³⁹⁾: twenty subjects per group were required to obtain a power of 80 % and an α of 0.05. The overall study was composed of two distinct parts: (1) supplementation with mushrooms and (2) supplementation with capsules. Both arms of these double-blind randomised placebo-controlled dietary trials were performed using identical protocols except for the treatment type. Using a computer-generated randomisation code, ninety participants were randomly assigned to receive daily: 15 µg vitamin D₂-enhanced mushroom powder or placebo mushroom powder (mushroom study); 15 µg vitamin D₃ capsules or placebo capsules (capsule study). Participants were given enough supplements for 7 d of treatment at the beginning of each week and compliance was assessed by reporting the non-used mushroom sachets or capsules.

The mushroom powder was manufactured by Monaghan Mushrooms. Briefly, to naturally enhance the production of vitamin D, button mushrooms (*Agaricus bisporus*) were treated for 3 s with a UVB dose of 1.5 J/cm². The batch was pooled, lyophilised and analysed for vitamin D₂ content. The amount of powder containing 15 µg vitamin D₂ was computed. The treatment and placebo sachets were identical and weighed approximately 0.64 (SD 0.02) g. The vitamin D₂ placebo sachets contained no detectable level of vitamin D₂. Participants were instructed on the storage of the mushrooms (i.e. cool, dry place, hermetically closed) and were asked to consume the whole content of a sachet per d mixed with any meal. The vitamin D and placebo capsules containing 15 µg of vitamin D₃ were produced specifically for the study by Banner Pharmacaps and were ingested once per d with a beverage.

Blood samples were collected by a trained phlebotomist following a 12 h overnight fast at the beginning and following the 4-week



intervention. EDTA and lithium heparin tubes (BD Vacutainer) were used for plasma collection and directly placed on ice. Serum samples were collected in tubes with a clotting agent with or without a barrier gel (BD Vacutainer) and were allowed to clot for 30 min at room temperature before being placed on ice. All samples were centrifuged at 1800 g for 10 min at 4°C and samples were stored at -80°C until subsequent analysis.

Biochemical measurements

All measurements were made according to manufacturers' instructions. Serum levels of 25(OH)D₂ and 25(OH)D₃ were measured using LC/MS-MS at a laboratory certified by the Vitamin D External Quality Assessment Scheme (DEQAS) at the Institute of Aging and Chronic Disease, Liverpool, UK as detailed by Tolppanen *et al.*⁽⁴²⁾. Total 25(OH)D levels were computed by adding up the values of 25(OH)D₂ and 25(OH)D₃. A semi-automated biochip analyser (Metabolic Syndrome Array I, Randox Investigator; Randox) was used to determine serum levels of TNF-α, C-peptide, ferritin, IL-6, insulin, leptin, plasminogen activator inhibitor-1 (PAI-1) and resistin. Plasma hsC-reactive protein (hsCRP), glucose, TAG, NEFA, total and HDL-cholesterol, and serum Ca and albumin were measured on a RX Daytona™ automated analyser (Randox). Homeostasis model of assessment of insulin resistance (HOMA-IR) score was calculated from the glucose and insulin values as follows: HOMA-IR = fasting insulin (μU/ml) × fasting glucose (mmol/l) / 22.5⁽⁴³⁾. Total Ca was adjusted for plasma albumin level using the formula of Payne *et al.*⁽⁴⁴⁾: corrected Ca (mmol/l) = serum Ca (mmol/l) + 0.02 (40 - serum albumin (g/l)). Serum parathyroid hormone (PTH; MD Biosciences GmbH) and plasma adiponectin (Alpco Diagnostics) were measured using ELISA kits. Serum LDL-cholesterol was measured with enzymic colorimetric method (Wako Diagnostics).

Statistical analyses

Statistical analyses were performed using SPSS software for Windows (version 18.0; SPSS Inc.). The χ^2 test was used to analyse sex distribution across the groups. The comparisons between age, BMI and waist:hip ratio were performed with independent-samples *t* tests. General linear model (GLM) ANOVA adjusted for sex, age and BMI was used to compare the changes in the measurements (from baseline to after the 4-week intervention) between the placebo and treatment groups. Changes in post-intervention values were assessed using a GLM ANOVA controlling for baseline values, sex, age and BMI. Linear regression analysis were performed to test the relationships between serum 25(OH)D and the measurements. The results were considered significant when $P < 0.05$.

Results

Characteristics of participants

An overview of the study design and recruitment process is shown in Fig. 1. After the initial contact and screening for

eligibility criteria, ninety participants aged 40 to 65 years were recruited and randomly allocated to one of four treatments that made up the mushroom and the capsule studies. In all, five participants dropped out from the study: one due to a suspected mushroom allergy, two due to health problems, one for personal reasons and one who was already taking a vitamin D supplement. No adverse effects of supplementation were observed and the average compliance was 98 %, ranging from 97 % (vitamin D₂ mushroom group) to 99 % (vitamin D₃ capsules). The sex distribution of participants that completed the protocol was not significantly different between groups in the studies. However, the age of the participants in the mushroom study differed between the placebo and active mushroom groups (Tables 1 and 2).

At baseline, 56.5 % of the participants were vitamin D deficient (total serum 25(OH)D concentration less than 50 nmol/l), 11.8 % had sufficient total 25(OH)D levels above 75 nmol/l, while the remaining 31.8 % were classified as insufficient (total 25(OH)D levels between 50 and 75 nmol/l). Linear regression analysis indicated a negative relationship between baseline total 25(OH)D concentration and PTH levels (β coefficient -0.184; $P = 0.017$; R^2 0.260).

Serum 25-hydroxyvitamin D₂, 25-hydroxyvitamin D₃ and total 25-hydroxyvitamin D concentrations

Following daily supplementation with vitamin D-enhanced mushrooms the change in 25(OH)D₂ concentration was significantly higher than for the placebo mushroom group ($P = 0.007$) (Fig. 2(a)). The final 25(OH)D₂ concentrations were 8.9 (SD 5.8) and 4.3 (SD 2.2) nmol/l for the enhanced mushroom and the placebo group, respectively. There was no significant change in serum 25(OH)D₃ and total 25(OH)D following supplementation with vitamin D-enhanced mushrooms compared with placebo mushrooms. The post-intervention total 25(OH)D concentration was 36.8 (SD 16.6) and 30.6 (SD 15.1) nmol/l for enhanced and placebo mushrooms (Fig. 2(a) and 2(c)), respectively.

For participants consuming a daily vitamin D₃ capsule there was no significant change in 25(OH)D₂ (Fig. 2(b)). Both serum 25(OH)D₃ and total 25(OH)D increased significantly in the supplemented group ($P = 10.5 \times 10^{-6}$ and $P = 8.1 \times 10^{-5}$, respectively) compared with the placebo group (Fig. 2(b) and 2(c)). Following the intervention total 25(OH)D levels were 57.3 (SD 17.7) and 41.7 (SD 20.1) nmol/l for the supplemented and placebo groups, respectively.

Comparison of the concentrations across the four groups revealed that the 25(OH)D₂ concentration post-intervention was significantly higher in the vitamin D₂-enhanced mushroom compared with the placebo mushroom and the vitamin D₃ capsule group. The post-intervention values for 25(OH)D₃ and total 25(OH)D were significantly higher in the vitamin D₃ capsule group compared with all other groups (Table 3).

Blood pressure and markers of the metabolic syndrome

Analysis of the markers of the MetS was performed across the four groups and significant group effects were found for

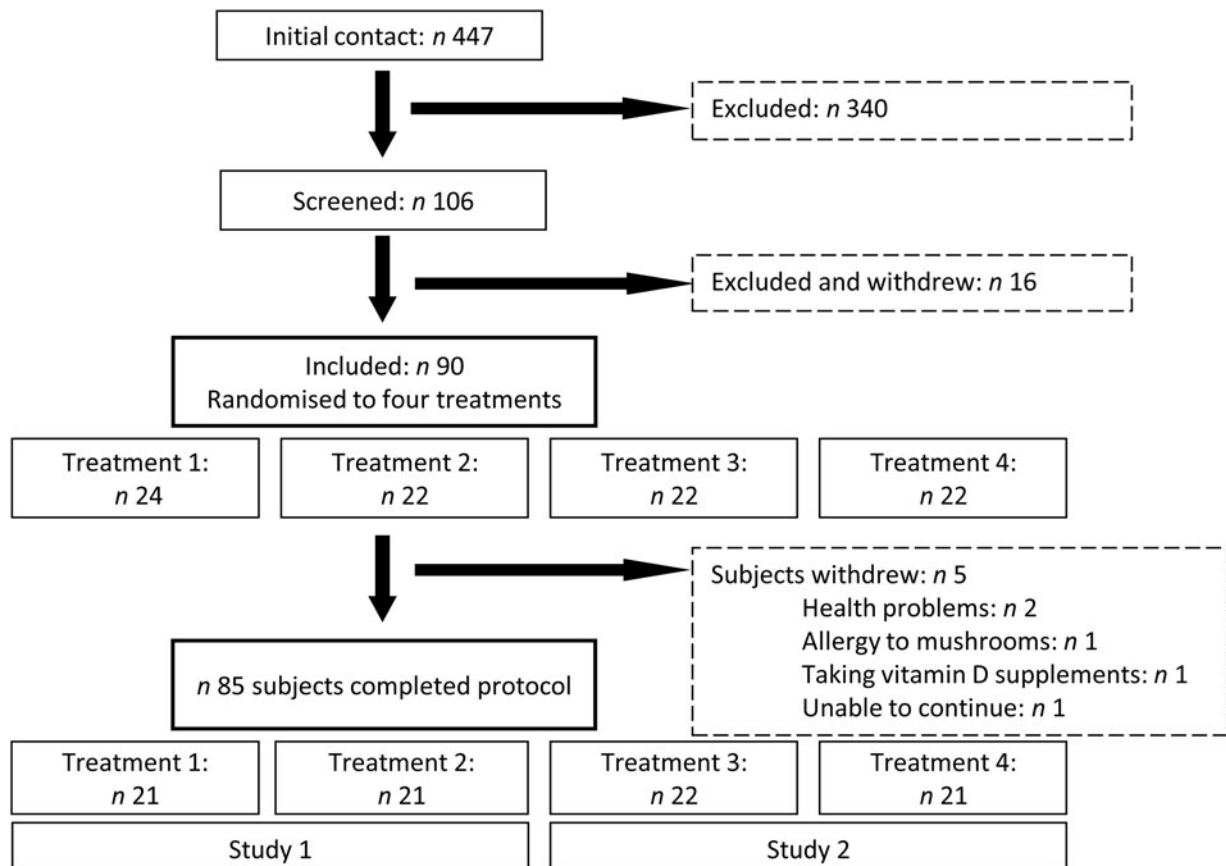


Fig. 1. Summary of study design. Due to an expected lower number of enrolled male participants, males were assigned to treatment groups 1 to 4 in that order following the randomisation code. Treatments: 1, vitamin D₂-enhanced mushrooms; 2, placebo mushrooms; 3, vitamin D₃ capsules; 4, placebo capsules.

hsCRP and PAI-1. A decrease in hsCRP was observed in the vitamin D₂ mushroom group compared with the vitamin D₃ capsule group. Subsequent analysis was performed separately for the mushroom and the capsule groups (Tables 4 and 5). Following 4 weeks of daily supplementation with mushrooms enhanced with 15 µg of vitamin D₂ a decrease in plasma PAI-1 concentration was observed ($P=0.038$). Comparison of the capsule groups revealed significant differences only in

C-peptide. However, there was no change in the supplemented group and a decrease in the placebo group.

Discussion

In the present study the effect of a daily intake for 4 weeks of 15 µg of vitamin D₂-enhanced mushrooms or vitamin D₃ in capsule form on serum 25(OH)D₂ and 25(OH)D₃ concentrations and markers of the MetS was investigated. The intake of each form of the vitamin significantly increased serum concentration of its own hydroxylated form, but only vitamin D₃ supplements positively affected vitamin D status, i.e. total 25(OH)D levels.

Vitamin D₂-enhanced mushrooms could serve as an alternative vegetarian source of vitamin D, which could be added to any meal, giving a broad choice for consumers. To date only four randomised controlled trials have investigated the impact of vitamin D₂-enhanced foods on vitamin D status and, contradictory to the present results, three of these showed a significant increase in total 25(OH)D concentration by 50–67%^(34,39,40). However, Stephensen *et al.*⁽⁴¹⁾ found no effect of supplementation with vitamin D₂-enhanced mushrooms on vitamin D status. Three of these studies used vitamin D₂-enriched mushrooms with levels of vitamin D₂ ranging from an equivalent daily dose of 8.8 to 100 µg. In addition to this, the baseline values of total 25(OH)D were dramatically different in the various studies. In the study group used by

Table 1. Characteristics of the participants assigned to the treatment and placebo groups in study 1 (Number of subjects; mean values, standard deviations and ranges)

	Vitamin D ₂ -enhanced mushrooms		Placebo mushrooms		<i>P</i> *
	Mean	SD	Mean	SD	
Subjects (n)	21		21		0.747
Female	14		13		
Male	7		8		
Age (years)	54.4	6.3	49.8	6.3	0.022
Range	40–65		40–63		
Height (m)	1.68	0.07	1.70	0.08	0.388
Weight (kg)	71.6	13.3	75.3	12.7	0.359
BMI (kg/m ²)	25.2	3.2	26.0	3.1	0.441
Waist:hip ratio	0.80	0.08	0.82	0.10	0.656

* χ^2 statistics were used to test sex distribution between the groups. Independent-samples *t* tests were used to determine differences between subject characteristics.



Table 2. Characteristics of the participants assigned to the treatment and placebo groups in study 2 (Number of subjects; mean values, standard deviations and ranges)

	Vitamin D ₃ capsules		Placebo capsules		P*
	Mean	SD	Mean	SD	
Subjects (n)		22		21	0.835
Female		14		15	
Male		8		7	
Age (years)	49.1	6.7	52.9	7.2	0.083
Range		40–63		40–64	
Height (m)	1.69	0.10	1.72	0.10	0.351
Weight (kg)	74.7	15.0	76.5	14.6	0.691
BMI (kg/m ²)	25.9	3.5	25.7	3.2	0.791
Waist:hip ratio	0.83	0.09	0.81	0.08	0.285

* χ^2 statistics were used to test sex distribution between the groups. Independent-samples *t* tests were used to determine differences between subject characteristics.

Stephensen *et al.*⁽⁴¹⁾, 94 % of participants had 25(OH)D concentrations > 50 nmol/l at the start of the study with a significant contribution from cutaneous synthesis, making it difficult to improve status via a dietary source. In contrast, in the study

by Outila *et al.*⁽³⁹⁾, a younger population with low baseline vitamin D status (25(OH)D < 60 nmol/l) was selected for the intervention. This contrasts with our older population with no pre-selection based on vitamin D status. In the present

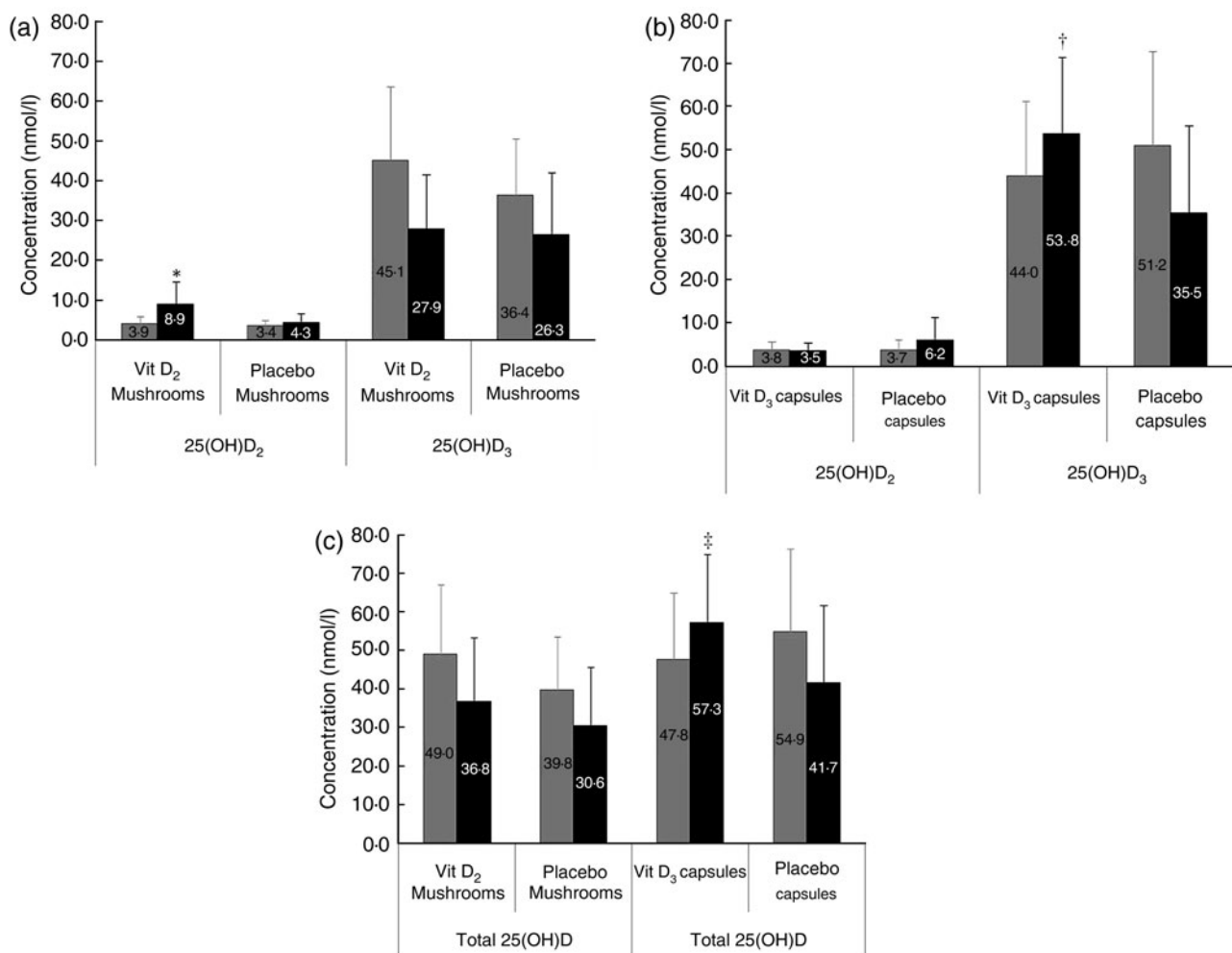


Fig. 2. Serum levels of 25-hydroxyvitamin D₂ (25(OH)D₂) and 25(OH)D₃ at baseline (■) and after a 4-week intervention (■) in (a) the mushroom study and (b) capsule study. (c) Serum levels of total 25(OH)D in the mushroom and capsule studies. Vit D₂, vitamin D₂; Vit D₃, vitamin D₃. Values are means, with standard deviations represented by vertical bars. A general linear model ANOVA was used, adjusted for sex, age and BMI ($P < 0.05$). * Significant difference in the change of 25(OH)D₂ was observed in the vitamin D₂-enhanced mushroom group *v.* placebo mushroom group ($P = 0.007$). There was no difference in the change of 25(OH)D₃. † Significant difference in the change of 25(OH)D₃ was observed in the vitamin D₃ capsule group *v.* placebo capsule group ($P < 0.0003$). There was no difference in the change of 25(OH)D₂. ‡ Significant difference in the change of total 25(OH)D between the supplemented and placebo groups in the capsule study ($P = 0.0003$). No difference in total 25(OH)D was observed in the mushroom study. Total 25(OH)D was computed as a sum of 25(OH)D₂ and 25(OH)D₃ forms.



Table 3. Concentrations (nmol/l) of 25-hydroxyvitamin D₂ (25(OH)D₂), 25(OH)D₃ and total 25(OH)D across the four treatment groups following the intervention

(Mean values and standard deviations)

	Vitamin D ₂ -enhanced mushrooms		Placebo mushrooms		Vitamin D ₃ capsules		Placebo capsules		P
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	
25(OH)D ₂	8.9†‡	5.8	4.3*	2.2	3.5*	1.8	6.2	5.2	0.001
25(OH)D ₃	27.9†	13.5	26.3†	15.7	53.8*†§	18.0	35.5†	18.4	<0.001
25(OH)D	36.8†	16.6	30.6†	15.1	57.3*†§	17.7	41.7†	20.1	<0.001

* Mean value was significantly different from that of the vitamin D₂-enhanced mushroom treatment group ($P < 0.05$).

† Mean value was significantly different from that of the placebo mushroom treatment group ($P < 0.05$).

‡ Mean value was significantly different from that of the vitamin D₃ capsule treatment group ($P < 0.05$).

§ Mean value was significantly different from that of the placebo capsule treatment group ($P < 0.05$).

|| P values are based on analysis of group effect for post-values adjusted for baseline values, sex, age and BMI ($P < 0.05$).

study, despite no changes in total 25(OH)D there was a significant increase in 25(OH)D₂ levels in the enhanced mushroom group, similarly to the results obtained by Stephensen *et al.*⁽⁴¹⁾. A number of reasons may explain the lack of changes in total 25(OH)D: first, 25(OH)D₂ only contributed to approximately 8 % of the total 25(OH)D at baseline, making it difficult for any change in 25(OH)D₂ levels to alter the overall status. Second, there was a large decrease in 25(OH)D₃ (38 %) due to the time of year, again making it very difficult to overcome this drop with supplementation of the minor contributor to total 25(OH)D. Although daily consumption of 15 µg of vitamin D₂-enhanced mushrooms did not significantly make an impact on vitamin D status, we have clearly demonstrated

that the vitamin was well absorbed, since it significantly increased 25(OH)D₂ levels.

In the current literature a debate exists as to whether vitamin D₃ or vitamin D₂ is more potent in improving vitamin D status. Recent systematic reviews have indicated that vitamin D₃ is more efficacious at increasing total 25(OH)D^(45,46). Previous work has suggested that vitamin D₂ supplementation may cause a reduction of serum 25(OH)D₃ levels^(41,47). One of the potential mechanisms by which this may occur is due to a competition of both forms of the vitamin for 25-hydroxylase or/and increased degradation of 25(OH)D₃⁽⁴⁸⁾. The results of the present study in conjunction with other studies^(27,34,49) do not concur with this hypothesis. Total serum 25(OH)D was

Table 4. Metabolic syndrome markers pre- and post-intervention in the vitamin D₂-enhanced and placebo mushroom groups (Mean values and standard deviations)

	Vitamin D ₂ -enhanced mushrooms				Placebo mushrooms				P*
	Pre		Post		Pre		Post		
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	
Systolic BP (mmHg)	117	13	115	13	121	18	122	18	0.143
Diastolic BP (mmHg)	79	8	77	10	83	11	84	14	0.101
HR (bpm)	72	11	70	9	75	12	72	14	0.702
Glucose (mmol/l)	5.73	0.45	5.65	0.41	5.95	0.69	5.68	0.56	0.753
Insulin (µIU/ml)	3.94	2.79	3.55	2.87	4.53	1.99	4.41	3.07	0.719
HOMA-IR	1.01	0.73	0.89	0.73	1.22	0.60	1.14	0.95	0.617
C-peptide (ng/ml)	1.50	1.38	1.29	1.06	1.21	0.68	1.34	0.89	0.307
TAG (mmol/l)	0.83	0.27	0.90	0.35	1.23	0.79	1.13	0.75	0.420
Total cholesterol (mmol/l)	5.33	1.42	5.61	1.06	5.86	1.20	6.07	1.41	0.195
HDL-cholesterol (mmol/l)	1.71	0.49	1.72	0.45	1.61	0.41	1.63	0.44	0.484
LDL-cholesterol (mmol/l)	3.77	0.64	3.84	0.63	3.73	0.72	3.99	0.79	0.107
NEFA (mmol/l)	0.59	0.32	0.55	0.31	0.73	0.27	0.53	0.19	0.398
PTH (pg/ml)	61.6	32.2	59.1	32.0	60.1	21.4	57.1	16.6	0.659
Corrected Ca (mmol/l)	2.36	0.10	2.36	0.13	2.34	0.12	2.32	0.12	0.750
Adiponectin (µg/ml)	8.28	3.17	7.36	2.48	6.76	4.01	5.67	4.00	0.210
Ferritin (ng/ml)	48.2	44.4	52.4	45.8	75.0	62.0	61.8	51.5	0.650
Leptin (ng/ml)	5.17	13.13	3.55	9.16	2.25	2.70	1.77	1.59	0.774
Resistin (ng/ml)	3.29	0.88	3.00	1.03	3.06	0.96	3.39	1.11	0.135
hsCRP (mg/l)	2.15	2.37	1.06	0.72	1.78	2.48	1.23	1.48	0.498
TNFα (pg/ml)	4.62	1.27	4.34	1.24	4.47	1.48	4.14	1.27	0.333
IL-6 (pg/ml)	0.92	0.63	0.90	0.47	1.36	1.38	0.77	0.47	0.066
PAI-1 (ng/ml)	15.43	4.07	11.46	4.83	14.39	3.75	15.19	6.33	0.038

BP, blood pressure; HR, heart rate; bpm, beats per min; HOMA-IR, homeostasis model of assessment of insulin resistance; PTH, parathyroid hormone; hsCRP, high-sensitivity C-reactive protein; PAI-1, plasminogen activator inhibitor-1.

* P values are based on analysis of group effect for post values adjusted for baseline values, sex, age and BMI ($P < 0.05$).



Table 5. Metabolic syndrome markers pre- and post-intervention in the vitamin D₃ and placebo capsule groups (Mean values and standard deviations)

	Vitamin D ₃ capsules				Placebo capsules				P*
	Pre		Post		Pre		Post		
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	
Systolic BP (mmHg)	119	12	115	11	113	13	117	13	0.073
Diastolic BP (mmHg)	85	11	80	10	78	8	79	9	0.507
HR (bpm)	76	14	73	13	71	9	69	10	0.583
Glucose (mmol/l)	5.92	0.43	5.68	0.43	5.76	0.48	5.59	0.46	0.830
Insulin (μU/ml)	4.57	2.35	4.23	2.99	4.32	3.59	3.17	1.63	0.081
HOMA-IR	1.21	0.63	1.09	0.81	1.14	1.05	0.80	0.44	0.064
C-peptide (ng/ml)	1.39	1.05	1.33	0.91	1.46	1.18	1.01	0.70	0.020
TAG (mmol/l)	1.30	0.69	1.24	0.72	1.07	0.43	0.95	0.39	0.153
Total cholesterol (mmol/l)	5.66	1.12	5.43	1.24	6.47	1.16	6.06	1.40	0.496
HDL-cholesterol (mmol/l)	1.57	0.33	1.51	0.35	1.81	0.63	1.76	0.66	0.957
LDL-cholesterol (mmol/l)	3.57	0.48	3.73	0.60	4.08	0.48	4.12	0.42	0.736
NEFA (mmol/l)	0.62	0.27	0.58	0.21	0.51	0.19	0.49	0.19	0.650
PTH (pg/ml)	59.9	25.3	54.0	20.1	54.5	25.8	57.0	21.3	0.588
Corrected Ca (mmol/l)	2.31	0.09	2.31	0.09	2.34	0.10	2.36	0.12	0.178
Adiponectin (μg/ml)	6.23	2.96	5.73	2.12	7.28	2.93	6.98	2.68	0.528
Ferritin (ng/ml)	68.4	47.0	63.5	46.6	70.0	47.9	64.4	46.0	0.712
Leptin (ng/ml)	2.21	2.12	2.51	2.99	1.70	1.35	1.81	1.44	0.466
Resistin (ng/ml)	3.34	0.96	3.24	0.90	3.40	1.94	3.59	1.44	0.326
hsCRP (mg/l)	1.92	1.61	2.08	2.44	1.54	2.18	1.68	1.94	0.434
TNFα (pg/ml)	4.59	1.14	4.24	1.02	4.85	1.49	4.40	1.59	0.970
IL-6 (pg/ml)	1.06	1.30	0.83	0.71	1.12	1.28	1.65	2.94	0.536
PAI-1 (ng/ml)	15.29	5.62	14.19	6.26	14.35	5.80	11.09	4.51	0.082

BP, blood pressure; HR, heart rate; bpm, beats per min; HOMA-IR, homeostasis model of assessment of insulin resistance; PTH, parathyroid hormone; hsCRP, high-sensitivity C-reactive protein; PAI-1, plasminogen activator inhibitor-1.

* P values are based on analysis of group effect for post values adjusted for baseline values, sex, age and BMI ($P < 0.05$).

higher in vitamin D₂ supplement users compared with the unsupplemented group without any change in 25(OH)D₃ levels in the study of Rapuri *et al.*⁽⁴⁹⁾ and Holick *et al.*⁽²⁷⁾. Biancuzzo *et al.*⁽³⁴⁾ observed a similar increase in total 25(OH)D after supplementation with vitamin D₂ or D₃. The present study demonstrated a depletion of 25(OH)D₃ levels in all three groups not supplemented with vitamin D₃ and this decrease was not greater in the vitamin D₂-enhanced mushroom group. Winter depletion and seasonal fluctuation of total 25(OH)D levels have been previously shown in northern latitudes. We also found, in agreement with several earlier studies^(40,50), no changes in Ca levels and a negative relationship between total 25(OH)D and PTH levels, with no response of PTH to vitamin D supplementation, underlying the safety of the supplementation.

A secondary objective of the study was to investigate the effect of vitamin D supplementation on MetS markers. In the present study the 4-week intake of vitamin D₂-enhanced mushrooms led to a decrease in PAI-1 concentration. To date, no clear consensus regarding the impact of vitamin D supplementation on MetS markers has emerged in the literature. Some intervention studies failed to report any changes in MetS biomarkers⁽¹⁰⁾, while others found positive changes in the components of MetS in vitamin D-supplemented groups, such as insulin secretion or sensitivity, β-cell function, inflammation markers and lipid profile^(7–9,11,12). In our randomised controlled trial, only PAI-1 was lowered by the intake of vitamin D₂. However, it is important to consider that the study population was healthy and the intervention was only

for 4 weeks, which may be too short to see any changes in markers of the MetS. Several authors have suggested that a high dose of vitamin D that increases total 25(OH)D to levels above 75 nmol/l may be needed to produce significant changes in disease biomarkers^(51,52); in the present study the final circulating 25(OH)D concentration was below this threshold. PAI-1, which is a risk marker for coronary artery disease, was altered following mushroom supplementation⁽⁵³⁾. Despite no changes in vitamin D status, a significant decrease in PAI-1 in the vitamin D₂-supplemented group was observed. In a recent study PAI-1 activity was associated with serum 2(OH)D levels⁽⁵⁴⁾. Furthermore, mushroom extracts have been shown to alter PAI-1 levels *in vitro*⁽⁵⁵⁾. To the best of our knowledge, this is the first report of alterations in PAI-1 following vitamin D supplementation. Further work will be needed to validate this finding and ascertain whether the changes are due to alterations in 25(OH)D₂ levels or due to other bioactives present in the mushrooms.

In agreement with other studies, no inflammatory markers were affected by vitamin D supplementation^(56,57). A unique aspect of the present study was the use of vitamin D₂-enhanced mushrooms as a vehicle for supplementation; all of the previous studies examining these biomarkers used only vitamin D₃. Mushrooms contain several antioxidant and anti-inflammatory compounds⁽⁵⁸⁾ and an interesting result with respect to hsCRP was observed in the present study. Analysis across the groups revealed a significant reduction in hsCRP in the vitamin D₂-enhanced mushroom group. However, more studies are needed to confirm prolonged



mushroom and/or vitamin D₂ intake on the systemic inflammation marker hsCRP. In conclusion, consumption of vitamin D₂-enhanced mushrooms did not alter total 25(OH)D levels but significantly increased serum 25(OH)D₂ levels, indicating that the vitamin was bioavailable in this form. Furthermore, supplementation with vitamin D₂ did not negatively affect the levels of 25(OH)D₃. Further studies are warranted to ascertain the effects of supplementation with vitamin D₂-enhanced mushrooms at higher levels of vitamin D₂ and for longer durations. These studies will be critical for the future development of vitamin D-enhanced mushrooms as a vitamin D food source.

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M. S., L. B. and A. P. N. prepared the manuscript. L. B., A. P. N. and M. J. G. designed the research. M. S., L. O. M. and A. O. S. performed the research. M. S., L. B. and W. D. F. performed the analysis. J. C. prepared and analysed the mushrooms for the study.

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