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25-Hydroxyvitamin D concentration and all-cause mortality: the Melbourne Collaborative Cohort Study

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Abstract

Objective: To investigate relationships between mortality and circulating 25-hydroxyvitamin D (25(OH)D), 25-hydroxycholecalciferol ($25(OH)D_3$) and 25-hydroxyergocalciferol ($25(OH)D_2$).

Design: Case–cohort study within the Melbourne Collaborative Cohort Study (MCCS). We measured $25(OH)D_2$ and $25(OH)D_3$ in archived dried blood spots by LC–MS/MS. Cox regression was used to estimate mortality hazard ratios (HR), with adjustment for confounders.

Setting: General community.

Subjects: The MCCS included 29 206 participants, who at recruitment in 1990–1994 were aged 40–69 years, had dried blood spots collected and no history of cancer. For the present study we selected participants who died by 31 December 2007 (*n* 2410) and a random sample (sub-cohort, *n* 2996).

Results: The HR per 25 nmol/l increment in concentration of 25(OH)D and $25(OH)D_3$ were 0·86 (95 % CI 0·78, 0·96; $P=0·007$) and 0·85 (95 % CI 0·77, 0·95; $P=0·003$), respectively. Of 5108 participants, sixty-three (1·2 %) had detectable $25(OH)D_2$; their mean 25(OH)D concentration was 11·9 (95 % CI 7·3, 16·6) nmol/l higher ($P<0·001$). The HR for detectable $25(OH)D_2$ was 1·80 (95 % CI 1·09, 2·97; $P=0·023$); for those with detectable $25(OH)D_2$, the HR per 25 nmol/l increment in 25(OH)D was 1·06 (95 % CI 0·87, 1·29; *P* interaction = 0·02). HR were similar for participants who reported being in good, very good or excellent health four years after recruitment.

Conclusions: Total 25(OH)D and $25(OH)D_3$ concentrations were inversely associated with mortality. The finding that the inverse association for 25(OH)D was restricted to those with no detectable $25(OH)D_2$ requires confirmation in populations with higher exposure to ergocalciferol.

Keywords
Ergocalciferol
Cholecalciferol
25-Hydroxyvitamin D
All-cause mortality

Many cohort studies have reported inverse associations between circulating 25-hydroxyvitamin D (25(OH)D) and mortality[1]. Vitamin D exists in two forms, ergocalciferol (vitamin D_2) and cholecalciferol (vitamin D_3); 25-hydroxyergocalciferol ($25(OH)D_2$) and 25-hydroxycholecalciferol ($25(OH)D_3$) can be quantified separately; and a large study of their relative prevalence in New York has been published[2]. However, no cohort studies of mortality have presented associations separately for $25(OH)D_2$ and $25(OH)D_3$; many used assays such as RIA and chemiluminescent immunoassays[1] that cannot quantify them separately.

Cholecalciferol is the natural form of vitamin D in man, endogenously synthesised following UVB irradiation of 7-dehydrocholesterol in skin[3]. Dietary sources of cholecalciferol include fatty fish, fortified foods and supplements[3]. Ergocalciferol, an exogenous form of vitamin D, is produced in fungi following UVB irradiation

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of ergosterol. People can obtain ergocalciferol from some fortified foods (e.g. margarine)[4] and some species of mushrooms, and it is used in supplements in some countries[3,5]. In Australia, margarine contains low levels of ergocalciferol (1 μg/100 g)[6].

A systematic review and meta-analysis of randomised controlled trials (RCT) comparing the effects of ergocalciferol and cholecalciferol supplementation on 25(OH)D concentrations found that cholecalciferol is more effective than ergocalciferol at increasing serum 25(OH)D[4], especially when given as large weekly or monthly doses. Ergocalciferol might also increase catabolism of cholecalciferol; several RCT have reported that ergocalciferol supplementation lowered $25(OH)D_3$ concentrations[7–13]. Reviews of RCT of the effects of vitamin D supplementation on mortality show that there was a modest decrease in mortality for trials of cholecalciferol supplementation, but no decrease for trials of ergocalciferol supplementation, although the ergocalciferol trials were few and of relatively low quality[1,5].

Given limited evidence from trials that ergocalciferol and cholecalciferol supplementation might have different effects on mortality, observational studies of 25(OH)D and mortality should separate the two forms. The advent of liquid chromatography–tandem mass spectrometry (LC–MS/MS) now makes this possible because it enables simultaneous separate measurement of $25(OH)D_2$ and $25(OH)D_3$. We report here analyses of 25(OH)D, $25(OH)D_3$ and $25(OH)D_2$ in relation to all-cause mortality in a prospective cohort study of middle-aged men and women. The large size of the study and the availability of data on self-reported health about four years after recruitment also enabled us to assess the potential impact of reverse causality, which has been a major limitation of existing cohort studies.

Methods

Study design

A case–cohort study to investigate vitamin D status and risk of cancer, diabetes and mortality was conducted within the Melbourne Collaborative Cohort Study (MCCS), a prospective cohort study of 41 514 participants (25 % Southern European migrants) aged predominantly between 40 and 69 years at study recruitment (1990–1994). Details of the study design, recruitment and procedures are published elsewhere[14].

At baseline participants were interviewed about lifestyle and medical history, completed a self-administered 121-item FFQ developed for the MCCS[15], and anthropometric measurements (height, weight, waist and hip circumferences) were performed according to a standard protocol. Blood samples were collected and from the second year of recruitment (approximately 75 % of participants), whole blood was spotted on to Guthrie cards that were air dried and stored in the dark at room temperature. About four years after baseline, participants were mailed a questionnaire that included a question about their general health status, rated as poor, fair, good, very good or excellent.

Case–cohort study

Eligibility for the case–cohort study was restricted to 29 206 participants who had dried blood spot samples and, to reduce the possibility of reverse causality, no cancer diagnosed before baseline. The present analysis was restricted to the 2410 people who died by 31 December 2007 and the sub-cohort, a sex-stratified random sample of 2996 participants, with sampling fractions of 7·8 % for women and 13·6 % for men, approximately double the number of incident breast cancer and prostate cancer cases, respectively (Fig. 1).

Measurement of 25-hydroxyvitamin D

The mean time between blood sampling and 25(OH)D measurement was 18·1 (SD 0·8) years. Concentration of $25(OH)D_3$ has previously been shown to be stable in dried blood spots stored for 8–22 years[16]. Measurements of $25(OH)D_2$ and $25(OH)D_3$ were made in the laboratory of D.W.E. using LC–MS/MS[16] over 15 months in thirty-one batches of approximately 230 samples each. This laboratory uses National Institute of Standards and Technology calibrants and participates in the Vitamin D External Quality Assessment Scheme. The absolute limits of detection were 1 nmol/l for $25(OH)D_3$ and 2 nmol/l for $25(OH)D_2$[16]. We have previously reported high reliability of dried blood spot 25(OH)D measurements using 493 replicates from the case–cohort study; within- and between-batch intraclass correlations were 0·82 (95 % CI 0·80, 0·85) and 0·73 (95 % CI 0·68, 0·78), respectively[17]. To estimate the reliability of the $25(OH)D_2$ measurements, we calculated the intraclass correlation coefficient for the presence *v.* absence of $25(OH)D_2$ for the 493 replicate measurements. For a binary variable with two replicates, the intraclass correlation is the same as Cohen's κ. We calculated bias-corrected CI using a bootstrap method with 10 000 replications. The intraclass correlation for detectability of $25(OH)D_2$ was 0·54 (95 % CI 0·25, 0·72).

Direct comparison between dried blood spot and serum/plasma 25(OH)D concentrations is problematic because 25(OH)D is excluded from erythrocytes. Therefore, for initial descriptions of the concentrations, we corrected the observed concentrations for mean sex-specific haematocrit[17]. For all other analyses of 25(OH)D and $25(OH)D_3$, we used a calibration equation to predict plasma concentration[17]. In a subset of sixty-two MCCS participants who had dried blood spot and matching plasma samples (two replicates for each biospecimen type), we found good agreement between dried blood spot 25(OH)D and plasma 25(OH)D concentrations ($R^2 = 0{\cdot}73$) and developed an equation to estimate plasma 25(OH)D concentration from the dried blood spot

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concentration[17]. Because few participants in that study had detectable 25(OH)D_2, the calibration equation applies to total 25(OH)D and 25(OH)D_3 only. The sensitivity and specificity of 25(OH)D_2 detection in the dried blood spot samples compared with the plasma samples was respectively 100·0 (95 % CI 29·0 100·0) % and 94·9 (95 % CI 85·9, 98·9) % for the first replicate, and 50·0 (95 % CI 7·0, 93·0) % and 100·0 (95 % CI 93·8, 100·0) % for the second replicate.

Ascertainment of deaths

Deaths to 31 December 2007 were identified by linkage to the Victorian Registry of Births, Deaths and Marriages and the National Death Index at the Australian Institute of Health and Welfare.

Statistical analysis

Association between 25-hydroxyergocalciferol and 25-hydroxycholecalciferol

One participant whose 25(OH)D_2 concentration was several times higher than the next highest value was excluded from these analyses. For participants with detectable 25(OH)D_2, we estimated Spearman's correlation between the raw 25(OH)D_3 and 25(OH)D_2 concentrations. We used linear regression to estimate the difference in mean plasma 25(OH)D and 25(OH)D_3 concentrations for participants with and without detectable 25(OH)D_2, with adjustment for sex. For the regression analyses, we used estimated plasma 25(OH)D and 25(OH)D_3 concentrations derived from our calibration equation[17], but did not remove season and batch effects. The adjustment for sex was necessary because men had higher 25(OH)D and 25(OH)D_3 concentrations and were more likely to have detectable 25(OH)D_2.

Association between vitamin D and mortality

Participants with missing data for covariates (n 44) and those with extreme total energy intakes (<1st and >99th sex-specific percentiles; n 100) were excluded (Fig. 1).

Total 25(OH)D and 25(OH)D_3 concentrations were adjusted for batch and season, then converted to plasma-equivalent values using our calibration equation[17]. To remove batch effects, we first fitted a mixed-effects linear regression model with a random effect for batch to the sub-cohort observations. Next, for all observations, the predicted batch-specific deviations from the overall mean were subtracted from the observed values. Seasonal variation was removed by fitting linear regression models for 25(OH)D and 25(OH)D_3 as a sinusoidal function of the time of year when blood was sampled[18], then standardising measurements to represent 25(OH)D and 25(OH)D_3 measurements on the dates when the annual mean was attained (15 May and 14 November; late autumn and late spring in Australia). Participants were grouped into sex-specific quintiles based on the distribution of batch- and season-adjusted 25(OH)D or 25(OH)D_3 in the sub-cohort. For 25(OH)D_2, batch and seasonal adjustments were not performed because there were too few participants with detectable 25(OH)D_2.

Cox regression was used to estimate hazard ratios (HR) and 95 % CI. Barlow's method, robust SE[19] and Wald tests were used to account for the case–cohort design. Age was the timescale and models were stratified by sex and country of birth. Follow-up began at baseline and ended at the date of death, date left Australia or 31 December 2007, whichever came first. The proportional hazards assumption was assessed by fitting interactions between each covariate separately and attained age. There was no evidence that any covariate violated the assumptions.

We estimated HR for all-cause mortality for each quintile of 25(OH)D and 25(OH)D_3 relative to the lowest quintile and for the presence *v.* absence of detectable 25(OH)D_2, adjusting for confounders described below. For 25(OH)D and 25(OH)D_3 we also estimated the HR per 25 nmol/l by using the continuous forms of the variables. We fitted models with each vitamin D variable alone, a model including continuous 25(OH)D and its interaction with detectable 25(OH)D_2, and a model including main effects for continuous 25(OH)D_3 and detectable 25(OH)D_2. The interaction model assesses whether the relationship between 25(OH)D and mortality depends on the source of the 25(OH)D. Effect modification by sex was assessed by fitting interactions between each vitamin D variable (continuous for 25(OH)D and 25(OH)D_3) and sex.

Confounders

The following confounders were selected using a causal diagram and *a priori* knowledge from existing literature: age, sex, country of birth (Australia/New Zealand/Northern Europe or Southern Europe), an area-based measure of socio-economic disadvantage (quintiles from most disadvantaged to least disadvantaged), highest education level attained (primary school, some secondary school, secondary school, tertiary qualification), total energy intake (sex-specific quartiles, kJ/d), Mediterranean diet score (three categories indicating dietary pattern, with the highest representing high adherence to a Mediterranean diet), alcohol (never, former and sex-specific tertiles of current intake), smoking (never, former, current), physical activity (four categories reflecting frequency and intensity of exercise in the past 6 months), waist circumference (sex-specific quartiles, centimetres), and self-reported history of diabetes mellitus, hypertension and CVD (angina, myocardial infarction or stroke).

Sensitivity analyses

We repeated the analyses for the continuous versions of 25(OH)D and 25(OH)D_3 after excluding participants whose values were greater than the 99th percentiles to assess whether outliers had undue influence on the HR. We also assessed non-linearity of the dose–response relationships by fitting restricted cubic spline models with four knots at fixed, equally spaced percentiles (5 %, 35 %, 65 %, 95 %).

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People in poor health may have had reduced sun exposure, leading to lower 25(OH)D_3. They may also have taken supplements, leading to higher 25(OH)D_2, since vitamin D supplements in Australia in the early 1990s contained ergocalciferol[20]. Thus, observed associations between vitamin D and mortality could potentially be explained by reverse causality. To assess this possibility, a sensitivity analysis was conducted by restricting the analysis to people who reported having good, very good or excellent health on the 4-year follow-up questionnaire. For that analysis, follow-up began on the date of completion of that questionnaire.

Analyses were performed using the statistical software package Stata version 13·1.

Results

Dried blood spots were not located for sixteen participants and seven deaths were identified after the study began, leaving 5108 for analysis (Fig. 1). All samples had detectable concentrations of 25(OH)D_3 (range for haematocrit-corrected values, 2·4–146·9 nmol/l; to convert 25(OH)D in nmol/l to ng/ml, divide by 2·496), whereas 25(OH)D_2 was detected in sixty-three samples (1·2 %; range for haematocrit-corrected values, 4·1–195·0 nmol/l). The median plasma-equivalent 25(OH)D concentration, adjusted for batch and season, was 48·4 nmol/l; 99 % of values were greater than 19·7 nmol/l and 1 % greater than 107·3 nmol/l.

Participants who were born in Southern Europe, had primary school education or less, were in the lowest energy intake quartile, did not consume alcohol (both lifetime abstainers and former drinkers), were current smokers, did not do any physical activity, were in the largest waist circumference quartile or had diabetes mellitus at baseline were more likely to have low 25(OH)D_3 concentrations (Table 1). These patterns were almost identical for 25(OH)D (data not shown). Participants with high alcohol consumption and current smokers were less likely to have 25(OH)D_2, while those who took multivitamins were more likely to have 25(OH)D_2 (Table 2).

The mean plasma 25(OH)D concentration was 11·9 (95 % CI 7·3, 16·6) nmol/l higher ($P<0·001$) when 25(OH)D_2 was detected than when it was not detected, whereas the mean plasma 25(OH)D_3 concentration was 4·9 (95 % CI −9·6, −0·3) nmol/l lower ($P=0·04$). For participants with detectable 25(OH)D_2 there was a weak, non-significant inverse correlation between their raw 25(OH)D_2 and 25(OH)D_3 concentrations (Spearman's correlation = −0·09, $P=0·50$).

Vitamin D and mortality

During a mean of 13·7 (SD 2·2) years of follow-up (maximum 16·9 years), 2410 (8·3 %) participants died. The present analysis included 4964 participants, 2307 of whom died (Fig. 1).

The HR for the highest compared with the lowest total 25(OH)D quintile was 0·67 (95 % CI 0·54, 0·84) and the HR per 25 nmol/l increment in 25(OH)D was 0·86 (95 % CI 0·78, 0·96; $P=0·007$; Table 3). There was no evidence that the splines fitted better than a linear trend ($P=0·44$) and the HR was unchanged after excluding outliers (data not shown).

Because few participants had 25(OH)D_2, the HR for 25(OH)D_3 were essentially identical to those for total 25(OH)D (Table 3). The splines fitted no better than a linear trend ($P=0·42$) and excluding outliers did not affect the HR (data not shown). The HR for the presence of detectable 25(OH)D_2 was 1·80 (95 % CI 1·09, 2·97; $P=0·02$; Table 3). One batch of measurements had a disproportionate number of samples with 25(OH)D_2; results were similar when this batch was excluded (HR = 1·88; 95 % CI 1·07, 3·28; $P=0·03$).

When 25(OH)D_3 and detectable 25(OH)D_2 were included in the same model, their HR changed little (Table 3). The interaction between the continuous 25(OH)D and 25(OH)D_2 was significant ($P=0·02$). From this model, the HR per 25 nmol/l increment in 25(OH)D concentration was 0·85 (95 % CI 0·77, 0·95) when there was no detectable 25(OH)D_2 and 1·06 (95 % CI 0·87, 1·29) when there was detectable 25(OH)D_2.

There was little evidence that the HR for any of the vitamin D variables varied by sex (interactions: 25(OH)D, $P=0·30$; 25(OH)D_3, $P=0·28$; 25(OH)D_2, $P=0·25$).

Vitamin D and mortality in people reporting good health

When restricted to the 3159 participants (1136 of whom died) who reported good, very good or excellent health four years after baseline blood sample collection, forty-six had detectable 25(OH)D_2. The HR per 25 nmol/l increment in 25(OH)D and 25(OH)D_3 were 0·85 (95 % CI 0·73, 0·98; $P=0·021$) and 0·83 (95 % CI 0·72, 0·95; $P=0·009$), respectively. The HR for detectable 25(OH)D_2 was 1·75 (95 % CI 0·98, 3·10; $P=0·06$). The HR from the model including 25(OH)D and the interaction with detectable 25(OH)D_2 were also similar to those for all participants (data not shown).

Discussion

Higher 25(OH)D_3 and 25(OH)D concentrations were associated with a moderately reduced risk of death, whereas the presence of 25(OH)D_2 was associated with a moderately increased risk of death, despite participants with detectable 25(OH)D_2 having higher mean 25(OH)D concentration. The inverse association between mortality and 25(OH)D was restricted to those with no detectable 25(OH)D_2. The HR for 25(OH)D_3 and detectable 25(OH)D_2 changed little when they were included in the same model. These associations were similar when the

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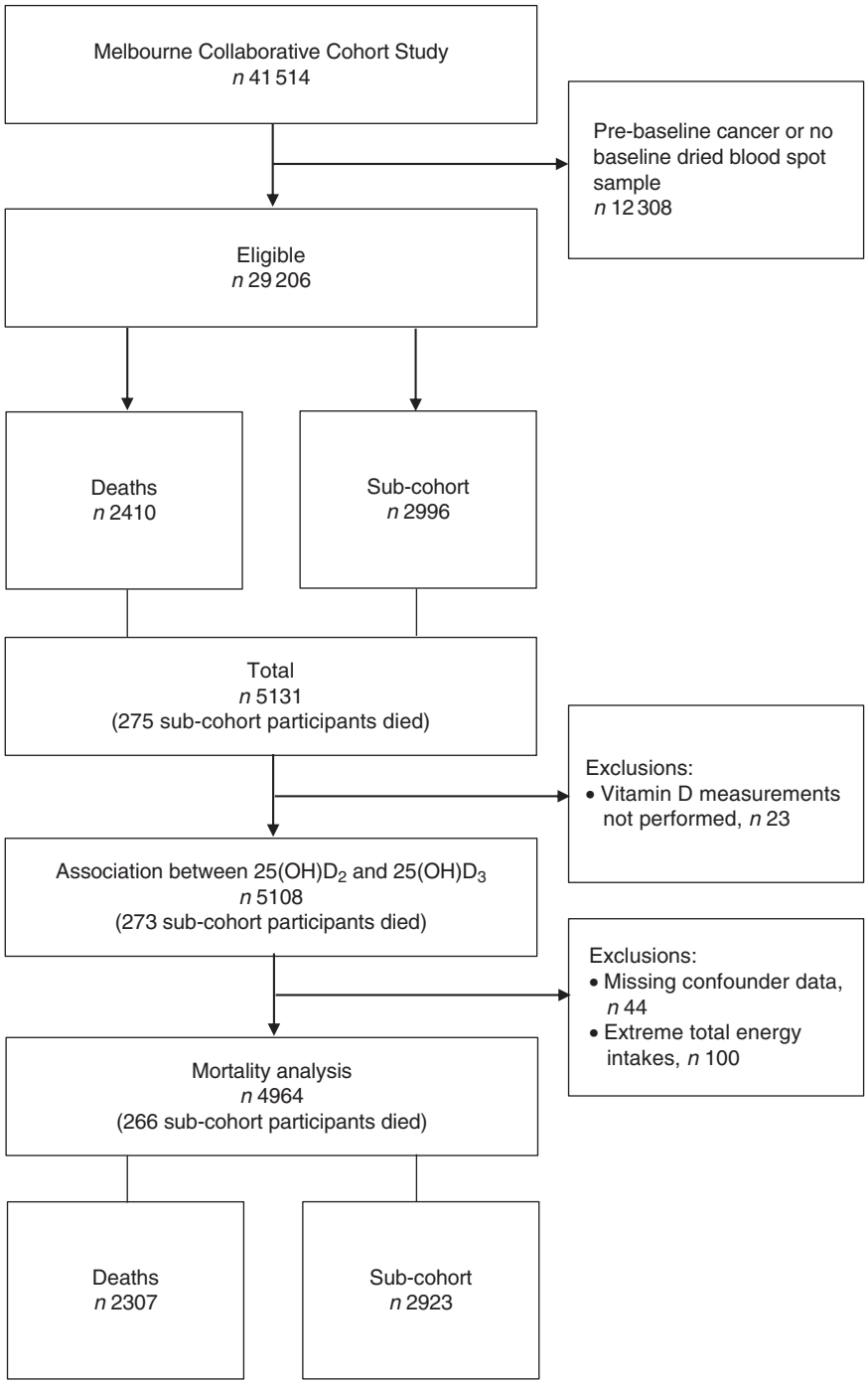

Fig. 1 Flow of participants in the present study. The sub-cohort was a sex-stratified random sample of eligible participants. Deaths were all deaths between baseline (1990–1994) and 31 December 2007 (25(OH)D_2, 25-hydroxyergocalciferol; 25(OH)D_3, 25-hydroxycholecalciferol)

analysis was restricted to people who reported being in good to excellent health about four years after blood draw.

Strengths of the current study include its prospective design, long follow-up, use of highly sensitive LC–MS/MS for separate measurement of both 25(OH)D forms, availability of extensive information on potential confounders, accurate and complete mortality data, and broad generalisability. Measurement error is unlikely to explain the associations: the samples were analysed in random order and blind to outcome status and so any measurement error would be non-differential and attenuate associations. We had no data on vitamin D supplementation, but only 16 % of participants reported using multivitamins at baseline (which at the time in Australia contained ergocalciferol only, but which now contain cholecalciferol only). We controlled for history of disease

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Table 1 Baseline characteristics of sub-cohort participants included in the mortality analyses (*n* 2923) according to quintiles of batch- and season-adjusted plasma 25-hydroxycholecalciferol ($25(OH)D_3$) concentrations (plasma concentration estimated from calibration equation[17]); Melbourne Collaborative Cohort Study

	$25(OH)D_3$ quintile (nmol/l)*									
	Quintile 1		Quintile 2		Quintile 3		Quintile 4		Quintile 5	
	(*n* 583; 20·0 %)		(*n* 585; 20·0 %)		(*n* 585; 20·0 %)		(*n* 585; 20·0 %)		(*n* 585; 20·0 %)	
	Median or *n*	IQR or %	Median or *n*	IQR or %	Median or *n*	IQR or %	Median or *n*	IQR or %	Median or *n*	IQR or %
Plasma $25(OH)D_3$ (nmol/l), median and IQR										
Overall	30·2	6·3	41·3	8·7	51·2	11·8	60·8	14·8	75·2	21·3
Females	29·1	4·9	36·5	3·4	42·9	3·4	50·6	4·5	62·7	10·3
Males	32·2	8·8	45·2	5·2	54·7	4·6	65·3	6·4	82·8	14·7
Male sex	322	20·0	323	20·0	323	20·0	323	20·0	323	20·0
Number of deaths										
Females	17	21·3	24	30·0	11	13·8	12	15·0	16	20·0
Males	45	24·2	37	19·9	40	21·5	33	17·7	31	16·7
Age (years), median and IQR	53·8	15·3	54·3	14·8	53·2	14·8	53·6	15·3	54·1	15·9
Country of birth										
Australia/NZ/Northern Europe	409	16·7	443	18·1	495	20·3	541	22·1	555	22·7
Southern Europe	174	36·3	142	29·6	90	18·8	44	9·2	30	6·3
Socio-economic disadvantage										
1st quintile (most disadvantage)	81	21·2	69	18·0	76	19·8	84	21·9	73	19·1
2nd quintile	101	21·1	104	21·8	91	19·0	98	20·5	84	17·6
3rd quintile	87	18·0	97	20·1	101	20·9	98	20·3	100	20·7
4th quintile	135	20·0	131	19·4	135	20·0	141	20·9	134	19·8
5th quintile (least disadvantage)	179	19·8	184	20·4	182	20·2	164	18·2	194	21·5
Education										
Primary school or less	95	28·7	102	30·8	55	16·6	45	13·6	34	10·3
Some secondary school	189	17·2	203	18·5	222	20·2	233	21·2	253	23·0
Secondary school	138	19·9	112	16·2	156	22·5	138	19·9	149	21·5
Tertiary	161	20·2	168	21·0	152	19·0	169	21·2	149	18·7
Energy intake (kJ/d, sex-specific quartiles)†										
1	174	23·8	164	22·5	138	18·9	130	17·8	124	17·0
2	144	19·7	137	18·7	147	20·1	147	20·1	156	21·3
3	144	19·7	127	17·4	152	20·8	151	20·6	158	21·6
4	121	16·6	157	21·5	148	20·3	157	21·5	147	20·1
Mediterranean diet score										
1 (low)	147	20·6	139	19·4	154	21·5	131	18·3	144	20·1
2 (medium)	370	20·2	355	19·4	358	19·6	380	20·8	367	20·1
3 (high)	66	17·5	91	24·1	73	19·3	74	19·6	74	19·6
Alcohol intake (g/d)‡										
Never	152	23·2	140	21·3	131	20·0	119	18·1	114	17·4
Former	35	27·3	26	20·3	25	19·5	20	15·6	22	17·2
Low	127	18·5	142	20·7	141	20·5	128	18·6	149	21·7
Medium	135	19·3	118	16·9	148	21·2	151	21·6	147	21·0
High	134	17·8	159	21·1	140	18·6	167	22·2	153	20·3
Smoking status										
Never	313	19·8	321	20·3	302	19·1	327	20·7	320	20·2
Former	190	18·7	196	19·3	214	21·0	200	19·7	217	21·3
Current	80	24·8	68	21·1	69	21·4	58	18·0	48	14·9
Physical activity score										
None	158	26·5	133	22·3	110	18·5	103	17·3	92	15·4
Low	124	21·7	126	22·1	115	20·1	105	18·4	101	17·7
Medium	199	19·9	201	20·1	209	20·9	189	18·9	200	20·0
High	102	13·5	125	16·5	151	19·9	188	24·8	192	25·3
Waist circumference (cm, sex-specific quartiles)‡,§										
1	127	17·7	147	20·5	126	17·6	149	20·8	169	23·5
2	124	16·9	135	18·4	153	20·9	157	21·4	164	22·4
3	142	19·1	151	20·4	168	22·6	153	20·6	128	17·3
4	190	26·0	152	20·8	138	18·9	126	17·3	124	17·0
History of diabetes mellitus	28	35·9	15	19·2	16	20·5	12	15·4	7	9·0
History of hypertension	101	19·0	113	21·2	107	20·1	93	17·5	118	22·2
History of CVD (angina, myocardial infarction or stroke)	35	19·0	43	23·4	35	19·0	27	14·7	44	23·9

IQR, interquartile range; NZ, New Zealand.
Values are median and interquartile range for $25(OH)D_3$ concentrations and age, or number and percentage for categorical variables.
*To convert 25(OH)D in nmol/l to ng/ml, divide by 2·496.
†Quartiles of total energy intake (kJ/d): 1, females = 3149–6301, males = 3755–7399; 2, females = 6302–8007, males = 7400–9192; 3, females = 8008–9827, males = 9193–11 397; 4, females = 9828–18 831, males = 11 398–22 150.
‡Categories of alcohol intake (g/d): low, females 0·1–4·2, males 0·1–9·2; medium, females 4·3–14·9, males 9·3–25·7; high, females 15·0–117·6, males 25·8–221·4.
§Quartiles of waist circumference (cm): 1, females = 52·7–70·6, males = 62·0–85·9; 2, females = 70·7–76·9, males = 86·0–91·9; 3, females = 77·0–85·9, males = 92·0–98·4; 4, females = 86·0–134·9, males = 98·5–131·0.

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Table 2 Presence of 25-hydroxyergocalciferol ($25(OH)D_2$) according to baseline characteristics of study participants; Melbourne Collaborative Cohort Study

		$25(OH)D_2$ present	
	N	*n*	%
Participants	5108	63	1·2
Sex			
Female	2255	22	1·0
Male	2853	41	1·4
Country of birth			
Australia/NZ/Northern Europe	4260	56	1·3
Southern Europe	848	7	0·8
Socio-economic disadvantage			
1st quintile (most disadvantage)	755	10	1·3
2nd quintile	940	15	1·6
3rd quintile	857	11	1·3
4th quintile	1111	13	1·2
5th quintile (least disadvantage)	1422	14	1·0
Education			
Primary school or less	729	9	1·2
Some secondary school	1988	16	0·8
Secondary school	1190	23	1·9
Tertiary	1200	15	1·3
Alcohol intake (g/d)*			
Never	1251	20	1·6
Former	245	2	0·8
Low	1169	16	1·4
Medium	1083	15	1·4
High	1352	10	0·7
Smoking status			
Never	2520	36	1·4
Former	1877	23	1·2
Current	710	4	0·6
Physical activity score			
None	1067	12	1·1
Low	964	10	1·0
Medium	1899	29	1·5
High	1177	12	1·0
Waist circumference (cm, sex-specific quartiles)†			
1	1127	17	1·5
2	1149	19	1·7
3	1295	10	0·8
4	1525	17	1·1
Multivitamin use			
No	4297	33	0·8
Yes	811	30	3·7
Mushroom consumption (frequency, categorized)			
Never or less than once per month	1565	24	1·5
Monthly	1624	17	1·1
Weekly	1919	22	1·2
Margarine intake (times/week, quartiles)			
0–0·4	1101	14	1·3
0·5–6·9	1363	17	1·3
7·0–17·4	1186	19	1·6
≥17·5	1450	13	0·9
History of hypertension			
No	3860	44	1·1
Yes	1246	18	1·4
History of CVD (angina, myocardial infarction or stroke)			
No	4563	56	1·2
Yes	544	7	1·3

NZ, New Zealand.
All values are number and percentage.
*Categories of alcohol intake (g/d): low, females = 0·1–4·2, males = 0·1–9·2; medium, females = 4·3–14·9, males = 9·3–25·7; high, females = 15·0–157·9, males = 25·8–232·2.
†Quartiles of waist circumference (cm): 1, females = 47·0–70·5, males = 62·0–85·9; 2, females = 70·6–76·9, males = 86·0–91·9; 3, females = 77·0–85·4, males = 92·0–98·3; 4, females = 85·5–153·6, males = 98·4–143·0.

and several risk factors, but cannot rule out the possibility of residual confounding. Given the small number of people with detectable $25(OH)D_2$, chance is also a potential explanation for its positive association with mortality.

Inclusion of middle-aged adults with no pre-baseline cancer reduced the possibility of reverse causality explaining the results. However, reverse causality might explain the positive association with $25(OH)D_2$ and inverse association with $25(OH)D_3$ if people in poor health were more likely to take supplements and spend less time outdoors, thus having lower concentrations of $25(OH)D_3$. Our sensitivity analysis, restricted to participants in good to excellent health several years after baseline, was designed to assess this possibility. While it is not possible to rule out reverse causality, the sensitivity analysis showed no evidence of its occurrence. The HR for total 25(OH)D and $25(OH)D_3$ were very similar; this might not be the case in populations with more ergocalciferol use, such as in the USA[2]. Finally, the absolute 25(OH)D values reported should be interpreted cautiously as these were plasma-equivalent 25(OH)D concentrations estimated from measurements of 25(OH)D in dried blood spots and with adjustment for batch and season.

A recent meta-analysis of cohort studies of vitamin D and all-cause mortality found a relative risk (RR) of 1·16 (95 % CI 1·08, 1·23) for a 10 ng/ml decrease in 25(OH)D, which is equivalent to 0·86 (95 % CI 0·81, 0·93) for a 25 nmol/l increase, and thus identical to the estimate from our study for 25(OH)D[1]. There was substantial heterogeneity between studies ($I^2 = 90\,\%$) that did not appear to be explained by any of the study characteristics used to form subgroups (no formal meta-regression was performed). The association was similar when stratified by latitude (≤40° *v.* >40°) and by age at baseline (<70 *v.* ≥70 years)[1]. It was slightly stronger for males, whereas we found similar associations for men and women. The association was stronger in the first five years after baseline[1]. One possible explanation for this finding is reverse causality – our study is one of few that have been able to assess this possibility by performing an analysis restricted to people in good health several years after baseline. Our results for 25(OH)D are also consistent with a recent meta-analysis of individual data from eight cohort studies from Europe and the USA, in which the RR for mortality in the lowest *v.* the highest quintile was 1·57 (95 % CI 1·36, 1·81), which is equivalent to RR of 0·64 (= 1/1·57) for the highest *v.* the lowest quintile (our corresponding HR was 0·67)[21]. In that study, there was also little heterogeneity by age and sex[21]. Some cohort studies have found U-shaped or reverse J-shaped relationships between 25(OH)D and all-cause mortality[22–25] but we found no such evidence. However, because few of our participants had concentrations above 100 nmol/l, our ability to assess curvilinear relationships was limited.

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Table 3 Hazard ratios (HR) and 95 % CI for all-cause mortality for total 25-hydroxyvitamin D (25(OH)D), 25-hydroxycholecalciferol ($25(OH)D_3$) and 25-hydroxyergocalciferol ($25(OH)D_2$); Melbourne Collaborative Cohort Study

	Concentration (nmol/l)*					Single-variable model			Two-variable model		
	Median	IQR	*n*	Deaths	Person-years†	HR‡	95 % CI	P_{trend}	HR‡	95 % CI	P_{trend}
Total plasma 25(OH)D§											
Quintile											
Q1	30·3	7·6	1067	545	7885	1·00	ref.	0·001			
Q2	41·3	8·9	1008	485	7977	0·85	0·68, 1·06				
Q3	51·6	11·8	992	458	8020	0·90	0·72, 1·11				
Q4	61·0	14·9	958	417	8056	0·80	0·64, 1·00				
Q5	77·0	21·1	939	402	8150	0·67	0·54, 0·84				
Per 25 nmol/l increase			4964	2307	40 088	0·86	0·78, 0·96	0·007			
$25(OH)D_2$ absent									0·85‖	0·77, 0·95	0·003
$25(OH)D_2$ present									1·06‖	0·87, 1·29	0·55
Plasma $25(OH)D_3$‡											
Quintile											
Q1	30·1	7·3	1066	545	7884	1·00	ref.	0·001			
Q2	41·0	8·7	1011	487	7988	0·86	0·69, 1·08				
Q3	51·4	11·6	991	457	8003	0·88	0·71, 1·10				
Q4	60·7	14·3	953	413	8050	0·77	0·62, 0·97				
Q5	76·2	20·9	943	405	8163	0·68	0·54, 0·85				
Per 25 nmol/l increase			4964	2307	40 088	0·85	0·77, 0·95	0·003	0·86¶	0·77, 0·95	0·004
Plasma $25(OH)D_2$											
Absent	0·0	0·0	4902	2273	39 653	1·00	ref.				
Present	13·7	9·2	62	34	436	1·80	1·09, 2·97	0·023	1·73¶	1·05, 2·87	0·032

IQR, interquartile range; ref., reference category.
*To convert 25(OH)D in nmol/l to ng/ml, divide by 2·496.
†Sub-cohort participants.
‡Adjusted for age, area-based index of socio-economic disadvantage, education, total energy intake, Mediterranean diet pattern, alcohol intake, smoking status, physical activity, waist measurement, diabetes mellitus at baseline, history of hypertension, history of angina, history of myocardial infarction, history of stroke, and stratified by sex and Southern European migrant status.
§Adjusted for batch and seasonal effects.
‖Interaction between 25(OH)D and $25(OH)D_2$.
¶Mutual adjustment for $25(OH)D_2$ and $25(OH)D_3$.

We are not aware of any cohort studies of $25(OH)D_2$ and $25(OH)D_3$ and mortality. According to a Cochrane review of RCT of vitamin D supplementation, cholecalciferol reduced all-cause mortality by 6 % (RR = 0·94; 95 % CI 0·91, 0·98; $P = 0·002$). Ergocalciferol had no apparent beneficial effect (RR = 1·02; 95 % CI 0·96, 1·08; $P = 0·54$), but in RCT involving participants with vitamin D insufficiency, the risk of mortality was elevated (RR = 1·20; 95 % CI 1·05, 1·37; $P = 0·008$)[5]. The authors commented that 'the effect of vitamin D_2 may be neutral or even detrimental'[5]. In another meta-analysis of RCT, cholecalciferol supplementation reduced all-cause mortality (RR = 0·89; 95 % CI 0·80, 0·99). Overall, ergocalciferol had no beneficial effect (RR = 1·04; 95 % CI 0·97, 1·11), but increased mortality risk in RCT using doses of 15–50 μg/d (600–2000 IU/d; RR = 1·20; 95 % CI 1·05, 1·38), in RCT with shorter mean intervention periods (<1·5 years; RR = 1·20; 95 % CI 1·05, 1·37) and in RCT with shorter follow-up (<2·5 years; RR = 1·19; 95 % CI 1·04, 1·36)[1]. The median follow-up from the included RCT was only 1·4 years, the median age of participants was 77 years, there were relatively few deaths in each study, and thirteen out of the twenty-two RCT involved participants with pre-existing chronic disease[1]. Thus, existing RCT are not necessarily generalisable to younger/middle-aged community-dwelling populations.

Our findings that participants with detectable $25(OH)D_2$ had higher mean 25(OH)D but lower mean $25(OH)D_3$ concentration is consistent with the Osteoporotic Fractures in Men Study (MrOS)[26]. These findings complement evidence from RCT showing that ergocalciferol might interfere with production of $25(OH)D_3$ and/or induce processes that degrade $25(OH)D_3$[7–13].

Several potential mechanisms could explain differences in effects of ergocalciferol and cholecalciferol. The presence of a methyl group on C24 of ergocalciferol metabolites may slightly alter their ability to bind to, or their affinity for, vitamin D binding protein[27], vitamin D hydroxylases and the vitamin D receptor[28]. The lower affinity of $25(OH)D_2$ for vitamin D binding protein relative to $25(OH)D_3$ means its free fraction in blood is likely to be higher, consistent with the faster turnover of $25(OH)D_2$[8,27]. The shorter circulating half-life of ergocalciferol metabolites may lead to up-regulation of enzymes that not only metabolise ergocalciferol, but also concomitantly induce accelerated catabolism and clearance of $25(OH)D_3$[8,11].

Differential specificities of the various cytochrome P450 (CYP) enzymes that metabolise vitamin D may also help to explain the apparent effect of ergocalciferol on $25(OH)D_3$ concentration. While the microsomal vitamin D 25-hydroxylase, CYP2R1, 25-hydroxylates both

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ergocalciferol and cholecalciferol equally well[29,30], mitochondrial CYP27A1 25-hydroxylates cholecalciferol[31], but not ergocalciferol[32].

Another enzyme, CYP3A4, 25-hydroxylates ergocalciferol and not cholecalciferol, but also 24-hydroxylates several vitamin D metabolites[33–35]. In particular, CYP3A4 plays a major role in 23- and 24-hydroxylation of the biologically active form of cholecalciferol, 1,25-dihydroxycholecalciferol ($1,25(OH)_2D_3$), in the liver and small intestine[35]. CYP3A4 is the most abundant liver cytochrome P450 enzyme and has broad specificity, catabolising more than 50 % of known drugs[34,36]. Many drugs inhibit or induce CYP3A4, thereby potentially increasing vitamin D turnover[33,35]. It is possible that ergocalciferol similarly induces CYP3A4, leading to enhanced 23- and 24-hydroxylation of $1,25(OH)_2D_3$; inactivating cholecalciferol and ultimately eliminating it from the body. Thus, a plausible explanation for the inverse association between circulating $25(OH)D_2$ and $25(OH)D_3$, and any potential detrimental effect of ergocalciferol on health, is enhanced CYP3A4-induced catabolism of $1,25(OH)_2D_3$ in the presence of ergocalciferol.

In summary, our results confirm those of previous studies that higher levels of 25(OH)D are associated with reduced mortality. For our study at least, this was due to the inverse association between $25(OH)D_3$ and mortality. Our findings with respect to $25(OH)D_2$ require confirmation in other populations with higher exposure to this form of vitamin D.

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