Cross-sectional and longitudinal associations between the active vitamin D metabolite (1,25 dihydroxyvitamin D) and haemoglobin levels in older Australian men: the Concord Health and Ageing in Men Project

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Abstract Anaemia and low 25 hydroxyvitamin D (25D) and 1,25 dihydroxyvitamin D (1,25D) levels are common in older people and may adversely affect morbidity and mortality. While there is some evidence for an association between low serum 25D levels and anaemia, there are limited studies among community-dwelling older people. In addition, the relationship between anaemia and the active vitamin D metabolite, 1,25D, has not been investigated. The aim of this study was to examine the associations between serum 25D and 1,25D with anaemia in community-living men aged ≥ 70 years. Population-based, cross-sectional analysis of the baseline phase and longitudinal analysis of the Concord Health and Ageing in Men Project (CHAMP), a large epidemiological study conducted in Sydney among men

aged 70 years and older, were performed; 1666 men were seen at baseline (2005–2007), 1314 men at a 2-year follow-up (2007–2009) and 917 at a 5-year follow-up (2012–2013). The main outcome measurement was haemoglobin levels as a continuous measure. Covariates included 25D and 1,25D, estimated glomerular filtration rate, demographic information, lifestyle measures, health conditions and medication information. The prevalence of anaemia ($\text{Hb} < 13.0 \text{ g/dL}$, WHO definition) was 14.6 %. In cross-sectional analysis, serum 25D concentrations were positively associated with haemoglobin levels in unadjusted analysis (β value 0.004; 95 % confidence interval (CI) 0.0009, 0.007; $p=0.01$), but the associations were no longer significant after multivariate adjustment. The association between

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1,25D levels and haemoglobin levels was significant in unadjusted analysis (β value 0.003; 95 % CI 0.002, 0.004; $p < 0.0001$) and remained significant in adjusted analysis (β value 0.001; 95 % CI 0.004, 0.003; $p = 0.01$). Serum 1,25D (but not 25D) levels at baseline were significantly associated with changes in haemoglobin over 2 and 5 years in unadjusted (β value 0.002; 95 % CI 0.0009, 0.003; $p < 0.0001$) and in fully adjusted analyses (β value 0.001; 95 % CI 0.0004, 0.002; $p = 0.001$). Serum 1,25D, but not 25D, concentrations are independently associated with haemoglobin levels in older men in both cross-sectional and longitudinal analyses. This raises the question whether vitamin D metabolites may influence anaemia states, mediated through different biological pathways, or represent a time-dependent biomarker of chronic ill health.

Keywords Vitamin D · Calcitriol · Anaemia older men · Population study

Introduction

Increasing evidence indicates that anaemia is common in older people and may adversely affect morbidity and mortality (Nissenson et al. 2003; Artz 2008). Studies examining anaemia in older populations have shown that about one third of cases are attributable to nutritional deficiency, one third to anaemia of inflammation or chronic disease and the other one third of cases remain “unexplained” (Guralnik et al. 2004).

Poor vitamin D status is common among older people and may be an increasingly important public health issue worldwide (Mithal et al. 2009). The importance of vitamin D in calcium absorption and metabolism for bone health is well known. The serum concentrations of 25 hydroxyvitamin D (25D), the major storage and circulating form of vitamin D, rise and fall with the supply of cholecalciferol (mainly from sunlight exposure) and ergocalciferol (from dietary sources). 25D is converted to its metabolically active form, 1,25 dihydroxyvitamin D (1,25D), in the kidney, although hydroxylation of 25D to 1,25D can also occur in many different tissues throughout the body, including bone and muscle (Hewison et al. 2007). Deficiency in 25D or 1,25D can lead to secondary hyperparathyroidism (through changes in serum ionised calcium levels). Parathyroid hormone

promotes the hydroxylation of 25D to 1,25D, which results in a compensatory rise in 1,25D concentrations to normal or elevated levels (Lips 2007). Low serum levels of 1,25D can occur due to impaired renal conversion of 25D, a process known to decline with age (Holick 2003).

Older adults are at an increased risk of vitamin D deficiency due to a range of factors including lack of sun exposure and an age-related decline in efficiency of vitamin D synthesis and metabolism. Thus, anaemia and low 25D and 1,25D levels are conditions that both increase with age and may result in significant morbidity (Holick 2003). While there is some evidence for an association between low serum 25D levels and anaemia (Sim et al. 2010), the majority of these studies were conducted among patients with chronic kidney disease (CKD) (Patel et al. 2010), end-stage renal disease (Kiss et al. 2011), congestive heart failure (Zittermann et al. 2010) or diabetes (Meguro et al. 2011). There are limited studies investigating this among community-dwelling older people (Perlstein et al. 2011). The relationship between haemoglobin levels and the active vitamin D metabolite, 1,25D, has not been investigated in any community-dwelling population group.

However, there are biologically plausible reasons for why 1,25D could be associated with anaemia. 1,25D regulates the process of erythropoiesis by stimulating erythroid progenitor cells in a synergistic fashion with other hormones and cytokines, including erythropoietin (EPO), and it has been reported that vitamin D metabolites are crucial for normal red blood cell production (Alon et al. 2002; Lucisano et al. 2014). Low 1,25D levels may therefore lead to anaemia. In addition, 1,25D mediates most of the known cellular actions of vitamin D. It modulates the level of systemic cytokine production, thus reducing the inflammatory milieu that leads to anaemia of chronic disease, and both in vivo and in vitro studies have demonstrated that 1,25D reduces cytokine production (Pedersen et al. 2007).

1,25D has not been investigated in other studies because the assay is expensive and requires expertise to produce reliable results. The aim of the current analysis was therefore to assess and compare the associations of both 25D and 1,25D status with haemoglobin levels in a cohort of older community-living men.

Methods

Population

The Concord Health and Ageing in Men Project (CHAMP) is an epidemiological study of a wide range of health issues in Australian men aged 70 years and over (Cumming et al. 2008). The population selection has been described in detail elsewhere (Cumming et al. 2008). Briefly, CHAMP involves men living in a defined urban geographical region (the Local Government Areas of Burwood, Canada Bay and Strathfield) near Concord Hospital in Sydney, Australia. The sampling frame was the New South Wales Electoral Roll, on which registration is compulsory in Australia. The only exclusion criterion was living in a residential aged care facility. Eligible men were sent a letter describing the study and, if they had a listed telephone number, were telephoned about 1 week later. Of the 2815 eligible men with whom contact was made, 1511 participated in the study (54 %). An additional 194 men aged 70 years or older living in the study area took part in the study before receiving the invitation letter; these men had been told about the study by friends or read reports in newspapers (Cumming et al. 2008).

Data collection

Baseline data were collected between January 2005 and June 2007. Men completed a questionnaire at home before coming to the study clinic at Concord Hospital. The clinic visit consisted of physical performance measures, biological measures, medication inventory and neuropsychological testing. Data were collected by fully trained staff, and the same equipment was used for all measurements and assessments, which were carried out in a single clinic. Two-year follow-up assessments were conducted between January 2007 and October 2009, and a 5-year follow-up was conducted between January 2012 and October 2013, using the same measures as at baseline. Of the 1705 subjects who completed the baseline assessments, a total of 1666 subjects were included in cross-sectional analyses. Thirty-nine men did not have a valid haemoglobin blood measurement; 1314 (79 %) had 2-year follow-up assessments, and 917 (55 %) had 5-year follow-up assessments. Death was the main reason for non-participation at 2 years (99 deaths) and at 5 years (382 deaths). The other main

reason for failure to attend the follow-up clinic visits was illness ($n=110$ at 2 years and $n=174$ at 5 years).

Measurement of serum 25D and 1,25D concentrations

Fasting blood samples were collected from participants on the morning of their clinic visit. Serum 25D and 1,25D levels were measured by radioimmunoassay (RIA) using single-batch reagents (DiaSorin Inc., Stillwater, MN), as described previously (Hirani et al. 2013). The assay for 25D has a sensitivity of <3.75 nmol/L with an intra-assay precision of 7.6 % and an inter-assay precision of 9.0 %. The assay for 1,25D has a sensitivity of <5.2 pmol/L, an intra-assay precision of 7.7 % and an inter-assay precision of 12.3 %. All assays were carried out in duplicates. Of note, due to complete cross-reactivity of the antibody, the assays measure total circulating vitamin D levels, including both vitamin D2 and vitamin D3. The laboratory participates in regular quality assurance (QA) protocols, including the vitamin D external Quality Assessment Scheme (DEQAS). Regular QA checks were performed with any adjustment to assays as necessary.

Blood tests

Blood tests were performed at the Diagnostic Pathology Unit of Concord Hospital, which is a National Australian Testing Authority (NATA)-accredited pathology service, using a MODULAR Analytics system (Roche Diagnostics, Castle Hill, Australia). Haemoglobin was measured by absorption spectrophotometry. The World Health Organization (WHO) criteria for anaemia in men (<13 g/dL) were used to ascertain the prevalence of anaemia (World Health Organization 1968).

Serum albumin level was included in the analysis as a continuous variable. White blood cell count (WBC) analysis was done by laser flow cytometry. In this study, WBC of 4–10,000 cells/ μ L was categorised as normal, <4000 cells/ μ L as low and $\geq 10,000$ cells/ μ L as high (Cheng et al. 2004).

Socioeconomic measures

Socioeconomic measures included source of income which was categorised as reliant on a government pension only versus other sources of income.

Lifestyle factors

Smoking status was classified as never, past (ex-smoker) or current. Physical activity was measured using the Physical Activity Scale for the Elderly (PASE), a method that scores the level of physical activity in individuals aged 65 years or older (Washburn et al. 1993a). The total PASE score was computed by multiplying the amount of time spent in each activity (hours/week) or participation (yes/no) in an activity by empirically derived item weights and summing over all activities.

Height and weight were measured, and body mass index (BMI) was calculated as kilograms per square metre and categorised as underweight (less than 20), normal weight (20–24.9), overweight (25.0–29.9) and obese (30.0 or over).

Vitamin D supplement use was coded as “yes” if participants reported currently taking them. Supplements included ergocalciferol-D2, cholecalciferol-D3, alfacalcidol and Ostevit-D (providing 25 µg/1000 IU of vitamin D).

Season

Season of blood sampling was categorised into summer (December–February), autumn (March–May), winter (June–August) and spring (September–November).

Health status

The comorbidity score (continuous) was calculated by summing the presence of the following self-reported conditions: diabetes, thyroid dysfunction, osteoporosis, Paget’s disease, stroke, Parkinson’s disease, epilepsy, hypertension, heart attack, angina, congestive heart failure, intermittent claudication, chronic obstructive lung disease, liver disease, cancer (excluding non-melanoma skin cancers), osteoarthritis and gout. Data on self-rated general health were obtained and dichotomized into excellent/good versus fair/poor/very poor. Depressive symptoms were evaluated by the Geriatric Depression Scale, short form (GDS) (Shiekh and Yesavage 1986).

Frailty

Frailty was defined as described previously in detail (Hirani et al. 2013) according to the frailty criteria used in the Cardiovascular Health Study (CHS) (Fried et al.

2001). Subjects were considered frail if they had three or more of the following frailty components: weight loss, weakness/reduced muscular strength, slow walking speed, exhaustion and low activity level. Adapted criteria were used for weight loss, exhaustion and low activity, as the exact measurements used in the CHS were not available in this study. Frailty scores were calculated from the five frailty components using baseline study variables as follows:

1. Weight loss/shrinking. Defined as current weight lower by 15 % or more than self-reported heaviest. Current weight was measured at the clinic visit.
2. Weakness. Defined as being in the lowest CHS study quintile for grip strength, adjusted for BMI. Maximal grip strength was measured using a Jamar dynamometer. Participants performed two trials on each side. The mean value of the measures was used.
3. Exhaustion. Defined according to responses to the following question from the Medical Outcomes Survey Short Form (SF12) (Ware et al. 1996): “How much of the time during the past 4 weeks did you have a lot of energy?” Men who chose the response options “a little” or “none of the time” were classified as exhausted.
4. Slowness. Defined as being in the lowest CHS study quintile for walking speed, adjusted for height (measured using a Harpenden stadiometer). Walking speed was measured on a 6-m course at usual pace. The mean value of two trials was used in this study.
5. Low activity. Defined as being in the lowest quintile on the PASE (cutoff score <73) (Washburn et al. 1993b). The PASE score was derived from questionnaire items that assess the frequency of activities of varying levels of exertion in several areas of daily life (recreational sport, leisure activities, and home and work activities) over a 1-week recall period.

Participants meeting none of the CHS criteria were considered “robust,” whereas those meeting 1–2 or 3+ criteria were termed “intermediate/prefrail” or “frail,” respectively.

Parathyroid hormone analysis

Parathyroid hormone (PTH) measures were important to include in the analysis since the activity of the enzyme

1 α hydroxylase that converts 25D to 1,25D in the kidneys is stimulated by PTH and inhibited by high 1,25D concentrations. Serum levels of intact PTH were determined by a two-site chemiluminescent ELISA on an Immulite 1000 analyser (Diagnostic Products, Los Angeles, CA), which measures the intact PTH molecule. The sensitivity of this assay is 1 pg/mL, and cross-reactivity to PTH fragments and related compounds is low. The assay has a typical intra-assay precision of 5.5 % and an inter-assay precision of 7.9 %, and the laboratory reference range is 23–66 pg/mL.

Renal function

Impaired renal function in older populations can lead to reduced conversion of 25D to 1,25D and anaemia. Serum creatinine levels were used to estimate glomerular filtration rate (eGFR), a measure of renal (kidney) function, using the Modification of Diet in Renal Disease (MDRD) formulae for men (Levey et al. 2005): $eGFR = 175 \times (\text{Serum creatinine}/88.4) - 1.154 \times \text{age}^{-0.203}$.

Medication assessment

Trained personnel conducted a medication inventory of each participant during the baseline clinic visit. Participants were instructed to bring all the prescription and over-the-counter medications they were taking to the clinic visit for review. They were also asked whether they had taken any prescription or non-prescription medications during the past month. Details of all medications and prescription patterns were recorded. Reported medicines were coded using the Iowa Drug Information Service code numbers (Division of Drug Information Service 2011).

Non-steroidal anti-inflammatory drugs

Non-steroidal anti-inflammatory drugs (NSAIDs) are the most frequently prescribed medications used to treat a variety of conditions that cause pain, inflammation and increased risk of anaemia (Day and Graham 2013; Artz 2008). We categorised participants as “NSAIDs users” versus “non-users.” NSAIDs users were classified as users in the month prior to the baseline visit either prescribed NSAIDs or obtained over the counter.

Statistical analysis

Analysis was carried out using STATA v12 (Stata Corp., College Station, TX). Descriptive characteristics were expressed as means (SD) and percentages. We performed univariate analysis to confirm inclusion of significant ($p < 0.1$) independent variables in adjusted linear regression analysis. Multiple linear regression modelling was used to examine unadjusted and adjusted associations between continuous measures of haemoglobin levels (the dependent variable) and vitamin D measures (25D and 1,25D) as the independent variables in separate models, reporting unstandardized regression coefficients, controlling for the effects of confounders and covariates of clinical significance such as demographic factors, lifestyle factors, season of blood sampling, presence of comorbidities, as well as adjustments for frailty, physical activity, depressive symptoms, the respective other serum vitamin D measure (either 25D or 1,25D), PTH, eGFR, NSAIDs, white cell count and albumin (Tables 2 and 3). Logistic regression analyses was carried out with 25D and 1,25D concentrations of the sample divided into deciles to help identify graphically any specific thresholds for 25D and 1,25D levels associated with anaemia.

To study the longitudinal association between haemoglobin levels and vitamin D measures, we used generalised estimating equation (GEE) analyses (Liang and Zeger 1986). With GEE analysis, the association between the longitudinally measured variables can be studied using all longitudinal data simultaneously and adjusting for within-person correlations caused by repeated measurement on each participant by using robust estimation of the variances of the regression coefficients. In this analysis, we had measures at three time points for haemoglobin measures (outcome). Vitamin D measures were only measured at baseline. Models were initially unadjusted and then adjusted by confounders including sociodemographic, lifestyle factors, health conditions and measures of clinical significance such as eGFR (Tables 4 and 5, models 2 and 3).

Ethics approval and informed consent

All participants gave written informed consent. The study was approved by the Sydney South West Area Health Service Human Research Ethics Committee, Concord Repatriation General Hospital, Sydney, Australia.

Results

Characteristics of participants

Characteristics of the analysed sample are summarised in Table 1. A valid serum 25D sample was obtained from 97.3 % (1659) of the total sample of men (1705 men) who participated in the study. Serum 1,25D samples were obtained from 1570 men. Thirty-four participants with renal disease, defined as having eGFR less than 30 mL/min/1.73 m² (National Kidney Foundation 2002), were excluded from the analysis.

The mean age of the study population was 77 (range 70–97) years. Forty percent were aged between 70 and 74 years, 32 % were between 75 and 79 years and 28 % were between 90 and 97 years old. Serum 1,25D, but not 25D, levels declined significantly with age ($p_{\text{for trend}} < 0.0001$). Further analysis showed that the decline in 1,25D levels by age (β value -1.37 ; 95 % confidence interval (CI) $-0.30, -4.50$; $p > 0.0001$) remained after adjustment for eGFR (β value -0.74 ; 95 % CI $-0.39, -0.11$; $p = 0.02$), suggesting that the decline in 1,25D levels with age is partly due to inefficiencies in renal function. Using the US Institute of Medicine and the Australian position paper definitions for 25D deficiency (25D < 30 nmol/L) and insufficiency (25D < 50 nmol/L) (Working group of the Australian and New Zealand Bone and Mineral Society 2012; Ross et al. 2011), the prevalence of vitamin D deficiency was 10.5 % and for 44.7 % of men had levels below 50 nmol/L.

Fifty percent of men were born in Australia and around 20 % were born in Italy. Australian-borne men had significantly higher serum 25D levels than men born in Italy ($p < 0.0001$), but there were no differences in serum 1,25D levels by country of birth. The majority (93.5 %) of men were not taking vitamin D supplements. There were significant seasonal differences in mean levels of both 25D and 1,25D, with lower serum concentrations in winter than in summer and autumn ($p < 0.0001$ for both vitamin D measures).

The prevalence of anaemia was 13.9 % for men with valid 25D and 14.0 % for those with 1,25D measures (Table 1). There were significant differences in mean levels of both 25D and 1,25D, with lower serum concentrations among men classified as anaemic compared with those who were not anaemic ($p < 0.0001$ for both vitamin D measures). The prevalence of anaemia by deciles of 1,25 and 25D measures are shown in Figs. 1

and 2. The prevalence of anaemia was higher in the lowest deciles of 25D below 52.9 nmol/L. Similarly, the prevalence of anaemia was higher in the lowest deciles (below 95.9 pmol/L) of 1,25D measures. When the presence of anaemia was examined by 25D and 1,25D deciles, a linear relationship was observed in cross-sectional analysis of baseline data adjusted by age. We identified a threshold for 25D concentrations associated with anaemia to be below 52.9 nmol/L and for 1,25D to be below 66.9 pmol/L (data not shown).

Correlation of 25D levels with 1,25D

The correlation between 25D and 1,25D levels (univariate analysis) was modest but statistically significant ($r = 0.30$, $p = 0.001$).

Further analysis shows that PTH itself was also associated with anaemia, although the association with 1,25D is independent of PTH (data not shown).

Cross-sectional analysis

The linear associations between 25D levels and haemoglobin levels were significant in unadjusted analyses (β value 0.004; 95 % CI 0.0009, 0.007; $p = 0.01$), but the statistical significance of these associations was lost with multivariate adjustment (Table 2, model 3).

The linear association between 1,25D levels and haemoglobin levels was found to be significant in unadjusted analysis (β value 0.003; 95 % CI 0.002, 0.004; $p < 0.0001$) and remained significant in fully adjusted analysis (β value 0.001; 95 % CI 0.001, 0.003; $p = 0.003$) (Table 3, model 3).

Further analysis showed that the association between 1,25D and haemoglobin levels, categorised into < 13 and ≥ 13 g/dL (the referent category), was found to be significant: for unadjusted (β value -22.4 ; 95 % CI $-32.00, -12.8$; $p < 0.0001$) and adjusted analyses (β value -11.1 ; 95 % CI $-20.82, -1.34$; $p = 0.03$). 25D levels were associated with anaemia in unadjusted analysis (β value -3.89 ; 95 % CI $-7.05, -0.73$; $p = 0.02$), but the significance was lost with adjustment by covariates (β value -1.53 ; 95 % CI $-4.66, 1.61$; $p = 0.34$, data not shown).

Longitudinal analysis

The longitudinal associations between the two vitamin D measures at baseline and haemoglobin using GEE are shown in Tables 4 and 5. 1,25D was significantly

Table 1 Descriptive characteristics of men aged 70 years and over participating in the CHAMP (2005–2007), with serum 25D and 1,25D measures

Variables	N (%)	Serum 25D measures (nmol/L)		N (%)	Serum 1,25D measures (pmol/L)	
		Mean	SD		Mean	SD
Age group	1625			1536		
70–74	652 (40.1)	56.0	22.5	612 (39.8)	116.4	64.5
75–79	518 (31.9)	56.2	21.6	490 (31.9)	112.6	68.5
80–84	294 (18.1)	56.0	21.2	281 (18.3)	102.2	64.8
85+	161 (9.9)	55.0	24.4	153 (10.0)	97.0	64.0
Income	1638			1515		
Other income	874 (54.4)	56.1	21.6	696 (45.9)	110.9	68.3
Pension	730 (45.5)	56.2	22.8	819 (54.1)	110.8	64.6
Country of birth	1625			1536		
Australia	804 (49.5)	59.0	22.9	748 (49.2)	110.0	65.6
Italy	325 (20.0)	53.9	20.7	310 (20.2)	114.3	68.3
UK	69 (4.2)	58.6	20.9	63 (4.1)	114.7	65.2
Greece	64 (3.9)	50.3	19.5	60 (3.9)	107.0	54.8
China	44 (2.7)	54.2	17.8	44 (2.9)	114.9	66.2
Other	319 (19.6)	51.3	22.0	303 (19.7)	-	-
Taking vitamin D supplements	1625			1523		
No	1519 (93.5)	55.8	22.0	1436 (93.5)	111.3	65.9
Yes	106 (6.5)	58.9	24.2	100 (6.5)	98.9	69.2
Taking NSAIDS	1619			1566		
No	1489 (92.0)	56.0	22.1	1447 (92.4)	110.6	66.7
Yes	130 (8.0)	54.9	22.2	119 (7.6)	100.0	57.9
Season	1625			1536		
Summer: December to February	337 (20.7)	58.0	20.0	294 (19.1)	151.8	78.6
Autumn: March to May	462 (28.4)	65.3	23.8	442 (28.8)	104.7	63.1
Winter: June to August	399 (24.6)	49.8	21.5	392 (25.5)	90.2	48.7
Spring: September to November	427 (26.3)	50.0	18.7	408 (26.6)	106.6	61.3
Cigarette smoking status	1607			1518		
Never smoked	605 (37.6)	57.6	22.3	577 (38.0)	111.5	67.0
Ex-smoker	906 (56.4)	55.6	21.8	850 (56.0)	110.3	66.3
Current smoker	96 (6.0)	52.5	23.6	91 (6.0)	114.5	59.1
Sun exposure (avoids direct sunshine)	1601			1512		
Never	576 (36.0)	57.0	23.4	542 (35.9)	110.1	66.2
Usually	868 (54.2)	56.3	21.6	819 (54.2)	112.3	67.2
Always	157 (9.8)	51.1	19.8	151 (10.0)	108.3	60.5
BMI status (kg/m ²)	1632			1512		
<20	27 (1.7)	59.7	22.5	339 (22.4)	121.5	71.4
20–24.9	358 (22.4)	59.1	24.3	736 (48.7)	107.7	64.6
25–29.9	784 (49.0)	56.3	21.9	412 (27.3)	108.1	62.4
30 and over	432 (27.0)	52.6	20.0	25 (1.7)	94.0	81.2
Self-rated general health	1604			1515		
Excellent/good	1134 (70.7)	57.7	22.2	1060 (70.0)	113.2	65.7
Fair/poor/very poor	470 (29.3)	52.3	21.7	455 (30.0)	105.0	66.7

Table 1 (continued)

Variables	N (%)	Serum 25D measures (nmol/L)		N (%)	Serum 1,25D measures (pmol/L)	
		Mean	SD		Mean	SD
Doctor-diagnosed conditions	1609			1520		
0–3 conditions	1205 (74.9)	57.3	22.7	1133 (74.5)	111.8	64.5
≥4 conditions	404 (25.1)	52.6	20.2	387 (24.5)	108.1	71.1
Depression	1602			1513		
No	1372 (85.6)	57.0	22.2	1298 (85.8)	112.6	66.3
Yes	230 (14.4)	51.5	21.6	215 (14.2)	99.8	63.9
Frailty	1593			1505		
Robust (not frail)	1475 (91.5)	56.9	21.9	1376 (91.4)	112.6	65.9
Yes	136 (8.5)	48.4	23.9	129 (8.6)	93.4	65.6
PTH	1616	56.0	22.2	1527	110.7	66.2
GFR	1625	56.0	22.2	1536	110.5	66.1
Haemoglobin	1617	56.0	22.2	1528	110.5	66.1
Anaemia	1617			1528		
No	1393 (86.2)	56.5	21.7	1314 (86.0)	113.8	66.4
Yes	224 (13.9)	52.8	24.9	214 (14.0)	90.6	60.4
Albumin	1625	56.0	22.2	1536	110.5	66.1

associated with changes in haemoglobin with time (baseline to 2-year follow-up to 5-year follow-up) in unadjusted (β value 0.002; 95 % CI 0.0009, 0.003; $p < 0.0001$) and in fully adjusted analysis (β value 0.001; 95 % CI 0.0004, 0.002; $p = 0.001$). 25D was not significantly associated with changes in haemoglobin

with time in unadjusted or adjusted analysis. There was no interaction between time and haemoglobin levels.

Additional adjustment by the respective other vitamin D metabolite (i.e. adjusting for 25D when analysing associations between 1,25D with haemoglobin levels

Fig. 1 Prevalence of anaemia by 25D, nanomoles per litre in deciles

Fig. 2 Prevalence of anaemia by 1, 25D, picomoles per litre in deciles

and vice versa) in cross-sectional or longitudinal analysis did not significantly change the results (data not shown).

Discussion

In this large epidemiological study, we examine cross-sectional and longitudinal associations between vitamin D status and haemoglobin levels in community-dwelling older men. There has been some evidence to suggest that 25D levels influence haemoglobin levels (Sim et al. 2010; Perlstein et al. 2011). Two cross-sectional studies have shown associations between low 25D levels and anaemia in older people (Sim et al. 2010;

Perlstein et al. 2011). However, our study is the first study to investigate a possible link between the biologically active form of vitamin D, 1,25D, and the prevalence of anaemia in older community-dwelling men. We found that after adjusting for known potential confounders, serum 1,25D levels, but not 25D, were significantly associated with haemoglobin levels.

Our findings raise the possibility of the potential role of 1,25D bioactivity in the pathogenesis of anaemia among older men. It has also been suggested that 1,25D is a modulator of chronic inflammatory responses (Guillot et al. 2010; Wu and Sun 2011). It is biologically plausible that 1,25D is involved in downregulating inflammatory markers, conferring an anti-proliferative effect, and involved in cytokine-induced interaction in chronic inflammatory diseases (Oelzner et al. 1997).

Table 2 Unadjusted and adjusted linear regression analyses for cross-sectional associations between 25D levels and haemoglobin among CHAMP men

Outcome per unit increase in 25D	Serum 25D nmol/L B (95 % CI)	<i>p</i> value
Model 1 ^a	0.004 (0.0009, 0.007)	0.01
Model 2 ^b	0.004 (0.001, 0.007)	0.01
Model 3 ^c	−0.001 (−0.004, 0.003)	0.80

^a Unadjusted

^b Adjusted for age and eGFR

^c Model 2 plus season, income, BMI physical activity, smoking, health conditions, self-rated general health, white cell count, albumin, NSAIDs, frailty, depressive symptoms, cancer, chronic heart failure

Table 3 Unadjusted and adjusted linear regression analyses for cross-sectional associations between 1,25D levels and haemoglobin among CHAMP men

Outcome per unit increase in 1,25D	Serum 1,25D pmol/L B (95 % CI)	<i>p</i> value
Model 1 ^a	0.003 (0.002, 0.004)	<0.0001
Model 2 ^b	0.002 (0.001, 0.003)	0.003
Model 3 ^c	0.001 (0.004, 0.003)	0.01

^a Unadjusted

^b Adjusted for age and eGFR

^c Model 2 plus season, income, BMI physical activity, smoking, health conditions, self-rated general health, white cell count, albumin, NSAIDs, frailty, depressive symptoms, cancer, chronic heart failure

Table 4 Unadjusted and adjusted linear regression (GEE) analyses for longitudinal associations between 25D levels and changes in haemoglobin levels over three time points among CHAMP men

Outcome per unit increase in 25D	Serum 25D nmol/L B (95 % CI)	<i>p</i> value
Model 1 ^a	0.003 (−0.0002, 0.005)	0.07
Model 2 ^b	0.002 (−0.0002, 0.005)	0.07
Model 3 ^c	−0.003 (−0.007, 0.002)	0.30

^a Unadjusted^b Adjusted for age and eGFR^c Model 2 plus, season, income, BMI, physical activity, smoking, health conditions, self-rated general health, white cell count, albumin, NSAIDs, frailty, depressive symptoms, cancer, chronic heart failure

The association between anaemia and low 1,25D levels may therefore indirectly reflect the effect of low-grade inflammation which is thought to play a major role in the development of anaemia of chronic disease in older adults (Perlstein et al. 2011; Roubenoff 2003). Alternatively, the association between low 1,25D status and anaemia may be attributable to the stimulatory effect of locally produced 1,25D in the development of red blood cells in bone marrow (Norman 2006). 1,25D may also affect haematopoietic tissue by directly activating erythroid precursor cells (Aucella et al. 2003). Further research is required to determine if 1,25D has the anti-inflammatory and/or stimulatory effects to the extent that it could influence haemoglobin levels.

We found a decline in mean serum 1,25D levels with age, a change that was not seen in serum 25D levels.

Table 5 Unadjusted and adjusted linear regression (GEE) analyses for longitudinal associations between 1,25D levels and changes in haemoglobin levels over three time points among CHAMP men

Outcome per unit increase in 1,25D	Serum 1,25D pmol/L B (95 % CI)	<i>p</i> value
Model 1 ^a	0.002 (0.0009, 0.003)	<0.0001
Model 2 ^b	0.001 (0.0005, 0.002)	0.001
Model 3 ^c	0.001 (0.0004, 0.002)	0.001

^a Unadjusted^b Adjusted for age and eGFR^c Model 2 plus, season, income, BMI physical activity, smoking, health conditions, self-rated general health, white cell count, albumin, NSAIDs, frailty, depressive symptoms, cancer, chronic heart failure

Further analysis showed that this is partly due to age-dependent decline in the efficiency of renal conversion of 25D to the active form, 1,25D. Therefore, older men may be at risk of 1,25D deficiency due to a progressive decline in renal vitamin D hydroxylation, even in the presence of normal serum 25D concentrations (Slovik et al. 1981); although the measure of eGFR was calculated from creatinine, it may not be a good estimator of renal function in old age because it can be confounded by muscle mass (Fliser 2008). The biological effect of 1,25D, however, is also influenced by other age-related factors, including impaired vitamin D receptor function or reduced receptor expression (Slovik et al. 1981; Bischoff-Ferrari et al. 2004).

Serum 25D concentrations were not associated with haemoglobin levels after adjusting for potential confounders. In contrast to our findings, two earlier cross-sectional studies among older people have shown a relationship between 25D deficiency and anaemia (Sim et al. 2010; Perlstein et al. 2011). One study, among 554 ambulatory participants with a mean age of 65 years showed a significant association between more severe anaemia (Hb<11 g/dL) and vitamin D insufficiency [25D<75 nmol/L] (Sim et al. 2010). The other study was in a cohort of 9675 non-institutionalised participants aged over 60 years (Perlstein et al. 2011). 25D deficiency was associated with anaemia (odds ratio 1.47; 95 % CI 1.06–2.05). The conflicting findings in our study could be due to our study participants being older than participants in these studies and also due to the ability for us to be able to account for the full range of confounders, which these studies were unable to do.

In our study, we showed that the cross-sectional association between serum 25D levels and haemoglobin levels was significant in unadjusted analysis, but with full adjustment by confounders and covariates of clinical relevance, the association was no longer significant.

This highlights the importance of accounting for potential confounders such as physical activity that could be associated with vitamin D and haemoglobin. Anaemia and hypoxia diminish tissue oxygenation, resulting in impaired muscle performance which is associated with physical decline, impaired functional performance and weakness (Penninx et al. 2004). It is conceivable therefore that older adults with low haemoglobin levels may be less mobile and decrease their physical activity, resulting in less time outdoors which in turn means less sunlight exposure and thus low levels of vitamin D.

Our study is the first to provide evidence of a longitudinal association between serum 1,25D and haemoglobin levels. Longitudinal studies have the advantage of being able to investigate temporal aspects of potentially causal associations, unlike cross-sectional designs which cannot ascribe causation. They are particularly important in ageing research, in allowing investigation into changes in health outcomes over time. The limitations of longitudinal studies are subject attrition (loss at follow-up), due to non-participation or mortality, a potential source of bias (Bhamra et al. 2008). However, analysis of attrition in our data showed that there were no significant differences between the demographic and self-reported baseline characteristics of those followed-up and participants lost to follow-up.

Our study raises the provocative question about whether vitamin D replacement in older people with iron deficiency anaemia could improve their anaemia status; however, it is not possible to answer this question based on current evidence (Sooragonda et al. 2014).

The main strengths of our study are that it involves a large, representative sample of community-dwelling Australian men aged 70 years and over. The age distribution of the men in the CHAMP study is similar to that of the census population in the study area during the same time period (Cumming et al. 2008) and the prevalence of self-reported disease in CHAMP participants is very similar to that found in an Australian national telephone survey of men's health (Holden et al. 2005). Recent literature only includes 25D as the preferred assay, related to the fact that 1,25D levels can be normal or even elevated in the presence of secondary hyperparathyroidism. We also have the advantage of having data on 1,25D, the biologically active form of vitamin D, as well as PTH, eGFR, medications and a range of confounding measures that have allowed us to investigate and adjust for these variables in examining associations between haemoglobin with the two vitamin D metabolites.

There are also study limitations. We did not have any data on vitamin B12, folate and ferritin to enable us to examine the associations between vitamin D metabolites and specific subtypes of anaemia. We did not have data on inflammatory cytokines such as TNF- α and IL-6. However, we did have measures for some inflammatory markers such as WCC and albumin which we were

able to adjust for in the analysis. We did not measure dietary vitamin D intake, nor did we obtain data on exact times spent outdoors or sunscreen use, which is important since UVB exposure is the primary source of vitamin D. Our study utilised the Diasorin assay for measurement of serum 25D and 1,25D levels. As all immunoassays, this method has inherent limitations as regards to bias, although it should be noted that in contrast to many other vitamin D immunoassays, the antibody used in the Diasorin assay has 100 % cross-reactivity for vitamin D2 and D3. Furthermore, we have thoroughly tested the Diasorin and other commercially available vitamin D assays against HPLC and liquid chromatography tandem mass spectrometry (LC/MS-MS) methodologies and found that the Diasorin assay delivered results that were comparable to those produced by LC/MS-MS (Herrmann et al. 2010). We therefore feel confident that the results presented in our study are reliable, in particular given the large number of participants. However, in future studies, the use of mass spectrometry should be considered for the measurement of vitamin D metabolites. Other limitations include loss to follow-up, which is unavoidable in longitudinal studies of community-based older people. Finally, it remains possible that both anaemia and low 1,25D are biomarkers of progressive ill health influenced by other unmeasured aspects of the comorbidities of ageing.

Conclusion

Serum 1,25D, but not 25D, concentrations are independently associated with haemoglobin levels in older men in both cross-sectional and longitudinal analyses. This raises the question whether vitamin D metabolites may influence anaemia states, mediated through different biological pathways, or represent a time-dependent biomarker of chronic ill health.

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Conflict of interest None

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Author contributions VH had the idea for the paper, conducted the analyses, interpreted the results and wrote the first draft. All authors contributed to revising the earlier drafts and approved the final paper.

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