Associations of total and free 25OHD and 1,25(OH)2D with serum markers of inflammation in older men

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Abstract:

Purpose: Vitamin D is hypothesized to suppress inflammation, and circulating 25-hydroxyvitamin D (25OHD) and inflammatory markers are inversely correlated. However, total serum 25OHD may not be the best indicator of biologically active vitamin D.

Methods: We tested serum total 25OHD, total 1,25(OH)2D, vitamin D binding protein (DBP), and estimated free 25OHD and free 1,25(OH)2D associations with inflammatory markers serum IL-6, TNFα and their soluble receptors, IL-10 and CRP as continuous outcomes and the presence of ≥2 inflammatory markers in the highest quartile as a dichotomous outcome, in a random subcohort of 679 men in the Osteoporotic Fractures in Men (MrOS) study.

Results: IL-6 was lower in men with higher 25OHD (-0.23 μg/mL per SD increase in 25OHD, 95% CI: -0.07 to -0.38 μg/mL) and with higher 1,25(OH)2D (-0.20 μg/mL, 95% CI: -0.0004 to -0.39 μg/mL); free D associations were slightly stronger. 25OHD and DBP, but not 1,25(OH)2D, were independently associated with IL-6. TNFα soluble receptors were inversely associated with 1,25(OH)2D but positively associated with 25OHD, and each had independent effects. The strongest association with ≥2 inflammatory markers in the highest quartile was for free 1,25(OH)2D (OR: 0.70, 95% CI: 0.54 to 0.89 per SD increase in free 1,25(OH)2D).

Conclusions: Associations of 1,25(OH)2D and free 25OHD with IL-6 mirrored those of 25OHD, suggesting that 1,25(OH)2D and free D do not improve upon 25OHD in population-based IL-6 studies. However, associations for the two metabolites diverged

	for TNFα soluble receptor, warranting examination of both metabolites in studies of TNFα and its antagonists.
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Author Comments:	January 25, 2016 Drs. R. Lindsay and J. Kanis Editors-in-Chief Osteoporosis International Dear Drs. Lindsay and Kanis, Thank you for the opportunity to resubmit "Associations of total and free 25OHD and 1,25(OH)2D with serum markers of inflammation in older men". We have addressed each reviewer's comment, as described in the attachment, and believe the revisions have resulted in a much stronger manuscript. Sincerely, Carrie Nielson, PhD, MPH

Response to Reviewers:	COMMENTS FOR THE AUTHOR: Reviewer #1: This manuscript explores the relationships between measures of vitamin D and inflammatory markers in a cross-sectional analysis. The main concern is the considerable number of associations assessed with no account taken of multiple testing. Could the authors please estimate the number of different associations assessed in the various tables and then attempt to take this into account appropriately to reduce the risk of type II error. Response: We are examining associations between five vitamin D metabolites – total 25(OH)D, total 1,25(OH)2D, free 25(OH)D, free 1,25(OH)2D and vitamin D binding protein (DBP) and six inflammatory markers (including soluble receptors) – IL-6, IL-6sR, TNFα, TNFα-sRI, TNFα-sRII and CRP, and, one anti-inflammatory marker – IL-10. That sums up to thirty-five associations of interest. The Bonferroni adjusted alpha is 0.001 (0.05/35). We have added this to the statistical methods section (lines 201-203) and a footnote to all tables for significant associations at the Bonferroni adjusted alpha. On a similar point, in addition to the multiple inflammatory markers assessed, the authors produce a composite score of inflammatory markers. This appears fairly arbitrary. Please could you authors justify the use of this outcome (including the thresholds chosen) if it is to remain in the analysis. Response: This is an attempt to examine the overall profile of a subject that may have elevated levels of more than one inflammatory marker. The profile of an individual having elevated levels of only one inflammatory marker might be different from the profile of an individual who might have elevated levels of more than one inflammatory marker. We do realize that there might be more than one method of computing a composite inflammatory score like a z-score (Hopkins, M.F., (2012), Associations of Circulating Inflammatory Biomarkers with Risk Factors for Colorectal Cancer in Colorectal Adenoma Patients, Biomarker Insights). The method we presented has also been previously published in multiple studies (Inflammatory markers and incident fracture risk in older men and women: the Health Aging and Body Composition Study. J Bone Miner Res 22:1088-1095, Inflammatory markers and risk of hip fracture in older white women: the study of osteoporotic fractures. J Bone Miner Res 29(9):2057-64, Inflammatory markers and the risk of hip fracture: the Women's Health Initiative. J Bone Miner Res 27(5):1167-76, Inflammatory markers and incident mobility limitation in the elderly. J Am Geriatr Soc 52(7):1105-13) and represents a more specific indicator of systemic inflammation than a high level of just one inflammatory marker. We have added this limitation to the Discussion (line 306-309). Why have the authors assessed correlations and then linear regressions for relationships between inflammatory markers and vitamin D? Furthermore, why have they then presented them in different parts of the results as if they are completely separate analyses? Perhaps the objective of the study in the introduction could be expanded and made more specific than just "examine associations" to justify why correlations and regressions are both necessary (if they are both needed). Response: We provide correlations as a supplemental table in order to show correlations among the independent variables and among the dependent variables, whereas the association analyses show the relationship between each independent and dependent variable. Although correlations among vitamin D variables are moderate to strong, and among inflammatory markers most correlations are weak to moderate, the correlations between them are weak (Results section lines 211 to 215). Correlations are only able to assess unadjusted strength of association, whereas linear regression modelling provides multiply adjusted estimates of magnitude of associations. Hence, the decision to examine correlations and linear regression modelling to quantify inflammatory markers and vitamin D associations. Supplementary table 1 is very confusing. Could the variables not be listed in a more

intuitive way? This will depend on the research question you are trying to answer (see above).

Response: The table has been rearranged to have all vitamin D metabolites together and all inflammatory markers together so it has better readability.

In table 1, the superscript "a" is used to mean two different things.

Response: Thank you for pointing out the error. It has been corrected.

Please justify presenting the raw data points in figures 2 and 3 but adding the adjusted line of best fit.

Response: The data points in figures 2 and 3 are not raw data points. They are actually predicted values from our fully adjusted linear regression model with the adjusted line of best fit added to it. We have clarified this better by adding it to the footnote of figures 2 and 3.

Could the authors comment on the generalisability of their results? Is the cohort in this specific analysis, similar to the US men in general?

Response: These results could be generalized to a predominantly non-Hispanic, white population of older men in the US. We have added a discussion of generalizability and comparison to other studies that examined these associations in SLE and RA patients to lines 293-295.

Reviewer #2: This is a interesting and well conducted study with the objective to examine associations of total and free 25OHD and 1,25(OH)2D with serum markers of inflammation. The topic is current and the results might have important clinical implications (measurement of 1,25(OH)2D as independent predictor of inflammatory state ?) and interesting speculations (dichotomous functions for the two vitamin D metabolites in TNFα pathway).

I have only few comments:
1. I suggest to expand the background adding some clinical example about the correlation between vitamin D deficiency and increase inflammation: Rossini M, Maddali Bongi S, La Montagna G, et al. Vitamin D deficiency in rheumatoid arthritis: prevalence, determinants and associations with disease activity and disability. Arthritis Res Ther 2010; 12:R216. Lange, U., Jung, O., Teichmann, J. & Neeck, G. Relationship between disease activity and serum levels of vitamin D metabolites and parathyroid hormone in ankylosing spondylitis. Osteoporos. Int. 12, 1031-1035 (2001). Amital H, Szekanecz Z, Szucs G, et al. Serum concentrations of 25-OH vitamin D in patients with systemic lupus erythematosus (SLE) are inversely related to disease activity: is it time to routinely supplement patients with SLE with vitamin D? Ann Rheum Dis 2010; 69:1155-1157.

Response: Thank you for the references. The background has been expanded with these clinical examples.

2. About the major and correctly recognized limitation of this study on the impossibility to address the causality relationship between inflammation and vitamin D, I suggest to add in the references the first reporting of Ried D, Toole BJ, Knox S et al. The relation between acute changes in the systemic inflammatory response and plasma 25-hydroxyvitamin D concentrations after elective knee arthroplasty. Am J Clin Nutr 2011;93:1006-11

Response: Thank you. We have added this reference to line 304.

3. In consideration of the interesting divergent associations of vitamin D metabolites with TNFα soluble receptors, have you any comment about the results of this study "Welsh P, Peters MJ, McInnes IB, et al. Vitamin D deficiency is common in patients with RA and linked to disease activity, but circulating levels are unaffected by TNFalpha blockade: results from a prospective cohort study. Ann Rheum Dis 2011; 70:1165-1167" ?.

	Response: The Welsh et al. study is an interesting study which we suggest supports, at least in part, our proposed explanation for the divergent effects of 25OHD and 1,25(OH)2D on soluble TNFα receptors. The Welsh et al. study assumes that TNFα is linked to vitamin D through effects on serum 25OHD levels. Instead we contend that the relationship is the other way round – low serum 25OHD may drive higher TNFα. However, as we outline in the Discussion of our manuscript, TNFα levels may indeed be subject to regulation by the active form of vitamin D, 1,25(OH)2D. This was not measured in the study by Welsh et al. but, as shown in our study, higher 1,25(OH)2D is associated with higher soluble receptor for TNFα. Conversion of 25OHD to 1,25(OH)2D is known to be stimulated by cytokines such as TNFα, so the induction of soluble TNFα receptors following the synthesis of 1,25(OH)2D may be part of a feedback control linking vitamin D and TNFα. It will certainly be interesting to assess the effects of TNFα blockade on serum levels of 1,25(OH)2D as well as 25OHD, and this is something that we are planning for future studies.

Associations of total and free 25OHD and 1,25(OH)$_2$D with serum markers of inflammation in older men

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Abbreviated Title: Associations of total and free 25OHD and 1,25(OH)$_2$D with inflammation

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Word count: *3,587 (OI limit: 5,000 words)*

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ABSTRACT (Between 150 & 250 words) *(Current number of words = 248)*

Purpose: Vitamin D is hypothesized to suppress inflammation, and circulating 25-hydroxyvitamin D (25OHD) and inflammatory markers are inversely correlated. However, total serum 25OHD may not be the best indicator of biologically active vitamin D.

Methods: We tested serum total 25OHD, total $1,25(OH)_2D$, vitamin D binding protein (DBP), and estimated free 25OHD and free $1,25(OH)_2D$ associations with inflammatory markers serum IL-6, TNFα and their soluble receptors, IL-10 and CRP as continuous outcomes and the presence of ≥2 inflammatory markers in the highest quartile as a dichotomous outcome, in a random subcohort of 679 men in the Osteoporotic Fractures in Men (MrOS) study.

Results: IL-6 was lower in men with higher 25OHD (-0.23 µg/mL per SD increase in 25OHD, 95% CI: -0.07 to -0.38 µg/mL) and with higher $1,25(OH)_2D$ (-0.20 µg/mL, 95% CI: -0.0004 to -0.39 µg/mL); free D associations were slightly stronger. 25OHD and DBP, but not $1,25(OH)_2D$, were *independently* associated with IL-6. TNFα soluble receptors were inversely associated with $1,25(OH)_2D$ but positively associated with 25OHD, and each had independent effects. The strongest association with ≥2 inflammatory markers in the highest quartile was for free $1,25(OH)_2D$ (OR: 0.70, 95% CI: 0.54 to 0.89 per SD increase in free $1,25(OH)_2D$).

Conclusions: Associations of $1,25(OH)_2D$ and free 25OHD with IL-6 mirrored those of 25OHD, suggesting that $1,25(OH)_2D$ and free D do not improve upon 25OHD in population-based IL-6 studies. However, associations for the two metabolites diverged for TNFα soluble receptor, warranting examination of both metabolites in studies of TNFα and its antagonists.

Mini Abstract

Vitamin D is hypothesized to suppress inflammation. We tested total and free vitamin D metabolites and their association with inflammatory markers. Interleukin-6 levels were lower with higher 25-hydroxyvitamin D. 1,25-dihydroxyvitamin D and free 25OHD associations mirrored those of 25OHD. However, associations for the two metabolites diverged for TNFα soluble receptors.

BACKGROUND

Chronic low-grade inflammation is a contributor to age-associated frailty, mortality and morbidity, including osteoporosis [1]. Inflammatory markers such as interleukin-6 (IL-6) and tumor necrosis factor alpha (TNFα) are implicated in the process of vascular calcification and regulation of bone remodeling [2, 3] and have been linked to incident fracture [4] and BMD loss [5].

Vitamin D has direct effects on bone health and may also act on bone by modulating inflammation [6, 7]. We have recently shown that low 1,25(OH)2D and 25OHD are independently associated with hip fracture in older men, but only 25OHD was independently associated with BMD loss [8]. $1,25(OH)_2D_3$ may also play a role in regulating both the inflammatory process and bone turnover. Decreased $1,25(OH)_2D_3$ levels may contribute to inhibition of bone formation and suppress activated T cells and cell proliferation, which may accelerate the inflammation process in those with conditions such as ankylosing spondylitis (AS) [9].

In vitro and *in vivo* evidence suggests that the biologically active form of vitamin D, $1,25(OH)_2D$, has several immunomodulatory functions, including suppression of pro-inflammatory marker expression and regulation of immune cell activity [10]. Treatment of fibroblast cultures with $1,25(OH)_2D_3$ inhibits IL-6 and interleukin-8 (IL-8) [11]. Vitamin D inhibits the activation of the TNFα converting enzyme and subsequent inflammation on multiple levels [10, 12, 13]. Studies of vitamin D and inflammatory diseases, such as rheumatoid arthritis (RA) and systemic lupus erythematosus, suggest a role for vitamin D deficiency in inflammation [14-17]. However, little is known about the relationship between inflammation and vitamin D in the general population.

The objective of this study was to examine associations of total and free circulating 25OHD and $1,25(OH)_2D$ and vitamin D binding protein (DBP) with inflammatory markers that include CRP, IL-6, TNFα, IL-6 and TNFα soluble receptors, and one anti-inflammatory cytokine – interleukin-10 (IL-10) in a cohort of older men.

METHODS

Study Design

The Osteoporotic Fractures in Men Study (MrOS) is a prospective observational cohort study designed to determine risk factors for osteoporosis and age-related medical conditions in men 65 years of age and older. This study is conducted at six centers in the United States: Birmingham, Alabama; Minneapolis, Minnesota; Palo Alto, California; Pittsburgh, Pennsylvania; Portland, OR; and San Diego, California. Participants were recruited by

mailings to the Department of Motor Vehicles (DMV), voter registration and participant databases, community and senior newspaper features and advertisements, and targeted presentations, from March 2000 through April 2002. Exclusion criteria were (1) inability to walk without assistance from another person, (2) bilateral hip replacements, (3) inability to provide self-reported data, (4) residence not near a study site, (5) judged by an investigator to have a medical condition that would result in early death, (6) and inability to understand and sign informed consent [18, 19].

Vitamin D Measurements

Serum concentrations of both the inactive (25OHD) and active ($1,25(OH)_2D$) forms of vitamin D were analyzed in a random sample of men from the baseline visit of the MrOS study. Additional assays were carried out to measure vitamin D binding protein (DBP), the major serum protein carrier of vitamin D metabolites. Assays were completed in December 2012 using stored serum collected at the MrOS baseline visit. At the baseline visit, fasting morning blood samples were collected; serum was separated immediately after phlebotomy, and then stored at −70°C. All samples for total 25OHD remained frozen in foil wrapped vials to reduce UV exposure until assay.

Measures for $25OHD_2$ (derived from ergocalciferol) and $25OHD_3$ (derived from cholecalciferol) were performed at the Mayo Clinic using mass spectrometry as previously described [20, 21]. Deuterated stable isotope (d3-25OHD) was added to a 0.2-ml serum sample as internal standard. $25OHD_2$, $25OHD_3$, and the internal standard were extracted using acetonitrile precipitation. Extracts were further purified online and analyzed by liquid chromatography/tandem mass spectrometry using multiple reaction monitoring. $25OHD_2$ and $25OHD_3$ were reported individually. The minimum detectable limit was 4 ng/ml for $25OHD_2$ and 2 ng/ml for $25OHD_3$. Aliquots of a single serum pool were included in alternate assay runs. Using the pooled serum, the interassay coefficient of variation (CV) for $25OHD_3$ was 4.4%, and the intraassay CV was 4.9%.

Total $1,25(OH)_2D$ was measured at the University of Leuven in Belgium, using LC-MS/MS without derivatization [22]. The lower limit of quantitation (LLQ) was 4.3 pg/mL for $1,25(OH)_2D_2$ and 6 pg/mL for $1,25(OH)_2D_3$. Inter-assay CV of pooled serum at low and high serum concentrations, respectively, were 10.1% for serum with mean concentration of 7.16 pg/mL and 5.9% for serum with mean concentration of 55.8 pg/mL [23].

DBP concentration in serum was measured by a two-site polyclonal ELISA (Genway Biotech, San Diego, CA) at the OHSU Clinical and Translational Research Institute laboratory. Intra-assay CV was 3%. Because no gold standard for DBP exists, we also measured DBP by a monoclonal ELISA (mELISA; R&D Systems,

Minneapolis, MN) and by polyclonal radial immunodiffusion assay (Laboratory of Clinical and Experimental Endocrinology, KU Leuven, Belgium), which had intra-assay CVs of 2-4% [24].

Free 25OHD concentrations were calculated using published mathematical models that incorporates serum concentrations of 25OHD, 1,25$(OH)_2$D, DBP, and albumin. Primary analyses were performed with estimated free 25OHD that assumed constant binding affinity across GC genotypes; however, *GC*-genotype-specific affinity estimates were also calculated for comparison [25].

Inflammatory Markers

Cytokine assays were measured in MrOS baseline samples utilizing a random sampling scheme. The assays were completed between December, 2009, and August, 2010, using archived serum collected at baseline on 1530 MrOS men as part of a MrOS ancillary study. Cytokine measures used in this analysis include CRP, IL-6, TNFα, tumor necrosis factor alpha soluble receptors (TNFα-sRI, TNFα-sRII) and interleukin-6 soluble receptor (IL6-sR). IL-10 was also measured as an anti-inflammatory measure.

All cytokine assays were performed at the Laboratory for Cytokine Biochemistry, University of Vermont. The samples were thawed at 37°C and briefly centrifuged. 300 µl of serum was placed into one cryovial for testing of TNFα, TNF-αsRI and sRII, IL-10 and CRP. Approximately 230 µl were plated into two plates for IL-6 and IL-6sR. The plates were refrozen at -80°C until assaying. IL-6 was measured using a high sensitivity ELISA (R&D Systems, Minneapolis, MN). The assay range is 0.16 – 12.0 pg/mL. Inter-assay CVs range from 6.11 to 8.47%. IL-6sR was measured using ELISA (R&D Systems, Minneapolis, MN). [26] The assay range is 3120 – 200,000 pg/mL. Inter-assay CVs range from 4.68 to 8.83%. TNFα was measured using the Human Serum CVD3 Multiplex Kit (Millipore Corp., Billerica, MA) which is run by flow cytometry on the Bio-Rad BioPlex 200 Luminex instrument. The assay range is 0.13-2000 pg/mL. Inter-assay CVs range from 4.93 to 9.13%. TNF-αsRI and sRII were measured with an ELISA (R&D Systems, Minneapolis, MN). The normal range for TNF- αsRI in serum is 479 – 966 pg/mL and for TNF-αsRII in serum is 1003 – 3170 pg/mL. Inter-assay CVs range from 5.42% to 8.59% for TNF-αsRI and 2.87 to 3.54% for TNF-αsRII. CRP was measured using the BNII nephelometer from Dade Behring utilizing a particle enhanced immunonepholometric assay. The assay range is 0.16 – 1100 ug/mL. Expected values for CRP in normal, healthy individuals are ≤ 3 ug/mL. Inter-assay CVs ranged from 1.52 to 3.68%.

Covariates

Demographic characteristics such as age, race/ethnicity, clinical site, and lifestyle factors including weekly alcohol consumption and smoking history were determined at baseline by questionnaire. Physical activity was assessed with the Physical Activity Score for the Elderly (PASE) [27]. Height (centimeters) was measured on Harpenden stadiometers, and weight (kilograms) was measured on standard balance beam or digital scales. Body mass index (BMI) was calculated as kilograms per meter squared (kg/m^2). Prevalent cardiovascular disease was defined as self-report of heart attack, congestive heart failure (CHF) or angina. Diabetes, stroke history, self-reported health, surgical removal of stomach/intestine and rheumatoid arthritis at baseline were also from self-report. Participants brought in all medications they used within the last 30 days. All prescription medications recorded by the clinics were stored in an electronic medications inventory database (San Francisco Coordinating Center, San Francisco, CA). Each medication was matched to its ingredient(s) based on the Iowa Drug Information Service (IDIS) Drug Vocabulary (College of Pharmacy, University of Iowa, Iowa City, IA) [28].

Serum creatinine was measured using a variation of the Jaffe enzymatic method. Renal function was expressed as estimated glomerular filtration rate (eGFR) in ml/min/1.73 m^2 using a standardized serum-creatinine based formula [29]. Total fat mass was measured from dual-energy X-ray absorptiometry (DXA) scans using Hologic QDR 4500 scanners (Hologic, Inc., Bedford, MA).

Statistical Analysis

All vitamin D measurements were standardized by subtracting the mean from each value and dividing by the standard deviation to facilitate comparison across measures. Correlations among inflammatory markers and between the inflammatory markers and the vitamin D measures were assessed using Spearman's correlation coefficients. We used linear regression modeling with robust standard errors to examine the effect of standardized vitamin D measurements on each inflammatory marker. Although the inflammatory markers are right-skewed, least-squares regression methods perform well with 500 or more observations and provide 95% confidence interval coverage for all regression coefficients [30]. Betas (β) and 95% confidence intervals (CI) from the model are reported as mean difference in the inflammatory markers per standard deviation (SD) change in vitamin D measurements. To identify any nonlinear associations between each vitamin D measure and inflammatory marker, we examined loess plots. We created an inflammatory index by summing the number of pro-inflammatory markers in the highest quartile (CRP, IL-6, TNFα, TNFα-sRI, TNFα-sRII and IL-6sR). We then dichotomized this index into those having ≥2 inflammatory markers in the highest quartile in comparison with those having <2 inflammatory

markers in the highest quartile [4]. Logistic regression modeling was used to obtain odds ratios (OR) and corresponding 95% CI.

No nonlinear associations were detected between any vitamin D measurement and inflammatory marker. The base model included age, race, clinical site, and season. We used stepwise modeling with a probability of removal at > 0.10, forcing the base model covariates of age, site, race, season and the vitamin D measure into the model and to include all covariates that were significantly associated with each inflammatory marker. All covariates that were significantly associated with any inflammatory marker were included in the final model for all inflammatory markers. An age-squared term was added to each model to check for non-linear association with age. There are thirty-five associations of interest (five vitamin D metabolites by six inflammatory markers and one anti-inflammatory marker). Thus, we have added a footnote to our tables with a Bonferroni adjusted p-value of ≤ 0.001 ($0.05/35 = 0.001$).

All analyses were conducted using SAS 9.3 (Cary, NC) and STATA release 12 (StataCorp, College Station, TX).

RESULTS

Description and correlations

Men with inflammatory markers and vitamin D measures (Figure 1) had a mean age of 74 ±6 years and mean BMI of 27 ±4 kg/m^2. Most (91%) were non-Hispanic white, and 85% reported excellent or good health status. 16% were taking NSAIDs, and 27% reported a history of heart attack, CHF, or angina (Table 1).

All correlations between inflammatory markers and vitamin D measures were weak. IL-6 was negatively correlated with total and free 25OHD and 1,25$(OH)_2$D measures ($r = -0.21$ to -0.25, $p<0.001$). TNFα and its soluble receptors were significantly negatively correlated with total and free 1,25$(OH)_2$D measures ($r = -0.14$ to -0.35, $p<0.001$) but not with 25OHD. The strongest correlation between CRP and vitamin D measures was with DBP ($r = 0.23$, $p<0.001$) (Supplemental Table 1).

Inflammatory marker associations with 25OHD and 1,25$(OH)_2$D

There was a significant association between lower IL-6 and higher 25OHD (0.23 pg/mL lower IL-6 per SD increase in 25OHD, 95% CI: 0.07 to 0.38 pg/mL), and this was independent of 1,25$(OH)_2$D and DBP (Table 2). Mean TNFα was 0.21 pg/mL lower per SD increase in 25OHD but this association was not statistically significant (95% CI: -0.64 pg/mL to 0.21 pg/mL). Results did not change after adjusting for 1,25$(OH)_2$D and DBP (Table 2).

Associations of IL-6 with 25OHD and $1,25(OH)_2D$ were similar (Figure 2), but statistically significant only for 25OHD. Mean IL-6 levels were significantly lower per SD increase in 25OHD (-0.23 pg/mL per SD, 95% CI: -0.38 to -0.07) and remained significant after adjusting for $1,25(OH)_2D$ and DBP. Mean IL-6 levels were lower by 0.20 pg/mL (95% CI: 0.0004 to 0.39 pg/mL lower) per SD increase in $1,25(OH)_2D$. This association was attenuated to after 25OHD and DBP adjustment and was no longer significant.

TNFα soluble receptors I and II were positively associated with 25OHD and inversely associated with $1,25(OH)_2D$ (Figure 3). Average TNFα soluble receptors I and II were significantly lower by- 62.05 pg/mL (95% CI: 26.09 to 98.01 pg/mL) for TNFα-sRI and -88.83 pg/mL (95% CI: 30.64 to 147.02 pg/mL for TNFα-sRII) per SD increase in $1,25(OH)_2D$. This association was strengthened with adjustment of 25OHD and DBP (Table 2). TNFα soluble receptors I and II were higher by 34.66 pg/mL (95% CI: -4.17 to 73.48 pg/mL) and 38.32 pg/mL (95% CI: -31.14, 107.78 pg/mL) per SD increase in 25OHD. This association was also strengthened with $1,25(OH)_2D$ and DBP adjustment (Table 2).

Odds of having ≥2 inflammatory markers in the highest quartile decreased by 25% (95% CI: 3% to 42% decrease) per SD increase in total $1,25(OH)_2D$ and was slightly strengthened with 25OHD and DBP adjustment (Table 2). However, 25OHD itself was not associated with odds of having ≥2 inflammatory markers in the highest quartile.

Inflammatory marker associations with free 25OHD, free $1,25(OH)_2D$ and DBP

Average CRP was significantly higher for each SD increase in DBP (1.11 ug/mL higher, 95% CI: 0.45 to 1.76 ug/mL higher) (Table 3). This association did not change after adjusting for 25OHD and $1,25(OH)_2D$ (Table 2). Although there was a significant negative correlation between CRP and free 25OHD ($r=-0.12$, $p<0.05$), there was no significant association in regression analysis ($\beta = 0.16$ ug/mL; $p=0.31$) (Supplemental Table 1 and Table 3). Mean IL-6 levels were lower by 0.35 pg/mL (95% CI: 0.15 to 0.55 pg/mL lower) for each SD increase in free 25OHD and by 0.22 pg/mL (95% CI: 0.04 to 0.39 pg/mL lower) per each SD increase in free $1,25(OH)_2D$. TNFα soluble receptor I levels were lower by 61.51 pg/mL (95% CI: 26.28 to 96.73 pg/mL lower) and TNFα soluble receptor II levels were lower by 78.72 pg/mL (95% CI: 15.72 to 141.71 pg/mL) per SD increase in free $1,25(OH)_2D$.

Odds of having ≥2 inflammatory markers in the highest quartile decreased by 30% (95% CI: 11% to 46% decrease) for each SD increase in free $1,25(OH)_2D$. There was no significant association with free 25OHD (Table 3).

CRP and TNFα soluble receptors' associations with free measures of 25OHD and $1,25(OH)_2D$ and DBP from other assays (monoclonal ELISA (R&D Systems, Minneapolis, MN) and radioimmunodiffusion assay (RID)) were similar to the polyclonal ELISA (Genway Biotech, San Diego, CA) assay, but slightly weaker. The CRP-DBP associations were also somewhat weaker but still statistically significant. IL-6 associations were significant for the free D measures from the RID assay, though slightly weaker, but not significant for the free D measures from the monoclonal ELISA assay (Supplemental Table 2). Use of *GC*-genotype-specific binding affinities, rather than constant affinities, in the free D estimating equations did not make a substantial difference in associations.

DISCUSSION

In this study of older men, 25OHD and $1,25(OH)_2D$ were negatively associated with IL-6 with similar magnitudes. On the other hand, associations with TNFα soluble receptors were positive for 25OHD and negative for $1,25(OH)_2D$. DBP was positively associated with CRP, and had a weak positive association with IL-6. Perhaps for this reason, free vitamin D measures, which incorporate DBP, had slightly stronger associations with IL-6 than total vitamin D measures. We did not observe any significant associations with TNFα, IL-10, or IL-6sR. These results indicate that $1,25(OH)_2D$ and free D do not improve upon 25OHD in population-based IL-6 studies. However, examination of both 25OHD and $1,25(OH)_2D$ are warranted in studies of TNFα soluble receptors.

Our results support the role of serum IL-6 as a marker of the proposed anti-inflammatory effects of vitamin D. The observed associations of 25OHD and IL-6 were consistent with previous reports in older Irish adults [31], although we observed somewhat higher median IL-6 levels across the 25OHD range. The inverse correlation between IL-6 and both total and free forms of both vitamin D metabolites supports previous reports that this cytokine is a target for vitamin D within the immune system. This is supported by mechanistic studies [32], for example, demonstrating that $1,25(OH)_2D$ treatment in cell cultures inhibited p38 and lowered downstream production of IL-6 [33].

CRP is also an established systemic marker of inflammation, but it was not associated with either 25OHD or $1,25(OH)_2D$ in our study but instead was associated with levels of their serum carrier, DBP. CRP was shown to be associated with 25OHD among older Irish adults [31] and with $1,25(OH)_2D_3$ in ankylosing spondylitis patients [9]. But CRP levels were much higher in those studies, while in MrOS, CRP remained low across the range of 25OHD and $1,25(OH)_2D$ (<1.5 μg/ml). Similar to our results, adults in the Framingham Offspring Study had no difference in CRP by 25OHD concentration [34]. We can speculate that the association between CRP and DBP may

reflect the potential impact of systemic inflammatory cytokines on liver production of DBP [35], although a link between inflammation, CRP and DBP has not been demonstrated in other studies [36].

Soluble TNFα receptors are another important marker of inflammation in that they represent potential antagonists of TNFα function. In this study, 25OHD was positively associated and $1,25(OH)_2D$ was negatively associated with TNFα SRI and II, suggesting dichotomous functions for the two vitamin D metabolites. We speculate that soluble TNFα receptors may be an important novel target for 25OHD as an anti-inflammatory agent. Specifically, the upregulated TNFα sRI and II may provide a sensitive mechanism by which localized conversion of 25OHD to $1,25(OH)_2D$ can abrogate inflammatory TNFα responses.

An alternative hypothesis for the negative correlations between inflammatory markers and vitamin D metabolites could be that circulating cytokines regulate serum vitamin D metabolites. The renal vitamin D-activating enzyme 1α-hydroxylase (CYP27B1) is mainly regulated by serum PTH and FGF23 but extra renal production of $1,25(OH)_2D$ by CYP247B1 is known to be induced by inflammatory cytokines such as TNFα [37-40]. Further characterization of this novel component of vitamin D and inflammation will be important in future studies of vitamin D and inflammation in the elderly, especially in those with increased inflammatory disease activity such as RA patients [41].

This is the first study to compare multiple measures of vitamin D and their associations with inflammatory markers in older adults. While other studies have examined Vitamin D metabolite levels in those with SLE and RA [14, 15], the MrOS study represents a predominantly healthy older male population, non-Hispanic white population with a very low prevalence of RA (5%). If $1,25(OH)_2D$ is confirmed to be an independent predictor of inflammatory state, it may be a useful marker in supplementation studies and for clinical detection of vitamin D deficiency. In the current study, $1,25(OH)_2D$ was more strongly associated than 25OHD with TNFα soluble receptors and with having ≥2 inflammatory markers in the top quartile.

We note limitations in our study. A substantial barrier to interpretation of vitamin D and inflammation studies is the question of whether inflammation also affects vitamin D. Due to the cross-sectional, observational nature of this analysis, we are unable to address the directionality. It is possible that inflammation affects vitamin D, rather than the reverse. For example, a recent study of patients undergoing elective hip or knee surgery recruited from orthopedic outpatient clinics showed orthopedic surgery patients had decreases in $25OHD_3$, 25(OH)D and

$1,25(OH)_2D$ as a systemic inflammatory response [42, 43]. However, RCTs [6, 44-47] and *in vitro* evidence also support a role for $1,25(OH)_2D_3$ on inflammation [12, 13, 25, 48] through inhibition of IL-6 and IL-8 synthesis [11].

The choice of a composite inflammatory score is somewhat arbitrary, and there are multiple methods of computing a composite inflammatory score [49]. The method we presented in this paper of examining those with two or more inflammatory markers in the top quartile has also been published previously and represents a more specific indicator of systemic inflammation than a high level of just one inflammatory marker [4, 50-52].

As in any observational study, the possibility for residual confounding remains. The MrOS cohort is well characterized in terms of body composition and used an extensive medical history questionnaire and medication inventory to capture potentially confounding variables. In this analysis, we adjusted for multiple variables known or suspected to be associated with inflammatory disease and vitamin D status. However, misclassification and unmeasured confounders could still have blunted or magnified associations between vitamin D and inflammation.

Estimations and direct measurements of circulating free 25OHD are not as yet standardized, and there is no gold standard for either DBP or free 25OHD assays. This limits our ability to conclude whether free 25OHD or free $1,25(OH)_2D$ can improve the prediction of inflammatory markers or their downstream effects on health outcomes. However, our inclusion of multiple DBP measures and their estimates of free vitamin D provides the most thorough analyses of this question to date and suggests that further studies of free 25OHD and its role in inflammation are warranted.

In conclusion, IL-6 associations with 25OHD have been consistent in several population-based and clinical studies, and we observed no added information in considering free 25OHD or $1,25(OH)_2D$. In contrast, we observed consistently divergent associations with TNFα soluble receptors for these metabolites. Considering the importance of TNFα action in osteoclastic maturation [7, 53, 54], future studies of vitamin D should include investigations of the effects of each metabolite.

Table 1. Baseline characteristics, MrOS

Characteristic	Overall (N=679) Mean ±SD, Median (IQR) or n(%)
Age	74 ± 6
Race	
White	616 (90.72)
African American	22 (3.24)
Asian	16 (2.36)
Hispanic	17 (2.50)
Other	8 (1.18)
Vitamin D	
25OHD (ng/ml)	25.95 ±7.98
Free 25OHD (nmol/L)	0.03 ±0.01
$1,25(OH)_2D$ (pg/ml)	64.24 ±71.72
Free $1,25(OH)_2D$ (nmol/L)	0.0015 ±0.0004
Vitamin D binding protein (μM)	4.36 ±0.75
Season of blood draw	
Winter	134 (19.73)
Spring	174 (45.36)
Summer	198 (29.16)
Fall	173 (25.48)
BMI (kg/m^2)	27 ±4
Total Fat Mass (kg) [§]	22 ±7
Inflammatory Markers	
CRP (ug/mL)[§ a]	1.44 (2.1)
IL-6 (pg/mL)[§ a]	2.37 (1.97)
TNFα (pg/mL)[§ a]	3.96 (2.54)
Soluble Receptors	
TNFα-sRI (pg/mL)[§ a]	1940.60 (593.40)
TNFα-sRII (pg/mL)[§ a]	3521.80 (938.90)
IL-6sR (ng/ml)[§ a]	49.09 (18.25)
Anti-Inflammatory Marker	
IL-10 (pg/mL) [§ a]	8.85 (6.93)
Alcohol (per week)	
0 drinks	238 (35.05)
1-7 drinks	323 (47.57)
>7 drinks	118 (17.38)
Self-reported quality of health*	
Excellent/Good	576 (84.96)
Fair/Poor/Very Poor	102 (15.04)
PASE score[†]	147 ±66
NSAIDS use[§]	107 (16.49)
Corticosteroid use[§]	53 (8.17)
Cox-II inhibitor use	51 (7.86)
CVD[b]	182 (26.80)
Stroke	51 (7.51)
Diabetes	83 (12.22)
Surgical removal of stomach or intestine	56 (8.25)
Rheumatoid Arthritis	34 (5.01)
Renal Function	
eGFR ($ml/min/(1.73m^2)$ [§]	77 ±19
Serum creatinine (mg/dl) [§]	1.02 ±0.30

* How would you rate your overall health?

† Physical activity score for the elderly

[a] median, inter-quartile range (IQR)

[§] 5 missing total fat mass, 36 missing lipids, 80 missing CRP, 83 missing IL-6, 81 missing TNFα, 66 missing, TNFα-sRI, 72 missing TNFα-sRII, 66 missing IL-6sR, 71 missing IL-10, 36 missing eGFR, 36 missing serum creatinine, 30 missing NSAIDS use, 30 missing corticosteroid use, 30 missing Cox-II inhibitor

[b] defined as self-report of previous heart attack, congestive heart failure or angina

Table 2. Associations with each inflammatory marker (β^c, 95% CI) per SD increase in total vitamin D measure, MrOS

	N	25OHD (SD=7.98 ng/ml)	$1,25(OH)_2D$ (SD=71.72 pg/ml)	25OHD, $1,25(OH)_2D$, and DBP in the same model		
		25OHD	**$1,25(OH)_2D$**	**25OHD**	**$1,25(OH)_2D$**	**DBP**
IL-6 (pg/mL)	557	-0.23 (-0.38, -0.07)**	-0.20 (-0.39, -0.0004)*	-0.21 (-0.37, -0.04)*	-0.14 (-0.35, 0.06)	0.15 (0.007, 0.30)*
IL-6sR (ng/mL)	571	0.39 (-.88, 1.67)	0.20 (-1.00, 1.42)	0.26 (-1.11, 1.64)	0.04 (-1.23, 1.31)	0.54 (-0.50, 1.58)
TNFα (pg/mL)	556	-0.23 (-0.65, 0.18)	-0.19 (-0.43, 0.06)	-0.21 (-0.64, 0.21)	-0.12 (-0.34, 0.10)	0.11 (-0.09, 0.30)
TNFα-sRI (pg/mL)	571	34.66 (-4.17, 73.48)	-62.05 (-98.01, -26.09)***	62.30 (21.33, 103.28)**	-86.53 (-124.18, -48.87)**	16.20 (-15.36, 47.76)
TNFα-sRII (pg/mL)	565	38.32 (-31.14, 107.78)	-88.83 (-147.02, -30.64)**	79.20 (5.53, 152.88)*	-118.75 (-180.45, -57.06)**	9.75 (-45.92, 65.42)
IL-10 (pg/mL)	566	-1.18 (-4.33, 1.97)	-1.77 (-3.74, 0.19)	-0.77 (-3.96, 2.41)	-1.62 (-3.49, 0.25)	0.83 (-0.38, 2.04)
CRP (μg/mL)	557	0.48 (-0.44, 1.40)	0.07 (-0.35, 0.48)	0.33 (-0.58, 1.24)	-0.20 (-0.66, 0.27)	1.08 (0.50, 1.65)**
≥2 inflammatory markers in highest quartile[§] (N=571)	571	0.98 (0.77, 1.26)	0.75 (0.58, 0.97)*	1.03 (0.79, 1.35)	0.72 (0.55, 0.95)*	1.29 (1.04, 1.59)*

[c]Adjusted for age, site, race, total fat mass, serum creatinine, eGFR, smoking, alcohol, season, self-reported health, diabetes, stroke, cox-II inhibitor use, surgical removal of stomach or intestine.

[§]Among CRP, IL-6, TNFα, TNFα-sRI, TNFα-sRII, IL-6sR. Effect measure = odds ratios (95%CI). **p≤0.01, *p≤0.05, ***p≤0.001 (Bonferroni-corrected alpha)

Table 3. Associations with each inflammatory marker (β^c, 95% CI) per SD increase in free vitamin D and binding protein (DBP) measures, MrOS

	N	**Free 25OHD** (SD=0.01 nmol/L)	**Free 1,25(OH)2D** (SD=0.0004 nmol/L)	**DBP** (SD=0.75 µM)
IL-6 (pg/mL)	557	-0.35 (-0.55, -0.15)**	-0.22 (-0.39, -0.04)*	0.11 (-0.04, 0.26)
IL-6sR (ng/mL)	571	0.20 (-1.55, 1.96)	-0.02 (-1.23, 1.18)	0.59 (-0.41, 1.59)
TNFα (pg/mL)	556	-0.36 (-0.93, 0.20)	-0.21 (-0.45, 0.03)	0.06 (-0.14, 0.25)
TNFα-sRI (pg/mL)	571	39.67 (-13.42, 92.76)	-61.51 (-96.73, -26.28)**	16.70 (-14.91, 48.32)
TNFα-sRII (pg/mL)	565	51.01 (-44.53, 146.55)	-78.72 (-141.71, -15.72)*	9.86 (-44.12, 63.83)
IL-10 (pg/mL)	566	-2.15 (-6.18, 1.88)	-1.93 (-3.81, -0.05)*	0.54 (-0.76, 1.84)
CRP (µg/mL)	557	0.16 (-0.98, 0.67)	-0.40 (-0.79, -0.02)*	1.11 (0.45, 1.76)***
≥2 inflammatory markers in highest quartile[§] (N=571)	571	0.85 (0.61, 1.19)	0.70 (0.54, 0.89)**	1.26 (1.03, 1.55)*

[c]Adjusted for age, site, race, total fat mass, serum creatinine, eGFR, smoking, alcohol, season, self-reported health, diabetes, stroke, cox-II inhibitor use, surgical removal of stomach or intestine.

[§]Among CRP, IL-6, TNFα, TNFα-sRI, TNFα-sRII, IL-6sR. Effect measure = odds ratios (95%CI). **p≤0.01, *p≤0.05, ***p≤0.001 (Bonferroni-corrected alpha)

Figure 1. MrOS analytic sample size, randomly selected from the full MrOS cohort (N=5994)

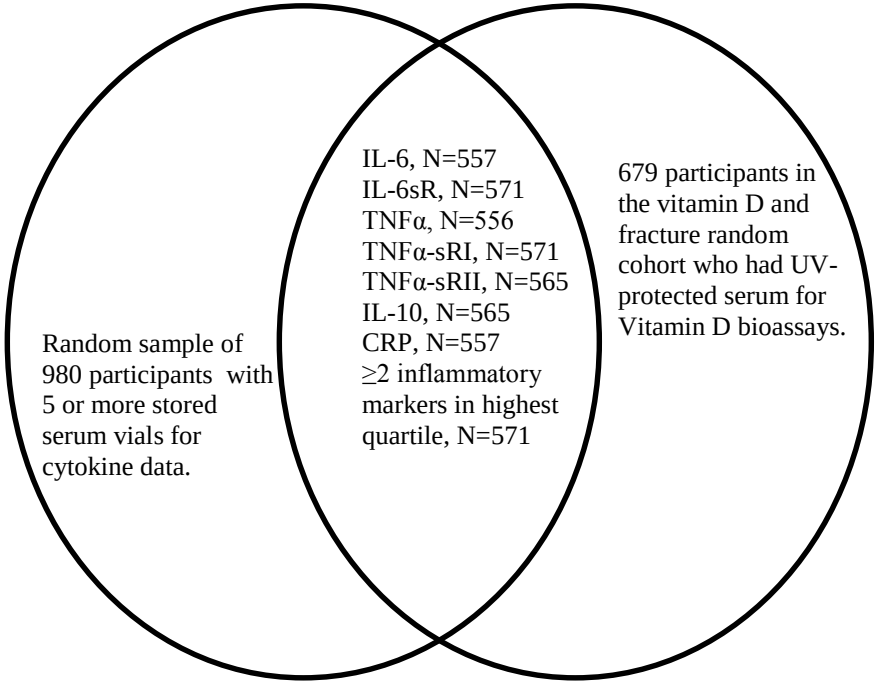
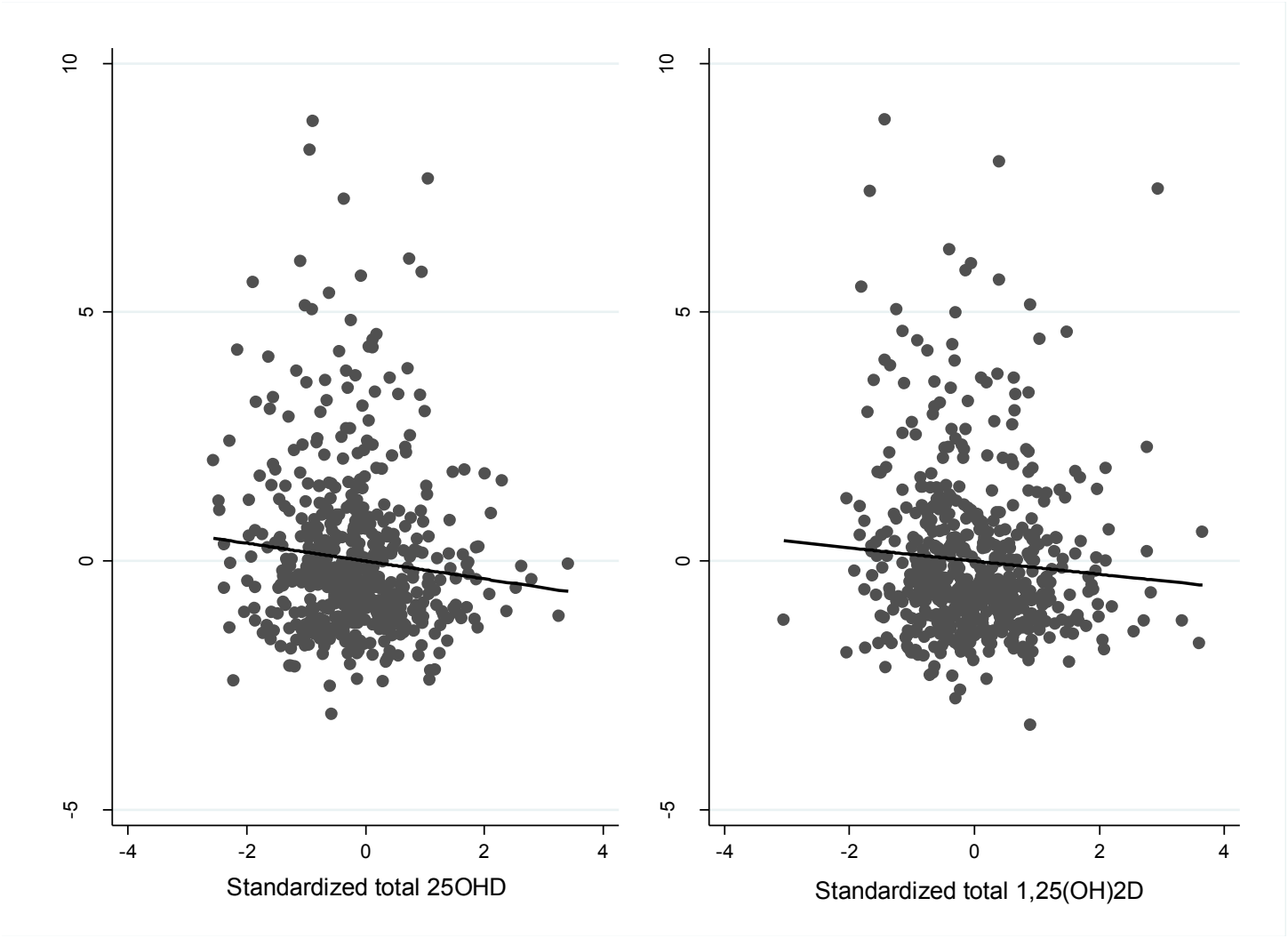
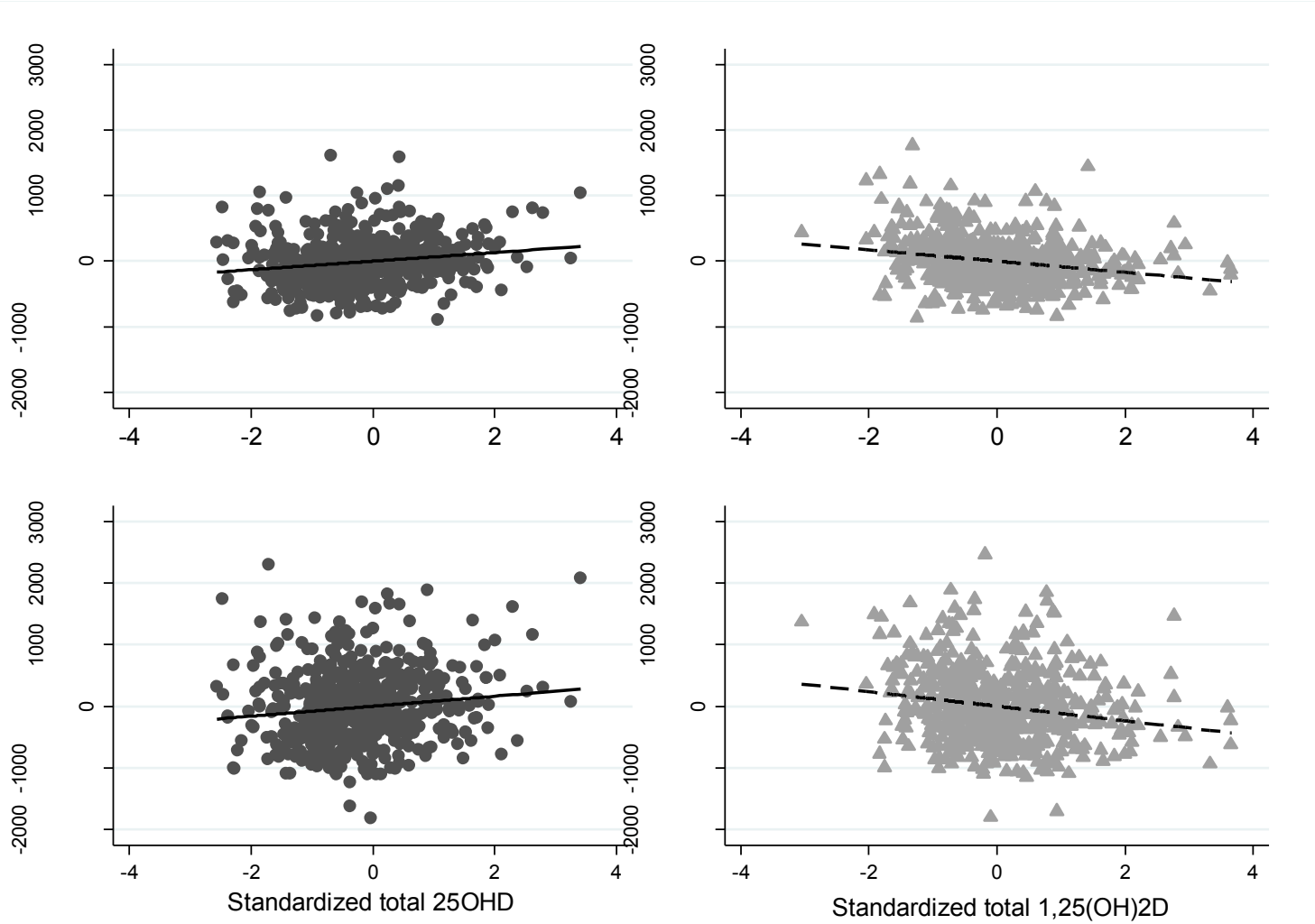


Figure 2. The IL-6 association with standardized 25OHD was nearly identical to the association with $1,25(OH)_2D$. Data points are predicted values[b].



[b] Data points and lines are from a fully adjusted regression model (adjusted for age, site, race, total fat mass, serum creatinine, eGFR, smoking, alcohol, season, self-reported health, diabetes, stroke, cox-II inhibitor use, surgical removal of stomach or intestine) with both vitamin D measures and DBP in the same model.

Figure 3. The TNFα soluble receptors I (top panels) and II (lower panels) associations with standardized 25OHD and 1,25$(OH)_2$D, were in *opposite directions*. Data points are predicted values[b].



[b] Data points and lines are from a fully adjusted regression model (adjusted for age, site, race, total fat mass, serum creatinine, eGFR, smoking, alcohol, season, self-reported health, diabetes, stroke, cox-II inhibitor use, surgical removal of stomach or intestine) with both vitamin D measures and DBP in the same model.

Supplemental Tables

Supplemental Table 1: Spearman correlations among Vitamin D measures and inflammatory markers

	Total 25OHD	Free 25OHD	Total 1,25(OH)$_2$D	Free 1,25(OH)$_2$D	VDBP (polyclonal ELISA)	VDBP (mono-clonal ELISA)	VDBP (RIA)	IL-6	TNFα	TNFα-sRI	TNFα-sRII	IL-6sR
Total 25OHD					0.17***	0.19***	0.23***					
Free 25OHD	0.87***				-0.27***	-0.59***	-0.21***					
Total 1,25(OH)$_2$D	0.35***	0.32***			0.09	0.09*	0.17***					
Free 1,25(OH)$_2$D	0.28***	0.43***	0.91***		-0.29***	-0.62***	-0.20***					
IL-6	-0.21***	-0.21***	-0.25***	-0.24***	0.03	-0.01	-0.06					
TNFα	-0.03	-0.04	-0.14***	-0.15***	0.01	0.08	0.04	0.24***				
TNFα-sRI	-0.01	-0.01	-0.35***	-0.34***	0.03	0.04	-0.07	0.43***	0.38***			
TNFα-sRII	-0.02	-0.02	-0.31***	-0.29***	-0.004	0.03	-0.09*	0.40***	0.42***	0.84***		
IL-6sR	0.01	-0.01	-0.05	-0.06	0.05	0.03	0.12***	0.13***	0.11***	0.15***	0.15***	
CRP	-0.04	-0.12*	-0.08	-0.16***	0.23***	0.10*	0.16***	0.45***	0.18***	0.29***	0.26***	0.05
IL-10	0.09	0.06	0.002	-0.007	0.02	0.13***	0.05	0.09*	0.38***	0.22***	0.24***	0.03

* p<0.05, ** p<0.01, ***p<0.001.

Supplement Table 2. Associations with inflammatory markers (β, 95% CI) for each SD increase in free vitamin D and binding protein (DBP measures, MrOS)

	Free 25OHD		**Free 1,25(OH)$_2$D**		**DBP**	
	(Using DBP from monoclonal ELISA)	(Using DBP from RIA)	(Using DBP from monoclonal ELISA)	(Using DBP from RIA)	(Using DBP from monoclonal ELISA)	(Using DBP from RIA)
IL-6 (pg/mL) (N=557)	-0.03 (-0.20, 0.15)	-0.31 (-0.51, -0.10)**	-.0.09 (-0.27, 0.09)	-0.18 (-0.36, 0.01)	0.05 (-0.10, 0.20)	0.01 (-0.13, 0.16)
IL-6sR (ng/mL) (N=571)	0.90 (-0.19, 1.99)	-0.21 (-1.95, 1.52)	0.73 (-0.33, 1.78)	-0.31 (-1.50, 0.88)	0.59 (-0.51, 1.69)	1.31 (0.31, 2.32)*
TNFα (pg/mL) (N=556)	0.01 (-0.34, 0.36)	-0.42 (-0.97, 0.12)	0.0003 (-0.25, 0.25)	-0.25 (-0.47, -0.03)*	0.005 (-0.43, 0.44)	0.15 (-0.03, 0.34)
TNFα-sRI (pg/mL) (N=571)	53.24 (17.40, 89.08)	41.79 (-11.01, 94.60)	-5.98 (-42.05, 30.71)	-64.66 (-97.47, -31.85)**	35.58 (0.58, 70.58)*	16.95 (-18.36, 52.26)
TNFα-sRII (pg/mL) (N=565)	2.05 (-59.55, 63.66)	57.97 (-37.49, 153.42)	-52.32 (-106.80, 2.16)	-79.96 (-139.29, -20.62)**	46.27 (-11.82, 104.35)	0.76 (-53.54, 55.06)
IL-10 (pg/mL) (N=566)	2.71 (-1.50, 6.92)	-2.22 (-6.44, 2.00)	1.56 (-0.86, 3.98)	-2.11 (-3.86, -0.37)*	-0.66 (-4.42, 3.11)	0.92 (-0.46, 2.31)
CRP (ug/mL) (N=557)	0.27 (-0.39, 0.93)	0.24 (-1.01, 1.49)	0.09 (-0.43, 0.61)	-0.19 (-0.61, 0.23)	0.44 (-0.10, 0.98)	0.70 (0.35, 1.04)***
≥2 inflammatory markers in highest quartile[§] (N=571)	1.09 (0.86, 1.39)	0.89 (0.63, 1.25)	0.85 (0.68, 1.07)	0.71 (0.55, 0.91)**	1.21 (0.97, 1.51)	1.20 (0.97, 1.49)

Adjusted for age, site, race, total fat mass, serum creatinine, eGFR, smoking, alcohol, season, self-reported health, diabetes, stroke, cox-II inhibitor use, surgical removal of stomach or intestine.

[§]Among CRP, IL-6, TNFα, TNFα-sRI, TNFα-sRII, IL-6sR. Effect measure = odds ratios.

**p≤0.01, *p<0.05, ***p≤0.001 (Bonferroni-corrected alpha)

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Associations of total and free 25OHD and 1,25(OH)$_2$D with serum markers of inflammation in older men

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ABSTRACT (Between 150 & 250 words) *(Current number of words = 248)*

Purpose: Vitamin D is hypothesized to suppress inflammation, and circulating 25-hydroxyvitamin D (25OHD) and inflammatory markers are inversely correlated. However, total serum 25OHD may not be the best indicator of biologically active vitamin D.

Methods: We tested serum total 25OHD, total $1,25(OH)_2D$, vitamin D binding protein (DBP), and estimated free 25OHD and free $1,25(OH)_2D$ associations with inflammatory markers serum IL-6, TNFα and their soluble receptors, IL-10 and CRP as continuous outcomes and the presence of ≥2 inflammatory markers in the highest quartile as a dichotomous outcome, in a random subcohort of 679 men in the Osteoporotic Fractures in Men (MrOS) study.

Results: IL-6 was lower in men with higher 25OHD (-0.23 µg/mL per SD increase in 25OHD, 95% CI: -0.07 to -0.38 µg/mL) and with higher $1,25(OH)_2D$ (-0.20 µg/mL, 95% CI: -0.0004 to -0.39 µg/mL); free D associations were slightly stronger. 25OHD and DBP, but not $1,25(OH)_2D$, were *independently* associated with IL-6. TNFα soluble receptors were inversely associated with $1,25(OH)_2D$ but positively associated with 25OHD, and each had independent effects. The strongest association with ≥2 inflammatory markers in the highest quartile was for free $1,25(OH)_2D$ (OR: 0.70, 95% CI: 0.54 to 0.89 per SD increase in free $1,25(OH)_2D$).

Conclusions: Associations of $1,25(OH)_2D$ and free 25OHD with IL-6 mirrored those of 25OHD, suggesting that $1,25(OH)_2D$ and free D do not improve upon 25OHD in population-based IL-6 studies. However, associations for the two metabolites diverged for TNFα soluble receptor, warranting examination of both metabolites in studies of TNFα and its antagonists.

Mini Abstract

Vitamin D is hypothesized to suppress inflammation. We tested total and free vitamin D metabolites and their association with inflammatory markers. Interleukin-6 levels were lower with higher 25-hydroxyvitamin D. 1,25-dihydroxyvitamin D and free 25OHD associations mirrored those of 25OHD. However, associations for the two metabolites diverged for TNFα soluble receptors.

BACKGROUND

Chronic low-grade inflammation is a contributor to age-associated frailty, mortality and morbidity, including osteoporosis [1]. Inflammatory markers such as interleukin-6 (IL-6) and tumor necrosis factor alpha (TNFα) are implicated in the process of vascular calcification and regulation of bone remodeling [2, 3] and have been linked to incident fracture [4] and BMD loss [5].

Vitamin D has direct effects on bone health and may also act on bone by modulating inflammation [6, 7]. We have recently shown that low 1,25(OH)2D and 25OHD are independently associated with hip fracture in older men, but only 25OHD was independently associated with BMD loss [8]. $1,25(OH)_2D_3$ may ~~is also~~ play a ~~possible candidate for mediatory function~~ role in regulating both the inflammatory process and bone turnover. Decreased $1,25(OH)_2D_3$ levels may contribute to inhibition of bone formation and suppress activated T cells and cell proliferation, which may accelerate the inflammation process in those with conditions such as ankylosing spondylitis (AS) [9].

In vitro and *in vivo* evidence suggests that the biologically active form of vitamin D, $1,25(OH)_2D$, has several immunomodulatory functions, including suppression of pro-inflammatory marker expression and regulation of immune cell activity [10]. ~~(ref Zhang).~~ Treatment of fibroblast cultures with $1,25(OH)_2D_3$ inhibits IL-6 and interleukin-8 (IL-8) [11]. Vitamin D inhibits the activation of the TNFα converting enzyme and subsequent inflammation on multiple levels [10, 12, 13]. Studies of vitamin D and inflammatory diseases, such as rheumatoid arthritis (RA) and systemic lupus erythematosus, suggest a role for vitamin D deficiency in However, little is known about the relationship between inflammation and vitamin D in the general population.

The objective of this study was to examine associations of total and free circulating 25OHD and $1,25(OH)_2D$ and vitamin D binding protein (DBP) with inflammatory markers that include CRP, IL-6, TNFα, IL-6 and TNFα soluble receptors, and one anti-inflammatory cytokine – interleukin-10 (IL-10) in a cohort of older men.

METHODS

Study Design

The Osteoporotic Fractures in Men Study (MrOS) is a prospective observational cohort study designed to determine risk factors for osteoporosis and age-related medical conditions in men 65 years of age and older. This study is conducted at six centers in the United States: Birmingham, Alabama; Minneapolis, Minnesota; Palo Alto,

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California; Pittsburgh, Pennsylvania; Portland, OR; and San Diego, California. Participants were recruited by mailings to the Department of Motor Vehicles (DMV), voter registration and participant databases, community and senior newspaper features and advertisements, and targeted presentations, from March 2000 through April 2002. Exclusion criteria were (1) inability to walk without assistance from another person, (2) bilateral hip replacements, (3) inability to provide self-reported data, (4) residence not near a study site, (5) judged by an investigator to have a medical condition that would result in early death, (6) and inability to understand and sign informed consent [18, 19].

Vitamin D Measurements

Serum concentrations of both the inactive (25OHD) and active ($1,25(OH)_2D$) forms of vitamin D were analyzed in a random sample of men from the baseline visit of the MrOS study. Additional assays were carried out to measure vitamin D binding protein (DBP), the major serum protein carrier of vitamin D metabolites. Assays were completed in December 2012 using stored serum collected at the MrOS baseline visit. At the baseline visit, fasting morning blood samples were collected; serum was separated immediately after phlebotomy, and then stored at −70°C. All samples for total 25OHD remained frozen in foil wrapped vials to reduce UV exposure until assay.

Measures for $25OHD_2$ (derived from ergocalciferol) and $25OHD_3$ (derived from cholecalciferol) were performed at the Mayo Clinic using mass spectrometry as previously described [20, 21]. Deuterated stable isotope (d3-25OHD) was added to a 0.2-ml serum sample as internal standard. $25OHD_2$, $25OHD_3$, and the internal standard were extracted using acetonitrile precipitation. Extracts were further purified online and analyzed by liquid chromatography/tandem mass spectrometry using multiple reaction monitoring. $25OHD_2$ and $25OHD_3$ were reported individually. The minimum detectable limit was 4 ng/ml for $25OHD_2$ and 2 ng/ml for $25OHD_3$. Aliquots of a single serum pool were included in alternate assay runs. Using the pooled serum, the interassay coefficient of variation (CV) for $25OHD_3$ was 4.4%, and the intraassay CV was 4.9%.

Total $1,25(OH)_2D$ was measured at the University of Leuven in Belgium, using LC-MS/MS without derivatization [22]. The lower limit of quantitation (LLQ) was 4.3 pg/mL for $1,25(OH)_2D_2$ and 6 pg/mL for $1,25(OH)_2D_3$. Inter-assay CV of pooled serum at low and high serum concentrations, respectively, were 10.1% for serum with mean concentration of 7.16 pg/mL and 5.9% for serum with mean concentration of 55.8 pg/mL [23].

DBP concentration in serum was measured by a two-site polyclonal ELISA (Genway Biotech, San Diego, CA) at the OHSU Clinical and Translational Research Institute laboratory. Intra-assay CV was 3%. Because no

gold standard for DBP exists, we also measured DBP by a monoclonal ELISA (mELISA; R&D Systems, Minneapolis, MN) and by polyclonal radial immunodiffusion assay (Laboratory of Clinical and Experimental Endocrinology, KU Leuven, Belgium), which had intra-assay CVs of 2-4% [24].

Free 25OHD concentrations were calculated using published mathematical models that incorporates serum concentrations of 25OHD, 1,25$(OH)_2$D, DBP, and albumin. Primary analyses were performed with estimated free 25OHD that assumed constant binding affinity across GC genotypes; however, *GC*-genotype-specific affinity estimates were also calculated for comparison [25].

Inflammatory Markers

Cytokine assays were measured in MrOS baseline samples utilizing a random sampling scheme. The assays were completed between December, 2009, and August, 2010, using archived serum collected at baseline on 1530 MrOS men as part of a MrOS ancillary study. Cytokine measures used in this analysis include CRP, IL-6, TNFα, tumor necrosis factor alpha soluble receptors (TNFα-sRI, TNFα-sRII) and interleukin-6 soluble receptor (IL6-sR). IL-10 was also measured as an anti-inflammatory measure.

All cytokine assays were performed at the Laboratory for Cytokine Biochemistry, University of Vermont. The samples were thawed at 37°C and briefly centrifuged. 300 µl of serum was placed into one cryovial for testing of TNFα, TNF-αsRI and sRII, IL-10 and CRP. Approximately 230 µl were plated into two plates for IL-6 and IL-6sR. The plates were refrozen at -80°C until assaying. IL-6 was measured using a high sensitivity ELISA (R&D Systems, Minneapolis, MN). The assay range is 0.16 – 12.0 pg/mL. Inter-assay CVs range from 6.11 to 8.47%. IL-6sR was measured using ELISA (R&D Systems, Minneapolis, MN). [26] The assay range is 3120 – 200,000 pg/mL. Inter-assay CVs range from 4.68 to 8.83%. TNFα was measured using the Human Serum CVD3 Multiplex Kit (Millipore Corp., Billerica, MA) which is run by flow cytometry on the Bio-Rad BioPlex 200 Luminex instrument. The assay range is 0.13-2000 pg/mL. Inter-assay CVs range from 4.93 to 9.13%. TNF-αsRI and sRII were measured with an ELISA (R&D Systems, Minneapolis, MN). The normal range for TNF- αsRI in serum is 479 – 966 pg/mL and for TNF-αsRII in serum is 1003 – 3170 pg/mL. Inter-assay CVs range from 5.42% to 8.59% for TNF-αsRI and 2.87 to 3.54% for TNF-αsRII. CRP was measured using the BNII nephelometer from Dade Behring utilizing a particle enhanced immunonepholometric assay. The assay range is 0.16 – 1100 ug/mL. Expected values for CRP in normal, healthy individuals are ≤ 3 ug/mL. Inter-assay CVs ranged from 1.52 to 3.68%.

Covariates

Demographic characteristics such as age, race/ethnicity, clinical site, and lifestyle factors including weekly alcohol consumption and smoking history were determined at baseline by questionnaire. Physical activity was assessed with the Physical Activity Score for the Elderly (PASE) [27]. Height (centimeters) was measured on Harpenden stadiometers, and weight (kilograms) was measured on standard balance beam or digital scales. Body mass index (BMI) was calculated as kilograms per meter squared (kg/m^2). Prevalent cardiovascular disease was defined as self-report of heart attack, congestive heart failure (CHF) or angina. Diabetes, stroke history, self-reported health, surgical removal of stomach/intestine and rheumatoid arthritis at baseline were also from self-report. Participants brought in all medications they used within the last 30 days. All prescription medications recorded by the clinics were stored in an electronic medications inventory database (San Francisco Coordinating Center, San Francisco, CA). Each medication was matched to its ingredient(s) based on the Iowa Drug Information Service (IDIS) Drug Vocabulary (College of Pharmacy, University of Iowa, Iowa City, IA) [28].

Serum creatinine was measured using a variation of the Jaffe enzymatic method. Renal function was expressed as estimated glomerular filtration rate (eGFR) in ml/min/1.73 m^2 using a standardized serum-creatinine based formula [29]. Total fat mass was measured from dual-energy X-ray absorptiometry (DXA) scans using Hologic QDR 4500 scanners (Hologic, Inc., Bedford, MA).

Statistical Analysis

All vitamin D measurements were standardized by subtracting the mean from each value and dividing by the standard deviation to facilitate comparison across measures. Correlations among inflammatory markers and between the inflammatory markers and the vitamin D measures were assessed using Spearman's correlation coefficients. We used linear regression modeling with robust standard errors to examine the effect of standardized vitamin D measurements on each inflammatory marker. Although the inflammatory markers are right-skewed, least-squares regression methods perform well with 500 or more observations and provide 95% confidence interval coverage for all regression coefficients [30]. Betas (β) and 95% confidence intervals (CI) from the model are reported as mean difference in the inflammatory markers per standard deviation (SD) change in vitamin D measurements. To identify any nonlinear associations between each vitamin D measure and inflammatory marker, we examined loess plots. We created an inflammatory index by summing the number of pro-inflammatory markers in the highest quartile (CRP, IL-6, TNFα, TNFα-sRI, TNFα-sRII and IL-6sR). We then dichotomized this index into those having ≥2 inflammatory markers in the highest quartile in comparison with those having <2 inflammatory

markers in the highest quartile [4]. Logistic regression modeling was used to obtain odds ratios (OR) and corresponding 95% CI.

No nonlinear associations were detected between any vitamin D measurement and inflammatory marker. The base model included age, race, clinical site, and season. We used stepwise modeling with a probability of removal at > 0.10, forcing the base model covariates of age, site, race, season and the vitamin D measure into the model and to include all covariates that were significantly associated with each inflammatory marker. All covariates that were significantly associated with any inflammatory marker were included in the final model for all inflammatory markers. An age-squared term was added to each model to check for non-linear association with age. There are thirty ~~-~~five associations of interest (~~with~~ five vitamin D metabolites by ~~,~~ six inflammatory markers and one anti-inflammatory marker). ~~,~~ ~~t~~Thus, we have added a footnote to our tables with a Bonferroni adjusted p-value of ≤0.001 (0.05/35 = 0.001).

All analyses were conducted using SAS 9.3 (Cary, NC) and STATA release 12 (StataCorp, College Station, TX).

RESULTS

Description and correlations

Men with inflammatory markers and vitamin D measures (Figure 1) had a mean age of 74 ±6 years and mean BMI of 27 ±4 kg/m^2. Most (91%) were non-Hispanic white, and 85% reported excellent or good health status. 16% were taking NSAIDs, and 27% reported a history of heart attack, CHF, or angina (Table 1).

All correlations between inflammatory markers and vitamin D measures were weak. IL-6 was negatively correlated with total and free 25OHD and 1,25$(OH)_2$D measures (r = -0.21 to -0.25, p<0.001). TNFα and its soluble receptors were significantly negatively correlated with total and free 1,25$(OH)_2$D measures (r = -0.14 to -0.35, p<0.001) but not with 25OHD. The strongest correlation between CRP and vitamin D measures was with DBP (r = 0.23, p<0.001) (Supplemental Table 1).

Inflammatory marker associations with 25OHD and 1,25$(OH)_2$D

There was a significant association between lower IL-6 and higher 25OHD (0.23 pg/mL lower IL-6 per SD increase in 25OHD, 95% CI: 0.07 to 0.38 pg/mL), and this was independent of 1,25$(OH)_2$D and DBP (Table 2). Mean TNFα was 0.21 pg/mL lower per SD increase in 25OHD but this association was not statistically significant (95% CI: -0.64 pg/mL to 0.21 pg/mL). Results did not change after adjusting for 1,25$(OH)_2$D and DBP (Table 2).

Associations of IL-6 with 25OHD and 1,25$(OH)_2$D were similar (Figure 2), but statistically significant only for 25OHD. Mean IL-6 levels were significantly lower per SD increase in 25OHD (-0.23 pg/mL per SD, 95% CI: -0.38 to -0.07) and remained significant after adjusting for 1,25$(OH)_2$D and DBP. Mean IL-6 levels were lower by 0.20 pg/mL (95% CI: 0.0004 to 0.39 pg/mL lower) per SD increase in 1,25$(OH)_2$D. This association was attenuated to after 25OHD and DBP adjustment and was no longer significant.

TNFα soluble receptors I and II were positively associated with 25OHD and inversely associated with 1,25$(OH)_2$D (Figure 3). Average TNFα soluble receptors I and II were significantly lower by- 62.05 pg/mL (95% CI: 26.09 to 98.01 pg/mL) for TNFα-sRI and -88.83 pg/mL (95% CI: 30.64 to 147.02 pg/mL for TNFα-sRII) per SD increase in 1,25$(OH)_2$D. This association was strengthened with adjustment of 25OHD and DBP (Table 2). TNFα soluble receptors I and II were higher by 34.66 pg/mL (95% CI: -4.17 to 73.48 pg/mL) and 38.32 pg/mL (95% CI: -31.14, 107.78 pg/mL) per SD increase in 25OHD. This association was also strengthened with 1,25$(OH)_2$D and DBP adjustment (Table 2).

Odds of having ≥2 inflammatory markers in the highest quartile decreased by 25% (95% CI: 3% to 42% decrease) per SD increase in total 1,25$(OH)_2$D and was slightly strengthened with 25OHD and DBP adjustment (Table 2). However, 25OHD itself was not associated with odds of having ≥2 inflammatory markers in the highest quartile.

Inflammatory marker associations with free 25OHD, free 1,25$(OH)_2$D and DBP

Average CRP was significantly higher for each SD increase in DBP (1.11 ug/mL higher, 95% CI: 0.45 to 1.76 ug/mL higher) (Table 3). This association did not change after adjusting for 25OHD and 1,25$(OH)_2$D (Table 2). Although there was a significant negative correlation between CRP and free 25OHD ($r=-0.12$, $p<0.05$), there was no significant association in regression analysis ($\beta = 0.16$ ug/mL; $p=0.31$) (Supplemental Table 1 and Table 3). Mean IL-6 levels were lower by 0.35 pg/mL (95% CI: 0.15 to 0.55 pg/mL lower) for each SD increase in free 25OHD and by 0.22 pg/mL (95% CI: 0.04 to 0.39 pg/mL lower) per each SD increase in free 1,25$(OH)_2$D. TNFα soluble receptor I levels were lower by 61.51 pg/mL (95% CI: 26.28 to 96.73 pg/mL lower) and TNFα soluble receptor II levels were lower by 78.72 pg/mL (95% CI: 15.72 to 141.71 pg/mL) per SD increase in free 1,25$(OH)_2$D.

Odds of having ≥2 inflammatory markers in the highest quartile decreased by 30% (95% CI: 11% to 46% decrease) for each SD increase in free 1,25$(OH)_2$D. There was no significant association with free 25OHD (Table 3).

CRP and TNFα soluble receptors' associations with free measures of 25OHD and $1,25(OH)_2D$ and DBP from other assays (monoclonal ELISA (R&D Systems, Minneapolis, MN) and radioimmunodiffusion assay (RID)) were similar to the polyclonal ELISA (Genway Biotech, San Diego, CA) assay, but slightly weaker. The CRP-DBP associations were also somewhat weaker but still statistically significant. IL-6 associations were significant for the free D measures from the RID assay, though slightly weaker, but not significant for the free D measures from the monoclonal ELISA assay (Supplemental Table 2). Use of *GC*-genotype-specific binding affinities, rather than constant affinities, in the free D estimating equations did not make a substantial difference in associations.

DISCUSSION

In this study of older men, 25OHD and $1,25(OH)_2D$ were negatively associated with IL-6 with similar magnitudes. On the other hand, associations with TNFα soluble receptors were positive for 25OHD and negative for $1,25(OH)_2D$. DBP was positively associated with CRP, and had a weak positive association with IL-6. Perhaps for this reason, free vitamin D measures, which incorporate DBP, had slightly stronger associations with IL-6 than total vitamin D measures. We did not observe any significant associations with TNFα, IL-10, or IL-6sR. These results indicate that $1,25(OH)_2D$ and free D do not improve upon 25OHD in population-based IL-6 studies. However, examination of both 25OHD and $1,25(OH)_2D$ are warranted in studies of TNFα soluble receptors.

Our results support the role of serum IL-6 as a marker of the proposed anti-inflammatory effects of vitamin D. The observed associations of 25OHD and IL-6 were consistent with previous reports in older Irish adults [31], although we observed somewhat higher median IL-6 levels across the 25OHD range. The inverse correlation between IL-6 and both total and free forms of both vitamin D metabolites supports previous reports that this cytokine is a target for vitamin D within the immune system. This is supported by mechanistic studies [32], for example, demonstrating that $1,25(OH)_2D$ treatment in cell cultures inhibited p38 and lowered downstream production of IL-6 [33].

CRP is also an established systemic marker of inflammation, but it was not associated with either 25OHD or $1,25(OH)_2D$ in our study but instead was associated with levels of their serum carrier, DBP. CRP was shown to be associated with 25OHD among older Irish adults [31] and with $1,25(OH)_2D_3$ in ankylosing spondylitis patients [9]. ~~(ref Lange).~~ ~~but~~ But CRP levels were much higher in ~~the vitamin D deficient group in~~ those~~at~~ studies~~y~~, while in MrOS, CRP remained low across the range of 25OHD and $1,25(OH)_2D$ (<1.5 µg/ml). Similar to our results, adults in the Framingham Offspring Study had no difference in CRP by 25OHD concentration [34]. We can speculate that

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the association between CRP and DBP may reflect the potential impact of systemic inflammatory cytokines on liver production of DBP [35], although a link between inflammation, CRP and DBP has not been demonstrated in other studies [36].

Soluble TNFα receptors are another important marker of inflammation in that they represent potential antagonists of TNFα function. In this study, 25OHD was positively associated and $1,25(OH)_2D$ was negatively associated with TNFα SRI and II, suggesting dichotomous functions for the two vitamin D metabolites. We speculate that soluble TNFα receptors may be an important novel target for 25OHD as an anti-inflammatory agent. Specifically, the upregulated TNFα sRI and II may provide a sensitive mechanism by which localized conversion of 25OHD to $1,25(OH)_2D$ can abrogate inflammatory TNFα responses.

An alternative hypothesis for the negative correlations between inflammatory markers and vitamin D metabolites could be that circulating cytokines regulate serum vitamin D metabolites. The renal vitamin D-activating enzyme 1α-hydroxylase (CYP27B1) is mainly regulated by serum PTH and FGF23 but extra renal production of $1,25(OH)_2D$ by CYP247B1 is known to be induced by inflammatory cytokines such as TNFα [37-40]. Further characterization of this novel component of vitamin D and inflammation will be important in future studies of vitamin D and inflammation in the elderly, especially in those with increased inflammatory disease activity such as RA patients [41].

This is the first study to compare multiple measures of vitamin D and their associations with inflammatory markers in older adults. While other studies have examined Vitamin D metabolite levels in those with SLE and RA [14, 15], the MrOS study represents a predominantly healthy older male population, non-Hispanic white population with a very low prevalence of RA (5%). If $1,25(OH)_2D$ is confirmed to be an independent predictor of inflammatory state, it may be a useful marker in supplementation studies and for clinical detection of vitamin D deficiency. In the current study, $1,25(OH)_2D$ was more strongly associated than 25OHD with TNFα soluble receptors and with having ≥2 inflammatory markers in the top quartile.

We note limitations in our study. A substantial barrier to interpretation of vitamin D and inflammation studies is the question of whether inflammation also affects vitamin D. Due to the cross-sectional, observational nature of this analysis, we are unable to address the directionality. It is possible that inflammation affects vitamin D, rather than the reverse. For example, a recent study of patients undergoing elective hip or knee surgery recruited from orthopedic outpatient clinics showed orthopedic surgery patients had decreases in $25OHD_3$, 25(OH)D and

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1,25(OH)$_2$D as a systemic inflammatory response [42, 43]. However, RCTs [6, 44-47] and *in vitro* evidence also support a role for 1,25(OH)$_2$D$_3$ on inflammation [12, 13, 25, 48] through inhibition of IL-6 and IL-8 synthesis [11].

The choice of a c~~C~~omposite inflammatory score is somewhat arbitrary, and ~~. T~~there ~~might be more than one~~ are multiple methods of computing a composite inflammatory score ~~like a z-score~~ [49]. The method we presented in this paper of examining those with two or more inflammatory markers in the top quartile has also been published previously and represents a more specific indicator of systemic inflammation than a high level of just one inflammatory marker [4, 50-52]~~. (ref Hopkins).~~

~~While other studies have examined Vitamin D metabolite levels in those with SLE and RA [14, 15], the MrOS study a male population, non-Hispanic white population with a very low prevalence of RA.(5%, n=34 men)~~

Estimations and direct measurements of circulating free 25OHD are not as yet standardized, and there is no gold standard for either DBP or free 25OHD assays. This limits our ability to conclude whether free 25OHD or free 1,25(OH)$_2$D can improve the prediction of inflammatory markers or their downstream effects on health outcomes. However, our inclusion of multiple DBP measures and their estimates of free vitamin D provides the most thorough analyses of this question to date and suggests that further studies of free 25OHD and its role in inflammation are warranted.

In conclusion, IL-6 associations with 25OHD have been consistent in several population-based and clinical studies, and we observed no added information in considering free 25OHD or 1,25(OH)$_2$D. In contrast, we observed consistently divergent associations with TNFα soluble receptors for these metabolites. Considering the importance of TNFα action in osteoclastic maturation [7, 53, 54], future studies of vitamin D should include investigations of the effects of each metabolite.

Table 1. Baseline characteristics, MrOS

Characteristic	Overall (N=679) Mean ±SD, Median (IQR) or n(%)
Age	74 ± 6
Race	
White	616 (90.72)
African American	22 (3.24)
Asian	16 (2.36)
Hispanic	17 (2.50)
Other	8 (1.18)
Vitamin D	
25OHD (ng/ml)	25.95 ±7.98
Free 25OHD (nmol/L)	0.03 ±0.01
$1,25(OH)_2D$ (pg/ml)	64.24 ±71.72
Free $1,25(OH)_2D$ (nmol/L)	0.0015 ±0.0004
Vitamin D binding protein (μM)	4.36 ±0.75
Season of blood draw	
Winter	134 (19.73)
Spring	174 (45.36)
Summer	198 (29.16)
Fall	173 (25.48)
BMI (kg/m^2)	27 ±4
Total Fat Mass (kg) [§]	22 ±7
Inflammatory Markers	
CRP (ug/mL)[§ a]	1.44 (2.1)
IL-6 (pg/mL)[§ a]	2.37 (1.97)
TNFα (pg/mL)[§ a]	3.96 (2.54)
Soluble Receptors	
TNFα-sRI (pg/mL)[§ a]	1940.60 (593.40)
TNFα-sRII (pg/mL)[§ a]	3521.80 (938.90)
IL-6sR (ng/ml)[§ a]	49.09 (18.25)
Anti-Inflammatory Marker	
IL-10 (pg/mL) [§ a]	8.85 (6.93)
Alcohol (per week)	
0 drinks	238 (35.05)
1-7 drinks	323 (47.57)
>7 drinks	118 (17.38)
Self-reported quality of health*	
Excellent/Good	576 (84.96)
Fair/Poor/Very Poor	102 (15.04)
PASE score[†]	147 ±66
NSAIDS use[§]	107 (16.49)
Corticosteroid use[§]	53 (8.17)
Cox-II inhibitor use	51 (7.86)
CVD[b]	182 (26.80)
Stroke	51 (7.51)
Diabetes	83 (12.22)
Surgical removal of stomach or intestine	56 (8.25)
Rheumatoid Arthritis	34 (5.01)
Renal Function	
eGFR ($ml/min/(1.73m^2)$ [§]	77 ±19
Serum creatinine (mg/dl) [§]	1.02 ±0.30

* How would you rate your overall health?

[†] Physical activity score for the elderly

[a] median, inter-quartile range (IQR)

§ 5 missing total fat mass, 36 missing lipids, 80 missing CRP, 83 missing IL-6, 81 missing TNFα, 66 missing, TNFα-sRI, 72 missing TNFα-sRII, 66 missing IL-6sR, 71 missing IL-10, 36 missing eGFR, 36 missing serum creatinine, 30 missing NSAIDS use, 30 missing corticosteroid use, 30 missing Cox-II inhibitor

[b] defined as self-report of previous heart attack, congestive heart failure or angina

Table 2. Associations with each inflammatory marker (β^c, 95% CI) per SD increase in total vitamin D measure, MrOS

	N	25OHD (SD=7.98 ng/ml)	$1,25(OH)_2D$ (SD=71.72 pg/ml)	25OHD, $1,25(OH)_2D$, and DBP in the same model		
		25OHD	**$1,25(OH)_2D$**	**25OHD**	**$1,25(OH)_2D$**	**DBP**
IL-6 (pg/mL)	557	-0.23 (-0.38, -0.07)**	-0.20 (-0.39, -0.0004)*	-0.21 (-0.37, -0.04)*	-0.14 (-0.35, 0.06)	0.15 (0.007, 0.30)*
IL-6sR (ng/mL)	571	0.39 (-.88, 1.67)	0.20 (-1.00, 1.42)	0.26 (-1.11, 1.64)	0.04 (-1.23, 1.31)	0.54 (-0.50, 1.58)
TNFα (pg/mL)	556	-0.23 (-0.65, 0.18)	-0.19 (-0.43, 0.06)	-0.21 (-0.64, 0.21)	-0.12 (-0.34, 0.10)	0.11 (-0.09, 0.30)
TNFα-sRI (pg/mL)	571	34.66 (-4.17, 73.48)	-62.05 (-98.01, -26.09)**	62.30 (21.33, 103.28)**	-86.53 (-124.18, -48.87)**	16.20 (-15.36, 47.76)
TNFα-sRII (pg/mL)	565	38.32 (-31.14, 107.78)	-88.83 (-147.02, -30.64)**	79.20 (5.53, 152.88)*	-118.75 (-180.45, -57.06)**	9.75 (-45.92, 65.42)
IL-10 (pg/mL)	566	-1.18 (-4.33, 1.97)	-1.77 (-3.74, 0.19)	-0.77 (-3.96, 2.41)	-1.62 (-3.49, 0.25)	0.83 (-0.38, 2.04)
CRP (µg/mL)	557	0.48 (-0.44, 1.40)	0.07 (-0.35, 0.48)	0.33 (-0.58, 1.24)	-0.20 (-0.66, 0.27)	1.08 (0.50, 1.65)**
≥2 inflammatory markers in highest quartile[§] (N=571)	571	0.98 (0.77, 1.26)	0.75 (0.58, 0.97)*	1.03 (0.79, 1.35)	0.72 (0.55, 0.95)*	1.29 (1.04, 1.59)*

[c]Adjusted for age, site, race, total fat mass, serum creatinine, eGFR, smoking, alcohol, season, self-reported health, diabetes, stroke, cox-II inhibitor use, surgical removal of stomach or intestine.

[§]Among CRP, IL-6, TNFα, TNFα-sRI, TNFα-sRII, IL-6sR. Effect measure = odds ratios (95%CI). **p≤0.01, *p≤0.05, ***p≤0.001 (Bonferroni-corrected alpha)

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Table 3. Associations with each inflammatory marker (β[c], 95% CI) per SD increase in free vitamin D and binding protein (DBP) measures, MrOS

	N	Free 25OHD (SD=0.01 nmol/L)	Free 1,25(OH)2D (SD=0.0004 nmol/L)	DBP (SD=0.75 µM)
IL-6 (pg/mL)	557	-0.35 (-0.55, -0.15)**	-0.22 (-0.39, -0.04)*	0.11 (-0.04, 0.26)
IL-6sR (ng/mL)	571	0.20 (-1.55, 1.96)	-0.02 (-1.23, 1.18)	0.59 (-0.41, 1.59)
TNFα (pg/mL)	556	-0.36 (-0.93, 0.20)	-0.21 (-0.45, 0.03)	0.06 (-0.14, 0.25)
TNFα-sRI (pg/mL)	571	39.67 (-13.42, 92.76)	-61.51 (-96.73, -26.28)**	16.70 (-14.91, 48.32)
TNFα-sRII (pg/mL)	565	51.01 (-44.53, 146.55)	-78.72 (-141.71, -15.72)*	9.86 (-44.12, 63.83)
IL-10 (pg/mL)	566	-2.15 (-6.18, 1.88)	-1.93 (-3.81, -0.05)*	0.54 (-0.76, 1.84)
CRP (µg/mL)	557	0.16 (-0.98, 0.67)	-0.40 (-0.79, -0.02)*	1.11 (0.45, 1.76)**
≥2 inflammatory markers in highest quartile[§] (N=571)	571	0.85 (0.61, 1.19)	0.70 (0.54, 0.89)**	1.26 (1.03, 1.55)*

[c]Adjusted for age, site, race, total fat mass, serum creatinine, eGFR, smoking, alcohol, season, self-reported health, diabetes, stroke, cox-II inhibitor use, surgical removal of stomach or intestine.

[§]Among CRP, IL-6, TNFα, TNFα-sRI, TNFα-sRII, IL-6sR. Effect measure = odds ratios (95%CI). **p≤0.01, *p≤0.05, ***p≤0.001 (Bonferroni-corrected alpha)

Figure 1. MrOS analytic sample size, randomly selected from the full MrOS cohort (N=5994)

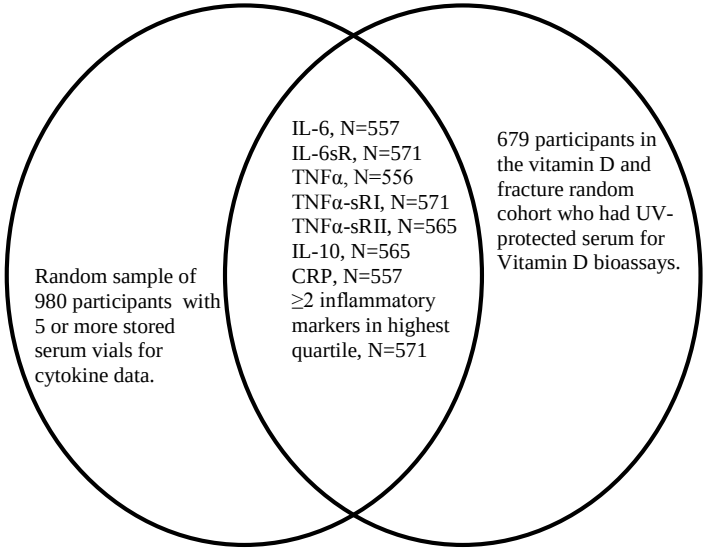
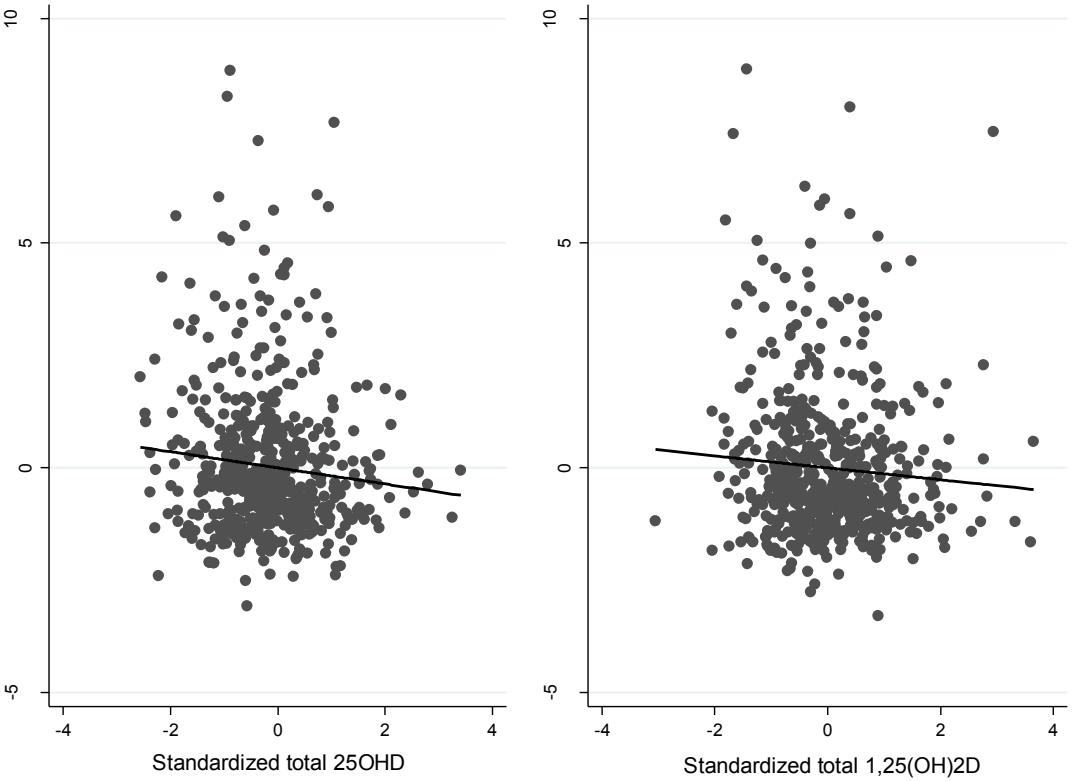
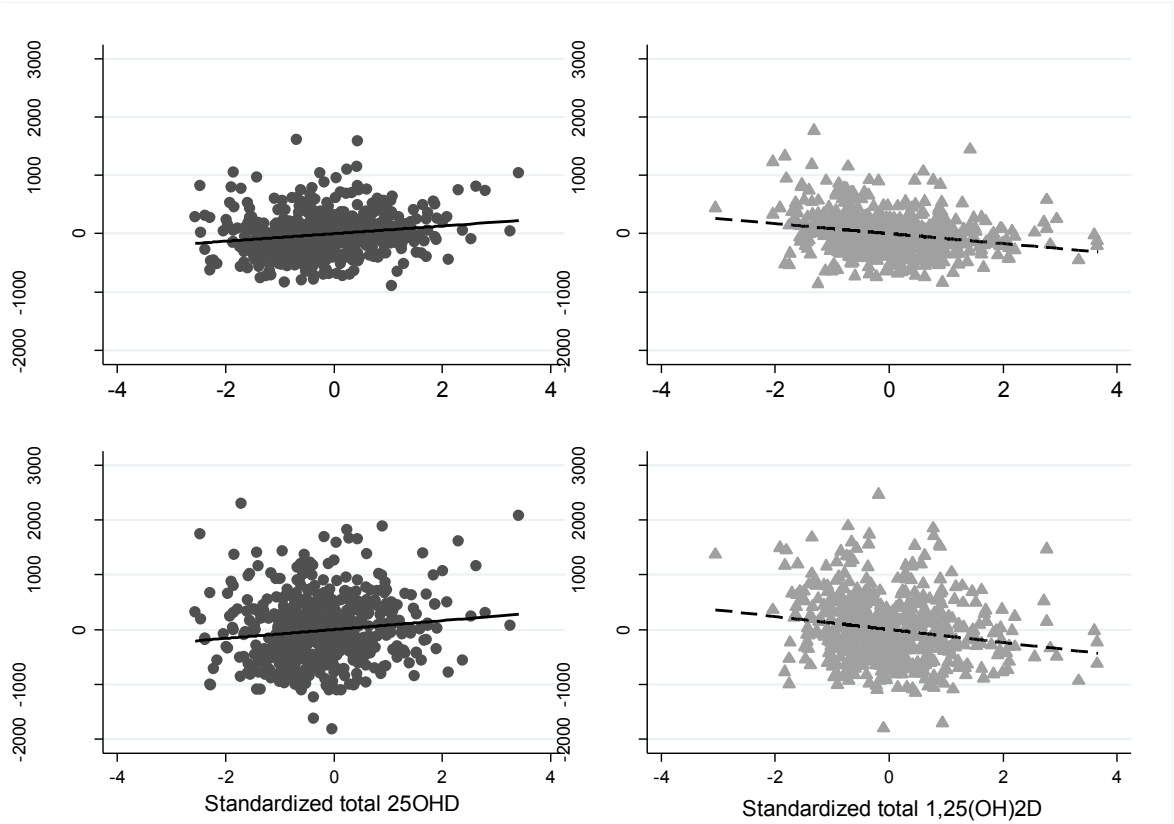


Figure 2. The IL-6 association with standardized 25OHD was nearly identical to the association with $1,25(OH)_2D$. Data points are predicted values[b].



[b] Data points and lines are from a fully adjusted regression model (adjusted for age, site, race, total fat mass, serum creatinine, eGFR, smoking, alcohol, season, self-reported health, diabetes, stroke, cox-II inhibitor use, surgical removal of stomach or intestine) with both vitamin D measures and DBP in the same model.

Figure 3. The TNFα soluble receptors I (top panels) and II (lower panels) associations with standardized 25OHD and 1,25(OH)$_2$D, were in *opposite directions*. Data points are predicted values[b].



[b] Data points and lines are from a fully adjusted regression model (adjusted for age, site, race, total fat mass, serum creatinine, eGFR, smoking, alcohol, season, self-reported health, diabetes, stroke, cox-II inhibitor use, surgical removal of stomach or intestine) with both vitamin D measures and DBP in the same model.

Supplemental Tables

Supplemental Table 1: Spearman correlations among Vitamin D measures and inflammatory markers

	Total 25OHD	Free 25OHD	Total $1,25(OH)_2D$	Free $1,25(OH)_2D$	VDBP (polyclonal ELISA)	VDBP (mono-clonal ELISA)	VDBP (RIA)	IL-6	TNFα	TNFα-sRI	TNFα-sRII	IL-6sR
Total 25OHD					0.17***	0.19***	0.23***					
Free 25OHD	0.87***				-0.27***	-0.59***	-0.21***					
Total $1,25(OH)_2D$	0.35***	0.32***			0.09	0.09*	0.17***					
Free $1,25(OH)_2D$	0.28***	0.43***	0.91***		-0.29***	-0.62***	-0.20***					
IL-6	-0.21***	-0.21***	-0.25***	-0.24***	0.03	-0.01	-0.06					
TNFα	-0.03	-0.04	-0.14***	-0.15***	0.01	0.08	0.04	0.24***				
TNFα-sRI	-0.01	-0.01	-0.35***	-0.34***	0.03	0.04	-0.07	0.43***	0.38***			
TNFα-sRII	-0.02	-0.02	-0.31***	-0.29***	-0.004	0.03	-0.09*	0.40***	0.42***	0.84***		
IL-6sR	0.01	-0.01	-0.05	-0.06	0.05	0.03	0.12***	0.13***	0.11***	0.15***	0.15***	
CRP	-0.04	-0.12*	-0.08	-0.16***	0.23***	0.10*	0.16***	0.45***	0.18***	0.29***	0.26***	0.05
IL-10	0.09	0.06	0.002	-0.007	0.02	0.13***	0.05	0.09*	0.38***	0.22***	0.24***	0.03

	~~VDBP (polyclonal ELISA)~~	~~VDBP (mono-clonal ELISA)~~	~~VDBP (RIA)~~	~~IL-6~~	~~TNFα~~	~~TNFα-sRI~~	~~TNFα-sRII~~	~~IL-6sR~~	~~Total 25OHD~~	~~Free 25OHD~~	~~Total $1,25(OH)_2D$~~	~~Free $1,25(OH)_2D$~~
~~Total 25OHD~~	~~0.17***~~	~~0.19***~~	~~0.23***~~	~~-0.21***~~	~~-0.03~~	~~-0.01~~	~~-0.02~~	~~0.01~~				
~~Free 25OHD~~	~~-0.27***~~	~~-0.59***~~	~~-0.21***~~	~~-0.21***~~	~~-0.04~~	~~-0.01~~	~~-0.02~~	~~-0.01~~	~~0.87***~~			
~~Total $1,25(OH)_2D$~~	~~0.09~~	~~-0.09*~~	~~0.17***~~	~~-0.25***~~	~~-0.14***~~	~~-0.35***~~	~~-0.31***~~	~~-0.05~~	~~0.35***~~	~~0.32***~~		
~~Free $1,25(OH)_2D$~~	~~-0.29***~~	~~-0.62***~~	~~-0.20***~~	~~-0.24***~~	~~-0.15***~~	~~-0.34***~~	~~-0.29***~~	~~-0.06~~	~~0.28***~~	~~0.43***~~	~~0.91***~~	
~~CRP~~	~~0.23***~~	~~0.10*~~	~~0.16***~~	~~0.45***~~	~~0.18***~~	~~0.29***~~	~~0.26***~~	~~0.05~~	~~-0.04~~	~~-0.12*~~	~~-0.08~~	~~-0.16***~~
~~IL-6~~	~~0.03~~	~~-0.01~~	~~-0.06~~		~~0.24***~~	~~0.43***~~	~~0.40***~~	~~0.13***~~				
~~TNFα~~	~~0.01~~	~~0.08~~	~~0.04~~	~~0.24***~~		~~0.38***~~	~~0.42***~~	~~0.11***~~				
~~TNFα sRI~~	~~0.03~~	~~0.04~~	~~-0.07~~	~~0.43***~~	~~0.38***~~		~~0.84***~~	~~0.15***~~				
~~TNFα sRII~~	~~-0.004~~	~~0.03~~	~~-0.09*~~	~~0.40***~~	~~0.42***~~	~~0.84***~~		~~0.15***~~				
~~IL-6sR~~	~~0.05~~	~~0.03~~	~~0.12**~~	~~0.13***~~	~~0.11***~~	~~0.15***~~	~~0.15***~~					
~~IL-10~~	~~0.02~~	~~0.13**~~	~~0.05~~	~~0.09*~~	~~0.38***~~	~~0.22***~~	~~0.24***~~	~~0.03~~	~~0.09~~	~~0.06~~	~~0.002~~	~~-0.007~~

* p<0.05, ** p<0.01, ***p<0.001.

Supplement Table 2. Associations with inflammatory markers (β, 95% CI) for each SD increase in free vitamin D and binding protein (DBP measures, MrOS)

	Free 25OHD		**Free 1,25(OH)$_2$D**		**DBP**	
	(Using DBP from monoclonal ELISA)	(Using DBP from RIA)	(Using DBP from monoclonal ELISA)	(Using DBP from RIA)	(Using DBP from monoclonal ELISA)	(Using DBP from RIA)
IL-6 (pg/mL) (N=557)	-0.03 (-0.20, 0.15)	-0.31 (-0.51, -0.10)**	-.0.09 (-0.27, 0.09)	-0.18 (-0.36, 0.01)	0.05 (-0.10, 0.20)	0.01 (-0.13, 0.16)
IL-6sR (ng/mL) (N=571)	0.90 (-0.19, 1.99)	-0.21 (-1.95, 1.52)	0.73 (-0.33, 1.78)	-0.31 (-1.50, 0.88)	0.59 (-0.51, 1.69)	1.31 (0.31, 2.32)*
TNFα (pg/mL) (N=556)	0.01 (-0.34, 0.36)	-0.42 (-0.97, 0.12)	0.0003 (-0.25, 0.25)	-0.25 (-0.47, -0.03)*	0.005 (-0.43, 0.44)	0.15 (-0.03, 0.34)
TNFα-sRI (pg/mL) (N=571)	53.24 (17.40, 89.08)	41.79 (-11.01, 94.60)	-5.98 (-42.05, 30.71)	-64.66 (-97.47, -31.85)**	35.58 (0.58, 70.58)*	16.95 (-18.36, 52.26)
TNFα-sRII (pg/mL) (N=565)	2.05 (-59.55, 63.66)	57.97 (-37.49, 153.42)	-52.32 (-106.80, 2.16)	-79.96 (-139.29, -20.62)**	46.27 (-11.82, 104.35)	0.76 (-53.54, 55.06)
IL-10 (pg/mL) (N=566)	2.71 (-1.50, 6.92)	-2.22 (-6.44, 2.00)	1.56 (-0.86, 3.98)	-2.11 (-3.86, -0.37)*	-0.66 (-4.42, 3.11)	0.92 (-0.46, 2.31)
CRP (ug/mL) (N=557)	0.27 (-0.39, 0.93)	0.24 (-1.01, 1.49)	0.09 (-0.43, 0.61)	-0.19 (-0.61, 0.23)	0.44 (-0.10, 0.98)	0.70 (0.35, 1.04)***
≥2 inflammatory markers in highest quartile[§] (N=571)	1.09 (0.86, 1.39)	0.89 (0.63, 1.25)	0.85 (0.68, 1.07)	0.71 (0.55, 0.91)**	1.21 (0.97, 1.51)	1.20 (0.97, 1.49)

Adjusted for age, site, race, total fat mass, serum creatinine, eGFR, smoking, alcohol, season, self-reported health, diabetes, stroke, cox-II inhibitor use, surgical removal of stomach or intestine.

[§]Among CRP, IL-6, TNFα, TNFα-sRI, TNFα-sRII, IL-6sR. Effect measure = odds ratios.

**p≤0.01, *p<0.05, ***p≤0.001 (Bonferroni-corrected alpha)

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