## UNIVERSITY<sup>OF</sup> BIRMINGHAM

**Research at Birmingham** 

# Associations of total and free 250HD and 1,25(OH)2D with serum markers of inflammation in older men

Srikanth, P; Chun, R F; Hewison, Martin; Adams, J S; Bouillon, R; Vanderschueren, D; Lane, N; Cawthon, P M; Dam, T; Barrett-Connor, E; Daniels, L B; Shikany, J M; Stefanick, M L; Cauley, J A; Orwoll, E S; Nielson, C M; Osteoporotic Fractures in Men (MrOS) Study Research Group

DOI: 10.1007/s00198-016-3537-3

*License:* None: All rights reserved

Document Version Peer reviewed version

Citation for published version (Harvard):

Srikanth, P, Chun, RF, Hewison, M, Adams, JS, Bouillon, R, Vanderschueren, D, Lane, N, Cawthon, PM, Dam, T, Barrett-Connor, E, Daniels, LB, Shikany, JM, Stefanick, ML, Cauley, JA, Orwoll, ES, Nielson, CM & Osteoporotic Fractures in Men (MrOS) Study Research Group 2016, 'Associations of total and free 25OHD and 1,25(OH)2D with serum markers of inflammation in older men', Osteoporosis International. https://doi.org/10.1007/s00198-016-3537-3

Link to publication on Research at Birmingham portal

#### **Publisher Rights Statement:**

The final publication is available at Springer via http://dx.doi.org/10.1007/s00198-016-3537-3

Checked Feb 2016

#### **General rights**

Unless a licence is specified above, all rights (including copyright and moral rights) in this document are retained by the authors and/or the copyright holders. The express permission of the copyright holder must be obtained for any use of this material other than for purposes permitted by law.

- Users may freely distribute the URL that is used to identify this publication.
- Users may download and/or print one copy of the publication from the University of Birmingham research portal for the purpose of private study or non-commercial research.

User may use extracts from the document in line with the concept of 'fair dealing' under the Copyright, Designs and Patents Act 1988 (?)
Users may not further distribute the material nor use it for the purposes of commercial gain.

Where a licence is displayed above, please note the terms and conditions of the licence govern your use of this document.

When citing, please reference the published version.

#### Take down policy

While the University of Birmingham exercises care and attention in making items available there are rare occasions when an item has been uploaded in error or has been deemed to be commercially or otherwise sensitive.

If you believe that this is the case for this document, please contact UBIRA@lists.bham.ac.uk providing details and we will remove access to the work immediately and investigate.

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

M	lanu	iscri	pt D	)raft
			~ ~ ~	

Manuscript Number:	OSIN-D-15-00747R1					
Full Title:	Associations of total and free 25OHD and 1,25(OH)2D with serum markers of inflammation in older men					
Article Type:	Original Article					
Funding Information:	National Institute on Aging (U01 AG027810)	Dr Eric Orwoll				
	National Institute on Aging (U01 AG042124)	Dr Eric Orwoll				
	National Institute on Aging (U01 AG042139)	Dr Jane Cauley				
	National Institute on Aging (U01 AG042140)	Dr James Shikany				
	National Institute on Aging (U01 AG042143)	Dr Marcia L Stefanick				
	National Institute on Aging (U01 AG042145)	Not applicable				
	National Institute on Aging (U01 AG042168)	Dr Elizabeth Barrett-Connor				
	National Institute of Arthritis and Musculoskeletal and Skin Diseases (U01 AR066160)	Not applicable				
	National Center for Advancing Translational Sciences (UL1 TR000128)	Not applicable				
	National Institute of Arthritis and Musculoskeletal and Skin Diseases (R01 AR063910)	Dr John S Adams				
	National Institute of Arthritis and Musculoskeletal and Skin Diseases (P60 AR054731)	Dr Jane Cauley				
	National Institute of Arthritis and Musculoskeletal and Skin Diseases (K01 AR062655)	Dr. Carrie Nielson				
	Merck (SRA-12-009)	Dr Eric Orwoll				
Abstract:	Purpose: Vitamin D is hypothesized to supp hydroxyvitamin D (25OHD) and inflammato However, total serum 25OHD may not be the vitamin D. Methods: We tested serum total 25OHD, to (DBP), and estimated free 25OHD and free inflammatory markers serum IL-6, TNFα and continuous outcomes and the presence of a quartile as a dichotomous outcome, in a ra Osteoporotic Fractures in Men (MrOS) stud Results: IL-6 was lower in men with higher 25OHD, 95% CI: -0.07 to -0.38 µg/mL) and CI: -0.0004 to -0.39 µg/mL); free D associate DBP, but not 1,25(OH)2D, were independe receptors were inversely associated with 1, 25OHD, and each had independent effects inflammatory markers in the highest quartile CI: 0.54 to 0.89 per SD increase in free 1,2 Conclusions: Associations of 1,25(OH)2D and papulation based the C studies. However, and	press inflammation, and circulating 25- ry markers are inversely correlated. the best indicator of biologically active tal 1,25(OH)2D, vitamin D binding protein e 1,25(OH)2D associations with id their soluble receptors, IL-10 and CRP as ≥2 inflammatory markers in the highest indom subcohort of 679 men in the ly. 25OHD (-0.23 µg/mL per SD increase in with higher 1,25(OH)2D (-0.20 µg/mL, 95% tions were slightly stronger. 25OHD and ntly associated with IL-6. TNFα soluble 25(OH)2D but positively associated with . The strongest association with ≥2 e was for free 1,25(OH)2D (OR: 0.70, 95% 5(OH)2D). and free 25OHD with IL-6 mirrored those of ree D do not improve upon 25OHD in				

	for TNF $\alpha$ soluble receptor, warranting examination of both metabolites in studies of TNF $\alpha$ and its antagonists.
Corresponding Author:	Carrie Nielson
	United States
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	
Corresponding Author's Secondary Institution:	
First Author:	Priya Srikanth
First Author Secondary Information:	
Order of Authors:	Priya Srikanth
	Rene F Chun
	Martin Hewison
	John S Adams
	Roger Bouillon
	Dirk Vanderschueren
	Nancy E Lane
	Peggy Cawthon
	Tien Dam
	Elizabeth Barrett-Connor
	Lori B Daniels
	James Shikany
	Marcia L Stefanick
	Jane Cauley
	Eric Orwoll
	Carrie Nielson
Order of Authors Secondary Information:	
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

#### 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, TNF $\alpha$ , TNF $\alpha$ -sRI, TNF $\alpha$ -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 TNF $\alpha$  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 TNF $\alpha$  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 TNF $\alpha$  receptors. The Welsh et al. study assumes that TNF $\alpha$  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 TNF $\alpha$ . However, as we outline in the Discussion of our manuscript, TNF $\alpha$  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 TNF $\alpha$ . Conversion of 25OHD to 1,25(OH)2D is known to be stimulated by cytokines such as TNF $\alpha$ , so the induction of soluble TNF $\alpha$  receptors following the synthesis of 1,25(OH)2D may be part of a feedback control linking vitamin D and TNF $\alpha$ . It will certainly be interesting to assess the effects of TNF $\alpha$  blockade on serum levels of 1,25(OH)2D as well as 25OHD, and this is something that we are planning for future studies.

#### Click here to view linked References

<u>±</u>

1 2		
3 4 5	1	Associations of total and free 25OHD and 1,25(OH) <sub>2</sub> D with
5 6 7	2	serum markers of inflammation in older men
8	3	Priya Srikanth <sup>1</sup> , Rene F. Chun <sup>2</sup> , Martin Hewison <sup>3</sup> , John S. Adams <sup>2</sup> , Roger Bouillon <sup>4</sup> , Dirk Vanderschueren <sup>4</sup> , Nancy
10 11	4	Lane <sup>5</sup> , Peggy M. Cawthon <sup>6</sup> , Tien Dam <sup>7</sup> , Elizabeth Barrett-Connor <sup>8</sup> , Lori B. Daniels <sup>8,9</sup> , James M. Shikany <sup>10</sup> , Marcia
12 13	5	L. Stefanick <sup>11</sup> , Jane A. Cauley <sup>12</sup> , Eric S. Orwoll <sup>13</sup> , Carrie M. Nielson <sup>1,13</sup> for the Osteoporotic Fractures in Men
14 15	6	(MrOS) Study Research Group
16 17	7	<sup>1</sup> Department of Public Health and Preventive Medicine, Oregon Health & Science University
18 19	8	<sup>2</sup> Department of Orthopaedic Surgery and Orthopaedic Hospital Research Center, David Geffen School of Medicine,
20 21	9	UCLA
22 23	10	<sup>3</sup> Centre for Endocrinology, Diabetes and Metabolism, University of Birmingham, UK
24 25	11 12	<sup>4</sup> Clinical and Experimental Endocrinology, Department of Clinical and Experimental Medicine, KU Leuven,
26 27	13	Belgium, Department of Endocrinology, University Hospital, Leuven
28 29	14	<sup>5</sup> Division of Rheumatology, University of California, Davis
30 31	15	<sup>6</sup> California Pacific Medical Center Research Institute, San Francisco, California
32 33	16	<sup>7</sup> Department of Medicine, Division of Geriatric Medicine and Aging, Columbia University
34 35	17	<sup>8</sup> Division of Epidemiology, Department of Family and Preventive Medicine, University of California, San Diego, La
36 37	18	Jolla, California
38 39	19	<sup>9</sup> Division of Cardiology, Department of Medicine, University of California, San Diego
40 41	20	<sup>10</sup> University of Alabama at Birmingham, Birmingham Alabama
42 43	21	<sup>11</sup> Stanford Prevention Research Center, School of Medicine, Stanford University
44 45	22	<sup>12</sup> Department of Epidemiology, Graduate School of Public Health, University of Pittsburgh, Pittsburgh,
46 47	23	Pennsylvania
48 49	24	<sup>13</sup> Bone and Mineral Unit, Oregon Health & Science University
50 51	25	Abbreviated Title: Associations of total and free 25OHD and 1,25(OH) <sub>2</sub> D with inflammation
52 53	26	Key Terms: Inflammation, total 25OHD, free 25OHD, total 1,25(OH) <sub>2</sub> D, free 1,25(OH) <sub>2</sub> D, elderly, men
54 55	27	Word count: 3,587 (OI limit: 5,000 words)
56 57	28	Number of figures and tables: 3 tables + 3 figures + 2 supplemental tables (OI limit: 6 figures and tables)
58 59 60	29	Corresponding author and person to whom reprint requests should be addressed:
62 63 64		1

Eric S. Orwoll, MD Oregon Health & Science University 3181 SW Sam Jackson Park Rd, CR113 Portland, OR USA 97239 Phone: 503-494-0225 Fax: 503-494-4816 orwoll@ohsu.edu Disclosure Statement: Roger Bouillon received lecture fees from Amgen, Novartis, Novo Nordisk, Chugai and Teijin and gave a license to a university patent on Vitamin D analogs to Hybrigenix (France). Eric S. Orwoll consults for and has received research support from Merck, Lilly and Amgen. Carrie Nielson, Priya Srikanth, Rene F Chun, Martin Hewison, John S Adams, Dirk Vanderschueren, Nancy E Lane, Peggy Cawthon, Tien Dam, Elizabeth Barrett-Connor, Lori B Daniels, James Shikany, Marcia L Stefanick, and Jane Cauley declare that they have no conflict of interest. Acknowledgments: The Osteoporotic Fractures in Men (MrOS) Study is supported by National Institutes of Health funding. The following institutes provide support: the National Institute on Aging (NIA), the National Institute of Arthritis and Musculoskeletal and Skin Diseases (NIAMS), the National Center for Advancing Translational Sciences (NCATS), and NIH Roadmap for Medical Research under the following grant numbers: U01 AG027810, U01 AG042124, U01 AG042139, U01 AG042140, U01 AG042143, U01 AG042145, U01 AG042168, U01 AR066160, and UL1 TR000128. Funding for this study was supported in part by the following NIH grants: NIAMS R01 AR063910 (PIs Martin Hewison and John Adams), P60 AR054731 (PI Jane Cauley), and NIAMS K01 AR062655 (PI Carrie Nielson). Supported in part by an independent investigator grant (SRA-12-009) from Merck &Co, Inc. 

#### **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.

59 Methods: We tested serum total 25OHD, total 1,25(OH)<sub>2</sub>D, vitamin D binding protein (DBP), and 60 estimated free 25OHD and free 1,25(OH)<sub>2</sub>D associations with inflammatory markers serum IL-6, TNF $\alpha$  and their 61 soluble receptors, IL-10 and CRP as continuous outcomes and the presence of  $\geq$ 2 inflammatory markers in the 62 highest quartile as a dichotomous outcome, in a random subcohort of 679 men in the Osteoporotic Fractures in Men 63 (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)<sub>2</sub>D (-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)<sub>2</sub>D, were *independently* associated with IL-6.
TNFα soluble receptors were inversely associated with 1,25(OH)<sub>2</sub>D 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)<sub>2</sub>D (OR: 0.70, 95% CI: 0.54 to 0.89 per SD increase in free 1,25(OH)<sub>2</sub>D).

Conclusions: Associations of 1,25(OH)<sub>2</sub>D and free 25OHD with IL-6 mirrored those of 25OHD,
suggesting that 1,25(OH)<sub>2</sub>D 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 (TNF $\alpha$ ) 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)<sub>2</sub>D<sub>3</sub> 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)<sub>2</sub>D, 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 TNF $\alpha$  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)<sub>2</sub>D and vitamin D binding protein (DBP) with inflammatory markers that include CRP, IL-6, TNFa, IL-6 and TNFa 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].

#### 116 Vitamin D Measurements

Serum concentrations of both the inactive (25OHD) and active (1,25(OH)<sub>2</sub>D) 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  $-70^{\circ}$ C. All samples for total 25OHD remained frozen in foil wrapped vials to reduce UV exposure until assay. Measures for 25OHD<sub>2</sub> (derived from ergocalciferol) and 25OHD<sub>3</sub> (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<sub>2</sub>, 25OHD<sub>3</sub>, 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<sub>2</sub> and 25OHD<sub>3</sub> were reported individually. The minimum detectable limit was 4 ng/ml for 25OHD<sub>2</sub> and 2 ng/ml for 25OHD<sub>3</sub>. Aliquots of a single serum pool were included in alternate assay runs. Using the pooled serum, the interassay coefficient of variation (CV) for 25OHD<sub>3</sub> was 4.4%, and the intraassay CV was 4.9%.

Total 1,25(OH)<sub>2</sub>D 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)<sub>2</sub>D<sub>2</sub> and 6 pg/mL for
133 1,25(OH)<sub>2</sub>D<sub>3</sub>. 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,

138Minneapolis, MN) and by polyclonal radial immunodiffusion assay (Laboratory of Clinical and Experimental

139 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)<sub>2</sub>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].

144 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 TNFa, TNF-asRI 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%. TNFa 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- asRI in serum is 479 – 966 pg/mL and for TNF-asRII in serum is 1003 – 3170 pg/mL. Inter-assay CVs range from 5.42% to 8.59% for TNF-asRI and 2.87 to 3.54% for TNF-αsRII. CRP was measured using the BNII nephelometer from Dade Behring utilizing a **162** particle enhanced immunonepholometric assay. The assay range is 0.16 - 1100 ug/mL. Expected values for CRP in normal, healthy individuals are  $\leq 3$  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<sup>2</sup>). 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<sup>2</sup> 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).

180 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 ( $\beta$ ) 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, TNFa, TNFa-sRI, TNFa-sRII and IL-6sR). We then dichotomized this index into those having  $\geq 2$  inflammatory markers in the highest quartile in comparison with those having  $\leq 2$  inflammatory

markers in the highest quartile [4]. Logistic regression modeling was used to obtain odds ratios (OR) andcorresponding 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  $\leq 0.001$ (0.05/35 = 0.001).

205 TX).

206 RESULTS

#### 207 Description and correlations

208 Men with inflammatory markers and vitamin D measures (Figure 1) had a mean age of 74 ±6 years and
209 mean BMI of 27 ±4 kg/m<sup>2</sup>. Most (91%) were non-Hispanic white, and 85% reported excellent or good health status.
210 16% were taking NSAIDs, and 27% reported a history of heart attack, CHF, or angina (Table 1).

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

211 All correlations between inflammatory markers and vitamin D measures were weak. IL-6 was negatively 212 correlated with total and free 25OHD and  $1,25(OH)_2D$  measures (r = -0.21 to -0.25, p<0.001). TNF $\alpha$  and its soluble 213 receptors were significantly negatively correlated with total and free  $1,25(OH)_2D$  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).

#### 51 216 Inflammatory marker associations with 25OHD and 1,25(OH)<sub>2</sub>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)<sub>2</sub>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)<sub>2</sub>D and DBP (Table 2).

Associations of IL-6 with 25OHD and 1,25(OH)<sub>2</sub>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)<sub>2</sub>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)<sub>2</sub>D. This association was attenuated to after 25OHD and DBP adjustment and was no longer significant.

TNF $\alpha$  soluble receptors I and II were positively associated with 25OHD and inversely associated with 1,25(OH)<sub>2</sub>D (Figure 3). Average TNF $\alpha$  soluble receptors I and II were significantly lower by- 62.05 pg/mL (95% CI: 26.09 to 98.01 pg/mL) for TNF $\alpha$ -sRI and -88.83 pg/mL (95% CI: 30.64 to 147.02 pg/mL for TNF $\alpha$ -sRII) per SD increase in 1,25(OH)<sub>2</sub>D. This association was strengthened with adjustment of 25OHD and DBP (Table 2). TNF $\alpha$ 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)<sub>2</sub>D and DBP adjustment (Table 2).

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

#### 237 Inflammatory marker associations with free 25OHD, free 1,25(OH)<sub>2</sub>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)<sub>2</sub>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)<sub>2</sub>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)<sub>2</sub>D. Odds of having  $\geq 2$  inflammatory markers in the highest quartile decreased by 30% (95% CI: 11% to 46%) decrease) for each SD increase in free 1,25(OH)<sub>2</sub>D. There was no significant association with free 25OHD (Table 3). 

249 CRP and TNF $\alpha$  soluble receptors' associations with free measures of 25OHD and 1,25(OH)<sub>2</sub>D and DBP 250 from other assays (monoclonal ELISA (R&D Systems, Minneapolis, MN) and radioimmunodiffusion assay (RID)) 251 were similar to the polyclonal ELISA (Genway Biotech, San Diego, CA) assay, but slightly weaker. The CRP-DBP 252 associations were also somewhat weaker but still statistically significant. IL-6 associations were significant for the 253 free D measures from the RID assay, though slightly weaker, but not significant for the free D measures from the 254 monoclonal ELISA assay (Supplemental Table 2). Use of *GC*-genotype-specific binding affinities, rather than 255 constant affinities, in the free D estimating equations did not make a substantial difference in associations.

#### 256 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 TNF $\alpha$  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 TNF $\alpha$ , 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 TNF $\alpha$  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)<sub>2</sub>D 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)<sub>2</sub>D 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)<sub>2</sub>D<sub>3</sub> in ankylosing spondylitis patients
[9]. But CRP levels were much higher in those studies, while in MrOS, CRP remained low across the range of
275 25OHD and 1,25(OH)<sub>2</sub>D (<1.5 µg/ml). Similar to our results, adults in the Framingham Offspring Study had no</li>
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 linkbetween 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)<sub>2</sub>D 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
250HD to 1,25(OH)<sub>2</sub>D 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 Dactivating enzyme 1 $\alpha$ -hydroxylase (CYP27B1) is mainly regulated by serum PTH and FGF23 but extra renal production of 1,25(OH)<sub>2</sub>D by CYP247B1 is known to be induced by inflammatory cytokines such as TNF $\alpha$  [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)<sub>2</sub>D 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)<sub>2</sub>D was more strongly associated than 25OHD with TNF $\alpha$  soluble receptors and with having  $\geq$ 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 250HD<sub>3</sub>, 25(OH)D and

1,25(OH)<sub>2</sub>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)<sub>2</sub>D<sub>3</sub> 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)<sub>2</sub>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)<sub>2</sub>D. In contrast, we observed consistently divergent associations with  $TNF\alpha$  soluble receptors for these metabolites. Considering the importance of TNFa action in osteoclastic maturation [7, 53, 54], future studies of vitamin D should include investigations of the effects of each metabolite.

C	heresteristic	Overall $(N-670)$
C.	naracteristic	Mean + SD Median (IOR)  or  n
Δ	ο Α	$\frac{1}{74 + 6}$
R		74±0
K	White	616 (90 72)
	A friegen A morigen	(90.72)
	Asian	22 (3.24)
		10 (2.30)
	Hispanic Other	17 (2.50)
V		8 (1.18)
V		25.05.700
	250HD (ng/m)	$25.95 \pm 7.98$
	Free 250HD (nmol/L) $1.25(OLD) D (mol/L)$	$0.03 \pm 0.01$
	$1,25(OH)_2D$ (pg/ml)	64.24 ±/1./2
_	Free 1,25(OH) <sub>2</sub> D (nmol/L)	0.0015 ±0.0004
	Vitamin D binding protein ( $\mu$ M)	4.36 ±0.75
Se	eason of blood draw	
	Winter	134 (19.73)
	Spring	174 (45.36)
	Summer	198 (29.16)
	Fall	173 (25.48)
В	MI (kg/m <sup>2</sup> )	27 ±4
Te	otal Fat Mass (kg) <sup>§</sup>	$22 \pm 7$
In	flammatory Markers	
	CRP (ug/mL) <sup>§ a</sup>	1.44 (2.1)
	IL-6 $(pg/mL)^{\$a}$	2.37 (1.97)
	$TNF\alpha (pg/mL)^{\$a}$	3.96 (2.54)
Se	oluble Receptors	
	$TNF\alpha$ -sRI (pg/mL) <sup>§ a</sup>	1940.60 (593.40)
	$TNF\alpha$ -sRII (pg/mL) <sup>§ a</sup>	3521.80 (938.90)
	IL-6sR $(ng/ml)^{\$a}$	49.09 (18.25)
A	nti-Inflammatory Marker	()
	IL-10 $(pg/mL)^{\$a}$	8.85 (6.93)
А	lcohol (per week)	
	0 drinks	238 (35.05)
	1-7 drinks	323 (47 57)
	7 drinks	118(17.38)
Se	olf-reported quality of health*	110 (17.50)
50	Excellent/Good	576 (84 96)
	Excenent/Good	102(15.04)
D	A SE score <sup>†</sup>	102(15.04) $147 \pm 66$
		$147\pm00$ 107 (16 40)
IN C	SAIDS use	107 (10.49) 52 (8 17)
C		51 (7.86)
	UX-II INNIDILOF USE	51 (7.80)
C	VD <sup>3</sup>	182 (20.80)
St	roke	51 (7.51)
D	labetes	83 (12.22)
Su	argical removal of stomach or intestine	56 (8.25)
R	heumatoid Arthritis	34 (5.01)
R	enal Function	
	eGFR $(ml/min/(1.73m^2))^{\$}$	$77 \pm 19$
	Serum creatinine (mg/dl) §	1.02 ±0.30
327 * H	Iow would you rate your overall health?	
328 † F	hysical activity score for the elderly	
329 <sup>a</sup> n	edian, inter-quartile range (IQR)	

	1
	2
	3
	4
	5
	б
	7
	8
	9
1	0
1	1
1	2
1	3
1	Λ

1	-	
2	2	
3	3	
4	± 330	<sup>§</sup> 5 missing total fat mass, 36 missing lipids, 80 missing CRP, 83 missing IL-6, 81 missing TNFα, 66 missing,
5	331	TNFα-sRI, 72 missing TNFα-sRII, 66 missing IL-6sR, 71 missing IL-10, 36 missing eGFR, 36 missing serum
6	332	creatinine, 30 missing NSAIDS use, 30 missing corticosteroid use, 30 missing Cox-II inhibitor
/ c	333	<sup>b</sup> defined as self-report of previous heart attack, congestive heart failure or angina
2 C	2	
10	)	
11	)	
12	-	
13	2	
14	ļ	
15	-	
16	5	
17	7	
18	3	
19	)	
20	)	
21	-	
22	2	
23	3	
24	Ł	
25	5	
26	5	
27	7	
28	3	
29	9	
30	)	
31	_	
32	2	
33	3	
34	Ł	
35	-	
36	2	
31	/ >	
38	5	
35	<i>i</i>	
4U /11	J	
41 / 1	- )	
12 43		
т. ДД	, L	
45	-	
46	- 	
47	7	
48	3	
49	)	
50	)	
51	_	
52	2	
53	3	
54	Ł	
55	5	
56	5	
57	7	
58	3	
50	۵	

- 60 61 62 63 64

	Ν	250HD	1,25(OH)2D	25OHD	1,25(OH)2D	DBP
		(SD=7.98 ng/ml)	(SD=71.72 pg/ml)	250HD, 1,	25(OH) <sub>2</sub> D, and DBP in the same	model
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 ( $\mu g/mL$ )	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 <sup>§</sup> (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)*
		11 u-5KI, 1141 u-5KII, IL-05I	x. Effect measure = odds ratios (	95%C1). **p≤0.01, *p≤0.05	, ***p≤0.001 (Bonferroni-correc	eted alpha)
		1 u-3NI, 1141 u-3NII, 1L-031	x. Effect measure = odds ratios (	95%C1). **p≤0.01, *p≤0.05	, ***p≤0.001 (Bonferroni-correc	ted alpha)
		1 u-SNI, 1141 u-SNII, 11-051	x. Effect measure = odds ratios (	95%C1). **p≤0.01, *p≤0.05	, ***p≤0.001 (Bonferroni-correc	eted alpha)
		1 u-3NI, 1141 u-3NII, 1L-031	x. Effect measure = odds ratios (	95%C1). **p≤0.01, *p≤0.05	, ***p≤0.001 (Bonferroni-correc	eted alpha)
		u u-sixi, 11vi u-sixii, 12-0si	x. Effect measure = odds ratios (	95%C1). **p≤0.01, *p≤0.05	, ***p≤0.001 (Bonferroni-correc	eted alpha)
		1 u-3Ki, 1141 u-3Kii, 12-03i	x. Effect measure = odds ratios (	95%C1). **p≤0.01, *p≤0.05	, ***p≤0.001 (Bonferroni-correc	eted alpha)
		u u-siki, 1141 u-sikii, 12-0si	x. Effect measure = odds ratios (	95%C1). **p≤0.01, *p≤0.05	, ***p≤0.001 (Bonferroni-correc	eted alpha)
		u u-siki, 11vi u-sikii, 12-osi	X. Effect measure = odds ratios (	95%C1). **p≤0.01, *p≤0.05	, ***p≤0.001 (Bonferroni-correc	eted alpha)
		u u-siki, 11vi u-sikii, 12-osi	X. Effect measure = odds ratios (	95%C1). **p≤0.01, *p≤0.05	, ***p≤0.001 (Bonferroni-correc	eted alpha)
			X. Effect measure = odds ratios (	95%C1). **p≤0.01, *p≤0.05	, ***p≤0.001 (Bonferroni-correc	eted alpha)
			X. Effect measure = odds ratios (	95%C1). **p≤0.01, *p≤0.05	, ***p≤0.001 (Bonferroni-correc	eted alpha)
			X. Effect measure = odds ratios (	95%C1). **p≤0.01, *p≤0.05	, ***p≤0.001 (Bonferroni-correc	eted alpha)
			X. Effect measure = odds ratios (	95%C1). **p≤0.01, *p≤0.05	, ***p≤0.001 (Bonferroni-correc	eted alpha)
			C. Effect measure = odds ratios (	95%C1). **p≤0.01, *p≤0.05	, ***p≤0.001 (Bonferroni-correc	eted alpha)
			X. Effect measure = odds ratios (	95%C1). **p≤0.01, *p≤0.05	, ***p≤0.001 (Bonferroni-correc	eted alpha)

IL-6 (pg/mL) IL-6sR (ng/mL)	19	FILE 250HD		
IL-6 (pg/mL) IL-6sR (ng/mL)		(SD=0.01 nmol/L)	(SD=0.0004 nmol/L)	(SD=0.75 µM)
IL-6sR (ng/mL)	557	-0.35 (-0.55, -0.15)**	-0.22 (-0.39, -0.04)*	0.11 (-0.04, 0.26)
-	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 ( $\mu$ g/mL) $\geq$ 2 inflammatory markers	557	0.16 (-0.98, 0.67)	-0.40 (-0.79, -0.02)*	1.11 (0.45, 1.76)***
in highest quartile <sup>§</sup> (N=571)	571	0.85 (0.61, 1.19)	0.70 (0.54, 0.89)**	1.26 (1.03, 1.55)*

\_\_\_







20 357 Figure 3. The TNFa soluble receptors I (top panels) and II (lower panels) associations with standardized 25OHD and 1,25(OH)<sub>2</sub>D, were in *opposite directions*. 21 358 Data points are predicted values<sup>b</sup>.

<sup>b</sup> 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

		1		0			2						
23		Total 250HD	Free	Total	Free	VDBP	VDBP	VDBP	IL-6	TNFα	TNFα-	TNFα-sRII	IL-6sR
24			25OHD	1,25(OH)	1,25(OH)	(polyclon	(mono-	(RIA)			sRI		
25				2 <b>D</b>	2 <b>D</b>	al	clonal						
26						ELISA)	ELISA)						
27	Total 25OHD					0.17***	0.19***	0.23***					
28	Free 25OHD	0.87***				-0.27***	-0.59***	-0.21***					
29	Total 1,25(OH) <sub>2</sub> D	0.35***	0.32***			0.09	0.09*	0.17***					
30	Free 1,25(OH) <sub>2</sub> D	0.28***	0.43***	0.91***		-0.29***	-0.62***	-0.20***					
3⊥ 20	IL-6	-0.21***	-0.21***	-0.25***	-0.24***	0.03	-0.01	-0.06					
32	TNFα	-0.03	-0.04	-0.14***	-0.15***	0.01	0.08	0.04	0.24***				
34	TNFα-sRI	-0.01	-0.01	-0.35***	-0.34***	0.03	0.04	-0.07	0.43***	0.38***			
35	TNFα-sRII	-0.02	-0.02	-0.31***	-0.29***	-0.004	0.03	-0.09*	0.40***	0.42***	0.84***		
36	IL-6sR	0.01	-0.01	-0.05	-0.06	0.05	0.03	0.12***	0.13***	0.11***	0.15***	0.15***	
37	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
38	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
39 <b>366</b>	* p<0.05, ** p<0.01,	***p<0.001.		•		•	•	-	•	•	•	•	-

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

41 **367** 

**368** 

51

15	
16	
17	
18	
19	
20	36
21	

;9 Supplement Table 2. Associations with inflammatory markers ( $\beta$ , 95% CI) for each SD increase in free vitamin D and binding protein (DBP measures, MrOS) Free 250HD Free 1.25(OH)2D DBP ᄼᆂ 22 (Using DBP from 23 monoclonal ELISA) monoclonal ELISA) monoclonal ELISA) RIA) RIA) RIA) 24 IL-6 (pg/mL)-0.03 (-0.20, 0.15) -0.31 (-0.51. --.0.09 (-0.27, 0.09) -0.18 (-0.36, 0.01) 0.05 (-0.10, 0.20) 0.01 (-0.13, 0.16) 25 0.10)\*\* (N=557) 26 0.90 (-0.19, 1.99) -0.21 (-1.95, 1.52) IL-6sR (ng/mL) 0.73 (-0.33, 1.78) -0.31 (-1.50, 0.88) 0.59 (-0.51, 1.69) 1.31 (0.31, 2.32)\* 27 (N=571) 28 TNF $\alpha$  (pg/mL) 0.01 (-0.34, 0.36) -0.42 (-0.97, 0.12) 0.0003 (-0.25, 0.25) -0.25 (-0.47, -0.005 (-0.43, 0.44) 0.15 (-0.03, 0.34) 29 (N=556) 0.03)\* 30 TNFα-sRI 53.24 (17.40, 89.08) 41.79 (-11.01, -5.98 (-42.05, 30.71) -64.66 (-97.47, -35.58 (0.58, 70.58)\* 16.95 (-18.36, 52.26) 31 94.60) 31.85)\*\* (pg/mL) (N=571) 32 2.05 (-59.55, 63.66) 57.97 (-37.49. -52.32 (-106.80, 2.16) 46.27 (-11.82. TNFα-sRII -79.96 (-139.29, -0.76 (-53.54, 55.06) 33 20.62)\*\* (pg/mL) (N=565) 153.42) 104.35) 34 -0.66 (-4.42, 3.11) IL-10 (pg/mL)2.71 (-1.50, 6.92) -2.22 (-6.44, 2.00) 1.56 (-0.86, 3.98) -2.11 (-3.86, -0.92 (-0.46, 2.31) 35 0.37)\* (N=566) 36 CRP (ug/mL) 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)\*\*\* 37 (N=557) 38  $\geq 2$  inflammatory 39 markers in 40 highest quartile§ 41 (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) 42 Adjusted for age, site, race, total fat mass, serum creatinine, eGFR, smoking, alcohol, season, self-reported health, diabetes, stroke, cox-II inhibitor use, surgical 370 43 371 removal of stomach or intestine. 44  $Among CRP, IL-6, TNF\alpha, TNF\alpha-sRI, TNF\alpha-sRII, IL-6sR. Effect measure = odds ratios.$ 372 45 \*\*p≤0.01, \*p<0.05, \*\*\*p≤0.001 (Bonferroni-corrected alpha) 373 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61 62 22 63 64

#### References

1. Franceschi C (2007) Inflammaging as a major characteristic of old people: can it be prevented or cured? Nutr Rev 65:S173-176

2. Collin-Osdoby P, Rothe L, Anderson F, Nelson M, Maloney W, Osdoby P (2001) Receptor activator of NF-kappa B and osteoprotegerin expression by human microvascular endothelial cells, regulation by inflammatory cytokines, and role in human osteoclastogenesis. J Biol Chem 276:20659-20672

3. Doherty TM, Asotra K, Fitzpatrick LA, Qiao JH, Wilkin DJ, Detrano RC, Dunstan CR, Shah PK, Rajavashisth TB (2003) Calcification in atherosclerosis: bone biology and chronic inflammation at the arterial crossroads. Proc Natl Acad Sci U S A 100:11201-11206

4. Cauley JA, Danielson ME, Boudreau RM, Forrest KY, Zmuda JM, Pahor M, Tylavsky FA, Cummings SR, Harris TB, Newman AB (2007) 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

5. Ding C, Parameswaran V, Udayan R, Burgess J, Jones G (2008) Circulating levels of inflammatory markers predict change in bone mineral density and resorption in older adults: a longitudinal study. J Clin Endocrinol Metab 93:1952-1958

6. Arnson Y, Itzhaky D, Mosseri M, Barak V, Tzur B, Agmon-Levin N, Amital H (2013) Vitamin D inflammatory cytokines and coronary events: a comprehensive review. Clin Rev Allergy Immunol 45:236-247

7. Inanir A, Ozoran K, Tutkak H, Mermerci B (2004) The effects of calcitriol therapy on serum interleukin-1, interleukin-6 and tumour necrosis factor-alpha concentrations in post-menopausal patients with osteoporosis. J Int Med Res 32:570-582

8. Swanson CM, Srikanth P, Lee CG, Cummings SR, Jans I, Cauley JA, Bouillon R, Vanderschueren D, Orwoll ES, Nielson CM (2015) Associations of 25-Hydroxyvitamin D and 1,25-Dihydroxyvitamin D With Bone Mineral Density, Bone Mineral Density Change, and Incident Nonvertebral Fracture. J Bone Miner Res 30:1403-1413

9. Lange U, Jung O, Teichmann J, Neeck G (2001) Relationship between disease activity and serum levels of vitamin D metabolites and parathyroid hormone in ankylosing spondylitis. Osteoporos Int 12:1031-1035

10. Zhang Y, Leung DY, Richers BN, Liu Y, Remigio LK, Riches DW, Goleva E (2012) Vitamin D inhibits monocyte/macrophage proinflammatory cytokine production by targeting MAPK phosphatase-1. J Immunol 188:2127-2135

11. Rostkowska-Nadolska B, Sliupkas-Dyrda E, Potyka J, Kusmierz D, Fraczek M, Krecicki T, Kubik P, Zatonski M, Latocha M (2010) Vitamin D derivatives: calcitriol and tacalcitol inhibits interleukin-6 and interleukin-8 expression in human nasal polyp fibroblast cultures. Adv Med Sci 55:86-92

12. Querfeld U (2013) Vitamin D and inflammation. Pediatr Nephrol 28:605-610

Ding C, Wilding JP, Bing C (2013) 1,25-dihydroxyvitamin D3 protects against macrophage-induced activation of NFkappaB and MAPK signalling and chemokine release in human adipocytes. PLoS One 8:e61707
 Amital H, Szekanecz Z, Szucs G, et al. (2010) 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 69:1155-1157

15. Rossini M, Maddali Bongi S, La Montagna G, Minisola G, Malavolta N, Bernini L, Cacace E, Sinigaglia L, Di Munno O, Adami S (2010) Vitamin D deficiency in rheumatoid arthritis: prevalence, determinants and associations with disease activity and disability. Arthritis Res Ther 12:R216

Merlino LA, Curtis J, Mikuls TR, Cerhan JR, Criswell LA, Saag KG (2004) Vitamin D intake is inversely associated with rheumatoid arthritis: results from the Iowa Women's Health Study. Arthritis Rheum 50:72-77
Munger KL, Zhang SM, O'Reilly E, Hernan MA, Olek MJ, Willett WC, Ascherio A (2004) Vitamin D

intake and incidence of multiple sclerosis. Neurology 62:60-65

18. Blank JB, Cawthon PM, Carrion-Petersen ML, Harper L, Johnson JP, Mitson E, Delay RR (2005) Overview of recruitment for the osteoporotic fractures in men study (MrOS). Contemp Clin Trials 26:557-568

19. Orwoll E, Blank JB, Barrett-Connor E, et al. (2005) Design and baseline characteristics of the osteoporotic fractures in men (MrOS) study--a large observational study of the determinants of fracture in older men. Contemp Clin Trials 26:569-585

20. Orwoll E, Nielson CM, Marshall LM, Lambert L, Holton KF, Hoffman AR, Barrett-Connor E, Shikany JM, Dam T, Cauley JA (2009) Vitamin D deficiency in older men. J Clin Endocrinol Metab 94:1214-1222
21. Singh RJ, Taylor RL, Reddy GS, Grebe SK (2006) C-3 epimers can account for a significant proportion of

total circulating 25-hydroxyvitamin D in infants, complicating accurate measurement and interpretation of vitamin D status. J Clin Endocrinol Metab 91:3055-3061

22. Vanderschueren D, Pye SR, O'Neill TW, et al. (2013) Active vitamin D (1,25-dihydroxyvitamin D) and bone health in middle-aged and elderly men: the European Male Aging Study (EMAS). The Journal of clinical endocrinology and metabolism 98:995-1005

23. Vanderschueren D, Pye SR, O'Neill TW, et al. (2013) Active vitamin D (1,25-dihydroxyvitamin D) and bone health in middle-aged and elderly men: the European Male Aging Study (EMAS). J Clin Endocrinol Metab 98:995-1005

24. Bouillon R, van Baelen H, de Moor P (1977) The measurement of the vitamin D-binding protein in human serum. J Clin Endocrinol Metab 45:225-231

25. Chun RF, Peercy BE, Adams JS, Hewison M (2012) Vitamin D binding protein and monocyte response to
25-hydroxyvitamin D and 1,25-dihydroxyvitamin D: analysis by mathematical modeling. PLoS One 7:e30773
26. Jones SA, Horiuchi S, Topley N, Yamamoto N, Fuller GM (2001) The soluble interleukin 6 receptor:

26. Jones SA, Horiuchi S, Topley N, Yamamoto N, Fuller GM (2001) The soluble interleukin 6 receptor: mechanisms of production and implications in disease. FASEB J 15:43-58

27. Washburn RA, Smith KW, Jette AM, Janney CA (1993) The Physical Activity Scale for the Elderly (PASE): development and evaluation. J Clin Epidemiol 46:153-162

28. Pahor M, Chrischilles EA, Guralnik JM, Brown SL, Wallace RB, Carbonin P (1994) Drug data coding and analysis in epidemiologic studies. Eur J Epidemiol 10:405-411

29. Levey AS, Coresh J, Greene T, Marsh J, Stevens LA, Kusek JW, Van Lente F (2007) Expressing the Modification of Diet in Renal Disease Study equation for estimating glomerular filtration rate with standardized serum creatinine values. Clin Chem 53:766-772

30. Lumley T, Diehr P, Emerson S, Chen L (2002) The importance of the normality assumption in large public health data sets. Annu Rev Public Health 23:151-169

31. Laird E, McNulty H, Ward M, et al. (2014) Vitamin d deficiency is associated with inflammation in older irish adults. J Clin Endocrinol Metab 99:1807-1815

32. Wobke TK, Sorg BL, Steinhilber D (2014) Vitamin D in inflammatory diseases. Front Physiol 5:244
33. Nonn L, Peng L, Feldman D, Peehl DM (2006) Inhibition of p38 by vitamin D reduces interleukin-6
production in normal prostate cells via mitogen-activated protein kinase phosphatase 5: implications for prostate cancer prevention by vitamin D. Cancer Res 66:4516-4524

34. Shea MK, Booth SL, Massaro JM, et al. (2008) Vitamin K and vitamin D status: associations with inflammatory markers in the Framingham Offspring Study. Am J Epidemiol 167:313-320

35. Bratke K, Wendt A, Garbe K, Kuepper M, Julius P, Lommatzsch M, Virchow JC (2014) Vitamin D binding protein and vitamin D in human allergen-induced endobronchial inflammation. Clin Exp Immunol 177:366-372

36. Toss G, Sorbo B (1986) Serum concentrations of 25-hydroxyvitamin D and vitamin D-binding protein in elderly people. Effects of institutionalization, protein-energy malnutrition and inflammation. Acta Med Scand 220:273-277

37. Zehnder D, Bland R, Chana RS, Wheeler DC, Howie AJ, Williams MC, Stewart PM, Hewison M (2002) Synthesis of 1,25-dihydroxyvitamin D(3) by human endothelial cells is regulated by inflammatory cytokines: a novel autocrine determinant of vascular cell adhesion. J Am Soc Nephrol 13:621-629

 Edfeldt K, Liu PT, Chun R, et al. (2010) T-cell cytokines differentially control human monocyte antimicrobial responses by regulating vitamin D metabolism. Proc Natl Acad Sci U S A 107:22593-22598
 Hewison M, Burke F, Evans KN, Lammas DA, Sansom DM, Liu P, Modlin RL, Adams JS (2007) Extra-

renal 25-hydroxyvitamin D3-1alpha-hydroxylase in human health and disease. J Steroid Biochem Mol Biol 103:316-321

40. Hummel DM, Fetahu IS, Groschel C, Manhardt T, Kallay E (2014) Role of proinflammatory cytokines on expression of vitamin D metabolism and target genes in colon cancer cells. J Steroid Biochem Mol Biol 144PA:91-

41. Welsh P, Peters MJ, McInnes IB, et al. (2011) 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 70:1165-1167

42. Waldron JL, Ashby HL, Cornes MP, Bechervaise J, Razavi C, Thomas OL, Chugh S, Deshpande S, Ford C, Gama R (2013) Vitamin D: a negative acute phase reactant. J Clin Pathol 66:620-622

43. Reid D, Toole BJ, Knox S, Talwar D, Harten J, O'Reilly DS, Blackwell S, Kinsella J, McMillan DC, Wallace AM (2011) The relation between acute changes in the systemic inflammatory response and plasma 25hydroxyvitamin D concentrations after elective knee arthroplasty. Am J Clin Nutr 93:1006-1011

44. Hopkins MH, Owen J, Ahearn T, Fedirko V, Flanders WD, Jones DP, Bostick RM (2011) Effects of supplemental vitamin D and calcium on biomarkers of inflammation in colorectal adenoma patients: a randomized, controlled clinical trial. Cancer Prev Res (Phila) 4:1645-1654

45. Peake JM, Kukuljan S, Nowson CA, Sanders K, Daly RM (2011) Inflammatory cytokine responses to progressive resistance training and supplementation with fortified milk in men aged 50+ years: an 18-month randomized controlled trial. Eur J Appl Physiol 111:3079-3088

46. Pittas AG, Harris SS, Stark PC, Dawson-Hughes B (2007) The effects of calcium and vitamin D supplementation on blood glucose and markers of inflammation in nondiabetic adults. Diabetes Care 30:980-986

47. Shab-Bidar S, Neyestani TR, Djazayery A, Eshraghian MR, Houshiarrad A, Kalayi A, Shariatzadeh N, Khalaji N, Gharavi A (2012) Improvement of vitamin D status resulted in amelioration of biomarkers of systemic inflammation in the subjects with type 2 diabetes. Diabetes Metab Res Rev 28:424-430

48. Verstuyf A, Carmeliet G, Bouillon R, Mathieu C (2010) Vitamin D: a pleiotropic hormone. Kidney Int 78:140-145

49. Hopkins MH, Flanders WD, Bostick RM (2012) Associations of circulating inflammatory biomarkers with risk factors for colorectal cancer in colorectal adenoma patients. Biomark Insights 7:143-150

50. Barbour KE, Boudreau R, Danielson ME, et al. (2012) Inflammatory markers and the risk of hip fracture: the Women's Health Initiative. J Bone Miner Res 27:1167-1176

51. Barbour KE, Lui LY, Ensrud KE, Hillier TA, LeBlanc ES, Ing SW, Hochberg MC, Cauley JA (2014) Inflammatory markers and risk of hip fracture in older white women: the study of osteoporotic fractures. J Bone Miner Res 29:2057-2064

52. Penninx BW, Kritchevsky SB, Newman AB, Nicklas BJ, Simonsick EM, Rubin S, Nevitt M, Visser M, Harris T, Pahor M (2004) Inflammatory markers and incident mobility limitation in the elderly. J Am Geriatr Soc 52:1105-1113

53. Karim Y, Turner C, Dalton N, Roplekar R, Sankaralingam A, Ewang M, Fogelman I, Hampson G (2013) The relationship between pro-resorptive inflammatory cytokines and the effect of high dose vitamin D supplementation on their circulating concentrations. Int Immunopharmacol 17:693-697

54. Willis KS, Smith DT, Broughton KS, Larson-Meyer DE (2012) Vitamin D status and biomarkers of inflammation in runners. Open Access J Sports Med 3:35-42

## Click here to view linked References

≛

4		
5		
6		
/ 8		
9		
10		
11 1	Associations of total and free 25OHD and 1,25(OH) <sub>2</sub> D with	Formatted: Numbering: Continuous
$\frac{12}{12}$	serum markers of inflammation in older men	
13 14 3	Priya Srikanth <sup>1</sup> , Rene F. Chun <sup>2</sup> , Martin Hewison <sup>3</sup> , John S. Adams <sup>2</sup> , Roger Bouillon <sup>4</sup> , Dirk Vanderschueren <sup>4</sup> , Nancy	
15 16 4	Lane <sup>5</sup> , Peggy M. Cawthon <sup>6</sup> , Tien Dam <sup>7</sup> , Elizabeth Barrett-Connor <sup>8</sup> , Lori B. Daniels <sup>8,9</sup> , James M. Shikany <sup>10</sup> , Marcia	
16 17 5	L. Stefanick <sup>11</sup> , Jane A. Caulev <sup>12</sup> , Eric S. Orwoll <sup>13</sup> , Carrie M. Nielson <sup>1,13</sup> for the Osteoporotic Fractures in Men	
18	(MrOS) Study Research Group	
19 - 20 7	<sup>1</sup> Department of Public Health and Preventive Medicine. Oregon Health & Science University	
21	<sup>2</sup> Department of Orthonoodia Surgery and Orthonoodia Hespital Passarah Center, David Coffan School of Medicine	
22 ° 23 o	Light A	
24 24	UCLA	
25 10 25 11	<sup>3</sup> Centre for Endocrinology, Diabetes and Metabolism, University of Birmingham, UK	
<sup>26</sup> 12 27	<sup>4</sup> Clinical and Experimental Endocrinology, Department of Clinical and Experimental Medicine, KU Leuven,	
28 13	Belgium, Department of Endocrinology, University Hospital, Leuven	
<sup>29</sup> 14 30	<sup>5</sup> Division of Rheumatology, University of California, Davis	
31 15	<sup>6</sup> California Pacific Medical Center Research Institute, San Francisco, California	
32 33 <sup>16</sup>	<sup>7</sup> Department of Medicine, Division of Geriatric Medicine and Aging, Columbia University	
34 17 25	<sup>8</sup> Division of Epidemiology, Department of Family and Preventive Medicine, University of California, San Diego, La	
35 36 <sup>18</sup>	Jolla, California	
37 <u>1</u> 9	<sup>9</sup> Division of Cardiology, Department of Medicine, University of California, San Diego	
30 39 <sup>20</sup>	<sup>10</sup> University of Alabama at Birmingham, Birmingham Alabama	
40 21	<sup>11</sup> Stanford Prevention Research Center, School of Medicine, Stanford University	
42 <sup>22</sup>	<sup>12</sup> Department of Epidemiology, Graduate School of Public Health, University of Pittsburgh, Pittsburgh,	
43 23	Pennsylvania	
45 <sup>24</sup>	<sup>13</sup> Bone and Mineral Unit, Oregon Health & Science University	
46 47	Abbreviated Title: Associations of total and free 25OHD and 1,25(OH) <sub>2</sub> D with inflammation	
48 26	Key Terms: Inflammation, total 25OHD, free 25OHD, total 1,25(OH) <sub>2</sub> D, free 1,25(OH) <sub>2</sub> D, elderly, men	
49 50	Word count: 3, <u>587338</u> (OI limit: 5,000 words)	
51 28	Number of figures and tables: 3 tables + 3 figures + 2 supplemental tables (OI limit: 6 figures and tables)	
52 53 <sup>29</sup>	Corresponding author and person to whom reprint requests should be addressed:	
54		
55	1	
56		
57		
с 20 20		
60		
61		
62		
63		
64		

1	
2	
3	
4	
5	
6	
7	
8	
q	
10	
1130	Fric S. Orwell MD
1 2 31	Oregon Health & Science University
1232	3181 SW Sam Jackson Park Rd, CR113
1 4 2 4	Portland, OR USA 97239
⊥4:34 1 ⊏ 35	Phone: 503-494-0225 Fax: 503-494-4816
1535	orwoll@ohsu.edu
1 <sup>6</sup> 37	
17 38 18	Disclosure Statement: Roger Bouillon received lecture fees from Amgen, Novartis, Novo Nordisk, Chugai and
19 <sup>39</sup>	Teijin and gave a license to a university patent on Vitamin D analogs to Hybrigenix (France). Eric S. Orwoll
20 40 21	consults for and has received research support from Merck, Lilly and Amgen.
22 <sup>41</sup> 23 <sub>42</sub>	Carrie Nielson, Priya Srikantn, Rene F Chun, Martin Hewison, John S Adams, Dirk Vanderschueren, Nancy E Lane,
24	Peggy Cawthon, Hen Dam, Enzadeth Barrett-Connor, Lori B Dameis, James Snikany, Marcia L Steranick, and Jane
25 <sup>43</sup> 26 44	Cauley declare that they have no contrict of interest.
27	funding. The following institutes provide supports the National Institute on Aging (NLA), the National Institute of
<sup>28</sup> <sup>45</sup> <sup>29</sup> <sub>46</sub>	Arthritis and Musculoskeletal and Skin Diseases (NLAMS), the National Center for Advancing Translational
30	Sciences (NCATS) and NIH Roadman for Medical Research under the following grant numbers: 101 AG027810
32 48	LIQ1 AG042124 LIQ1 AG042139 LIQ1 AG042140 LIQ1 AG042143 LIQ1 AG042145 LIQ1 AG042168 LIQ1
33 <sup>40</sup> 34 49	AR066160 and III 1 TR000128
35 <sub>50</sub>	Funding for this study was supported in part by the following NIH grants: NIAMS R01 AR063910 (PIs Martin
36 <sup>30</sup> 37 <sub>51</sub>	Hewison and John Adams) P60 AR054731 (PI Jane Cauley) and NIAMS K01 AR062655 (PI Carrie Nielson)
38	Supported in part by an independent investigator grant (SPA 12,000) from Marck & Co. Inc.
39 <sup>52</sup> 40 52	Supported in part by an independent investigator grant (SKA-12-009) noin Merck &Co, inc.
41	
42	
43	
44	
45	
46	
47	
48	
49	
50	
51	
52	
53	
54	
55	2
56	-
57	
58	
59	
60	
61	
62	
63	
64	

54	
5	<b>ABSTRACT (Between 150 &amp; 250 words)</b> (Current number of words = 248)
6	Purpose: Vitamin D is hypothesized to suppress inflammation, and circulating 25-hydroxyvitamin D
57	(250HD) and inflammatory markers are inversely correlated. However, total serum 250HD may not be the best
8	indicator of biologically active vitamin D.
9	Methods: We tested serum total 25OHD, total 1,25(OH) <sub>2</sub> D, vitamin D binding protein (DBP), and
60	estimated free 25OHD and free $1,25(OH)_2D$ associations with inflammatory markers serum IL-6, TNF $\alpha$ and their
51	soluble receptors, IL-10 and CRP as continuous outcomes and the presence of $\geq 2$ inflammatory markers in the
52	highest quartile as a dichotomous outcome, in a random subcohort of 679 men in the Osteoporotic Fractures in Men
53	(MrOS) study.
54	Results: IL-6 was lower in men with higher 25OHD (-0.23 $\mu$ g/mL per SD increase in 25OHD, 95% CI: -
55	0.07 to -0.38 $\mu$ g/mL) and with higher 1,25(OH) <sub>2</sub> D (-0.20 $\mu$ g/mL, 95% CI: -0.0004 to -0.39 $\mu$ g/mL); free D
66	associations were slightly stronger. 250HD and DBP, but not 1,25(OH) <sub>2</sub> D, were <i>independently</i> associated with IL-6.
57	$TNF\alpha$ soluble receptors were inversely associated with $1,25(OH)_2D$ but positively associated with 25OHD, and
68	each had independent effects. The strongest association with $\geq 2$ inflammatory markers in the highest quartile was
69	for free 1,25(OH) <sub>2</sub> D (OR: 0.70, 95% CI: 0.54 to 0.89 per SD increase in free 1,25(OH) <sub>2</sub> D).
0	<b>Conclusions:</b> Associations of 1,25(OH) <sub>2</sub> D and free 25OHD with IL-6 mirrored those of 25OHD,
'0 '1	<b>Conclusions:</b> Associations of 1,25(OH) <sub>2</sub> D and free 25OHD with IL-6 mirrored those of 25OHD, suggesting that 1,25(OH) <sub>2</sub> D and free D do not improve upon 25OHD in population-based IL-6 studies. However,
20 21 22	<b>Conclusions:</b> 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 TNF $\alpha$ soluble receptor, warranting examination of both
70 71 72 73	<b>Conclusions:</b> 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 TNF $\alpha$ soluble receptor, warranting examination of both metabolites in studies of TNF $\alpha$ and its antagonists.
70 71 72 73 74	<b>Conclusions:</b> 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 TNF $\alpha$ soluble receptor, warranting examination of both metabolites in studies of TNF $\alpha$ and its antagonists.
20 21 22 23 24	<b>Conclusions:</b> 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 TNF $\alpha$ soluble receptor, warranting examination of both metabolites in studies of TNF $\alpha$ and its antagonists.
20 21 22 23 24	<b>Conclusions:</b> 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 TNF $\alpha$ soluble receptor, warranting examination of both metabolites in studies of TNF $\alpha$ and its antagonists.
20 21 22 23 24	<b>Conclusions:</b> 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 TNF $\alpha$ soluble receptor, warranting examination of both metabolites in studies of TNF $\alpha$ and its antagonists.
21 22 23 24	<b>Conclusions:</b> 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 TNF $\alpha$ soluble receptor, warranting examination of both metabolites in studies of TNF $\alpha$ and its antagonists.
20 21 22 23 24	<b>Conclusions:</b> 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 TNF $\alpha$ soluble receptor, warranting examination of both metabolites in studies of TNF $\alpha$ and its antagonists.
20 21 22 23 24	<b>Conclusions:</b> 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 TNF $\alpha$ soluble receptor, warranting examination of both metabolites in studies of TNF $\alpha$ and its antagonists.
20 21 22 23 24	<b>Conclusions:</b> Associations of 1,25(OH) <sub>2</sub> D and free 25OHD with IL-6 mirrored those of 25OHD, suggesting that 1,25(OH) <sub>2</sub> D 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.
20 21 22 23 24	<b>Conclusions:</b> 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 TNF $\alpha$ soluble receptor, warranting examination of both metabolites in studies of TNF $\alpha$ and its antagonists.
20 21 22 23 24	Conclusions: Associations of 1,25(OH) <sub>2</sub> D and free 25OHD with L-6 mirrored those of 25OHD, suggesting that 1,25(OH) <sub>2</sub> D 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.
20 21 22 23 24	Conclusions: Associations of 1,25(OH) <sub>2</sub> D and free 25OHD with IL-6 mirrored those of 25OHD, suggesting that 1,25(OH) <sub>2</sub> D 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.

$1 \\ 2 \\ 3 \\ 4 \\ 5 \\ 6 \\ 7 \\ 8 \\ 9 \\ 10 \\ 11 \\ 2 \\ 13 \\ 14 \\ 15 \\ 16 \\ 17 \\ 18 \\ 9 \\ 20 \\ 22 \\ 24 \\ 25 \\ 27 \\ 28 \\ 20 \\ 10 \\ 22 \\ 24 \\ 25 \\ 27 \\ 28 \\ 20 \\ 10 \\ 22 \\ 24 \\ 25 \\ 27 \\ 28 \\ 20 \\ 10 \\ 10 \\ 10 \\ 10 \\ 10 \\ 10 \\ 10$	75 76 77 80 81
33 34 35 36	
37 38 39	
40 41 42	
43 44	
45 46 47	
48 49 50	
51 52 53	
54 55	
56 57 59	
59 60	
ьΤ	

Mini Abstract

metabolites diverged for  $\mbox{TNF}\alpha$  soluble receptors.

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. 1,25dihydroxyvitamin D and free 250HD associations mirrored those of 250HD. However, associations for the two

#### 

1		
2		
5 4		
5		
6 7		Formatted: Subscript
8		Formatted: Subscript
9		
10	RACKCROUND	
12		
13 83	Chronic low-grade inflammation is a contributor to age-associated fraility, mortality and morbidity, including	
1484 15	osteoporosis [1]. Inflammatory markers such as interleukin-6 (IL-6) and tumor necrosis factor alpha (TNFa) are	
16 <sup>85</sup>	implicated in the process of vascular calcification and regulation of bone remodeling [2, 3] and have been linked to	
17 86	incident fracture [4] and BMD loss [5].	
18 19 <sup>87</sup>	Vitamin D has direct effects on bone health and may also act on bone by modulating inflammation [6, 7]. We	
20 88	have recently shown that low 1,25(OH)2D and 25OHD are independently associated with hip fracture in older men,	
21 21 89	but only 250HD was independently associated with BMD loss [8]. <u>1,25(OH)<sub>2</sub>D<sub>3</sub> may is also play a possible</u>	
23 <sub>90</sub>	candidate for mediatory function role in regulating both the inflammatory process and hone turnover. Decreased	
24	1.25/(OID) D. Jacob men contribute to individual of here formation and engrance estimated T calls and call	
25 91	1,25(OH)2D3 levels may contribute to inmotion of bone formation and suppress activated 1 cens and cen	
27 92	proliferation, which may accelerate the inflammation process in those with conditions such as ankylosing spondylitis	
28 93	<u>(AS)[9].</u>	
<sup>29</sup> 94 30	In vitro and in vivo evidence suggests that the biologically active form of vitamin D, 1,25(OH) <sub>2</sub> D, has several	
31 95	immunomodulatory functions, including suppression of pro-inflammatory marker expression and regulation of	
~ ~		
32 33 <sup>96</sup>	immune cell activity [10], (ref-Zhang), Treatment of fibroblast cultures with 1,25(OH) <sub>2</sub> D <sub>3</sub> inhibits IL-6 and	Formatted: Highlight
32 33 34 97	immune cell activity [10] <sub>3</sub> (ref Zhang). Treatment of fibroblast cultures with 1,25(OH) <sub>2</sub> D <sub>3</sub> inhibits IL-6 and interleukin-8 (IL-8) [11]. <u>Vitamin D inhibits the activation of the TNFα converting enzyme and subsequent</u>	Formatted: Highlight
32 33 34 97 35 36 98	immune cell activity [10] <sub>3</sub> ( <del>ref Zhang),</del> Treatment of fibroblast cultures with 1,25(OH) <sub>2</sub> D <sub>3</sub> inhibits IL-6 and interleukin-8 (IL-8) [11]. <u>Vitamin D inhibits the activation of the TNFα converting enzyme and subsequent</u> <u>inflammation on multiple levels [10, 12, 13]. Studies of vitamin D and inflammatory diseases, such as rheumatoid</u>	Formatted: Highlight Field Code Changed
32 33 34 97 35 36 98 37 99	immune cell activity [10], (ref Zhang), Treatment of fibroblast cultures with 1,25(OH) <sub>2</sub> D <sub>3</sub> inhibits IL-6 and interleukin-8 (IL-8) [11]. <u>Vitamin D inhibits the activation of the TNFα converting enzyme and subsequent</u> inflammation on multiple levels [10, 12, 13]. <u>Studies of vitamin D and inflammatory diseases</u> , such as rheumatoid arthritis (RA) and systemic lupus erythematosus, suggest a role for vitamin D deficiency in	Formatted: Highlight Field Code Changed
32 96 33 97 34 97 35 98 36 98 37 99 38 39 <sup>100</sup>	<ul> <li>immune cell activity [10], (ref Zhang). Treatment of fibroblast cultures with 1,25(OH)<sub>2</sub>D<sub>3</sub> inhibits IL-6 and</li> <li>interleukin-8 (IL-8) [11]. <u>Vitamin D inhibits the activation of the TNFα converting enzyme and subsequent</u></li> <li>inflammation on multiple levels [10, 12, 13]. <u>Studies of vitamin D and inflammatory diseases</u>, such as rheumatoid</li> <li>arthritis (RA) and systemic lupus erythematosus, suggest a role for vitamin D deficiency in</li> <li>However, little is known about the relationship between inflammation and vitamin D in the general population.</li> </ul>	Formatted: Highlight Field Code Changed
32 96 33 97 34 97 35 98 36 98 37 99 38 39100 40101	<ul> <li>immune cell activity [10]<sub>2</sub>(ref Zhang). Treatment of fibroblast cultures with 1,25(OH)<sub>2</sub>D<sub>3</sub> inhibits IL-6 and</li> <li>interleukin-8 (IL-8) [11]. <u>Vitamin D inhibits the activation of the TNFα converting enzyme and subsequent</u></li> <li><u>inflammation on multiple levels [10, 12, 13]</u>. <u>Studies of vitamin D and inflammatory diseases, such as rheumatoid</u></li> <li><u>arthritis (RA) and systemic lupus erythematosus</u>, <u>suggest a role for vitamin D deficiency in</u></li> <li><u>However</u>, little is known about the relationship between inflammation and vitamin D in the general population.</li> <li>The objective of this study was to examine associations of total and free circulating 25OHD and 1,25(OH)<sub>2</sub>D</li> </ul>	Formatted: Highlight Field Code Changed
32 33 34 37 35 36 37 99 38 39 30 40101 41 42102	<ul> <li>immune cell activity [10], (ref Zhang), Treatment of fibroblast cultures with 1,25(OH)<sub>2</sub>D<sub>3</sub> inhibits IL-6 and</li> <li>interleukin-8 (IL-8) [11]. <u>Vitamin D inhibits the activation of the TNFα converting enzyme and subsequent</u></li> <li>inflammation on multiple levels [10, 12, 13], <u>Studies of vitamin D and inflammatory diseases</u>, such as rheumatoid</li> <li>arthritis (RA) and systemic lupus erythematosus, suggest a role for vitamin D deficiency in</li> <li><u>However</u>, little is known about the relationship between inflammation and vitamin D in the general population.</li> <li>The objective of this study was to examine associations of total and free circulating 25OHD and 1,25(OH)<sub>2</sub>D</li> <li>and vitamin D binding protein (DBP) with inflammatory markers that include CRP, IL-6, TNFα, IL-6 and TNFα</li> </ul>	Formatted: Highlight Field Code Changed
32 96 33 97 35 98 37 99 38 39 <sup>100</sup> 40 <sub>101</sub> 41 42 <sup>102</sup> 43 <sub>103</sub>	<ul> <li>immune cell activity [10]<sub>3</sub>(ref Zhang). Treatment of fibroblast cultures with 1,25(OH)<sub>2</sub>D<sub>3</sub> inhibits IL-6 and</li> <li>interleukin-8 (IL-8) [11]. <u>Vitamin D inhibits the activation of the TNFα converting enzyme and subsequent</u></li> <li>inflammation on multiple levels [10, 12, 13]. <u>Studies of vitamin D and inflammatory diseases</u>, such as rheumatoid</li> <li>arthritis (RA) and systemic lupus erythematosus, suggest a role for vitamin D deficiency in</li> <li>However, little is known about the relationship between inflammation and vitamin D in the general population.</li> <li>The objective of this study was to examine associations of total and free circulating 25OHD and 1,25(OH)<sub>2</sub>D</li> <li>and vitamin D binding protein (DBP) with inflammatory markers that include CRP, IL-6, TNFα, IL-6 and TNFα</li> <li>soluble receptors, and one anti-inflammatory cytokine – interleukin-10 (IL-10) in a cohort of older men.</li> </ul>	Formatted: Highlight Field Code Changed
32 96 33 97 35 98 36 98 39100 40101 41 42102 43103 44 45104	<ul> <li>immune cell activity [10]<sub>3</sub>(ref Zhang), Treatment of fibroblast cultures with 1,25(OH)<sub>2</sub>D<sub>3</sub> inhibits IL-6 and</li> <li>interleukin-8 (IL-8) [11]. <u>Vitamin D inhibits the activation of the TNFα converting enzyme and subsequent</u></li> <li><u>inflammation on multiple levels [10, 12, 13].</u> <u>Studies of vitamin D and inflammatory diseases, such as rheumatoid</u></li> <li>arthritis (RA) and systemic lupus erythematosus, suggest a role for vitamin D deficiency in</li> <li><u>However</u>, little is known about the relationship between inflammation and vitamin D in the general population.</li> <li>The objective of this study was to examine associations of total and free circulating 25OHD and 1,25(OH)<sub>2</sub>D</li> <li>and vitamin D binding protein (DBP) with inflammatory markers that include CRP, IL-6, TNFα, IL-6 and TNFα</li> <li>soluble receptors, and one anti-inflammatory cytokine – interleukin-10 (IL-10) in a cohort of older men.</li> </ul>	Formatted: Highlight Field Code Changed
32 96 33 97 35 98 37 99 38 39 <sup>100</sup> 40 <sup>101</sup> 41 42 <sup>102</sup> 43 <sup>103</sup> 44 45 <sup>104</sup> 46 <sup>105</sup>	<ul> <li>immune cell activity [10]<sub>2</sub>(ref Zhang)<sup>-</sup> Treatment of fibroblast cultures with 1,25(OH)<sub>2</sub>D<sub>3</sub> inhibits IL-6 and</li> <li>interleukin-8 (IL-8) [11]. <u>Vitamin D inhibits the activation of the TNFα converting enzyme and subsequent</u></li> <li>inflammation on multiple levels [10, 12, 13]Studies of vitamin D and inflammatory diseases, such as rheumatoid</li> <li>arthritis (RA) and systemic lupus erythematosus, suggest a role for vitamin D deficiency in</li> <li>However, little is known about the relationship between inflammation and vitamin D in the general population.</li> <li>The objective of this study was to examine associations of total and free circulating 25OHD and 1,25(OH)<sub>2</sub>D</li> <li>and vitamin D binding protein (DBP) with inflammatory markers that include CRP, IL-6, TNFα, IL-6 and TNFα</li> <li>soluble receptors, and one anti-inflammatory cytokine – interleukin-10 (IL-10) in a cohort of older men.</li> </ul>	Formatted: Highlight Field Code Changed
32 96 33 97 35 98 37 99 38 39 <sup>100</sup> 40 <sup>101</sup> 41 42 <sup>102</sup> 43 <sub>103</sub> 44 45 <sup>104</sup> 46 <sub>105</sub> 47 48106	<ul> <li>immune cell activity [10]<sub>3</sub>(ref Zhang). Treatment of fibroblast cultures with 1,25(OH)<sub>2</sub>D<sub>3</sub> inhibits IL-6 and</li> <li>interleukin-8 (IL-8) [11]. <u>Vitamin D inhibits the activation of the TNFα converting enzyme and subsequent</u></li> <li><u>inflammation on multiple levels [10, 12, 13]</u>. <u>Studies of vitamin D and inflammatory diseases, such as rheumatoid</u></li> <li><u>arthritis (RA) and systemic lupus erythematosus</u>, <u>suggest a role for vitamin D deficiency in</u></li> <li><u>However</u>, little is known about the relationship between inflammation and vitamin D in the general population.</li> <li>The objective of this study was to examine associations of total and free circulating 25OHD and 1,25(OH)<sub>2</sub>D</li> <li>and vitamin D binding protein (DBP) with inflammatory markers that include CRP, IL-6, TNFα, IL-6 and TNFα</li> <li>soluble receptors, and one anti-inflammatory cytokine – interleukin-10 (IL-10) in a cohort of older men.</li> </ul>	Formatted: Highlight Field Code Changed
32 96 34 97 35 98 36 98 37 99 38 39100 40101 41 42102 43103 445104 45104 46105 47 48106 49107	<ul> <li>immune cell activity [10], <u>teef Zhang</u>, Treatment of fibroblast cultures with 1,25(OH)<sub>2</sub>D<sub>3</sub> inhibits IL-6 and</li> <li>interleukin-8 (IL-8) [11]. <u>Vitamin D inhibits the activation of the TNFα converting enzyme and subsequent</u></li> <li><u>inflammation on multiple levels [10, 12, 13].</u> <u>Studies of vitamin D and inflammatory diseases, such as rheumatoid</u></li> <li>arthritis (RA) and systemic lupus erythematosus, <u>suggest a role for vitamin D deficiency in</u></li> <li><u>However, little is known about the relationship between inflammation and vitamin D in the general population.</u></li> <li>The objective of this study was to examine associations of total and free circulating 25OHD and 1,25(OH)<sub>2</sub>D</li> <li>and vitamin D binding protein (DBP) with inflammatory markers that include CRP, IL-6, TNFα, IL-6 and TNFα</li> <li>soluble receptors, and one anti-inflammatory cytokine – interleukin-10 (IL-10) in a cohort of older men.</li> </ul>	Formatted: Highlight Field Code Changed
32 96 33 97 35 98 36 98 37 99 38 39100 40101 41 42102 43103 44 45104 46105 47 48106 49 107 50	immune cell activity [10], iref-Zhang). Treatment of fibroblast cultures with 1,25(OH) <sub>2</sub> D <sub>3</sub> inhibits IL-6 and interleukin-8 (IL-8) [11]. <u>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)<sub>2</sub>D 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 between inflammaticine provide the principal of the true for the principal of the principal of the principal of the true for the principal of </u>	Formatted: Highlight Field Code Changed
32 96 33 97 35 98 36 98 37 99 38 39100 40101 41 42102 43103 44 45104 46105 47 48106 49107 50 51108 52.22	immune cell activity [10], gref-Zhang): Treatment of fibroblast cultures with 1,25(OH) <sub>2</sub> D <sub>3</sub> inhibits IL-6 and interleukin-8 (IL-8) [11]. <u>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)<sub>2</sub>D 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</u>	Formatted: Highlight Field Code Changed
32 96 33 97 35 98 37 99 38 37 99 38 39 <sup>100</sup> 40 <sup>101</sup> 41 42 <sup>102</sup> 43 <sup>103</sup> 44 45 <sup>104</sup> 46 <sup>105</sup> 47 48 <sup>106</sup> 49 <sup>107</sup> 50 51 <sup>108</sup> 52 <sup>109</sup> 53 <sup>109</sup>	immune cell activity [10], <u>iref Zhang</u> : Treatment of fibroblast cultures with 1,25(OH) <sub>2</sub> D <sub>3</sub> inhibits IL-6 and interleukin-8 (IL-8) [11]. <u>Vitamin D inhibits the activation of the TNFα converting enzyme and subsequent</u> inflammation on multiple levels [10, 12, 13], <u>Studies of vitamin D and inflammatory diseases, such as rheumatoid</u> arthritis (RA) and systemic lupus erythematosus, <u>suggest a role for vitamin D deficiency in</u> 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) <sub>2</sub> D 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. <b>METHODS Study Design</b> 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,	Formatted: Highlight Field Code Changed
32 96 33 97 35 98 37 99 38 39 40101 41 42102 43103 44 45104 45104 45104 46105 47 48106 49107 50 51108 52 53 54 55	immune cell activity [10], teef Zhangy. Treatment of fibroblast cultures with 1,25(OH) <sub>2</sub> D <sub>3</sub> inhibits IL-6 and interleukin-8 (IL-8) [11]. <u>Vitamin D inhibits the activation of the TNFα converting enzyme and subsequent</u> 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) <sub>2</sub> D 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. <b>METHODS</b> <b>Study Design</b> 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,	Formatted: Highlight Field Code Changed
32 96 33 97 35 98 37 99 38 37 99 38 39 <sup>100</sup> 40 <sup>101</sup> 41 42 <sup>102</sup> 43 <sup>103</sup> 44 45 <sup>104</sup> 45 <sup>104</sup> 46 <sup>105</sup> 47 48 <sup>106</sup> 49 <sup>107</sup> 51 <sup>108</sup> 52 <sup>109</sup> 53 54 55 56	inmune cell activity [10] <u>fref Zhang</u> ; Treatment of fibroblast cultures with 1,25(OH) <sub>2</sub> D <sub>3</sub> inhibits IL-6 and interleukin-8 (IL-8) [11]. <u>Vitamin D inhibits the activation of the TNFα converting enzyme and subsequent</u> inflammation on multiple levels [10, 12, 13]_Studies of vitamin D and inflammatory diseases, such as rheumatoid arthritis (RA) and systemic lupus erythematosus, <u>suggest a role for vitamin D deficiency in</u> 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 250HD and 1,25(OH) <sub>2</sub> D 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. <b>METHODS</b> <b>Daty Design</b> 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,	Formatted: Highlight Field Code Changed
32 96 33 97 35 98 37 99 38 39 40101 41 42102 43103 44 45104 45104 45104 46105 47 48106 49107 51108 52 53 54 55 56 57 57 57 57 57 57 57 57 57 57	immune cell activity [10], gef Zhang). Treatment of fibroblast cultures with 1,25(OH);D;D inhibits IL-6 and interleukin-8 (IL-8) [11]. <u>Vitamin D inhibits the activation of the TNFa converting enzyme and subsequent</u> inflammation on multiple levels [10, 12, 13]. <u>Studies of vitamin D and inflammatory diseases</u> , such as rheumatoid arthritis (RA) and systemic lupus erythematosus, <u>suggest a role for vitamin D deficiency in</u> 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);D and vitamin D binding protein (DBP) with inflammatory markers that include CRP, IL-6, TNFa, IL-6 and TNFa soluble receptors, and one anti-inflammatory cytokine – interleukin-10 (IL-10) in a cohort of older men. <b>METHODS Study Design</b> 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,	Formatted: Highlight Field Code Changed
32 96 33 97 35 98 37 99 38 37 99 38 39100 40101 41 42102 43103 44 45104 45104 46105 51108 52 109 53 54 55 56 57 58 59	immune cell activity [10], eef Zhang). Treatment of fibroblast cultures with 1,25(OH);D3 inhibits IL-6 and interleukin-8 (IL-8) [11]. <u>Vitamin D inhibits the activation of the TNFa converting enzyme and subsequent</u> inflammation on multiple levels [10, 12, 13]Studies of vitamin D and inflammatory diseases, such as rheumatoid arthritis (RA) and systemic lupus erythematosus, suegest 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);D3 and vitamin D binding protein (DBP) with inflammatory markers that include CRP, IL-6, TNFa, IL-6 and TNFa soluble receptors, and one anti-inflammatory cytokine – interleukin-10 (IL-10) in a cohort of older men. <b>METHODS</b> <b>Study Design</b> 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,	Formatted: Highlight Field Code Changed
32 96 33 97 35 98 37 99 36 98 37 99 38 39100 40101 41 42102 43103 44 45104 45104 45104 45104 45104 45104 45105 51108 52 53 55 56 57 58 59 60	innune cell activity [10] <u>tref Zhang</u> ? Treatment of fibroblast cultures with 1,25(OH) <sub>2</sub> D; inhibits IL-6 and interleukin-8 (IL-8) [11]. <u>Vitamin D inhibits the activation of the TNFa converting enzyme and subsequent</u> inflammation on multiple levels [10, 12, 13]_Studies of vitamin D and inflammatory diseases, such as rheumatoid arthritis (RA) and systemic lupus erythematosus, success a role for vitamin D deficiency in the general population. The objective of this study was to examine associations of total and free circulating 25OHD and 1,25(OH) <sub>2</sub> D and vitamin D binding protein (DBP) with inflammatory markers that include CRP, IL-6, TNFa, IL-6 and TNFa soluble receptors, and one anti-inflammatory cytokine – interleukin-10 (IL-10) in a cohort of older men. <b>METHODS Study Design</b> 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,	Formatted: Highlight Field Code Changed
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 (250HD) and active (1,25(OH)<sub>2</sub>D) 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 250HD remained frozen in foil wrapped vials to reduce UV exposure until assay.
Measures for 250HD<sub>2</sub> (derived from ergocalciferol) and 250HD<sub>3</sub> (derived from cholecalciferol) were
performed at the Mayo Clinic using mass spectrometry as previously described [20, 21]. Deuterated stable isotope
(d3-250HD) was added to a 0.2-ml serum sample as internal standard. 250HD<sub>2</sub>, 250HD<sub>3</sub>, 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. 250HD<sub>2</sub> and 250HD<sub>3</sub>. Aliquots
of a single serum pool were included in alternate assay runs. Using the pooled serum, the interassay coefficient of
variation (CV) for 250HD<sub>3</sub> was 4.4%, and the intraassay CV was 4.9%.

Total 1,25(OH)<sub>2</sub>D 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)<sub>2</sub>D<sub>2</sub> and 6 pg/mL for
1,25(OH)<sub>2</sub>D<sub>3</sub>. 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)<sub>2</sub>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, TNF $\alpha$ , tumor necrosis factor alpha soluble receptors (TNF $\alpha$ -sRI, TNF $\alpha$ -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 TNF $\alpha$ , TNF- $\alpha$ sRI 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%. TNF $\alpha$  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- $\alpha$ sRI and sRII were measured with an ELISA (R&D Systems, Minneapolis, MN). The normal range for TNF- $\alpha$ sRI in serum is 479 – 966 pg/mL and for TNF- $\alpha$ sRII in serum is 1003 – 3170 pg/mL. Inter-assay CVs range from 5.42% to 8.59% for TNF- $\alpha$ sRI and 2.87 to 3.54% for TNF- $\alpha$ sRII. 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  $\leq$  3 ug/mL. Inter-assay CVs ranged from 1.52 to 3.68%.

Covariates

65

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<sup>2</sup>). 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<sup>2</sup> 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 ( $\beta$ ) 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, TNF $\alpha$ , TNF $\alpha$ -sRI, TNF $\alpha$ -sRII and IL-6sR). We then dichotomized this index into those having  $\geq$ 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). +Thus, we have added a footnote to our tables with a Bonferroni adjusted p-value of  $\leq 0.001 \ (0.05/35 = 0.001).$ 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<sup>2</sup>. 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  $1,25(OH)_2D$  measures (r = -0.21 to -0.25, p<0.001). TNF $\alpha$  and its soluble receptors were significantly negatively correlated with total and free  $1,25(OH)_2D$  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)2D 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)<sub>2</sub>D and DBP (Table 2). Mean TNFa 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)<sub>2</sub>D and DBP (Table 2). 9

Associations of IL-6 with 25OHD and 1,25(OH)<sub>2</sub>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)<sub>2</sub>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)<sub>2</sub>D. This association was attenuated to after 25OHD and DBP adjustment and was no longer significant.

TNF $\alpha$  soluble receptors I and II were positively associated with 25OHD and inversely associated with 1,25(OH)<sub>2</sub>D (Figure 3). Average TNF $\alpha$  soluble receptors I and II were significantly lower by- 62.05 pg/mL (95% CI: 26.09 to 98.01 pg/mL) for TNF $\alpha$ -sRI and -88.83 pg/mL (95% CI: 30.64 to 147.02 pg/mL for TNF $\alpha$ -sRII) per SD increase in 1,25(OH)<sub>2</sub>D. This association was strengthened with adjustment of 25OHD and DBP (Table 2). TNF $\alpha$  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)<sub>2</sub>D and DBP adjustment (Table 2).

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

#### Inflammatory marker associations with free 25OHD, free 1,25(OH)<sub>2</sub>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)<sub>2</sub>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)<sub>2</sub>D. TNFa soluble receptor I levels were lower by 61.51 pg/mL (95% CI: 26.28 to 96.73 pg/mL lower) and TNFa 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)<sub>2</sub>D. Odds of having  $\geq$ 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)<sub>2</sub>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)<sub>2</sub>D 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 RID assay, though slightly weaker, but not significant for the free D measures from the 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 TNF $\alpha$  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 TNF $\alpha$ , 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 TNF $\alpha$  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)<sub>2</sub>D 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)<sub>2</sub>D 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)<sub>2</sub>D<sub>8</sub> in ankylosing spondylitis patients [9]. (ref Lange): but But CRP levels were much higher in the vitamin D deficient group in those studies, while in MrOS, CRP remained low across the range of 25OHD and 1,25(OH)<sub>2</sub>D (<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

Formatted: Subscript
Formatted: Subscript

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 TNF $\alpha$  receptors are another important marker of inflammation in that they represent potential antagonists of TNF $\alpha$  function. In this study, 25OHD was positively associated and 1,25(OH)<sub>2</sub>D was negatively associated with TNF $\alpha$  SRI and II, suggesting dichotomous functions for the two vitamin D metabolites. We speculate that soluble TNF $\alpha$  receptors may be an important novel target for 25OHD as an anti-inflammatory agent. Specifically, the upregulated TNF $\alpha$  sRI and II may provide a sensitive mechanism by which localized conversion of 25OHD to 1,25(OH)<sub>2</sub>D can abrogate inflammatory TNF $\alpha$  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 1 $\alpha$ -hydroxylase (CYP27B1) is mainly regulated by serum PTH and FGF23 but extra renal production of 1,25(OH)<sub>2</sub>D by CYP247B1 is known to be induced by inflammatory cytokines such as TNF $\alpha$  [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)<sub>2</sub>D 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)<sub>2</sub>D was more strongly associated than 25OHD with TNF $\alpha$  soluble receptors and with having  $\geq$ 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<sub>3</sub>, -25(OH)D and

**Commented** [CN2]: Tried to find a home for this sentence that was alone on the next page.

1.25(OH)<sub>2</sub>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)<sub>2</sub>D<sub>3</sub> on inflammation [12, 13, 25, 48] through inhibition of IL-6 and IL-8 synthesis [11]. The choice of a cComposite inflammatory score is somewhat arbitrary, and .--Tthere 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)<sub>2</sub>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  $1,25(OH)_2D$ . In contrast, we observed consistently divergent associations with TNF $\alpha$  soluble receptors for these metabolites. Considering the importance of TNF $\alpha$  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	Q11 (N(70)
Characteristic	Overall (N= $6/9$ ) Mean +SD Median (IOP) or
Age	74 + 6
Race	71±0
White	616 (90.72)
African American	22 (3.24)
Asian	16 (2.36)
Hispanic	17 (2.50)
Other Vitamin D	8 (1.18)
vitamin D 250HD $(ng/ml)$	25.05 .7.09
Eree 25OHD (nmol/L)	$23.95 \pm 7.98$ 0.03 ± 0.01
$1.25(OH)_2D (pg/ml)$	64.24 +71 72
Free 1,25(OH) <sub>2</sub> D (nmol/L)	$0.0015 \pm 0.0004$
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 $\mathbf{PMI}(ka/m^2)$	175 (25.48)
DMI (Kg/III <sup>-</sup> ) Total Fat Mass (kg) <sup>§</sup>	27 ±4 22 +7
Inflammatory Markers	$LL \pm l$
CRP (ug/mL) <sup>§ a</sup>	1.44 (2.1)
IL-6 (pg/mL) <sup>§ a</sup>	2.37 (1.97)
TNFα (pg/mL) <sup>§ a</sup>	3.96 (2.54)
Soluble Receptors	
TNF $\alpha$ -sRI (pg/mL) <sup>§ a</sup>	1940.60 (593.40)
$1 \text{ NF}\alpha$ -sRII (pg/mL) <sup>8 a</sup>	3521.80 (938.90)
IL-0SK (ng/mi) <sup>s</sup> " Anti-Inflammatory Marker	49.09 (18.25)
$IL-10 (ng/mL)^{\$a}$	8 85 (6 93)
Alcohol (per week)	0.00 (0.95)
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)
raif/Poor/very Poor PASE score <sup>†</sup>	102 (15.04) 147 ±66
NSAIDS use <sup>§</sup>	147 ±00 107 (16 40)
Corticosteroid use <sup>§</sup>	53 (8 17)
Cox-II inhibitor use	51 (7.86)
CVD <sup><u>b</u>#</sup>	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 $aCEP (m1/min/(1.72m^2))^{\$}$	77 . 10
$extrk (m/mm/(1.73m^2))$	//±19

61 62

1	
2	
3	
4	
5	
6	
7	
, 8	
0	
9	
1 1220	§ 5 mining to 1 for more 26 mining 15 it 00 mining CDD 02 mining H ( 01 mining TNT) (6 mining
⊥_330 1_331	* 5 missing total fat mass, 56 missing lipids, 80 missing CRP, 85 missing IL-6, 81 missing 1NF0, 66 missing, TNF0-sRI 72 missing TNF0-sRII 66 missing IL-6sR 71 missing IL-10, 36 missing eGFR 36 missing serum
12031	creatinine, 30 missing NSAIDS use, 30 missing corticosteroid use, 30 missing Cox-II inhibitor
13 <sub>333</sub>	be defined as self-report of previous heart attack, congestive heart failure or angina
14	
15	
16	
17	
18	
19	
20	
21	
22	
22	
2.5	
24	
25	
26	
27	
28	
29	
30	
31	
32	
33	
34	
35	
36	
37	
38	
39	
40	
но И1	
4 D	
т <u>⊿</u> //2	
45	
44 45	
45	
40	
4/	
48	
49	
50	
51	
52	
53	
54	
55	15
56	
57	
58	
59	
55	
61	
60 0 T	
02	
63	
64	

1	5
1	6
	_

	Ν	25OHD	1,25(OH) <sub>2</sub> D	250HD	1,25(OH) <sub>2</sub> D	DBP
		(SD=7.98 ng/ml)	(SD=71.72 pg/ml)	250HD, 1,	25(OH) <sub>2</sub> D, and DBP in the same	model
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)** <u>*</u>	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)
40 /						
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 <sup>§</sup> (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)*

<sup>e</sup>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.

<sup>§</sup>Among CRP, IL-6, TNF $\alpha$ , TNF $\alpha$ -sRI, TNF $\alpha$ -sRI, IL-6sR. Effect measure = odds ratios (95%CI). \*\*p≤0.01, \*p≤0.05, \*\*\*p≤0.001 (Bonferroni-corrected alpha)

1	5
1	б

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

	Ν	Free 25OHD	Free 1,25(OH)2D	DBP
		(SD=0.01 nmol/L)	(SD=0.0004 nmol/L)	(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)
$CRP (\mu g/mL)$	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 <sup>§</sup>	571	0.85 (0.61, 1.19)	0.70 (0.54, 0.89)**	1.26 (1.03, 1.55)*
(N=571)				

<sup>c</sup>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.

36<sup>342</sup> 36<sup>343</sup> 37<sup>344</sup> 38 <sup>8</sup>Among CRP, IL-6, TNFα, TNFα-sRI, TNFα-sRI, IL-6sR. Effect measure = odds ratios (95%CI). \*\*p≤0.01, \*p≤0.05, \*\*\*p≤0.001 (Bonferroni-corrected alpha)

14 15	
16	
17	
18	
19	
20	
20 21	
21 22	
22 23	
25 24215	Figure 1. MrOS analytic complection randomly selected from the full MrOS ashort (N=5004)
25346	Figure 1. MIOS analytic sample size, randomly selected from the full MIOS conort $(1-3994)$
26347	
20	
<sup>2</sup> ′ 348 28240	
20349	
30	II_6 N=557
31	IL-65R, N=571 679 participants in
32	TNF $\alpha$ , N=556 the vitamin D and fracture random
33	TNF $\alpha$ -sRI, N=571 cohort who had UV-
34	$1 \text{ NF}\alpha$ -sRII, N=565 protected serum for
35	Random sample of CRP, N=557 Vitamin D bioassays.
36	980 participants with $\geq 2$ inflammatory
37	5 or more stored markers in highest
38	serum vials for quartile, N=5/1
39	
40	
41	
42	
43	$\sim$ $\times$ $/$
44	
45	
46	
47	
48	
49	
50	
51 50	
52 52	
こう E 4	
54 EE	
55	
50	
50	19
59	10
59 60	
61	
62	
63	
64	
65	
	14 15 16 17 18 19 22 22 23 24 23 22 22 22 22 22 22 22 22 22





Figure 2. The IL-6 association with standardized 250HD was nearly identical to the association with 1,25(OH)<sub>2</sub>D. Data points are predicted values<sup>b</sup>.

<sup>b</sup> <u>Data points and l</u>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 TNF $\alpha$  soluble receptors I (top panels) and II (lower panels) associations with standardized 25OHD and 1,25(OH)<sub>2</sub>D, were in *opposite directions*. Data points are predicted values<sup>b</sup>.



17 18 19 20 21 22 23 24363 Supplemental Tables <u>25</u>364 26 365 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 Supplemental Table 1: Spearman correlations among Vitamin D measures and inflammatory markers Total 250HD Free Total Free VDBP VDBP 250HD 1,25(OH) 1,25(OH) (polyclon (mono-<u>2D</u> <u>2</u>D clonal al ELISA) ELISA Total 250HD 0.17\*\*\* 0.19\*\*\* Free 250HD 0.87\*\*\* -0.27\*\*\* -0.59\*\*\* Total 1,25(OH)2D 0.35\*\*\* 0.32\*\*\* 0.09 0.09\* Free 1,25(OH)<sub>2</sub>D 0.28\*\*\* 0.43\*\*\* 0.91\*\*\* -0.29\*\*\* -0.62\*\*\* IL-6 -0.21\*\*\* -0.21\*\*\* -0.25\*\*\* -0.24\*\*\* 0.03 -0.01 TNFα -0.03 -0.04 -0.14\*\*\* -0.15\*\*\* 0.01 0.08 -0.34\*\*\* TNFα-sRI -0.01 -0.01 -0.35\*\*\* 0.03 0.04 TNFα-sRI -0.02 -0.02 -0.31\*\*\* -0.29\*\*\* -0.004 0.03 IL-6sR 0.01 -0.01 -0.05 -0.06 0.05 0.03 CRP -0.04 -0.12\* -0.08 -0.16\*\*\* 0.23\*\*\* 0.10\* IL-10 0.09 0.06 0.002 -0.007 0.02 0.13\*\*\* VDBP **VDBP** TNFa TNFa **VDBP** IL-6 (RIA) <del>sRI</del> (polyclonal (mono-ELISA) <del>clonal</del> ELISA) Total 250HD 0.17\*\*\* 0.21\*\*\* 0.19\*\*\* 0.23\*\*\* 0.03 0.01 Free 250HD 0 27\*\*\* 0 59\*\*\* 0.21\*\*\* 0.21\*\*\* 0.04 0.01

14 15 16

0.25\*\*\*

0.24\*\*\*

0.45\*\*\*

0.24\*\*\*

0.43\*\*\*

0.40\*\*\*

0.13\*\*\*

0.00\*

<u>VDBP</u>

(RIA)

0.23\*\*\*

-0.21\*\*\*

0.17\*\*\*

-0.20\*\*\*

-0.06

0.04

-0.07

-0.09\*

0.12\*\*\*

0.16\*\*\*

TNFa

sRII

0.02

0.02

0.31\*\*\*

<del>-0.29\*\*\*</del>

0.26\*\*\*

0.40\*\*\*

0.42\*\*\*

0.84\*\*\*

0.15\*\*\*

0.24\*\*\*

0.05

0.35\*\*\*

-0.34\*\*\*

0.29\*\*\*

0.43\*\*\*

0.38\*\*\*

0.84\*\*\*

0.15\*\*\*

0.22\*\*\*

<u>IL-6</u>

0.24\*\*\*

0.43\*\*\*

0.40\*\*\*

0.13\*\*\*

0.45\*\*\*

IL 6sR

0.01

0.01

-0.05

-0.06

0.05

0.13\*\*\*

0.11\*\*\*

0.15\*\*\*

0.15\*\*\*

0.03

0.09\*

<u>TNFa</u>

0.38\*\*\*

0.42\*\*\*

0.11\*\*\*

0.18\*\*\*

0.38\*\*\*

Total

250HD

0.87\*\*\*

0.35\*\*\*

0.28\*\*\*

0.04

0.09

<u>TNFα-</u>

0.84\*\*\*

0.15\*\*\*

0.29\*\*\*

0.22\*\*\*

Free

250HD

0.32\*\*\*

0.43\*\*\*

0.12\*

0.06

sRI

TNFα-sRII

0.15\*\*\*

0.26\*\*\*

0.24\*\*\*

Total

1.25(OH)2D

0.91\*\*\*

0.002

0.08

IL-6sR

0.05

0.03

Free

1,25(OH)2

Ð

0.16\*\*\*

0.007

21

0.14\*\*\*

-0.15\*\*\*

0.18\*\*\*

0.24\*\*\*

0.38\*\*\*

0.42\*\*\*

0.11\*\*\*

0.38\*\*\*

0.17\*\*\*

-0.20\*\*\*

0.16\*\*\*

0.06

0.04

0.07

0.09\*

0.12\*\*

0.05

0.09\*

0.10\*

0.01

0.08

0.04

0.03

0.03

0 13\*\*

-0.62\*\*\*

0.09

0.03

0.01

0.03

0.004

0.05

0.02

<del>-0.29\*\*\*</del>

0.23\*\*\*

Total 1,25(OH)2D

Free 1,25(OH)<sub>2</sub>D

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

CRP

IL-6

 $TNF\alpha$ 

IL 6sR

II. 10

TNFa sRI

TNFa sRII

- 58 59
- 60

47

- 61
- 62
- 63
- 64
- 65

	Free 24	OHD	Free 1.25	(OH) <sub>2</sub> D	D	BP
	(Using DBP from	(Using DBP from	(Using DBP from	(Using DBP from	(Using DBP from	(Using DBP from
	monoclonal ELISA)	RIA)	monoclonal ELISA)	RIA)	monoclonal ELISA)	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)
TNF $\alpha$ -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)
TNF $\alpha$ -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)
(Pg/mL) 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)***
$\geq 2$ inflammatory						
markers in highest quartile <sup>§</sup>						
(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)
			22			
			22			
			22			
			22			

4 5 6 7 8 9 10 11 References Franceschi C (2007) Inflammaging as a major characteristic of old people: can it be prevented or 1. 12 cured? Nutr Rev 65:S173-176 13 Collin-Osdoby P, Rothe L, Anderson F, Nelson M, Maloney W, Osdoby P (2001) Receptor 2. 14 activator of NF-kappa B and osteoprotegerin expression by human microvascular endothelial cells, 15 regulation by inflammatory cytokines, and role in human osteoclastogenesis. J Biol Chem 276:20659-16 20672 17 Doherty TM, Asotra K, Fitzpatrick LA, Qiao JH, Wilkin DJ, Detrano RC, Dunstan CR, Shah PK, 3. 18 Rajavashisth TB (2003) Calcification in atherosclerosis: bone biology and chronic inflammation at the 19 arterial crossroads. Proc Natl Acad Sci U S A 100:11201-11206 Cauley JA, Danielson ME, Boudreau RM, Forrest KY, Zmuda JM, Pahor M, Tylavsky FA, Cummings 20 4. SR, Harris TB, Newman AB (2007) Inflammatory markers and incident fracture risk in older men and 21 women: the Health Aging and Body Composition Study. J Bone Miner Res 22:1088-1095 22 Ding C, Parameswaran V, Udayan R, Burgess J, Jones G (2008) Circulating levels of inflammatory 5. 23 markers predict change in bone mineral density and resorption in older adults: a longitudinal study. J 24 Clin Endocrinol Metab 93:1952-1958 25 Arnson Y, Itzhaky D, Mosseri M, Barak V, Tzur B, Agmon-Levin N, Amital H (2013) Vitamin D 6. 26 inflammatory cytokines and coronary events: a comprehensive review. Clin Rev Allergy Immunol 45:236-27 247 Inanir A, Ozoran K, Tutkak H, Mermerci B (2004) The effects of calcitriol therapy on serum 28 7. interleukin-1, interleukin-6 and tumour necrosis factor-alpha concentrations in post-menopausal 29 patients with osteoporosis. J Int Med Res 32:570-582 30 8 Swanson CM, Srikanth P, Lee CG, Cummings SR, Jans I, Cauley JA, Bouillon R, Vanderschueren D, 31 Orwoll ES, Nielson CM (2015) Associations of 25-Hydroxyvitamin D and 1,25-Dihydroxyvitamin D With 32 Bone Mineral Density, Bone Mineral Density Change, and Incident Nonvertebral Fracture. J Bone Miner 33 Res 30:1403-1413 34 Lange U, Jung O, Teichmann J, Neeck G (2001) Relationship between disease activity and serum 9 35 levels of vitamin D metabolites and parathyroid hormone in ankylosing spondylitis. Osteoporos Int 36 12:1031-1035 37 10. Zhang Y, Leung DY, Richers BN, Liu Y, Remigio LK, Riches DW, Goleva E (2012) Vitamin D inhibits monocyte/macrophage proinflammatory cytokine production by targeting MAPK phosphatase-1. J 38 Immunol 188:2127-2135 39 Rostkowska-Nadolska B, Sliupkas-Dyrda E, Potyka J, Kusmierz D, Fraczek M, Krecicki T, Kubik P, 11. 40 Zatonski M, Latocha M (2010) Vitamin D derivatives: calcitriol and tacalcitol inhibits interleukin-6 and 41 interleukin-8 expression in human nasal polyp fibroblast cultures. Adv Med Sci 55:86-92 42 Querfeld U (2013) Vitamin D and inflammation. Pediatr Nephrol 28:605-610 12. 43 13. Ding C, Wilding JP, Bing C (2013) 1,25-dihydroxyvitamin D3 protects against macrophage-44 induced activation of NFkappaB and MAPK signalling and chemokine release in human adipocytes. PLoS 45 One 8:e61707 Amital H, Szekanecz Z, Szucs G, et al. (2010) Serum concentrations of 25-OH vitamin D in 46 14. patients with systemic lupus erythematosus (SLE) are inversely related to disease activity: is it time to 47 routinely supplement patients with SLE with vitamin D? Ann Rheum Dis 69:1155-1157 48 Rossini M, Maddali Bongi S, La Montagna G, Minisola G, Malavolta N, Bernini L, Cacace E, 15. 49 Sinigaglia L, Di Munno O, Adami S (2010) Vitamin D deficiency in rheumatoid arthritis: prevalence, 50 determinants and associations with disease activity and disability. Arthritis Res Ther 12:R216 51 Merlino LA, Curtis J, Mikuls TR, Cerhan JR, Criswell LA, Saag KG (2004) Vitamin D intake is 52 inversely associated with rheumatoid arthritis: results from the Iowa Women's Health Study. Arthritis 53 Rheum 50:72-77 54 55 23 56 57 58 59 60 61 62

#### 63 64 65

3 4 5 6 7 8 9 10 11 Munger KL, Zhang SM, O'Reilly E, Hernan MA, Olek MJ, Willett WC, Ascherio A (2004) Vitamin D 17. 12 intake and incidence of multiple sclerosis. Neurology 62:60-65 Blank JB, Cawthon PM, Carrion-Petersen ML, Harper L, Johnson JP, Mitson E, Delay RR (2005) 18. 13 Overview of recruitment for the osteoporotic fractures in men study (MrOS). Contemp Clin Trials 14 26:557-568 15 19. Orwoll E, Blank JB, Barrett-Connor E, et al. (2005) Design and baseline characteristics of the 16 osteoporotic fractures in men (MrOS) study--a large observational study of the determinants of fracture 17 in older men. Contemp Clin Trials 26:569-585 18 Orwoll E, Nielson CM, Marshall LM, Lambert L, Holton KF, Hoffman AR, Barrett-Connor E, 20. 19 Shikany JM, Dam T, Cauley JA (2009) Vitamin D deficiency in older men. J Clin Endocrinol Metab 20 94:1214-1222 Singh RJ, Taylor RL, Reddy GS, Grebe SK (2006) C-3 epimers can account for a significant 21 21. proportion of total circulating 25-hydroxyvitamin D in infants, complicating accurate measurement and 22 interpretation of vitamin D status. J Clin Endocrinol Metab 91:3055-3061 23 Vanderschueren D, Pye SR, O'Neill TW, et al. (2013) Active vitamin D (1,25-dihydroxyvitamin D) 22. 24 and bone health in middle-aged and elderly men: the European Male Aging Study (EMAS). The Journal of 25 clinical endocrinology and metabolism 98:995-1005 26 Vanderschueren D, Pye SR, O'Neill TW, et al. (2013) Active vitamin D (1,25-dihydroxyvitamin D) 23. 27 and bone health in middle-aged and elderly men: the European Male Aging Study (EMAS). J Clin 28 Endocrinol Metab 98:995-1005 29 24. Bouillon R, van Baelen H, de Moor P (1977) The measurement of the vitamin D-binding protein in human serum. J Clin Endocrinol Metab 45:225-231 30 Chun RF, Peercy BE, Adams JS, Hewison M (2012) Vitamin D binding protein and monocyte 25. 31 response to 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D: analysis by mathematical modeling. 32 PLoS One 7:e30773 33 Jones SA, Horiuchi S, Topley N, Yamamoto N, Fuller GM (2001) The soluble interleukin 6 26. 34 receptor: mechanisms of production and implications in disease. FASEB J 15:43-58 35 27. Washburn RA, Smith KW, Jette AM, Janney CA (1993) The Physical Activity Scale for the Elderly 36 (PASE): development and evaluation. J Clin Epidemiol 46:153-162 37 28. Pahor M, Chrischilles EA, Guralnik JM, Brown SL, Wallace RB, Carbonin P (1994) Drug data 38 coding and analysis in epidemiologic studies. Eur J Epidemiol 10:405-411 39 29 Levey AS, Coresh J, Greene T, Marsh J, Stevens LA, Kusek JW, Van Lente F (2007) Expressing the Modification of Diet in Renal Disease Study equation for estimating glomerular filtration rate with 40 standardized serum creatinine values. Clin Chem 53:766-772 41 30. Lumley T, Diehr P, Emerson S, Chen L (2002) The importance of the normality assumption in 42 large public health data sets. Annu Rev Public Health 23:151-169 43 31. Laird E, McNulty H, Ward M, et al. (2014) Vitamin d deficiency is associated with inflammation in 44 older irish adults. J Clin Endocrinol Metab 99:1807-1815 45 Wobke TK, Sorg BL, Steinhilber D (2014) Vitamin D in inflammatory diseases. Front Physiol 5:244 32. 46 Nonn L, Peng L, Feldman D, Peehl DM (2006) Inhibition of p38 by vitamin D reduces interleukin-33. 47 6 production in normal prostate cells via mitogen-activated protein kinase phosphatase 5: implications for prostate cancer prevention by vitamin D. Cancer Res 66:4516-4524 48 Shea MK, Booth SL, Massaro JM, et al. (2008) Vitamin K and vitamin D status: associations with 34. 49 inflammatory markers in the Framingham Offspring Study. Am J Epidemiol 167:313-320 50 Bratke K, Wendt A, Garbe K, Kuepper M, Julius P, Lommatzsch M, Virchow JC (2014) Vitamin D 35. 51 binding protein and vitamin D in human allergen-induced endobronchial inflammation. Clin Exp 52 Immunol 177:366-372 53 54 55 24 56 57 58 59 60 61 62 63 64

65

1 2 3 4 5 6 7 8 9 10 11 Toss G, Sorbo B (1986) Serum concentrations of 25-hydroxyvitamin D and vitamin D-binding 36. 12 protein in elderly people. Effects of institutionalization, protein-energy malnutrition and inflammation. Acta Med Scand 220:273-277 13 Zehnder D, Bland R, Chana RS, Wheeler DC, Howie AJ, Williams MC, Stewart PM, Hewison M 37. 14 (2002) Synthesis of 1,25-dihydroxyvitamin D(3) by human endothelial cells is regulated by inflammatory 15 cytokines: a novel autocrine determinant of vascular cell adhesion. J Am Soc Nephrol 13:621-629 16 38. Edfeldt K, Liu PT, Chun R, et al. (2010) T-cell cytokines differentially control human monocyte 17 antimicrobial responses by regulating vitamin D metabolism. Proc Natl Acad Sci U S A 107:22593-22598 18 39. Hewison M, Burke F, Evans KN, Lammas DA, Sansom DM, Liu P, Modlin RL, Adams JS (2007) 19 Extra-renal 25-hydroxyvitamin D3-1alpha-hydroxylase in human health and disease. J Steroid Biochem 20 Mol Biol 103:316-321 Hummel DM, Fetahu IS, Groschel C, Manhardt T, Kallay E (2014) Role of proinflammatory 21 40. cytokines on expression of vitamin D metabolism and target genes in colon cancer cells. J Steroid 22 Biochem Mol Biol 144PA:91-95 23 Welsh P, Peters MJ, McInnes IB, et al. (2011) Vitamin D deficiency is common in patients with RA 41. 24 and linked to disease activity, but circulating levels are unaffected by TNFalpha blockade: results from a 25 prospective cohort study. Ann Rheum Dis 70:1165-1167 26 Waldron JL, Ashby HL, Cornes MP, Bechervaise J, Razavi C, Thomas OL, Chugh S, Deshpande S, 42. 27 Ford C, Gama R (2013) Vitamin D: a negative acute phase reactant. J Clin Pathol 66:620-622 28 Reid D, Toole BJ, Knox S, Talwar D, Harten J, O'Reilly DS, Blackwell S, Kinsella J, McMillan DC, 43. 29 Wallace AM (2011) The relation between acute changes in the systemic inflammatory response and plasma 25-hydroxyvitamin D concentrations after elective knee arthroplasty. Am J Clin Nutr 93:1006-30 1011 31 Hopkins MH, Owen J, Ahearn T, Fedirko V, Flanders WD, Jones DP, Bostick RM (2011) Effects of 44. 32 supplemental vitamin D and calcium on biomarkers of inflammation in colorectal adenoma patients: a 33 randomized, controlled clinical trial. Cancer Prev Res (Phila) 4:1645-1654 34 Peake JM, Kukulian S, Nowson CA, Sanders K, Daly RM (2011) Inflammatory cytokine responses 45. 35 to progressive resistance training and supplementation with fortified milk in men aged 50+ years: an 18-36 month randomized controlled trial. Eur J Appl Physiol 111:3079-3088 37 Pittas AG, Harris SS, Stark PC, Dawson-Hughes B (2007) The effects of calcium and vitamin D 46. 38 supplementation on blood glucose and markers of inflammation in nondiabetic adults. Diabetes Care 39 30:980-986 Shab-Bidar S, Neyestani TR, Djazayery A, Eshraghian MR, Houshiarrad A, Kalayi A, Shariatzadeh 47. 40 N, Khalaji N, Gharavi A (2012) Improvement of vitamin D status resulted in amelioration of biomarkers 41 of systemic inflammation in the subjects with type 2 diabetes. Diabetes Metab Res Rev 28:424-430 42 48. Verstuyf A, Carmeliet G, Bouillon R, Mathieu C (2010) Vitamin D: a pleiotropic hormone. Kidney 43 Int 78:140-145 44 Hopkins MH, Flanders WD, Bostick RM (2012) Associations of circulating inflammatory 49. 45 biomarkers with risk factors for colorectal cancer in colorectal adenoma patients. Biomark Insights 46 7:143-150 47 50. Barbour KE, Boudreau R, Danielson ME, et al. (2012) Inflammatory markers and the risk of hip fracture: the Women's Health Initiative. J Bone Miner Res 27:1167-1176 48 Barbour KE, Lui LY, Ensrud KE, Hillier TA, LeBlanc ES, Ing SW, Hochberg MC, Cauley JA (2014) 51. 49 Inflammatory markers and risk of hip fracture in older white women: the study of osteoporotic 50 fractures. J Bone Miner Res 29:2057-2064 51 52. Penninx BW, Kritchevsky SB, Newman AB, Nicklas BJ, Simonsick EM, Rubin S, Nevitt M, Visser M, 52 Harris T, Pahor M (2004) Inflammatory markers and incident mobility limitation in the elderly. J Am 53 Geriatr Soc 52:1105-1113 54 55 25 56 57 58 59 60

62

63 64

61

Karim Y, Turner C, Dalton N, Roplekar R, Sankaralingam A, Ewang M, Fogelman I, Hampson G
(2013) The relationship between pro-resorptive inflammatory cytokines and the effect of high dose
vitamin D supplementation on their circulating concentrations. Int Immunopharmacol 17:693-697
Willis KS, Smith DT, Broughton KS, Larson-Meyer DE (2012) Vitamin D status and biomarkers of
inflammation in runners. Open Access J Sports Med 3:35-42

Authorship and Disclosure Form OSTEOPOROSIS INTERNATIONAL

# D Springer

Authorship & Disclosure Form

MANUSCRIPT ID NUMBER (IF KNOWN)	
Author's signature PRIMA SRIKANTH 2/25/15 Printed name and date	Author's signature Printed name and date
Author's signature	Author's signature
Printed name and date	Printed name and date
Author's signature Printed name and date	Author's signature Printed name and date
Author's signature	Author's signature
Printed name and date	Printed name and date
Author's signature	Author's signature
Printed name and date	Printed name and date
Author's signature	Author's signature
Printed name and date	Printed name and date

Page 2 of 2- (signatures & dates required on page 2)



ASSOCIATIONS OF TOTAL AND FREE 25CH8 AND 1, 25(CH)2) Article Title (first few words) First Author: FRIMA SRIKANTH E-mail: NIELSOCA @OHSU. EBU

After submission of this agreement signed by all authors, changes of authorship or in the order of the authors listed will not be accepted by Springer.

### **AUTHORSHIP**

I, the undersigned author(s), certify that:

- I have seen and approved the final version of the manuscript, and all subsequent versions.
- I have made substantial contributions to conception and design, or acquisition of data, or analysis and interpretation of data;
- I have drafted the article or revised it critically for important intellectual content.

I accept public responsibility for it, and believe it represents valid work. As an author of this article, I also certify that none of the material in the manuscript has been previously published, nor is it included in any other manuscript. I certify that this manuscript is not under consideration for publication elsewhere, nor has it been submitted or accepted in another publication in any form. The rights or interest in the manuscript have not been assigned to any third party.

Moreover, should the editor of Osteoporosis International request the data upon which the manuscript is based, I shall produce it. I also certify that I have read and complied with the copyright information, as found on the Osteoporosis International home page website.

#### FINANCIAL DISCLOSURE/CONFLICT OF INTEREST

I certify that any financial interests such as employment, stock ownership, honoraria, paid expert testimony, as well as any personal relationships, academic competition, and intellectual passion which may inappropriately influence my actions, have been disclosed on a separate attachment.

All funding sources supporting the work and all institutional or corporate affiliations of mine are acknowledged in a footnote.

I have had full access to all the data in the study (if applicable) and thereby accept full responsibility for the integrity of the data and the accuracy of the data analysis.

By checking the box next to my signature I assert that there are no conflicts of interest (both personal and institutional) regarding specific financial interests that are relevant to the work conducted or reported in this manuscript.

#### **PLEASE NOTE**

1. Every author must sign the Authorship & Disclosure form.

2. It is possible to submit more than one form if the authors are in several locations.

3. All forms must be submitted at the same time.

4. Completed forms must be scanned and included as a pdf file during the online submission process as a supplemental file not for review.

#### Please email any gueries to the appropriate Managing Editor:

European Office: Fina Liu - oi.europe@iofbonehealth.org USA Office: Adrianne Tewksbury - tewksburya@helenhayeshosp.org

Page 1 of 2- (signatures & dates required on page 2)

<b>OSTEOPOROSIS INTERNATIONAL</b>	
Authorship & Disclosure Form	



René	F. Chem
Author's signature	

Author's signature

René F. Chun 2/20/2015

Printed name and date

Printed name and date



Associations of total and free 250HD and 1,25(0H)2D with serum markers of inflammation in older men

#### Article Title (first few words)

First Author:Priya SrikanthE-mail:srikanth@ohsu.edu

After submission of this agreement signed by all authors, changes of authorship or in the order of the authors listed will not be accepted by Springer.

#### AUTHORSHIP

I, the undersigned author(s), certify that:

- I have seen and approved the final version of the manuscript, and all subsequent versions.
- I have made substantial contributions to conception and design, or acquisition of data, or analysis and interpretation of data;
- I have drafted the article or revised it critically for important intellectual content.

I accept public responsibility for it, and believe it represents valid work. As an author of this article, I also certify that none of the material in the manuscript has been previously published, nor is it included in any other manuscript. I certify that this manuscript is not under consideration for publication elsewhere, nor has it been submitted or accepted in another publication in any form. The rights or interest in the manuscript have not been assigned to any third party.

Moreover, should the editor of Osteoporosis International request the data upon which the manuscript is based, I shall produce it. I also certify that I have read and complied with the copyright information, as found on the Osteoporosis International home page website.

### FINANCIAL DISCLOSURE/CONFLICT OF INTEREST

I certify that any financial interests such as employment, stock ownership, honoraria, paid expert testimony, as well as any personal relationships, academic competition, and intellectual passion which may inappropriately influence my actions, have been disclosed on a separate attachment.

All funding sources supporting the work and all institutional or corporate affiliations of mine are acknowledged in a footnote.

I have had full access to all the data in the study (if applicable) and thereby accept full responsibility for the integrity of the data and the accuracy of the data analysis.

By checking the box next to my signature I assert that there are no conflicts of interest (both personal and institutional) regarding specific financial interests that are relevant to the work conducted or reported in this manuscript.

### PLEASE NOTE

1. Every author must sign the Authorship & Disclosure form.

It is possible to submit more than one form if the authors are in several locations.
 All forms must be submitted at the same time.

4. Completed forms must be <u>scanned</u> and included as a pdf file during the <u>online</u> submission process as a supplemental file not for review.

#### Please email any queries to the appropriate Managing Editor:

European Office: Fina Liu - oi.europe@iofbonehealth.org

USA Office: Adrianne Tewksbury - tewksburya@helenhayeshosp.org



Unknown

MANUSCRIPT ID NUMBER (IF KNOWN)

× Parm 2/23/15	́	A
Author's signature /		Author's signature
John J. Adams		
Printed name and date		Printed name and date
Author's signature		Author's signature
Printed name and date		Printed name and date
[]		
Author's signature	L	Author's signature
Printed name and date		Printed name and date
Author's signature		Author's signature
Printed name and date		Printed name and date
Author's signature	L	Author's signature
		Drivted years and data
Printed name and date		rinteo name ano date
Author's signature		Author's signature

Printed name and date

Printed name and date



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

#### Article Title (first few words)

First Author: Priya Srikanth E-mail: srikanth@ohsu.edu

After submission of this agreement signed by all authors, changes of authorship or in the order of the authors listed will not be accepted by Springer.

#### AUTHORSHIP

I, the undersigned author(s), certify that

- I have seen and approved the final version of the manuscript, and all subsequent versions.
- I have made substantial contributions to conception and design, or acquisition of data, or analysis and interpretation of data;
- I have drafted the article or revised it critically for important intellectual content.

I accept public responsibility for it, and believe it represents valid work. As an author of this article, I also certify that none of the material in the manuscript has been previously published, nor is it included in any other manuscript. I certify that this manuscript is not under consideration for publication elsewhere, nor has it been submitted or accepted in another publication in any form. The rights or interest in the manuscript have not been assigned to any third party.

Moreover, should the editor of Osteoporosis International request the data upon which the manuscript is based, I shall produce it. I also certify that I have read and complied with the copyright information, as found on the Osteoporosis International home page website.

#### FINANCIAL DISCLOSURE/CONFLICT OF INTEREST

I certify that any financial interests such as employment, stock ownership, honoraria, paid expert testimony, as well as any personal relationships, academic competition, and intellectual passion which may inappropriately influence my actions, have been disclosed on a separate attachment.

All funding sources supporting the work and all institutional or corporate affiliations of mine are acknowledged in a footnote.

I have had full access to all the data in the study (if applicable) and thereby accept full responsibility for the integrity of the data and the accuracy of the data analysis.

By checking the box next to my signature I assert that there are no conflicts of interest (both personal and institutional) regarding specific financial interests that are relevant to the work conducted or reported in this manuscript.

#### PLEASE NOTE

- 1. Every author must sign the Authorship & Disclosure form.
- 2. It is possible to submit more than one form if the authors are in several locations.
- 3. All forms must be submitted at the same time.

 Completed forms must be <u>scanned</u> and included as a pdf file during the <u>online</u> submission process as a supplemental file not for review.

Please email any queries to the appropriate Managing Editor:

European Office: Fina Liu – <u>ci.europe@iofbonehealth.org</u> USA Office: Adrianne Tewksbury – <u>tewksburya@helenhayeshosp.org</u>

Page 1 of 2- (signatures & dates required on page 2)

ateb boe awan bater	eteb bne emen betning	
	entengie enormale	
wortheringin all solution		
stab bus ernan befruh	ateb bine aman batrih <sup>4</sup>	
enthor's signature	widengie e'rodhiA	
stab bre aman bench	atab bisa aman batritrif	
erutsogie e'sottuk	andangis anddala	
ateb bris omen bohih?	etab brie erran betriff	
	empeofie a monte	
enutergie enotatue		
	ateb brie emen batran	
stab bre erren betring	a string	States and the
enterdis stodaut	Author's algrature	
in and i		
109 34 10 . Jul eteb brie emain beholing	eteb bne aman betning	
Wannos wast	องการสนเป็นร. 6. เกมเวลาห	
Author's signatura		
(many)		
TTT)		
NAMBER OF BUILDING OF THE PARTY		
nwondaU		
sous and & Disclos	mioł 9	
Authorship & Disel	TVNOLLWN	19guinger
and 212040403T20		4

Authorship and Disclosure Form OSTEOPOROSIS INTERNATIONAL Authorship & Disclosure Form





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

### Article Title (first few words)

First Author: Priya Srikanth E-mail: srikanth@ohsu.edu

After submission of this agreement signed by all authors, changes of authorship or in the order of the authors listed will not be accepted by Springer.

#### AUTHORSHIP

I, the undersigned author(s), certify that:

- I have seen and approved the final version of the manuscript, and all subsequent versions.
- I have made substantial contributions to conception and design, or acquisition of data, or analysis and interpretation of data;
- I have drafted the article or revised it critically for important intellectual content.

I accept public responsibility for it, and believe it represents valid work. As an author of this article, I also certify that none of the material in the manuscript has been previously published, nor is it included in any other manuscript. I certify that this manuscript is not under consideration for publication elsewhere, nor has it been submitted or accepted in another publication in any form. The rights or interest in the manuscript have not been assigned to any third party.

Moreover, should the editor of Osteoporosis International request the data upon which the manuscript is based, I shall produce it. I also certify that I have read and complied with the copyright information, as found on the Osteoporosis International home page website.

### FINANCIAL DISCLOSURE/CONFLICT OF INTEREST

I certify that any financial interests such as employment, stock ownership, honoraria, paid expert testimony, as well as any personal relationships, academic competition, and intellectual passion which may inappropriately influence my actions, have been disclosed on a separate attachment.

All funding sources supporting the work and all institutional or corporate affiliations of mine are acknowledged in a footnote.

I have had full access to all the data in the study (if applicable) and thereby accept full responsibility for the integrity of the data and the accuracy of the data analysis.

By checking the box next to my signature I assert that there are no conflicts of interest (both personal and institutional) regarding specific financial interests that are relevant to the work conducted or reported in this manuscript.

### PLEASE NOTE

- 1. Every author must sign the Authorship & Disclosure form.
- 2. It is possible to submit more than one form if the authors are in several locations.
- 3. All forms must be submitted at the same time.

4. Completed forms must be <u>scanned</u> and included as a pdf file during the <u>online</u> submission process as a supplemental file not for review.

#### Please email any queries to the appropriate Managing Editor:

European Office: Fina Liu – <u>oi.europe@iofbonehealth.org</u>

USA Office: Adrianne Tewksbury - tewksburya@helenhayeshosp.org



Unknown

MANUSCRIPT ID NUMBER (IF KNOWN)	
Author's signature Author's signature Ames M. Sittik Ang 2/23/15 Printed name and date	Author's signature Printed name and date
Author's signature	Author's signature
Printed name and date	Printed name and date
Author's signature Printed name and date	Author's signature Printed name and date
Author's signature	Author's signature
Printed name and date	Printed name and date
Author's signature	Author's signature
Printed name and date	Printed name and date
Author's signature	Author's signature
Printed name and date	Printed name and date



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

### Article Title (first few words)

First Author:Priya SrikanthE-mail:srikanth@ohsu.edu

After submission of this agreement signed by all authors, changes of authorship or in the order of the authors listed will not be accepted by Springer.

#### **AUTHORSHIP**

I, the undersigned author(s), certify that:

- I have seen and approved the final version of the manuscript, and all subsequent versions.
- I have made substantial contributions to conception and design, or acquisition of data, or analysis and interpretation of data;
- I have drafted the article or revised it critically for important intellectual content.

I accept public responsibility for it, and believe it represents valid work. As an author of this article, I also certify that none of the material in the manuscript has been previously published, nor is it included in any other manuscript. I certify that this manuscript is not under consideration for publication elsewhere, nor has it been submitted or accepted in another publication in any form. The rights or interest in the manuscript have not been assigned to any third party.

Moreover, should the editor of Osteoporosis International request the data upon which the manuscript is based, I shall produce it. I also certify that I have read and complied with the copyright information, as found on the Osteoporosis International home page website.

#### FINANCIAL DISCLOSURE/CONFLICT OF INTEREST

I certify that any financial interests such as employment, stock ownership, honoraria, paid expert testimony, as well as any personal relationships, academic competition, and intellectual passion which may inappropriately influence my actions, have been disclosed on a separate attachment.

All funding sources supporting the work and all institutional or corporate affiliations of mine are acknowledged in a footnote.

I have had full access to all the data in the study (if applicable) and thereby accept full responsibility for the integrity of the data and the accuracy of the data analysis.

By checking the box next to my signature I assert that there are no conflicts of interest (both personal and institutional) regarding specific financial interests that are relevant to the work conducted or reported in this manuscript.

### PLEASE NOTE

1. Every author must sign the Authorship & Disclosure form.

2. It is possible to submit more than one form if the authors are in several locations.

3. All forms must be submitted at the same time.

4. Completed forms must be <u>scanned</u> and included as a pdf file during the <u>online</u> <u>submission process</u> as a supplemental file not for review.

### Please email any queries to the appropriate Managing Editor:

European Office: Fina Liu - oi.europe@iofbonehealth.org

USA Office: Adrianne Tewksbury – <u>tewksburya@helenhayeshosp.org</u>





Unknown

MANUSCRIPT ID NUMBER (IF KNOWN)

Author's signature	Author's signature
Jone A. Couley 2235 Printed name and date	Printed name and date
Author's signature	Author's signature
Printed name and date	Printed name and date
Author's signature	Author's signature
Printed name and date	Printed name and date
Author's signature	Author's signature
Printed name and date	Printed name and date
Author's signature	Author's signature
Printed name and date	Printed name and date
Author's signature	Author's signature
Printed name and date	Printed name and date



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

#### Article Title (first few words)

First Author:Priya SrikanthE-mail:srikanth@ohsu.edu

After submission of this agreement signed by all authors, changes of authorship or in the order of the authors listed will not be accepted by Springer.

#### AUTHORSHIP

I, the undersigned author(s), certify that:

- I have seen and approved the final version of the manuscript, and all subsequent versions.
- I have made substantial contributions to conception and design, or acquisition of data, or analysis and interpretation of data;
- I have drafted the article or revised it critically for important intellectual content.

I accept public responsibility for it, and believe it represents valid work. As an author of this article, I also certify that none of the material in the manuscript has been previously published, nor is it included in any other manuscript. I certify that this manuscript is not under consideration for publication elsewhere, nor has it been submitted or accepted in another publication in any form. The rights or interest in the manuscript have not been assigned to any third party.

Moreover, should the editor of *Osteoporosis International* request the data upon which the manuscript is based, I shall produce it. I also certify that I have read and complied with the copyright information, as found on the *Osteoporosis International* home page website.

#### FINANCIAL DISCLOSURE/CONFLICT OF INTEREST

I certify that any financial interests such as employment, stock ownership, honoraria, paid expert testimony, as well as any personal relationships, academic competition, and intellectual passion which may inappropriately influence my actions, have been disclosed on a separate attachment.

All funding sources supporting the work and all institutional or corporate affiliations of mine are acknowledged in a footnote.

I have had full access to all the data in the study (if applicable) and thereby accept full responsibility for the integrity of the data and the accuracy of the data analysis.

By checking the box next to my signature I assert that there are no conflicts of interest (both personal and institutional) regarding specific financial interests that are relevant to the work conducted or reported in this manuscript.

#### PLEASE NOTE

1. <u>Every author must sign the Authorship & Disclosure form.</u>

2. It is possible to submit more than one form if the authors are in several locations.

3. All forms must be submitted at the same time.

4. Completed forms must be <u>scanned</u> and included as a pdf file during the <u>online</u> submission process as a supplemental file not for review.

#### Please email any queries to the appropriate Managing Editor:

European Office: Fina Liu – <u>oi.europe@iofbonehealth.org</u> USA Office: Adrianne Tewksbury – <u>tewksburya@helenhayeshosp.org</u>



MANUSCRIPT ID NUMBER (IF KNOWN)	
Author's signature <u>Carrie Nielson Sept 11, 201</u> Printed name and date	Author's signature
Author's signature	Author's signature
Printed name and date	Printed name and date
Author's signature	Author's signature
Printed name and date Author's signature	Printed name and date           Author's signature
Printed name and date	Printed name and date
Author's signature	Author's signature
Printed name and date	Printed name and date
Author's signature	Author's signature
Printed name and date	Printed name and date

Authorship and Disclosure Form OSTEOPOROSIS INTERNATIONAL Authorship & Disclosure Form



Unknown

MANUSCRIPT ID NUMBER (IF KNOWN)

Dichan Author's signature <u>Tien bau 9(22/15</u> Printed name and date	Author's signature Printed name and date
Author's signature	Author's signature
Author's signature	Author's signature
Printed name and date	Printed name and date
Author's signature Printed name and date	Author's signature Printed name and date
Author's signature	Author's signature
Printed name and date	Printed name and date
Author's signature	Author's signature
Printed name and date	Printed name and date


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

#### Article Title (first few words)

First Author: Priya Srikanth E-mail: srikanth@ohsu.edu

After submission of this agreement signed by all authors, changes of authorship or in the order of the authors listed will not be accepted by Springer.

#### AUTHORSHIP

I, the undersigned author(s), certify that:

- I have seen and approved the final version of the manuscript, and all subsequent versions.
- I have made substantial contributions to conception and design, or acquisition of data, or analysis and interpretation of data;
- I have drafted the article or revised it critically for important intellectual content.

I accept public responsibility for it, and believe it represents valid work. As an author of this article, I also certify that none of the material in the manuscript has been previously published, nor is it included in any other manuscript. I certify that this manuscript is not under consideration for publication elsewhere, nor has it been submitted or accepted in another publication in any form. The rights or interest in the manuscript have not been assigned to any third party.

Moreover, should the editor of *Osteoporosis International* request the data upon which the manuscript is based, I shall produce it. I also certify that I have read and complied with the copyright information, as found on the *Osteoporosis International* home page website.

#### FINANCIAL DISCLOSURE/CONFLICT OF INTEREST

I certify that any financial interests such as employment, stock ownership, honoraria, paid expert testimony, as well as any personal relationships, academic competition, and intellectual passion which may inappropriately influence my actions, have been disclosed on a separate attachment.

All funding sources supporting the work and all institutional or corporate affiliations of mine are acknowledged in a footnote.

I have had full access to all the data in the study (if applicable) and thereby accept full responsibility for the integrity of the data and the accuracy of the data analysis.

By checking the box next to my signature I assert that there are no conflicts of interest (both personal and institutional) regarding specific financial interests that are relevant to the work conducted or reported in this manuscript.

## PLEASE NOTE

- 1. Every author must sign the Authorship & Disclosure form.
- 2. It is possible to submit more than one form if the authors are in several locations.
- 3. All forms must be submitted at the same time.
- 4. Completed forms must be <u>scanned</u> and included as a pdf file during the <u>online</u> submission process as a supplemental file not for review.

Please email any queries to the appropriate Managing Editor:

European Office: Fina Liu – oi.europe@iofbonehealth.org

USA Office: Adrianne Tewksbury - tewksburya@helenhayeshosp.org



Unknown		
MANUSCRIPT ID NUMBER (IF KNOWN)		
,		
Author's signature P. VANDERSCHUEREN 15-9-2015	Author's signature	
Printed name and date	Printed name and date	
Author's signature	Author's signature	
Printed name and date	Printed name and date	
Author's signature	Author's signature	
Printed name and date	Printed name and date	_
Author's signature	Author's signature	•
Printed name and date	Printed name and date	_
Author's signature	Author's signature	
Printed name and date	Printed name and date	_
Author's signature	Author's signature	5a
Printed name and date	Printed name and date	<u> </u>



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

### Article Title (first few words)

First Author:Priya SrikanthE-mail:srikanth@ohsu.edu

After submission of this agreement signed by all authors, changes of authorship or in the order of the authors listed will not be accepted by Springer.

#### **AUTHORSHIP**

I, the undersigned author(s), certify that:

- I have seen and approved the final version of the manuscript, and all subsequent versions.
- I have made substantial contributions to conception and design, or acquisition of data, or analysis and interpretation of data;
- I have drafted the article or revised it critically for important intellectual content.

I accept public responsibility for it, and believe it represents valid work. As an author of this article, I also certify that none of the material in the manuscript has been previously published, nor is it included in any other manuscript. I certify that this manuscript is not under consideration for publication elsewhere, nor has it been submitted or accepted in another publication in any form. The rights or interest in the manuscript have not been assigned to any third party.

Moreover, should the editor of Osteoporosis International request the data upon which the manuscript is based, I shall produce it. I also certify that I have read and complied with the copyright information, as found on the Osteoporosis International home page website.

### FINANCIAL DISCLOSURE/CONFLICT OF INTEREST

I certify that any financial interests such as employment, stock ownership, honoraria, paid expert testimony, as well as any personal relationships, academic competition, and intellectual passion which may inappropriately influence my actions, have been disclosed on a separate attachment.

All funding sources supporting the work and all institutional or corporate affiliations of mine are acknowledged in a footnote.

I have had full access to all the data in the study (if applicable) and thereby accept full responsibility for the integrity of the data and the accuracy of the data analysis.

By checking the box next to my signature I assert that there are no conflicts of interest (both personal and institutional) regarding specific financial interests that are relevant to the work conducted or reported in this manuscript.

# PLEASE NOTE

1. Every author must sign the Authorship & Disclosure form.

2. It is possible to submit more than one form if the authors are in several locations.

3. All forms must be submitted at the same time.

4. Completed forms must be <u>scanned</u> and included as a pdf file during the <u>online</u> <u>submission process</u> as a supplemental file not for review.

### Please email any queries to the appropriate Managing Editor:

European Office: Fina Liu – <u>oi.europe@iofbonehealth.org</u>

USA Office: Adrianne Tewksbury – tewksburya@helenhayeshosp.org





Unknown

MANUSCRIPT ID NUMBER (IF KNOWN)	
Author's signature EVIC OrWoll Printed name and date	Author's signature Printed name and date
Author's signature	Author's signature
Printed name and date	Printed name and date
Author's signature Printed name and date	Author's signature Printed name and date
Author's signature	Author's signature
Printed name and date	Printed name and date
Author's signature	Author's signature
Printed name and date	Printed name and date
Author's signature	Author's signature
Printed name and date	Printed name and date

**Authorship & Disclosure Form** 





Associations of total and free 250HD and 1,25(0H)2D with serum markers of inflammation in older men

#### Article Title (first few words)

First Author: Priya Srikanth E-mail: srikanth@ohsu.edu

After submission of this agreement signed by all authors, changes of authorship or in the order of the authors listed will not be accepted by Springer.

#### AUTHORSHIP

I, the undersigned author(s), certify that:

- I have seen and approved the final version of the manuscript, and all subsequent versions.
- I have made substantial contributions to conception and design, or acquisition of data, or analysis and interpretation of data;
- I have drafted the article or revised it critically for important intellectual content.

I accept public responsibility for it, and believe it represents valid work. As an author of this article, I also certify that none of the material in the manuscript has been previously published, nor is it included in any other manuscript. I certify that this manuscript is not under consideration for publication elsewhere, nor has it been submitted or accepted in another publication in any form. The rights or interest in the manuscript have not been assigned to any third party.

Moreover, should the editor of *Osteoporosis International* request the data upon which the manuscript is based, I shall produce it. I also certify that I have read and complied with the copyright information, as found on the *Osteoporosis International* home page website.

#### FINANCIAL DISCLOSURE/CONFLICT OF INTEREST

I certify that any financial interests such as employment, stock ownership, honoraria, paid expert testimony, as well as any personal relationships, academic competition, and intellectual passion which may inappropriately influence my actions, have been disclosed on a separate attachment.

All funding sources supporting the work and all institutional or corporate affiliations of mine are acknowledged in a footnote.

I have had full access to all the data in the study (if applicable) and thereby accept full responsibility for the integrity of the data and the accuracy of the data analysis.

By checking the box next to my signature I assert that there are no conflicts of interest (both personal and institutional) regarding specific financial interests that are relevant to the work conducted or reported in this manuscript.

### PLEASE NOTE

1. Every author must sign the Authorship & Disclosure form.

2. It is possible to submit more than one form if the authors are in several locations.

3. All forms must be submitted at the same time.

4. Completed forms must be <u>scanned</u> and included as a pdf file during the <u>online</u> <u>submission process</u> as a supplemental file not for review.

#### Please email any queries to the appropriate Managing Editor:

European Office: Fina Liu – <u>oi.europe@iofbonehealth.org</u> USA Office: Adrianne Tewksbury – tewksburya@helenhayeshosp.org





Unknown

MANUSCRIPT ID NUMBER (IF KNOWN)

Author's signature Nan certe Carp	□	Author's signature
Printed name and date 9/14/15		Printed name and date
Author's signature	L	Author's signature
Printed name and date		Printed name and date
• • • •	<b>[</b> ]	
Author's signature	L	Author's signature
Printed name and date		Printed name and date
Aumor's signature		Author's signature
Printed name and date		Printed name and date
Author's signature		Author's signature
Printed name and date		Printed name and date
Author's signature	□	Author's signature
Printed name and date		Printed name and date



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

### Article Title (first few words)

First Author: Priya Srikanth E-mail: srikanth@ohsu.edu

After submission of this agreement signed by all authors, changes of authorship or in the order of the authors listed will not be accepted by Springer.

#### AUTHORSHIP

I, the undersigned author(s), certify that:

- I have seen and approved the final version of the manuscript, and all subsequent versions.
- I have made substantial contributions to conception and design, or acquisition of data, or analysis and interpretation of data;
- I have drafted the article or revised it critically for important intellectual content.

I accept public responsibility for it, and believe it represents valid work. As an author of this article, I also certify that none of the material in the manuscript has been previously published, nor is it included in any other manuscript. I certify that this manuscript is not under consideration for publication elsewhere, nor has it been submitted or accepted in another publication in any form. The rights or interest in the manuscript have not been assigned to any third party.

Moreover, should the editor of Osteoporosis International request the data upon which the manuscript is based, I shall produce it. I also certify that I have read and complied with the copyright information, as found on the Osteoporosis International home page website.

#### FINANCIAL DISCLOSURE/CONFLICT OF INTEREST

I certify that any financial interests such as employment, stock ownership, honoraria, paid expert testimony, as well as any personal relationships, academic competition, and intellectual passion which may inappropriately influence my actions, have been disclosed on a separate attachment.

All funding sources supporting the work and all institutional or corporate affiliations of mine are acknowledged in a footnote.

I have had full access to all the data in the study (if applicable) and thereby accept full responsibility for the integrity of the data and the accuracy of the data analysis.

By checking the box next to my signature I assert that there are no conflicts of interest (both personal and institutional) regarding specific financial interests that are relevant to the work conducted or reported in this manuscript.

## PLEASE NOTE

- 1. Every author must sign the Authorship & Disclosure form.
- 2. It is possible to submit more than one form if the authors are in several locations.

3. All forms must be submitted at the same time.

4. Completed forms must be <u>scanned</u> and included as a pdf file during the <u>online</u> <u>submission process</u> as a supplemental file not for review.

Please email any queries to the appropriate Managing Editor:

European Office: Fina Liu – <u>oi.europe@iofbonehealth.org</u>

USA Office: Adrianne Tewksbury - tewksburya@helenhayeshosp.org



# Unknown

MANUSCRIPT ID NUMBER (IF KNOWN)

Author's signature Author's signature People, Courthon 9/16/15 Printed name and date	Author's signature Printed name and date
Author's signature	Author's signature
Author's signature	Printed name and date
Printed name and date	Printed name and date Author's signature
Printed name and date	Printed name and date Author's signature
Printed name and date	Printed name and date
Author's signature	Author's signature
Printed name and date	Printed name and date



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

#### Article Title (first few words)

First Author: Priya Srikanth E-mail: srikanth@ohsu.edu

After submission of this agreement signed by all authors, changes of authorship or in the order of the authors listed will not be accepted by Springer.

#### AUTHORSHIP

I, the undersigned author(s), certify that:

- I have seen and approved the final version of the manuscript, and all subsequent versions.
- I have made substantial contributions to conception and design, or acquisition of data, or analysis and interpretation of data;
- I have drafted the article or revised it critically for important intellectual content.

I accept public responsibility for it, and believe it represents valid work. As an author of this article, I also certify that none of the material in the manuscript has been previously published, nor is it included in any other manuscript. I certify that this manuscript is not under consideration for publication elsewhere, nor has it been submitted or accepted in another publication in any form. The rights or interest in the manuscript have not been assigned to any third party.

Moreover, should the editor of Osteoporosis International request the data upon which the manuscript is based, I shall produce it. I also certify that I have read and complied with the copyright information, as found on the Osteoporosis International home page website.

### FINANCIAL DISCLOSURE/CONFLICT OF INTEREST

I certify that any financial interests such as employment, stock ownership, honoraria, paid expert testimony, as well as any personal relationships, academic competition, and intellectual passion which may inappropriately influence my actions, have been disclosed on a separate attachment.

All funding sources supporting the work and all institutional or corporate affiliations of mine are acknowledged in a footnote.

I have had full access to all the data in the study (if applicable) and thereby accept full responsibility for the integrity of the data and the accuracy of the data analysis.

By checking the box next to my signature I assert that there are no conflicts of interest (both personal and institutional) regarding specific financial interests that are relevant to the work conducted or reported in this manuscript.

## PLEASE NOTE

1. Every author must sign the Authorship & Disclosure form.

2. It is possible to submit more than one form if the authors are in several locations.

3. All forms must be submitted at the same time.

4. Completed forms must be <u>scanned</u> and included as a pdf file during the <u>online submission</u> <u>process</u> as a supplemental file not for review.

#### Please email any queries to the appropriate Managing Editor:

European Office: Fina Liu - oi.europe@iofbonehealth.org

USA Office: Adrianne Tewksbury - tewksburya@helenhayeshosp.org



Unknown	37 - 37 17
MANUSCRIPT ID NUMBER (IF KNOWN)	
Author's signature	Author's signature
Marcia Stefanick 9/22/14	53 1000 Au
Printed name and date	Printed name and date
Author's signature	Author's signature
Printed name and date	Printed name and date
Author's signature	Author's signature
Printed name and date	Printed name and date
Author's signature	Author's signature
Printed name and date	Printed name and date
Author's signature	Author's signature
Printed name and date	Printed name and date
Author's signature	Author's signature
Printed name and date	Printed name and date

OSTEOPOROSIS INTER Authorship & Disclosu	NATIONAL 🖉 Springer re Form
Unknown	
MANUSCRIPT ID NUMBER (IF KNOWN)	
Author's signature	Author's signature
Printed name and date	Printed name and date
× Att.	
DR. MARTIN HEWISON 9/15	Author's signature
Printed name and date	Printed name and date
Author's signature	Author's signature
Printed name and date	Printed name and date
Author's signature	Author's signature
Printed name and date	Printed name and date
Author's signature	Author's signature
Printed name and date	Printed name and date
Author's signature	Author's signature
Printed name and date	Printed name and date

,



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

## Article Title (first few words)

First Author:Priya SrikanthE-mail:srikanth@ohsu.edu

After submission of this agreement signed by all authors, changes of authorship or in the order of the authors listed will not be accepted by Springer.

#### AUTHORSHIP

I, the undersigned author(s), certify that:

- I have seen and approved the final version of the manuscript, and all subsequent versions.
- I have made substantial contributions to conception and design, or acquisition of data, or analysis and interpretation of data;
- I have drafted the article or revised it critically for important intellectual content.

I accept public responsibility for it, and believe it represents valid work. As an author of this article, I also certify that none of the material in the manuscript has been previously published, nor is it included in any other manuscript. I certify that this manuscript is not under consideration for publication elsewhere, nor has it been submitted or accepted in another publication in any form. The rights or interest in the manuscript have not been assigned to any third party.

Moreover, should the editor of *Osteoporosis International* request the data upon which the manuscript is based, I shall produce it. I also certify that I have read and complied with the copyright information, as found on the *Osteoporosis International* home page website.

#### FINANCIAL DISCLOSURE/CONFLICT OF INTEREST

I certify that any financial interests such as employment, stock ownership, honoraria, paid expert testimony, as well as any personal relationships, academic competition, and intellectual passion which may inappropriately influence my actions, have been disclosed on a separate attachment.

All funding sources supporting the work and all institutional or corporate affiliations of mine are acknowledged in a footnote.

I have had full access to all the data in the study (if applicable) and thereby accept full responsibility for the integrity of the data and the accuracy of the data analysis.

By checking the box next to my signature I assert that there are no conflicts of interest (both personal and institutional) regarding specific financial interests that are relevant to the work conducted or reported in this manuscript.

### PLEASE NOTE

1. Every author must sign the Authorship & Disclosure form.

2. It is possible to submit more than one form if the authors are in several locations.

3. All forms must be submitted at the same time.

4. Completed forms must be <u>scanned</u> and included as a pdf file during the <u>online</u> submission process as a supplemental file not for review.

#### Please email any queries to the appropriate Managing Editor:

European Office: Fina Liu – <u>oi.europe@iofbonehealth.org</u> USA Office: Adrianne Tewksbury – tewksburya@helenhayeshosp.org





Unknown		
MANUSCRIPT ID NUMBER (IF KNOWN)		
$\boxtimes$	□	
Author's signature	Author's signature	
Lori B. Daniels 9/14/2015		
Printed name and date	Printed name and date	-
Author's signature	Author's signature	
Printed name and date	Printed name and date	-
—	_	
Author's signature	Author's signature	
Printed name and date	Printed name and date	-
Author's signature	Author's signature	
Printed name and date	Printed name and date	-
Author's signature	Author's signature	
Printed name and date	Printed name and date	-
Author's signature	Author's signature	
Printed name and date	Printed name and date	-