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Assay Variation Confounds the Diagnosis of Hypovitaminosis D: A Call for Standardization

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Endemic hypovitaminosis D contributes to osteoporosis development. However, variation in 25-hydroxyvitamin D (25OHD) measurement is reported and confounds the diagnosis of vitamin D insufficiency/deficiency. This report emphasizes the marked variability observed in serum 25OHD measurements between laboratories.

Initially, postmenopausal women had serum 25OHD determinations: 42 in laboratory A, 20 in laboratory B. Their mean (SEM) serum 25OHD concentrations were 46 (2.1) and 21 (2.3) ng/ml in laboratories A and B, respectively. Furthermore, there was little overlap in serum 25OHD among these clinically similar individuals. Specifically, 17% of those measured in laboratory A but 90% in laboratory B were below an arbitrary threshold value of 32 ng/ml.

Subsequently, serum was obtained from 10 healthy adults. Two aliquots from each individual, one of which was spiked with 20 ng/ml 25OHD, were sent to six laboratories. Substantial variability was noted between these six laboratories. The mean serum 25OHD concentration ranged from 17.1–35.6 ng/ml. Similarly, the mean increase produced by spiking with 20 ng/ml ranged from 7.7–18.0 ng/ml.

In conclusion, 25OHD assays yield markedly differing results; whether an individual is found to have low or normal vitamin D status is a function of the laboratory used. If the medical community is to make progress in correcting widespread hypovitaminosis D, 25OHD measurement must be standardized. (*J Clin Endocrinol Metab* 89: 3152–3157, 2004)

HYPOVITAMINOSIS D is common due to low dietary vitamin D intake coupled with sun avoidance or minimal exposure (1–3). As a result, vitamin D inadequacy is a relatively recently recognized, but common, cause of secondary hyperparathyroidism with consequent bone loss and osteoporosis (4, 5). Furthermore, vitamin D insufficiency is associated with muscle weakness, and its correction reduces the risk for falls (6). Consequently, simply correcting this vitamin inadequacy should lead to improved skeletal health. Consistent with this, provision of supplemental vitamin D (7) or vitamin D plus calcium (8, 9) reduces fracture risk. Moreover, evidence is accumulating that vitamin D inadequacy may have other adverse health consequences, including increased risk of malignancies, hypertension, and diabetes mellitus (10, 11). Not surprisingly, much work has focused on defining the optimal circulating concentration of 25-hydroxyvitamin D (25OHD) and determination of the required intake to achieve this concentration. In this regard, expert opinion suggests that this serum concentration is approximately 80 nmol/liter (32 ng/ml) (12, 13). Thus, it is often recommended that clinicians strive to maintain 25OHD concentrations above 32 ng/ml in their patients to maximize bone health. Although previously noted (14–17), it is perhaps less widely appreciated that the assays used to measure 25OHD may yield discrepant results. This study highlights variability in 25OHD measurements obtained using current assays in different laboratories.

Abbreviation: 25OHD, 25-Hydroxyvitamin D.

Subjects and Methods

Initial observation

Fifty-nine postmenopausal women were screened for potential participation in two osteoporosis clinical trials. A serum specimen from each woman was collected for 25OHD measurement; 42 samples were sent to laboratory A, and 20 were sent to laboratory B for analysis. Because three volunteers did not meet bone density criteria for the first study, they were subsequently screened for the second study.

Subsequent evaluation in healthy adults

Venous blood was obtained on April 8, 2003, from 10 healthy adult research staff and clinicians living in Madison, Wisconsin (~43° north latitude). Serum aliquots were prepared, and 20 ng/ml 25OHD (Amersham Searle, Arlington Heights, IL) was added to half of the aliquots (spiked specimen) from each volunteer. All aliquots were promptly frozen and maintained at −80 C until shipped for analysis. Spiked and unspiked aliquots from all individuals were sent to two clinical reference laboratories (C and D), two national central research laboratories (F and G), and two academic research laboratories (E and H) for 25OHD measurement. All laboratories were blinded to sample status, *i.e.* basal or spiked. Additionally, a local clinical laboratory measured serum calcium using a Cobas Integra 800 Autoanalyzer (Roche, Indianapolis, IN) and intact PTH with an immunochemiluminometric assay.

These studies were reviewed and approved by the University of Wisconsin Health Sciences Human Subjects Committee. Informed consent was obtained from all participants before any study-related procedure.

Vitamin D assay methodology

Table 1 lists the methods and normal range for 25OHD measurement in these laboratories.

TABLE 1. 25OHD assay methodology and normal range

Laboratory	Methodology	Normal range
A	Acetonitrile extraction followed by in-house RIA	10–55 ng/ml
B	DiaSorin RIA	10–40 ng/ml
C	Acetonitrile extraction followed by DiaSorin RIA	8–38 ng/ml
D	Chemiluminescent assay	20–57 ng/ml
E	Acetonitrile extraction followed by DiaSorin RIA	NA
F	Chemiluminescent assay	6–54 ng/ml
G	Chemiluminescent assay	10–68 ng/ml
H	Ethyl acetate extraction followed by normal phase HPLC	NA

NA, Not applicable.

TABLE 2. Patient demographics for specimens sent to laboratories A and B

Laboratory	N	Age (yr)	T-score	25OHD (ng/ml)
A	42	64.0 (1.5)	−2.6 (0.1)	46.0 (2.0) [20–71]
B	20	67.2 (1.3)	−2.7 (0.1)	21.0 (2.3) [10–48]

Data represent mean (SEM). Range is given in brackets.

Statistical analysis

For the purpose of analysis, HPLC measurement of 25OHD (laboratory H) was established as the gold standard to which the results from other laboratories/assays were compared. Serum 25OHD measured in laboratories C–H was compared in basal and spiked specimens using factorial ANOVA with subsequent Fisher's protected least significant difference testing using Statview software (Abacus Concepts, Cary, NC). Additionally, results of laboratories C–G were compared with laboratory H using Bland-Altman plots and Passing/Bablok regression analysis using Analyse-it software (Analyse-it Software Ltd., Leeds, UK).

Results

Demographics

Initial observation group. All of the subjects in both groups were Caucasian, postmenopausal women. Overall, their mean age was 65.0 ± 1.1 (SEM) yr (range, 49–86 yr). Osteoporosis was present in all of these women based on bone mineral density measurement at the lumbar spine and/or proximal femur using dual-energy x-ray absorptiometry. Their mean T score (lowest of the L1–L4 spine, total femur, femoral neck, or trochanter) was −2.7 ± 0.1. No differences were present in age, weight, or bone density between groups (Table 2).

Healthy laboratory personnel. All 10 individuals were Caucasian, and their mean age was 46.2 ± 3.7 yr (range, 25–57 yr). Serum calcium was normal in all (mean, 9.2 ± 0.1 mg/dl), but the intact PTH was elevated in two. Their mean serum PTH concentration was 37 ± 6.5 pg/ml and ranged from 13–73 pg/ml. The normal range in this laboratory is 7–53 pg/ml.

25OHD concentration/postmenopausal women

Despite the subjects in groups A and B being of similar age, geographic residence, and bone mass, there was almost no overlap in measured serum 25OHD concentration at these two national central laboratories (Fig. 1A). Additionally, if optimal serum 25OHD concentration is defined as being greater than 32 ng/ml, 90% of these women were insufficient at laboratory B, but only 17% in laboratory A. Furthermore, serum 25OHD concentration was measured in three women at both laboratories A and B with markedly differing results (Fig. 1B).

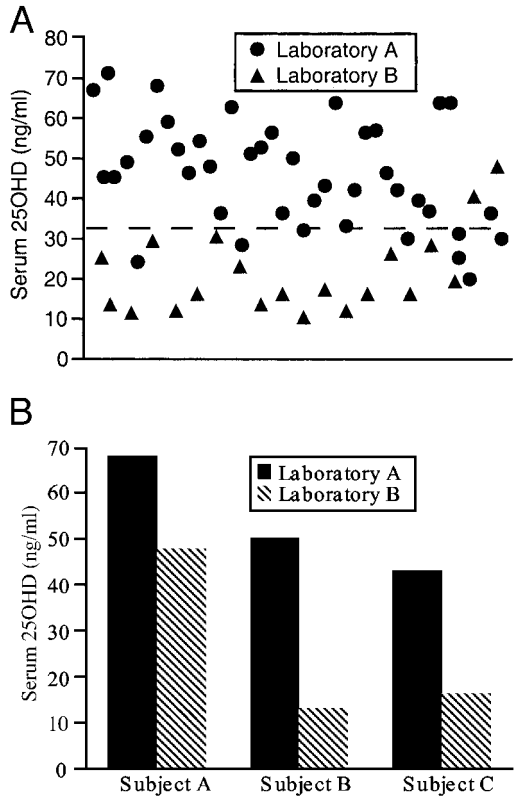

FIG. 1. Initial observation. A, Although these postmenopausal women were clinically similar, there was almost no overlap in measured serum 25OHD concentration at reference laboratories. Additionally, if optimal serum 25OHD concentration is defined as being greater than 32 ng/ml (*dashed line*), 90% of these women were insufficient at laboratory B, but only 17% were insufficient in laboratory A. B, Three women were measured in both laboratories A and B with markedly differing results.

25OHD concentration/healthy laboratory personnel

In these 10 healthy adults, the mean serum 25OHD varied widely from 17.1 (4.6) to 35.6 (5.2) ng/ml ($P < 0.005$) between laboratories (Fig. 2A). Similarly, for a given subject, the 25OHD result was substantially different depending on the laboratory used (Fig. 2B). Interestingly, laboratories C and D are the sites to which clinical specimens are routinely sent in our area. As evident from these observations, whether a patient is considered as being vitamin D insufficient (using 32 ng/ml as a threshold) depends, in large measure, on the laboratory used. For example, subjects 1, 4, 6, 7, and 9 (Fig. 2B) would be classified as insufficient in some laboratories and normal in others. Thus, 50% of these individuals might be classified as having a different vitamin D status (insufficient or normal) depending solely on the laboratory used.

25OHD concentration: effect of spiking with 25OHD

In the spiked specimens, the mean serum 25OHD also varied ($P < 0.05$) between laboratories from 27.4 (4.5) to 44.6

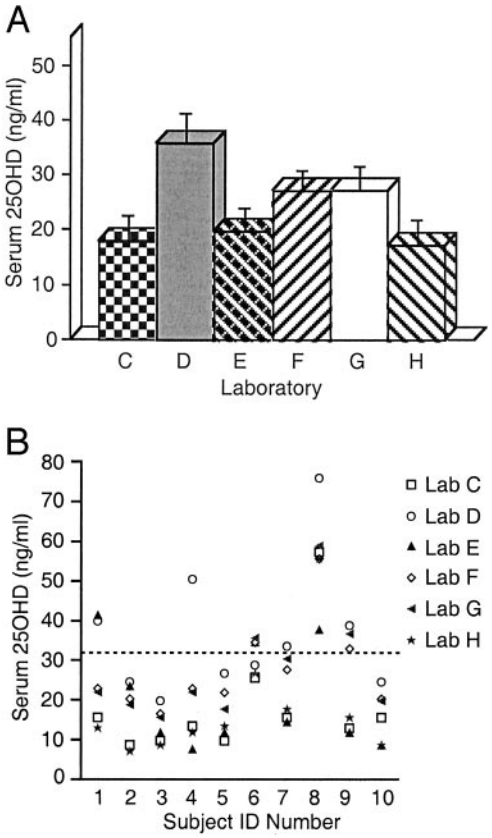

FIG. 2. Laboratory personnel basal serum 25OHD concentration. A, In these 10 individuals, the mean serum 25OHD varied widely [from 17.1 (4.6) to 35.6 (5.2) ng/ml; $P < 0.005$] between laboratories (*error bars* represent SEM). B, Similarly, marked within-individual variation was observed in 25OHD measurement in different laboratories. Whether an individual has hypovitaminosis D (arbitrary threshold, 32 ng/ml shown as *dashed line*) depends on which laboratory was used. Note that laboratory H used HPLC and was considered to be the "gold standard" against which others were compared.

(6.1) ng/ml (Fig. 3). Furthermore, the increase observed upon spiking with 20 ng/ml 25OHD was less than anticipated, *e.g.* from 7.7–18.0 ng/ml with the greatest increment detected by HPLC (Fig. 3).

Evaluation of results obtained in different laboratories

When compared with HPLC, all laboratories (with the exception of laboratory E) demonstrated a highly significant correlation when evaluated by Bablock regression analysis (Fig. 4). However, significant positive bias was observed in the basal samples measured in laboratories D, F, and G (Table 3 and Fig. 4). In the spiked specimens, significant positive bias was observed only in laboratory D (Table 3 and Fig. 4).

Discussion

This study illustrates that putative advances in methodology for assessment of vitamin D status have not eliminated the previously reported substantial laboratory variation in serum 25OHD measurement (15, 18, 19). Indeed, the mean serum 25OHD concentration in 10 subjects differs 2-fold between laboratories, and the proportion of subjects below an arbitrary threshold of insufficiency (32 ng/ml) (12) varies

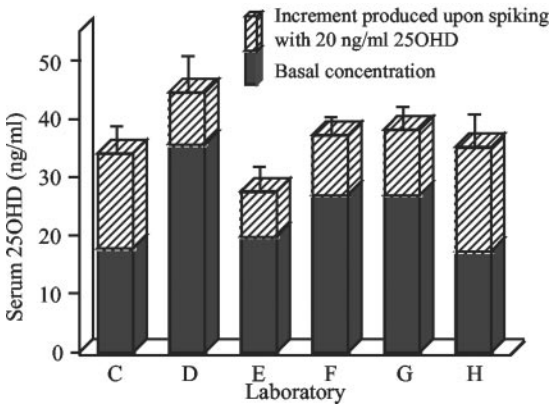

FIG. 3. Laboratory personnel effect of spiking with 25OHD on the measured concentration. In the spiked specimens, the mean serum 25OHD varied ($P < 0.05$) between laboratories from 27.4 (4.5) to 44.6 (6.1) ng/ml. Furthermore, the increment was less than anticipated and varied between laboratories ($P = 0.005$) from 7.7–18 ng/ml, with the greatest increment detected by HPLC. The *solid portion of the bars* depicts mean basal 25OHD concentration, and the *hatched portion* illustrates the measured increase from spiking with 20 ng/ml 25OHD. *Error bars* represent SEM.

from 17–90%. Furthermore, the measured increment produced by addition of a known quantity of 25OHD ranges from 17–95% of that expected, depending on the laboratory. Nevertheless, one facility measures 25OHD with excellent correlation to values obtained by HPLC and accurately measures the increment produced on sample spiking. Thus, accurate measurement of 25OHD is feasible in a commercial laboratory.

Vitamin D deficiency classically causes impairment of skeletal mineralization, resulting in rickets and osteomalacia in children or osteomalacia alone in adults. However, less severe degrees of vitamin D insufficiency contribute to bone loss without necessarily impairing bone mineralization. Specifically, vitamin D inadequacy leads to calcium malabsorption with resultant secondary hyperparathyroidism leading, over time, to bone loss and osteoporosis (5). In this context, optimal vitamin D status is defined as the serum 25OHD concentration above which PTH is not further suppressed. Applying this definition, hypovitaminosis D is extremely common due to low dietary intake and minimal cutaneous sunlight exposure (4, 13, 20–24). Additionally, vitamin D supplementation increases calcium absorption, improves bone density, reduces risk of falls, and decreases the likelihood of osteoporotic fracture (6, 8, 25–28). Finally, nonskeletal adverse effects related to inadequate vitamin D status, including such diverse consequences as hypertension, diabetes mellitus, cancer, and autoimmune disease, are receiving increased attention (29–32). As a result, there is an appropriately increasing emphasis on the measurement of 25OHD in the clinic and subsequent supplementation to maintain or enhance health status by attaining an optimal vitamin D status. In this regard, Chapuy *et al.* (13) and Heaney (12) have suggested that the optimal 25OHD serum concentration is above approximately 31–32 ng/ml (78–80 nmol/liter). However, our data indicate that widespread clinical application of a single target value, *e.g.* 32 ng/ml, is not appropriate at this time due to marked interlaboratory

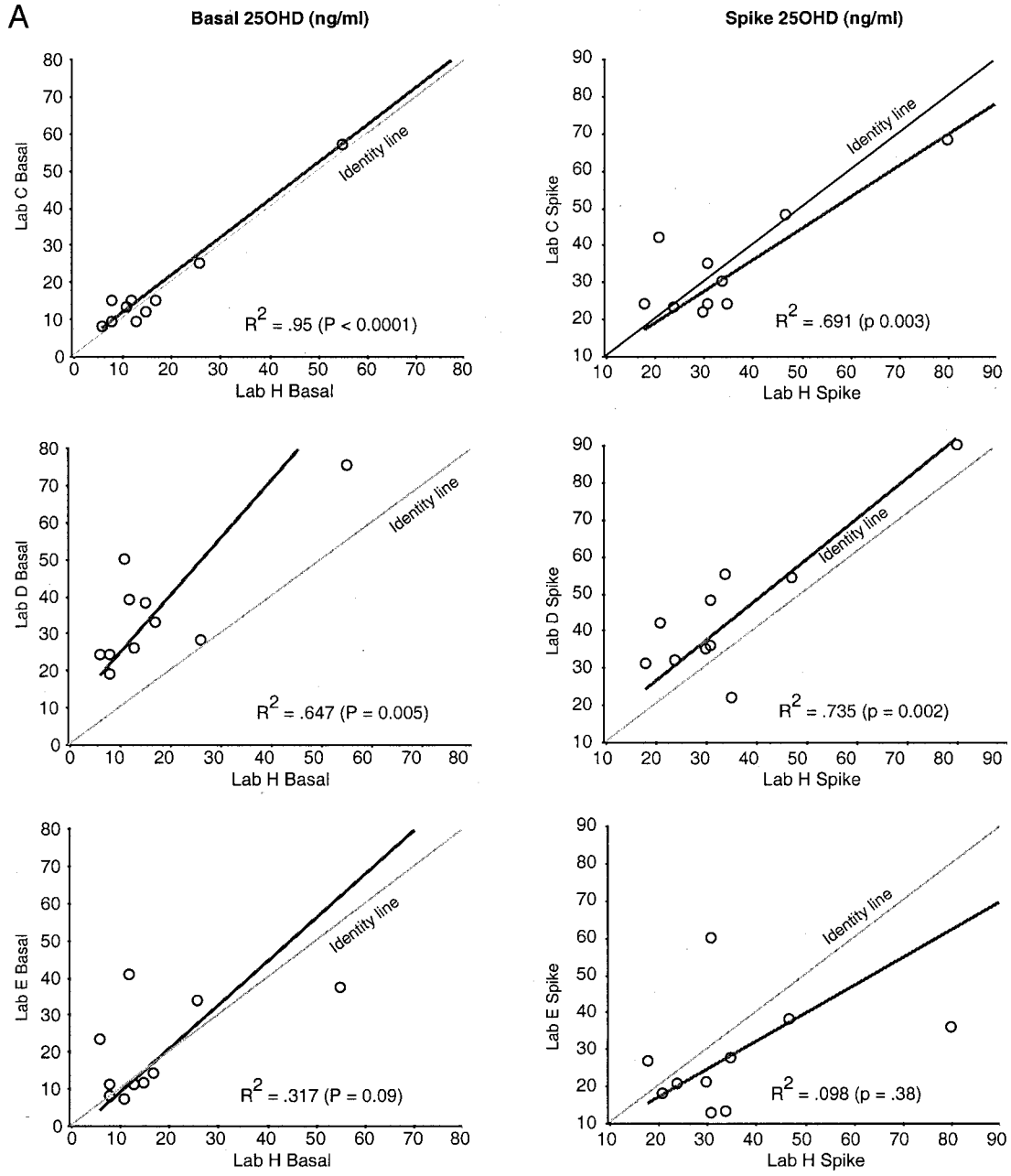

FIG. 4. Comparison with HPLC. Significant bias was observed in 25OHD measurement in laboratories D, F, and G compared with HPLC. This positive bias ranged from 9.8–18.5 ng/ml. Positive bias was present only in laboratory D in the spiked samples. Overall excellent correlation without significant bias was demonstrated in laboratory C.

variability. Thus, practicing clinicians do not have a reliable tool to detect hypovitaminosis D in their patients.

It is not surprising that discordant results are obtained because these laboratories use substantially different methodology for measurement of 25OHD, which, to this time, has not been standardized. For example, the chemiluminescent assay is, in essence, a protein binding assay that uses a reagent to separate vitamin D from its binding proteins without preparative chromatography (33). Thus, this procedure has similarities to protein binding assays that have been used for some time (34–36) and usually (14, 15, 37), but not always (17, 38), produce higher 25OHD results than other methodologies, perhaps reflecting the absence of a prior chromatographic separation (39). It is important to recognize that the competitive protein binding assays may use different sources of vitamin D binding proteins and modify the extraction or preliminary purification procedures and/or incubation conditions (33, 40). Thus, not all competitive protein binding assays are identical. Subsequently, RIA methodology using antibodies was developed (41) and documented to compare very well with an HPLC gold standard (42, 43). However, it is important to recognize that not all RIA assays are the same; some antibodies recognize both $25OHD_2$ and $25OHD_3$, whereas others underestimate $25OHD_2$ (16). Furthermore,

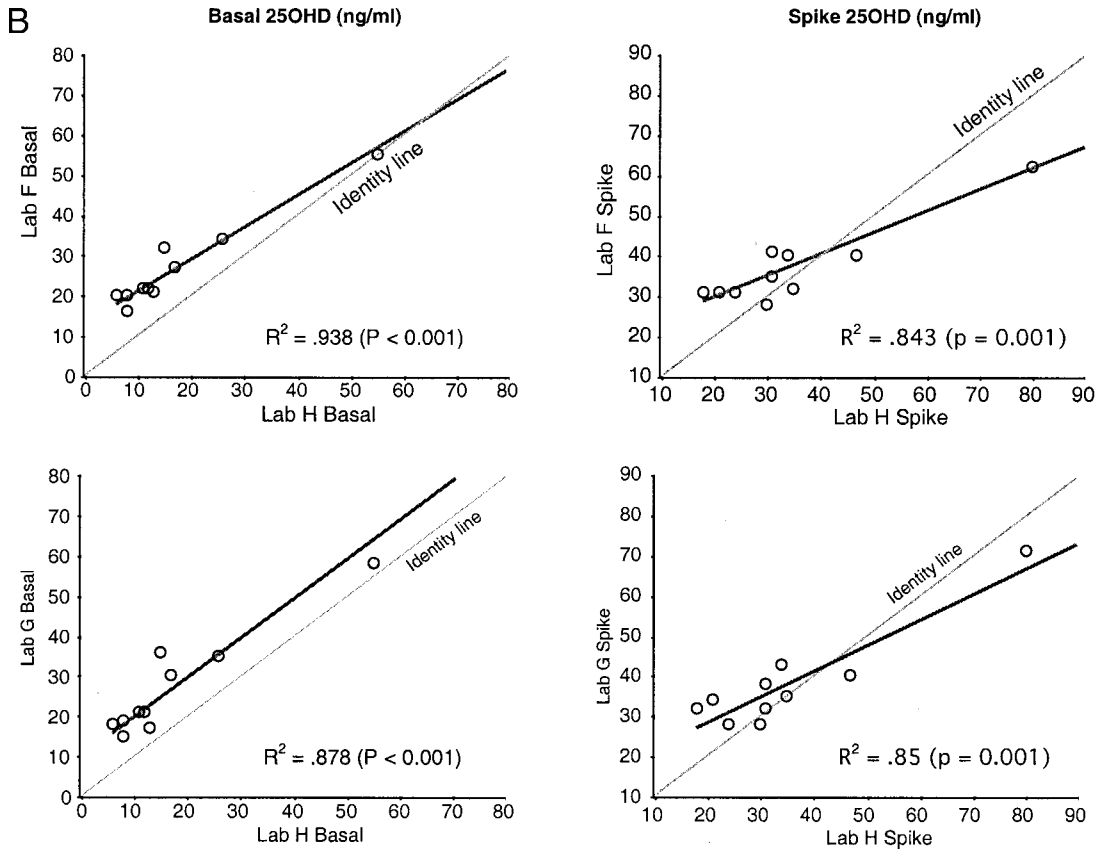

FIG. 4. Continued.

TABLE 3. Bias (nanograms/milliliter) observed when comparing laboratories with HPLC

Laboratory	Basal sample	Spiked sample
C	0.7 (−1.6 to 3.0)	−1.1 (−8.1 to 6.0)
D	18.5 (11.4 to 25.6)	9.4 (2.3 to 16.5)
E	2.6 (−6.6 to 11.8)	−7.9 (−21.5 to 5.8)
F	9.8 (6.6 to 13.0)	2.0 (−4.8 to 8.8)
G	9.9 (6.3 to 13.5)	3.0 (−2.6 to 8.6)

Data presented as bias (95% confidence interval).

the fact that an RIA, which has previously been reported to correlate well with HPLC, performed variably in this study likely indicates that this procedure is user-dependent and requires rigorous quality control to ensure reliable results. Finally, HPLC has long been recognized as the gold standard (44–46); however, despite recent advances to improve its clinical applicability (47), it is not widely available to clinicians.

We recognize that assay standardization is not a simple or inexpensive endeavor (48). However, given what appears to be an epidemic of hypovitaminosis D, the simplicity and low cost of its correction, and the potential beneficial skeletal and nonskeletal consequences of doing so, it is essential that the medical community define a threshold for optimal vitamin D status using accurate, reproducible assays. These assays must subsequently be internationally standardized and made available to practicing clinicians.

Limitations of this study include the small sample size and the fact that not every available methodology for 25OHD measurement was evaluated. However, these limitations do not negate the conclusion that widespread clinical application of a single threshold value to define optimal vitamin D status is impossible at this time.

In conclusion, marked between-laboratory variability exists in serum 25OHD measurement. As a result of this variability, an arbitrary definition of optimal serum 25OHD concentration is neither possible nor widely applicable. International standardization of 25OHD measurement is essential before elimination of endemic hypovitaminosis D is achievable. Given increased recognition of this situation by the National Institutes of Health (49), such standardization, perhaps through agencies such as the National Institute of Standards and Technology, seems attainable in the near future. In the interim, it is prudent that clinicians recommend routine vitamin D supplementation but, because of the difficulties inherent in measuring 25OHD, refrain from using a single 25OHD value to define optimal vitamin D status. Furthermore, the amount of supplemental vitamin D required remains controversial and likely varies with several factors, including age and severity of extant vitamin D deficiency. Although the recommended daily intake remains at 400–600 IU in the United States, recent data and, consequently, expert opinion suggest that substantially more (~1000 IU) may be required. Ideally, these higher doses should be monitored using an accurate 25OHD assay. Based on our study and those of others, clinicians should consider using RIA for 25OHD measurement to assess vitamin D status and response to supplementation until other methodologies are further validated.

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References

1. **Kudlacek S, Schneider B, Peterlik M, Leb G, Klaushofer K, Weber K, Woloszczuk W, Willvonseder R; Austrian Study Group on Normative Values of Bone Metabolism** 2003 Assessment of vitamin D and calcium status in healthy adult Austrians. Eur J Clin Invest 33:323–331
2. **Gomez-Alonso C, Naves-Diaz ML, Fernandez-Martin JL, Diaz-Lopez JB, Fernandez-Coto MT, Cannata-Andia JB** 2003 Vitamin D status and secondary hyperparathyroidism: the importance of 25-hydroxyvitamin D cut-off levels. Kidney Int 63(Suppl 85):S44–S48
3. **McKenna MJ, Freaney R** 1998 Secondary hyperparathyroidism in the elderly: means to defining hypovitaminosis D. Osteoporos Int 8(Suppl 2):S3–S6
4. **LeBoff MS, Kohlmeier L, Hurwitz S, Franklin J, Wright J, Glowacki J** 1999 Occult vitamin D deficiency in postmenopausal US women with acute hip fracture. JAMA 281:1505–1511
5. **Parfitt AM** 1990 Osteomalacia and related disorders. In: Avioli LV, Krane SM, eds. Metabolic bone disease and clinically related disorders. 2nd ed. Philadelphia: W. B. Saunders Co.; 329–396
6. **Bischoff HA, Stahelin HB, Dick W, Akos R, Knecht M, Salis C, Nebiker M, Theiler R, Pfeifer M, Bergerow B, Lew RA, Conzelmann M** 2003 Effects of vitamin D and calcium supplementation on falls: a randomized controlled trial. J Bone Miner Res 18:343–351
7. **Trivedi DP, Doll R, Khaw KT** 2003 Effect of four monthly oral vitamin D3 (cholecalciferol) supplementation on fractures and mortality in men and women living in the community: randomised double blind controlled trial. BMJ 326:469–474
8. **Chapuy MC, Pamphile R, Paris E, Kempf C, Schlichting M, Arnaud S, Garnero P, Meunier PJ** 2002 Combined calcium and vitamin D3 supplementation in elderly women: confirmation of reversal of secondary hyperparathyroidism and hip fracture risk: the Decalyos II Study. Osteoporos Int 13:257–264
9. **Chapuy MC, Arlot ME, Duboeuf F, Brun J, Crouzet B, Arnaud S, Delmas PD, Meunier PJ** 1992 Vitamin D3 and calcium to prevent hip fractures in elderly women. N Engl J Med 327:1637–1642
10. **Zittermann A** 2003 Vitamin D in preventive medicine: are we ignoring the evidence? Br J Nutr 89:552–572
11. **Holick MF** 2003 Vitamin D: a millennium perspective. J Cell Biochem 88:296–307
12. **Heaney RP** 2000 Vitamin D: how much do we need, and how much is too much? Osteoporos Int 11:553–555
13. **Chapuy MC, Preziosi P, Maamer M, Arnaud S, Galan P, Hercberg S, Meunier PJ** 1997 Prevalence of vitamin D insufficiency in an adult normal population. Osteoporos Int 7:439–443
14. **Vieth R** 2000 Problems with direct 25-hydroxyvitamin D assays, and the target amount of vitamin D nutrition desirable for patients with osteoporosis. Osteoporos Int 11:635–636
15. **Lips P, Chapuy MC, Dawson-Hughes B, Pols HAP, Holick MF** 1999 An international comparison of serum 25-hydroxyvitamin D measurements. Osteoporos Int 9:394–397
16. **Hollis BW** 2000 Comparison of commercially available ^{125}I-based RIA methods for the determination of circulating 25-hydroxyvitamin D. Clin Chem 46:1657–1661
17. **Glendenning P, Noble JM, Taranto M, Musk AA, McGuiness M, Goldswain PR, Fraser WD, Vasikaran SD** 2003 Issues of methodology, standardization and metabolite recognition for 25-hydroxyvitamin D when comparing the DiaSorin radioimmunoassay and the Nichols Advantage automated chemiluminescence protein-binding assay in hip fracture cases. Ann Clin Biochem 40:546–551
18. **Jongen MJM, van der Vijgh WJF, van Beresteyn ECH, van den Berg H, Bosch R, Hoogenboezem T, Visser TJ, Netelenbos JC** 1982 Interlaboratory variation of vitamin D1 metabolite measurements. J Clin Chem Clin Biochem 20:753–756
19. **Jongen MJM, Van Ginkel FCV, van der Vijgh WJF, Kuiper S, Netelenbos JC, Lips P** 1984 An international comparison of vitamin D metabolite measurements. Clin Chem 30:399–403
20. **Thomas MK, Lloyd-Jones DM, Thadhani RI, Shaw AC, Deraska DJ, Kitch BT, Vamvakas EC, Dick IM, Prince RL, Finkelstein JS** 1998 Hypovitaminosis D in medical inpatients. N Engl J Med 338:777–783
21. **Lamberg-Allardt CJE, Outila TA, Karkkainen MUM, Rita HJ, Valsta LM** 2001 Vitamin D deficiency and bone health in healthy adults in Finland: could this be a concern in other parts of Europe? J Bone Miner Res 16:2066–2073
22. **Vieth R, Cole DE, Hawker GA, Trang HM, Rubin LA** 2001 Wintertime vitamin D insufficiency is common in young Canadian women, and their vitamin D intake does not prevent it. Eur J Clin Nutr 55:1091–1097
23. **Rucker D, Allan JA, Fick GH, Hanley DA** 2002 Vitamin D insufficiency in a population of healthy western Canadians. CMAJ 166:1517–1524
24. **Looker AC, Dawson-Hughes B, Calvo MS, Gunter EW, Sahyoun NR** 2002 Serum 25-hydroxyvitamin D status of adolescents and adults in two seasonal subpopulations from NHANES III. Bone 30:771–777
25. **Lips P** 1996 Vitamin D deficiency and osteoporosis: the role of vitamin D deficiency and treatment with vitamin D and analogues in the prevention of osteoporosis-related fractures. Eur J Clin Invest 26:436–442
26. **Ooms ME, Roos JC, Bezemer PD, van der Vijgh WJF, Bouter LM, Lips P** 1995 Prevention of bone loss by vitamin D supplementation in elderly women: a randomized double-blind trial. J Clin Endocrinol Metab 80:1052–1058
27. **Peacock M, Liu G, Carey M, McClintock R, Ambrosius W, Hui S, Johnston CC** 2000 Effect of calcium or 25OH vitamin D3 dietary supplementation on bone loss at the hip in men and women over the age of 60. J Clin Endocrinol Metab 85:3011–3019
28. **Heaney RP, Dowell MS, Hale CA, Bendich A** 2003 Calcium absorption varies within the reference range for serum 25-hydroxyvitamin D. J Am Coll Nutr 22:142–146
29. **Hypponen E, Laara E, Reunanen A, Jarvelin MR, Virtanen SM** 2001 Intake of vitamin D and risk of type 1 diabetes: a birth-cohort study. Lancet 358:1500–1503
30. **Zittermann A, Schleithoff SS, Tenderich G, Berthold HK, Korfer R, Stehle P** 2003 Low vitamin D status: a contributing factor in the pathogenesis of congestive heart failure? J Am Coll Cardiol 41:105–112
31. **Guyton KZ, Kensler TW, Posner GH** 2003 Vitamin D and vitamin D analogs as cancer chemopreventive agents. Nutr Rev 61:227–238
32. **Eriksen EF, Glerup H** 2002 Vitamin D deficiency and aging: implications for general health and osteoporosis. Biogerontology 3:73–77
33. **Roth HJ, Zahn I, Alkier R, Schmidt H** 2001 Validation of the first automated chemiluminescence protein-binding assay for the detection of 25-hydroxycalciferol. Clin Lab 47:357–365
34. **Haddad JG, Chyu KJ** 1971 Competitive protein-binding radioassay for 25-hydroxycholecalciferol. J Clin Endocrinol Metab 33:992–995
35. **Skinner RK, Wills MR** 1977 Serum 25-hydroxyvitamin D assay. Evaluation of chromatographic and non-chromatographic procedures. Clin Chim Acta 80: 543–554
36. **Belsey RE, DeLuca HF, Potts Jr JT** 1974 A rapid assay for 25-OH-vitamin D3 without preparative chromatography. J Clin Endocrinol Metab 38:1046–1051
37. **Bouillon R, Van Herck E, Jans I, Tan BK, Van Baelen H, De Moor P** 1984 Two direct (nonchromatographic) assays for 25-hydroxyvitamin D. Clin Chem 30: 1731–1736
38. **Souberbielle JC, Lawson-Body E, Kindermans C** 2002 Authors' response: vitamin D status and redefining serum PTH reference range in the elderly. J Clin Endocrinol Metab 87:946–947 (Letter)
39. **Dorantes LM, Arnaud SB, Arnaud CD** 1978 Importance of the isolation of 25-hydroxyvitamin D before assay. J Lab Clin Med 91:791–796
40. **Schmidt-Gayk H, Armbruster FP, Bouillon R** 1990 Calcium regulating hormones, vitamin D metabolites, and cyclic AMP. Assays and their clinical application. Berlin, Heidelberg, New York: Springer
41. **Hollis BW, Napoli JL** 1985 Improved radioimmunoassay for vitamin D and its use in assessing vitamin D status. Clin Chem 31:1815–1819
42. **Horst RL, Littledike ET, Riley JL, Napoli JL** 1981 Quantitation of vitamin D and its metabolites and their plasma concentrations in five species of animals. Anal Biochem 116:189–203
43. **Hollis BW, Kamerud JQ, Selvaag SR, Lorenz JD, Napoli JL** 1993 Determination of vitamin D status by radioimmunoassay with an ^{125}I-labeled tracer. Clin Chem 39:529–533
44. **Lindback B, Berlin T, Bjorkhem I** 1987 Three commercial kits and one liquid-chromatographic method evaluated for determining 25-hydroxyvitamin D3 in serum. Clin Chem 33:1226–1227
45. **Gilbertson TJ, Stryd RP** 1977 High-performance liquid chromatographic assay for 25-hydroxyvitamin D3 in serum. Clin Chem 23:1700–1704
46. **Jones G** 1978 Assays of vitamins D2 and D3, and 25-hydroxyvitamins D2 and D3 in human plasma by high-performance liquid chromatography. Clin Chem 24:287–298
47. **Turpeinen U, Hohenthal U, Stenman UH** 2003 Determination of 25-hydroxyvitamin D in serum by HPLC and immunoassay. Clin Chem 49:1521–1524
48. **Stenman UH** 2001 Immunoassay standardization: is it possible, who is responsible, who is capable? Clin Chem 47:815–820
49. **Raiten DJ, Picciano MF,** Vitamin D and health in the 21st century: bone and beyond. National Institutes of Health Conference, Bethesda, MD, 2003