Over the last 20 years there have been numerous studies, including NHANES III (the National Health and Nutrition Examination Survey III), the Women’s Health Study, and the Nurses’ Health Study, showing an association between decreased 25-hydroxyvitamin D \([25(OH)D]\) concentrations in blood and the risks of cardiovascular disease, stroke, cancer, fractures, and mortality (see the Supplemental Reading List that accompanies the online version of this Q&A at http://www.clinchem.org/content/vol61/issue3). Approximately 10 years ago, these studies led to recommendations from multiple professional societies that the definition of 25(OH)D deficiency be changed from \(<20\) ng/mL (50 nmol/L) to \(<30\) ng/mL (75 nmol/L). In the US, we and other institutions saw the volume of 25(OH)D testing increase 5–6-fold between 2004 and 2007. Furthermore, the use of \(<30\) ng/mL (75 nmol/L) to define 25(OH)D deficiency results in almost half of the tested population in a large Midwestern US hospital, such as ours, as being vitamin D deficient.

In late 2010, the Institute of Medicine (IOM) issued a report that vitamin D supplementation was unlikely to be beneficial for any condition other than bone health and that blood concentrations of 20 ng/mL (50 nmol/L) or greater were sufficient for maintaining bone health. Since then, several metaanalyses have failed to show that low 25(OH)D concentrations are associated with risk for any of the above-mentioned nonskeletal chronic conditions, with the possible exception of fractures. Complicating the association of 25(OH)D blood concentrations with risk has been the poor agreement among 25(OH)D immunoassays (e.g., differences in recognition of 25(OH)D\(_2\) and 25(OH)D\(_3\), the 2 forms of commercial supplements of the vitamin), for which there are ongoing efforts to standardize 25(OH)D assays. Further complicating what we know and don’t know is the recent discovery of genetic polymorphisms in vitamin D binding proteins (VDBPs) that segregate well between blacks and whites, which may explain the paradox of blacks having lower 25(OH)D blood concentrations than whites but higher, or equivalent, bone density. These studies suggest that perhaps we should be looking at bioavailable 25(OH)D rather than total 25(OH)D. Here, 4 experts who have contributed to what we know about 25(OH)D address what we don’t know and where we might be headed.

**How should we define 25(OH)D reference intervals or deficiency? Central 95%? Outcomes? Which outcomes? By age? By race? By sex? Season? By biologic definitions such as a rise in parathyroid hormone?**

**Ian Reid:** I think it is appropriate to move back to defining the reference interval for 25(OH)D in the same way that we define other reference intervals, i.e., as the central 95% of the values found in a healthy normal population. Such a range would vary by race and season. If it is possible to reach consensus that adverse health outcomes are causally related to low concentrations of 25(OH)D, then these could be substituted for a conventionally derived interval. However, that consensus does not exist at present, other than for the outcome of osteomalacia, which occurs only in individuals whose 25(OH)D is \(<10\) ng/mL (25 nmol/L). The progressive increases of the reference interval based on associations of 25(OH)D with health outcomes has...
led to widespread use of vitamin D supplements, which is not supported by randomized control trials, and to massive expenditure on 25(OH)D assays, which is not justified by either the quality of the measurements produced or by demonstrable health benefits from that investment.

Michael F. Holick: It is generally agreed world-wide and supported by both the IOM and the Endocrine Society’s practice guidelines that 25(OH)D deficiency regarding bone health is defined as a 25(OH)D <20 ng/mL (50 nmol/L). The Endocrine Society’s practice guidelines defined 25(OH)D insufficiency as a concentration of 25(OH)D of 21–29 ng/mL (52–72 nmol/L). This is based on several lines of evidence, including the observation by Priemel (J Bone Miner Res 2010;25:305–12), who reported that 25(OH)D deficiency osteomalacia was not observed in any of 675 adult ileac crest biopsies, obtained at autopsy, when concentrations of 25(OH)D were >30 ng/mL (75 nmol/L). Furthermore, 24% of these presumed healthy adults with 25(OH)D 21–29 ng/mL (52–72 nmol/L) had evidence of 25(OH)D deficiency osteomalacia. Several studies relating declining parathyroid hormone (PTH) concentrations with 25(OH)D concentrations found that the PTH concentrations plateaued when the 25(OH)D concentrations were at least 30 ng/mL (75 nmol/L). Many association studies relating 25(OH)D status and chronic illness have suggested significant decreases in incidence when blood concentrations of 25(OH)D were >30 ng/mL (75 nmol/L). There is a seasonal variation in serum 25(OH)D concentrations. The highest concentrations are at the end of the summer and the lowest concentrations at the end of the winter. There is no firm evidence that concentrations of 25(OH)D should be any different based on age, ethnicity, and sex.

Karen Phinney: In methods with an extraction step, the 25(OH)D is typically isolated through the addition of organic solvents or other reagents that denature proteins in the sample and release protein-bound species. When developed and applied properly, such methods are not likely to be affected by sample-to-sample differences in VDBP concentration. In automated methods, details of the process for dissociation of 25(OH)D from the VDBP are generally proprietary to the assay platform. There has been at least one report of VDBP concentration affecting the results of automated assays. Heijboer et al. (N Engl J Med 2013;369:1991–2000) reported an inverse relationship between VDBP concentration and deviations from the LC-MS/MS comparison method for 4 of the 5 automated assays investigated. One factor limiting the interpretation of these results, however, is the absence of well-established methods for measurement of VDBP concentrations. In addition, genetic variants of the VDBP are believed to have different binding affinities for 25(OH)D. Further study is needed before additional conclusions can be drawn about the impact, if any, of VDBP concentrations on the performance of the many different methods used in clinical laboratories.

Michael F. Holick: Differences in the VDBP concentrations theoretically should not affect the ability of immunoassays to detect total 25(OH)D. However, it is well documented that these assays are often unable to quantitatively detect 25(OH)D$_2$. This may be due to either the concentration or binding capacity of the VDBP. This is not observed with LC-MS/MS.

Ravi Thadhani: Approximately 80% of total 25(OH)D is carried by VDBP. VDBP is a negative acute-phase reactant, with a short half-life (1–2 days). In acute conditions such as sepsis, liver production of VDBP goes down, and one should expect concentrations of total 25(OH)D to follow suit. Although most current assays will correctly measure 25(OH)D concentrations, one will more than likely find that patients with acute sepsis are 25(OH)D deficient. The question is whether these
patients are truly 25(OH)D deficient, or only appear to be transiently deficient until liver production returns to normal. In my experience, total concentrations of 25(OH)D return to baseline within 1–3 weeks of leaving the intensive care unit (ICU). This is not unlike defining hypothyroidism in patients in the ICU, from where the term “sick euthyroid” emanated.

Aside from acute changes in VDBP, there is some controversy as to the appropriate assay for VDBP. I suggest developing an assay that directly measures bioavailable 25(OH)D (albumin-bound and free 25(OH)D), which will bypass the need to measure VDBP, especially until the differences between assays are sorted out. That said, I believe the genetic differences in VDBP are real, and do account for some portion of the differences in 25(OH)D between blacks and whites. In other words, it is difficult to label all black patients as deficient, when in general their bone health on all fronts is better than that of whites. Therefore, how deficiency is defined should be reexamined, as well as which component of 25(OH)D is the appropriate measure to define 25(OH)D status.

**Ian Reid:** Yes. Current assays measure total 25(OH)D, which is made up of the free component and that bound to VDBP.

**Should we be measuring bioavailable 25(OH)D as opposed to total?**

**Michael F. Holick:** There is not enough information at this time to suggest that the bioavailable 25(OH)D is a better marker for 25(OH)D status as it relates to either bone health or other associated nonskeletal benefits.

**Ian Reid:** In theory, this would be preferable. However, there are very few VDBP assays available at present, and even fewer direct measurements of free 25(OH)D. There appear to be major technical hurdles to be overcome before such assays are freely available and affordable.

**Ravi Thadhani:** The concept put forward that bioavailable 25(OH)D is more physiologically important than total concentrations of 25(OH)D is one “borrowed” from the metabolism of other hormones with carrier proteins, such as testosterone and thyroid hormone. While I believe it is the component of 25(OH)D that should be measured, further studies using validated assays (not yet available) are needed before routine use of bioavailable 25(OH)D measurements can be advocated.

**Karen Phinney:** At the present time, few techniques are available for the direct measurement of either free or bioavailable 25(OH)D, and such techniques are not suited for routine use, nor has their accuracy been assessed. Mathematical approaches to estimation of bioavailable 25(OH)D have also been reported. These approaches use measured concentrations of VDBP and albumin along with published values for affinity constants for the 3 primary genetic variants of VDBP. The accuracy of methods for the measurement of VDBP has not yet been established, and indeed, the work of Powe et al. showed that there was no significant correlation between two different commercial assays for VDBP. In addition, these computational methods rely upon the availability of genotyping information that is not routinely available. Additional research is needed to demonstrate that measured or estimated bioavailable 25(OH)D is actually linked to particular health outcomes. Finally, given that 25(OH)D is also believed to enter cells through megalin-mediated endocytosis, consideration of only “free” 25(OH)D may overlook other mechanisms of action of 25(OH)D.

**Should the recent findings about VDBP polymorphisms affect interpretations about the association of 25(OH)D concentrations with cancer, diabetes, and cardiovascular disease mortality? Should such studies be repeated?**

**Karen Phinney:** The body of evidence supporting an association between VDBP polymorphisms and concentrations of circulating 25(OH)D is growing. There are 3 major variants of the VDBP (GC-1f, GC-1s, GC-2), which can be combined to form 6 diplotypes. Although genetic factors do appear to play a role in observed 25(OH)D concentrations, other factors such as vitamin D intake, season, and ancestry are probably equally important. For example, Gozdzik et al. (J Steroid Biochem Mol Biol 2011;127:405–12) examined young Canadian adults of various ancestries and found that GC-2 alleles were associated with lower 25(OH)D concentrations in East Asians in fall and winter, but no association was observed for South Asians. Malik et al. (Crit Rev Clin Lab Sci 2013;50:1–22) recently reviewed the available evidence linking variants of the VDBP gene with adverse health outcomes including diabetes, cancer, and infectious diseases. Perhaps not surprisingly, as described in Malik’s review, conflicting results have been obtained in some cases, suggesting that other factors such as environment, ethnicity, and lifestyle must be considered. In addition, many of the studies have lacked sufficient statistical power to draw meaningful conclusions. Nevertheless, VDBP polymorphisms clearly add additional complexity to the interpretation of 25(OH)D concentrations and their relationship to health and disease.

**Michael F. Holick:** There have been several studies suggesting that the polymorphisms for the VDBP as well as the vitamin D receptor may be related to bone health as well as nonskeletal chronic illnesses, including some can-
cerners. We need larger and better-designed studies to determine the importance of these preliminary observations. Furthermore, studies should be conducted to see whether increasing concentrations of 25(OH)D to >30 ng/mL (75 nmol/L) can overcome these polymorphic influences.

Ian Reid: It is highly likely that most of these associations between 25(OH)D concentrations and disease simply reflect the fact that people who are unwell, from whatever cause, spend less time exercising outdoors. There is also some evidence that inflammatory conditions reduce concentrations of VDBP, and this may also contribute to some disease associations. The reduced concentration of 25(OH)D in obesity is a further possible contributor. These factors are probably much more important than interracial differences in VDBP polymorphisms.

Ravi Thadhani: The studies should indeed be repeated. The data on genetic polymorphisms, differentiating blacks from whites, are important, and other studies have suggested that polymorphisms in the VDBP gene account for >10% of the variation of 25(OH)D concentrations in population studies. Genetic differences, in conjunction with accounting for acute changes in VDBP (a negative acute-phase reactant), must also be accounted for in studies of cancer, diabetes, and cardiovascular disease mortality.

Who should have 25(OH)D testing? General population screening? By certain age or sex groups? Based upon symptoms?

Michael F. Holick: The Endocrine Society and IOM both agree that general population screening should not be encouraged. However, children and adults who have risk factors for 25(OH)D deficiency should be screened and followed after being treated with vitamin D. Some of the risk factors include obesity, fat malabsorption syndromes, granulomatous disorders, pregnancy, and medications such as prednisone, antiseizure drugs, and HIV medications. Age and sex are not considered risk factors for 25(OH)D screening. People of color are at higher risk for 25(OH)D deficiency but do not need to be screened if provided an adequate amount of vitamin D supplementation. 25(OH)D deficiency symptoms are nonspecific and are not helpful for screening purposes.

Ian Reid: To provide an answer to this question, we need to reach agreement regarding concentrations of 25(OH)D that exert adverse effects on health. At present, the only persuasive data are for the prevention of osteomalacia. If 25(OH)D concentrations are maintained above 16 ng/mL (40 nmol/L), then osteomalacia is prevented with a considerable margin of safety. This is consistent with the recent recommendations from the IOM. The low reliability of the assays for 25(OH)D in widespread clinical use does not recommend them for routine patient assessment. It is probably much more cost-effective to target low-dose vitamin D supplements (e.g., 400–800 U per day) to individuals who have clinical risk factors for severe 25(OH)D deficiency (such as frailty, seldom venturing outdoors, dark skin, being habitually veiled) and not to measure 25(OH)D either before or after institution of supplementation.

Ravi Thadhani: Measurement of 25(OH)D should be performed in individuals at risk for deficiency, which includes, among other signs and symptoms, individuals with poor bone health, history or risk for fractures, muscle pain, and kidney disease.

Moderators
We want to thank the experts for their careful and thoughtful replies to our questions. Clearly, differences of opinion exist on what defines 25(OH)D deficiency, what should be measured to determine deficiency, and even whether 25(OH)D measurement is needed. We await future studies from the experts and others to elucidate what approach is most clinically relevant.

Author Contributions: All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.

Authors’ Disclosures or Potential Conflicts of Interest: Upon manuscript submission, all authors completed the author disclosure form. Disclosures and/or potential conflicts of interest:

Employment or Leadership: M.G. Scott, Clinical Chemistry, AACC; A.M. Gronowski, Clinical Chemistry, AACC.
Consultant or Advisory Role: None declared.
Stock Ownership: None declared.
Honoraria: R. Thadhani, Diasorin.
Research Funding: R. Thadhani, NIH.
Expert Testimony: None declared.
Patents: None declared.

Previously published online at DOI: 10.1373/clinchem.2014.222521