

Vitamin D₂ and D₃ Testing in Blood Spot



The Problem – Vitamin D Deficiency

Long known for its role in the prevention of childhood rickets¹ and in the intestinal absorption of dietary calcium, vitamin D has now been found to be important in protecting the body from a wide range of diseases. Disorders linked with vitamin D deficiency include stroke, cardiovascular disease, osteoporosis, osteomalacia, several forms of cancer, some autoimmune diseases such as multiple sclerosis, rheumatoid arthritis and type 1 diabetes, and even type 2 diabetes, depression and schizophrenia²⁻¹². A major culprit is inadequate sun exposure of the skin, usually for climatic or cultural reasons (even in countries near the equator, women in particular must have much of their skin area covered), and through the popular use of sunscreen. Vitamin D status is therefore an important screening test, especially for people who spend much of their time indoors, or who live in colder climates, and may also be used to monitor vitamin D supplementation to ensure that adequate blood levels are achieved.

Who is at Risk?

- **The Elderly**
Amounts of the vitamin D precursor in the skin decrease with age, therefore elderly people are particularly prone to deficiency¹³⁻¹⁶, and living in rest homes or becoming homebound can limit exposure to sunshine. Muscle weakness and osteoporosis associated with vitamin D deficiency make the elderly more susceptible to falling and fracture risk^{17,18} and trials indicate that vitamin D supplementation may decrease the risk of fractures¹⁹
- **Dark-Skinned People**
Higher melanin levels in dark skinned people block the action of sunlight on vitamin D precursors in the skin, requiring much longer sunlight exposure to generate adequate circulating vitamin D compared to fair-skinned people.
- **People with Limited Sunlight Exposure**
People living at northern latitudes or who have limited sunlight exposure because of their working environment or cultural dress rules may have low vitamin D levels
- **Musculoskeletal Pain Sufferers**
Patients with symptoms of hypothyroidism²⁰, non-specific musculoskeletal pain²¹, chronic low back pain²², or fibromyalgia²³ are frequently found to have low vitamin D levels and show clinical improvement after supplementation. Vitamin D screening is strongly recommended in patients presenting with musculoskeletal pain²⁴
- **Overweight or Obese People**
Vitamin D can be locked up in fat stores in obese patients, who have been found to have lower levels of circulating 25-hydroxy vitamin D and are at risk of deficiency²⁵

- **Breast-Fed Infants, and Children with Limited Sunlight Exposure**

All children require adequate circulating vitamin D to prevent rickets. Dark-skinned children and those who spend much of the day in indoor daycare centers are at risk of deficiency, and breast-fed children often receive inadequate amounts of vitamin D, particularly when their mothers are deficient. Maternal supplementation²⁶ or the use of cod liver oil or other vitamin D supplements in infants and children can avoid the risk of developing type 1 diabetes in childhood²⁷

- Routine vitamin D screening has been recommended as a routine part of the annual physical examination^{3,5}. Deficiency may be present even when there are no symptoms, yet it is simple to treat and may solve a number of subclinical health problems and reduce risk for more serious diseases

Why Measure D2 and D3?

Vitamin D3 (cholecalciferol) is technically a prohormone, and is produced from the action of ultraviolet light on 7-dehydrocholesterol in the skin. It is also found in cod liver oil and vitamin D supplements that state “cholecalciferol” in the ingredients. Vitamin D2 (ergocalciferol) is not found in animals, but is manufactured commercially by irradiating ergosterol, a component of fungal cell membranes, with ultraviolet light; it is the predominant form for prescription use in the US, especially in high dose preparations. Both are hydroxylated in the liver to form their 25-hydroxy metabolites, the major circulating form of the prohormone. The long half-life of 25-hydroxyvitamin D (> 2 weeks) allows for supplementation with large doses every few months in deficient individuals, since vitamin D is stored by the body in adipose tissue. The 25-hydroxy metabolites are further hydroxylated, primarily in the kidneys, to form 1,25-dihydroxy vitamin D2 and 1,25-dihydroxy vitamin D3, which are the highly active forms of the hormone that bind to specific vitamin D receptors in target tissues. Formation of 1,25-dihydroxy vitamin D is tightly regulated by the action of parathyroid hormone, and therefore has a very short half-life making it unsuitable for assessing vitamin D status. In vitamin D deficient states, there is in fact excess production of parathyroid hormone (secondary hyperparathyroidism) stimulating the kidneys to produce even more 1,25-dihydroxyvitamin D, such that levels can appear to be normal or even elevated. The 25-hydroxy metabolite, which reflects total body bioavailability of the prohormone, is therefore the commonly accepted measure of vitamin D status.

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Since D2 has been effective when used as a supplement to prevent rickets, it has historically been assumed to be equivalent to vitamin D3. However, more recently it has been found that D2 and D3 have different properties^{28,29}. 25-hydroxyvitamin D2 has a lower affinity than 25-hydroxyvitamin D3 for vitamin D binding protein, which carries the metabolites in the bloodstream, and a shorter half-life. Because of this, vitamin D2 is significantly less bioactive than D3, and must be given in much larger doses, although when given daily D2 is as effective as D3 in maintaining circulating levels of total 25-hydroxyvitamin D³⁰.

Vitamin D assays that measure only a "total" 25-hydroxyvitamin D do not distinguish how much is coming from the exogenous D2 and how much is biologically-identical D3 (whether from D3 supplementation or endogenous production). This is important when assessing vitamin D status in people supplementing with D2, which has a greater potential for toxicity than D3 if supplementation is not properly monitored with an accurate assay. The bioequivalence of D2 and D3 is an interesting area for research, particularly since trials showing reduced fractures with vitamin D supplementation have employed D3 at adequate levels¹⁹ rather than D2, and many vitamin producers are already switching to D3 in their over-the-counter preparations.

ZRT uses liquid chromatography/tandem mass spectrometry (LC-MS/MS) and not an immunoassay to measure 25-hydroxyvitamin D. Radioimmunoassays (RIA) typically do not differentiate between D2 and D3, and also don't respond equally to both. This is a problem because an RIA could seriously underestimate blood levels in people supplementing with D2, and lead to dangerous recommendations for additional supplementation. LC-MS/MS is the gold standard for determining 25-hydroxyvitamin D and differentiating between 25-hydroxyvitamin D2 and 25-hydroxyvitamin D3.

Advantages of a Simple Blood Spot Test

- No phlebotomist or centrifuging required, therefore less expensive and more convenient than conventional blood draws
- Particularly suitable for pediatric testing, since heelstick is already used in routine neonatal screening for phenylketonuria
- Convenience and privacy of collection at home using a nearly painless finger stick
- Excellent correlation with serum tests for 25-hydroxy vitamin D
- 25-hydroxy vitamin D is stable in dried blood spot at room temperature for weeks, allowing for worldwide shipment
- Safe handling and transport of samples, as infectious agents are inactivated by drying

Clinical Utility

Blood spot testing for 25-hydroxyvitamin D can help:

- Identify vitamin D deficiency as a potential cause of health problems – levels below 20 ng/mL indicate deficiency, while levels below 30 ng/mL are "low"; optimal levels are 30-60 ng/mL (research is ongoing to establish definitive recommendations)
- Monitor patient 25-hydroxyvitamin D levels during vitamin D supplementation to ensure adequate levels and protect against possible overdosing – toxicity may be expected at levels >150 ng/mL
- Recommend appropriate lifestyle changes for patients who may benefit from spending time or exercising outdoors to increase sunlight exposure
- Track patient progress with comparative history reports provided with follow-up testing

Reference

1. Rajakumar K. Vitamin D, cod-liver oil, sunlight, and rickets: a historical perspective. *Pediatrics*. 2003;112(2):e132-5.
2. Zittermann A. Vitamin D in preventive medicine: are we ignoring the evidence? *Br J Nutr*. 2003;89(5):552-72.
3. Holick MF. Sunlight and vitamin D for bone health and prevention of autoimmune diseases, cancers, and cardiovascular disease. *Am J Clin Nutr*. 2004;80(6 Suppl):1678S-88S.
4. Zittermann A, Schleithoff SS, Koerfer R. Putting cardiovascular disease and vitamin D insufficiency into perspective. *Br J Nutr*. 2005;94(4):483-92.
5. Holick MF. The vitamin D epidemic and its health consequences. *J Nutr*. 2005;135(11):2739S-48S.
6. Holick MF. Vitamin D: important for prevention of osteoporosis, cardiovascular heart disease, type 1 diabetes, autoimmune diseases, and some cancers. *South Med J*. 2005;98(10):1024-7.
7. Peterlik M, Cross HS. Vitamin D and calcium deficits predispose for multiple chronic diseases. *Eur J Clin Invest*. 2005;35(5):290-304.
8. Holick MF. High prevalence of vitamin D inadequacy and implications for health. *Mayo Clin Proc*. 2006;81(3):353-73.
9. Zittermann A. Vitamin D and disease prevention with special reference to cardiovascular disease. *Prog Biophys Mol Biol*. 2006;92(1):39-48.
10. Poole KE, Loveridge N, Barker PJ, Halsall DJ, Rose C, Reeve J, Warburton EA. Reduced vitamin D in acute stroke. *Stroke*. 2006;37(1):243-5.
11. Heaney RP. Vitamin D--the iceberg nutrient. *J Musculoskelet Neuronal Interact*. 2006;6(4):334-5.
12. Holick MF. Vitamin D deficiency. *N Engl J Med*. 2007;357(3):266-81.
13. MacLaughlin J, Holick MF. Aging decreases the capacity of human skin to produce vitamin D3. *J Clin Invest*. 1985;76(4):1536-8.
14. Need AG, Morris HA, Horowitz M, Nordin C. Effects of skin thickness, age, body fat, and sunlight on serum 25-hydroxyvitamin D. *Am J Clin Nutr*. 1993;58(6):882-5.
15. Need AG, Horowitz M, Morris HA, Nordin BC. Vitamin D status: effects on parathyroid hormone and 1, 25-dihydroxyvitamin D in postmenopausal women. *Am J Clin Nutr*. 2000;71(6):1577-81.
16. Need AG, O'Loughlin PD, Morris HA, Horowitz M, Nordin BE. The effects of age and other variables on serum parathyroid hormone in postmenopausal women attending an osteoporosis center. *J Clin Endocrinol Metab*. 2004;89(4):1646-9.
17. Gass M, Dawson-Hughes B. Preventing osteoporosis-related fractures: an overview. *Am J Med*. 2006;119(4 Suppl 1):S3-S11.
18. Boonen S, Bischoff-Ferrari HA, Cooper C, Lips P, Ljunggren O, Meunier PJ, Reginster JY. Addressing the musculoskeletal components of fracture risk with calcium and vitamin D: a review of the evidence. *Calcif Tissue Int*. 2006;78(5):257-70.
19. Bischoff-Ferrari HA, Willett WC, Wong JB, Giovannucci E, Dietrich T, Dawson-Hughes B. Fracture prevention with vitamin D supplementation: a meta-analysis of randomized controlled trials. *JAMA*. 2005;293(18):2257-64.
20. Faiz S, Panunti B, Andrews S. The epidemic of vitamin D deficiency. *J La State Med Soc*. 2007;159(1):17-20; quiz 20, 55.
21. Plotnikoff GA, Quigley JM. Prevalence of severe hypovitaminosis D in patients with persistent, nonspecific musculoskeletal pain. *Mayo Clin Proc*. 2003;78(12):1463-70.
22. Al Faraj S, Al Mutairi K. Vitamin D deficiency and chronic low back pain in Saudi Arabia. *Spine*. 2003;28(2):177-9.
23. Erkal MZ, Wilde J, Bilgin Y, Akinci A, Demir E, Bödeker RH, Mann M, Bretzel RG, Stracke H, Holick MF. High prevalence of vitamin D deficiency, secondary hyperparathyroidism and generalized bone pain in Turkish immigrants in Germany: identification of risk factors. *Osteoporos Int*. 2006;17(8):1133-40.
24. Lewis PJ. Vitamin D deficiency may have role in chronic low back pain. *BMJ*. 2005;331(7508):109.
25. Wortsman LA, Matsuoka LY, Chen TC, Lu Z, Holick MF. Decreased bioavailability of vitamin D in obesity. *Am J Clin Nutr*. 2000;72(3):690-3.
26. Hollis BW, Wagner CL. Vitamin D requirements during lactation: high-dose maternal supplementation as therapy to prevent hypovitaminosis D for both the mother and the nursing infant. *Am J Clin Nutr*. 2004;80(6 Suppl):1752S-8S.
27. The EURODIAB Substudy 2 Study Group. Vitamin D supplement in early childhood and risk for Type I (insulin-dependent) diabetes mellitus. *Diabetologia*. 1999;42(1):51-4.
28. Armas LA, Hollis BW, Heaney RP. Vitamin D2 is much less effective than vitamin D3 in humans. *J Clin Endocrinol Metab*. 2004;89(11):5387-91.
29. Houghton LA, Vieth R. The case against ergocalciferol (vitamin D2) as a vitamin supplement. *Am J Clin Nutr*. 2006;84(4):694-7.
30. Holick MF, Biancuzzo RM, Chen TC, Klein EK, Young A, Bibuld D, Reitz R, Salameh W, Ameri A, Tannenbaum AD. Vitamin D2 is as effective as vitamin D3 in maintaining circulating concentrations of 25-hydroxyvitamin D. *J Clin Endocrinol Metab*. 2007 Dec 18; [Epub ahead of print]