Interpreting the Laboratory Reports for Vit D

Devajit Sarmah*, Booloo Sharma**

Abstract

Importance for Vit D estimation has increased in the recent years due to its link to various diseases. Measurements of Vit D by different diagnostic laboratories is however not uniform. There is variation pertaining to assay methodology and also variation in the measurement of different metabolites of Vit D. There are also various confounders which influence Vit D assays and which in most instances are overlooked. Also a matter of concern regarding Vit D assays is the lack of assay standardisation. These factors contribute to the variation in the reports generated by the diagnostic laboratories. Therefore interpretation of Vit D reports needs proper understanding of these interfering factors and further reports need to be correlated substantially with the clinical findings.

Introduction

Sunshine Vitamin or Vitamin D has certainly emerged as one of the most discussed nutrients of the present century. Vitamin D (Vit D) an important fat soluble vitamin is synthesised in human body from cholesterol upon skin exposure to UVB sunlight or is available through dietary intake. The Vit D synthesised in the body is Vit D3 (cholecalciferol) and that availed from dietary sources are mainly Vit D2 (ergocalciferol) and some amount of Vit D3. Vit D3 is a 27-carbon molecule, whereas Vit D2 contains 28 carbons and differs from Vit D3 in the presence of an additional methyl group and a double bond between carbons 22 and 23. Vit D on hydroxylation in the liver form 25(OH) Vit D, which becomes biologically active 1,25-(OH)2 Vit D on further hydroxylation in the kidney.1,2 Vit D metabolism and actions in concert with calcium and phosphorus metabolism are regulated by parathyroid hormone. Vit D metabolites circulate in the body bound to Vit D binding proteins (VDBP).2,3 Recently there has been a manifold increase in vitamin D estimation which is because various research has linked vitamin D deficiency to chronic diseases such as cancer (breast, colon and prostate), cardiovascular disease, osteoporosis, osteomalacia and several autoimmune diseases among others. It is because of the possible non-skeletal benefits of Vit D that made it one of the hot and intriguing molecules for research in the 21st century.4 Different laboratories using different methodology estimates different metabolites of Vit D and hence a correct interpretation of the laboratory reports is essential.

Physiological Effects of the Two Forms of Vit D

As for the two forms of Vit D there is no existing evidence that vitamins D2 and D3 possess differing physiological effects. Thus, the clinical significance (if any) of differences between these two forms of Vit D and their respective metabolites remains unknown.5

Which Forms of Vitamin D is Ideal for Estimation?

1. The concentration of 1,25-(OH)2 Vit D is 1000 times lower than 25 (OH)Vit D. While 25(OH)Vit D is present in nanomolar (nm/ml) quantity 1,25-(OH)2 Vit D is present in picomolar (pm/ml)quantity.1,5
Due to its half life of 2–3 weeks, 25(OH) Vit D is the metabolite that is the most reliable clinical indicator of vitamin D status. 1,25-(OH)2 Vit D has a very short half life of about 4 hours and hence is often an unreliable clinical indicator of Vit D status.1,5

In case of hypovitaminosis D there is parathyroid hormone (PTH) elevation, which enhances renal 1-alpha hydroxylase activity, thereby promoting conversion of available 25(OH) Vit D to 1,25(OH)2 Vit D. As because 25(OH) Vit D is present in ng/mL quantities and 1,25(OH)2 Vit D in pg/mL quantities, it is apparent that even in the setting of low Vit D status, 1,25(OH)2 Vit D may be maintained within the normal range. Thus in the settings of hypovitaminosis D 1,25(OH)2 Vit D is not reliable.5,6

Measurement of 1,25(OH)2 Vit D is reserved for distinguishing some cases of primary hyperparathyroidism from hypercalcaemia of cancer and in the differential diagnosis of vitamin D-dependent rickets (type I vs type II).7,8

Also in patients with chronic renal disease, who may have normal 25(OH) Vit D levels 1,25(OH)2 Vit D is also useful for monitoring vitamin D therapy. As renal disease progresses, the ability of the kidney to produce 1,25(OH)2 Vit D decreases, because of inadequate 1-alpha hydroxylation of Vit D by the kidney. Supplementation with vitamin D is thus no longer effective, and patients require administration of 1,25(OH)2 Vit D.7

Although 1,25(OH)2 Vit D may be increased or decreased in a number of other disorders, levels are typically used for confirmation rather than diagnosis of these conditions.7,8 1,25(OH)2 Vit D estimation is done in conditions like inherited disorders in the metabolism of 25(OH)D and phosphate, hereditary phosphate-losing disorders, oncogenic osteomalacia, pseudovitamin D deficiency rickets, vitamin D-resistant rickets, as well as chronic granuloma forming disorders such as sarcoidosis and some lymphomas.7,9

Monitoring the effect of Vit D supplements is done by measuring 25(OH) Vit D. In certain case a clinician can ask for 25(OH) Vit D2 status to monitor the effect of Vit D2 supplementation, the most common form of Vit D supplementation.

So, barring few exceptions the thumb rule in Vit D estimation is that a total 25(OH) Vit D levels is sufficient. The total 25(OH) Vit D, i.e., 25(OH) D2 plus 25(OH)D3 is what physicians need to be aware of for their patients.

**Assays for vitamin D Assessment**

These assays can be placed into 3 general categories:

1. Competitive protein-binding assays (CPB),

2. Immunochemical assays (IC), and

3. Chromatographic procedures, which include gas chromatography/ mass spectrometry (GC-MS), high performance liquid chromatography (HPLC), and liquid chromatography-tandem mass spectrometry (LC-MS/MS).

LC-MS/MS is regarded as a “gold standard” for Vit D measurement. LC-MS/MS measures 25(OH) Vit D2 and D3 separately based on the difference of their molecular weights.5 HPLC also measures 25(OH) Vit D2 and D3 separately. The process of GC-MS and LC-MS/MS are however laborious and time consuming and their complexity and derivatisation requirements mitigate against its regular use. All modern immunoassays should have their results traceable to LC-MS/MS. For immunoassay to accurately measure both forms of Vit D, the antibody(s) used in the assay need to recognise 25(OH) Vit D2 and 25(OH) Vit D3 equally and there should not be cross reactivity between them. So, antibody specificity plays an important role in understanding the differences between assay results. For example, a specimen tested with an assay that measures only 80% of Vit D2 relative to Vit D3 will produce a different result when tested with an assay that measures 100% of both Vit D2 and Vit D3. Therefore, not all 25(OH) Vit D immunoassays show equal detection of serum 25(OH) Vit D2 and 25(OH) Vit D3 and some assays often underestimate the serum 25(OH) Vit D2 levels.5,9 The quality and source of materials that manufacturers use to standardise an assay also may differ and contribute to the observed discrepancies. So, there are some obvious differences in assay results between manufacturers. In one of the studies where a comparison of six laboratories were done, it was inferred that whether a patient is considered as being Vit D insufficient depends, in large measure, on the laboratory used.10 Thus the clinician sending Vit D investigation to a diagnostic laboratory should have a firsthand knowledge about the assay technique used by the laboratory.

HPLC is used in few laboratories across India and they give total Vit D by measuring both Vit D2 and D3 equally. However, presently most of the diagnostic laboratories across India are using immunoassays to measure Vit D. These immunoassays should have equal detection of Vit D2 and D3. This is especially important due to the fact that Vit D experts recommend the use of vitamin D assays with equal detection of Vit D2 and D3.10 Though many manufacturers claim to measure total Vit D (by equal detection of Vit D2 and Vit D3), presently immunoassay manufactured by DiaSorin Corporation, Siemens Healthcare and Ortho Clinical Diagnostic have been validated. Roche, BioMerieux and few other manufacturers have also launched such assays recently. Also as is with glycated haemoglobin, the use of standardisation material for Vit D is still a matter of concern in some laboratories.11
order to measure 25(OH) Vit D it is essential that it is treated with equal propensity. 15 Also in some samples, presence of other lipids in the serum or plasma changes the ability of the binding agent to react with other Vit D metabolites and can bias binding protein assays which are susceptible to cross-reactivity with other metabolites of 25(OH) Vit D. Most immunoassays is often found to significantly cross-react with 24,25-(OH)2 Vit D3, 25,26-(OH)2 Vit D3, and 25(OH)D3-26,23-lactone. It is however suggested that such interferences are clinically irrelevant. 15 But it is also a fact that 24,25-(OH) Vit D metabolites constitute about 10–15% of the 25(OH) Vit D concentration and their presence could slightly increase the 25(OH) Vit D concentration as measured by immunoassay and cannot be ignored completely.

### C3-Epimer:
Epimers are compounds that have identical molecular structure but differ in stereochemical configuration. It is the apparent stereochemistry of the hydroxyl (OH) group that produces two epimers of Vit D3, 3-epi-25(OH)D3 and 25(OH)D3 respectively. The physiological importance of 3-epi-25(OH)D3 is uncertain. It is a substrate for the 1α-hydroxylase enzyme, thus being converted to 3-epi-1, 25 dihydroxy Vit D3 and is capable of binding to the Vit D receptor. 16 The physiological effects of 3-epi-1, 25 dihydroxy Vit D3 appear to be variable as shown by certain animal model studies. 19 Although the physiological relevance of C-3 epimer is not yet established it could be an important confounder for Vit D measurement. This is even more apparent when a study confirmed the presence of C-3 epimer in considerable amount in adults, which was classically shown to be present only in neonates. 20

### Standardisation of Vit D assay:
Besides the above mentioned confounders, what has been more concern among clinicians and laboratory personal is the lack of assay standardisation for this “difficult” analyte. Until recently the lack of a reference material or a reference measurement procedure (RMP) against which assays could be standardised was a matter of concern. In 2009 there was introduction of the NIST (national institute of standards and technology) standard reference materials (SRM 972 and SRM 2972) which used ID-LC-MS/MS as the reference method for vitamin D estimation. 21, 22 Vitamin D external quality assurance scheme (DEQAS) used this reference material and has been helpful in improving the accuracy of LC-MS/MS analyses across laboratories. Laboratories across the globe were encouraged to participate in international Vitamin D External Quality Assessment Scheme (DEQAS), and gradually more and more laboratories undertook DEQAS. 23 Results submitted to the international DEQAS has been impressive and there has been a gradual reduction in interlaboratory imprecision (CV) in recent years—from 30% in 1995 to 15% in 2011. Although SRM 972 is suitable for LC-MS/MS methods, only Level 1 at 23.9 ng/mL is suitable for immunoassays and this variability persists inspite of an attempt of making immunoassay traceable to LC-MS/MS. But with release of new generation of human serum–based SRMs in 2012 this variability has been helpful in improving the accuracy of LC-MS/MS analyses across laboratories. Laboratories undertook DEQAS. 23 Results submitted to the international DEQAS has been impressive and there has been a gradual reduction in interlaboratory imprecision (CV) in recent years—from 30% in 1995 to 15% in 2011. Although SRM 972 is suitable for LC-MS/MS methods, only Level 1 at 23.9 ng/mL is suitable for immunoassays and this variability persists inspite of an attempt of making immunoassay traceable to LC-MS/MS. But with release of new generation of human serum–based SRMs in 2012 this variability is expected to reduce to a great extent. 24 Thus the greatest concern is the lack of standardisations for Vit D assays and until this issue is resolved Vit D assays result has to be interpreted with collaborative clinical discretions.

### Factors Influencing Vit D Assays

Vit D is often considered a “difficult” analyte and there has been a considerable debate regarding the measurements of Vit D. 5 For proper interpretation of Vit D laboratory reports, it is essential that the treating clinician be aware of the various factors that may affect the Vit D assays. Few of the confounders of Vit D assays are detailed below:

#### Matrix effect:
The lipophilic hydrophobic properties of Vit D, coupled with its tight affinity to Vit D binding protein, have made it challenging to measure. The most important type of matrix effect is any that occurs between the matrix in calibrants and patient samples. 14 Presence of other lipids in the serum or plasma changes the ability of the binding agent to associate with lipophilic 25(OH) Vit D in the sample and the standard with equal propensity. 15 Also in order to measure 25(OH) Vit D it is essential that it is released from VDBP. 15

#### Cross reactivity with other metabolites:
Early techniques to detect 25(OH) Vit D include competitive binding protein assays which are susceptible to cross-reactivity with other Vit D metabolites and can bias measurement of 25(OH) Vit D. 16, 17 Cross-reactivity with other metabolites of 25(OH) Vit D can lead to apparently higher concentrations of total 25(OH) Vit D. Most immunoassays is often found to significantly cross-react with 24,25-(OH)2 Vit D3, 25,26-(OH)2 Vit D3, and 25(OH)D3-26,23-lactone. It is however suggested that such interferences are clinically irrelevant. 15 But it is also a fact that 24,25-(OH) Vit D metabolites constitute about 10–15% of the 25(OH) Vit D concentration and their presence could slightly increase the 25(OH) Vit D concentration as measured by immunoassay and cannot be ignored completely.

### Table 1: Vit D levels as suggested by various societies

<table>
<thead>
<tr>
<th>Levels</th>
<th>Vitamin D Council</th>
<th>Endocrine Society</th>
<th>Food and Nutrition Board</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deficient</td>
<td>0-30 ng/ml</td>
<td>0-20 ng/ml</td>
<td>0-11 ng/ml</td>
</tr>
<tr>
<td>Insufficient</td>
<td>31-39 ng/ml</td>
<td>21-29 ng/ml</td>
<td>12-20 ng/ml</td>
</tr>
<tr>
<td>Sufficient</td>
<td>40-80 ng/ml</td>
<td>30-100 ng/ml</td>
<td>&gt; 20 ng/ml</td>
</tr>
<tr>
<td>Toxic</td>
<td>&gt; 150 ng/ml</td>
<td>&gt; 100 ng/ml</td>
<td></td>
</tr>
</tbody>
</table>

So for clinicians the message is “know your laboratory for Vit D estimation”.

### Vit D Range

Various organisations like the Vitamin D Council, Endocrine Society and Food and Nutrition Board Testing Laboratories have put forward ranges for Vit D. There is lack of consensus among these organisations, but it is the Endocrine society levels which are widely followed among medical fraternity. Moreover all manufacturers provide their own ranges which more or less are in consensus with the range provided by one of these organisations. The ranges for total Vit D as given by different organisations are shown in the Table 1.

### Standards of Vit D assay:
Besides the above mentioned confounders, what has been more concern among clinicians and laboratory personal is the lack of assay standardisation for this “difficult” analyte. Until recently the lack of a reference material or a reference measurement procedure (RMP) against which assays could be standardised was a matter of concern. In 2009 there was introduction of the NIST (national institute of standards and technology) standard reference materials (SRM 972 and SRM 2972) which used ID-LC-MS/MS as the reference method for vitamin D estimation.

## Table 1: Vit D levels as suggested by various societies

<table>
<thead>
<tr>
<th>Levels</th>
<th>Vitamin D Council</th>
<th>Endocrine Society</th>
<th>Food and Nutrition Board</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deficient</td>
<td>0-30 ng/ml</td>
<td>0-20 ng/ml</td>
<td>0-11 ng/ml</td>
</tr>
<tr>
<td>Insufficient</td>
<td>31-39 ng/ml</td>
<td>21-29 ng/ml</td>
<td>12-20 ng/ml</td>
</tr>
<tr>
<td>Sufficient</td>
<td>40-80 ng/ml</td>
<td>30-100 ng/ml</td>
<td>&gt; 20 ng/ml</td>
</tr>
<tr>
<td>Toxic</td>
<td>&gt; 150 ng/ml</td>
<td>&gt; 100 ng/ml</td>
<td></td>
</tr>
</tbody>
</table>
Interpretation of Laboratory Reports

Due to the confounders and wide variability in methodology the normal, low and toxic range for Vit D is many a time not well defined, and the range provided by diagnostic laboratories are often doubted. From the discussion above it remains clear that before interpreting a Vit D report generated by the laboratory clinicians should consider the following facts:

- The method that is used for estimation of Vit D assay.
- The metabolite of Vit D that is measured –Vit D2 or Vit D3.
- Whether the laboratory generates reports for total Vit D and if so whether it measures both Vit D2 and Vit D3 seperately.
- Whether the laboratory measures only 25 (OH) Vit D or 1, 25 (OH)2 Vit D or both.
- What is the extent of traceability of the generated result to ID-LC MS/MS generated result.

Clinician should make it clear that the diagnostic laboratory gives all the details regarding Vit D measurements. The interpretation of the report generated by the laboratory is at the clinician’s discretion. Especially important is the component of Vit D that the clinician wants to monitor. Needless to say that all components of Vit D have its own clinical importance as is discussed above.

Conclusion

Vit D estimation has increased manifold in the recent years and consequently more and more laboratories, both small and large are performing this test. But the methodology used for Vit D assays are not uniform and also different laboratories measures different metabolite of the sunshine Vit. There are various confounders for Vit assays as well. Lack of standardisation of Vit D assays is also a matter of concern. Hence the Vit D repsors that is generated from the diagnostic laboratory should always be interpreted in context with the methods used, the metabolite estimated and with proper clinical correlation.

References