Vitamin D Testing and the LC/MS/MS Advantage

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• Laboratory professionals may earn 1.0 contact hour for participation in today’s program

• Learning objectives: upon completion of this program, participants will be able to
  ✓ Discuss the risk factors for vitamin D deficiency and describe its impact on overall health
  ✓ Distinguish between vitamin D<sub>2</sub> and D<sub>3</sub> and summarize basic terminology used for vitamin D testing
  ✓ Compare and contrast commonly used methods of vitamin D testing
  ✓ Explain the advantages of the LC/MS/MS assay for assessing and monitoring vitamin D levels
Outline

- Vitamin D deficiency pandemic
- Impact of vitamin D deficiency
- Reference range determination
- Treatment approaches
- Vitamin D LC/MS/MS assay
- New frontiers in diagnosis and treatment
- Take home messages
Vitamin D Deficiency Pandemic

• Initial reasoning – it’s not an issue
  • Existence restricted to rickets in children and very rare

• Now deficiency worldwide problem
  • 1 billion people worldwide are either vitamin D deficient or insufficient
  • 40% to 100% of elderly US men and women are deficient
  • >50% of postmenopausal women on anti-osteoporosis therapy are not vitamin D sufficient
  • 52% of Hispanic and black adolescent boys in Maine and 48% of white preadolescent girls are deficient
  • Problem extends across globe with similar statistics in China, Mongolia, Europe, and Middle East
Risk Factors

- Lack of physical activity and sun exposure
- Dark skin pigmentation
- Heavy use of sunscreens
- Lack of dietary intake/appropriate supplementation
- Obesity
- Bariatric surgery and fat malabsorptive syndromes including celiac sprue
Risk Factors

• Drugs that interfere with vitamin D metabolism
  • Antiepileptics
  • Corticosteroids
  • Immunosuppressants
  • Antiretroviral drugs used in HIV

• Concomitant illnesses
  • Renal failure (all stages)
  • Primary hyperparathyroidism
  • Liver failure
Impact of Vitamin D Deficiency

• Usually asymptomatic but may present with bone pain, fatigue, muscle weakness, and falls

• Bone health
  • Vitamin D deficiency associated with
    • Increased osteopenia and osteoporosis
    • Sometime increased iPTH values
    • Increased fracture risk at all sites
  • Decreased risk of fall and Fx after therapy

Impacts of Vitamin D Deficiency

- Associated with increased risk of
  - Breast, prostate, and colon cancer
  - Diabetes
  - Autoimmune diseases including RA and multiple sclerosis
  - Increased risk of pre-eclampsia and eclampsia
  - Cardiovascular disease (incident MI and CHF) and hypertension

- Inhibits innate immune response to *Mycobacterium tuberculosis* (TB) and other infectious organisms

Limited Cumulative Evidence Supporting Non-classical Roles of 25(OH)D

Review of 2 meta analyses of all randomized control trials published in 2007 and 2009

• Examined cardiovascular events, cancer incidence (all types, breast, and colorectal), mortality in patients treated with vitamin D, calcium, or vitamin D + calcium

• Both analyses favored lower mortality with vitamin D supplementation, although neither was statistically significant

• Results for other health outcomes were inconsistent for both vitamin D and calcium supplementation

ACP Journal Club. 2009;151(5).
Questions About Vitamin D Testing

- What defines the standard method for vitamin D testing?
  - Analytical accuracy or historical use?

- Is there a bias in ability to measure 25(OH)D₃ and 25(OH)D₂?

- Is separate measurement of 25(OH)D₃ and 25(OH)D₂ clinically relevant or is it confusing to the physician?
Questions About Vitamin D Testing

- Is there a unique reference range independent of type and target of therapy, age, and sex?
- Is there a role for measurement of iPTH to define or tailor treatment to a particular patient?
How is Reference Range Determined?

Population-based reference range study – inadequate because of high incidence of vitamin D deficiency

Data on file at Quest Diagnostics.
Physiologically-based Endpoint: Impact of 25(OH) Vitamin D on iPTH Suppression

- 1 of criteria for sufficiency at bone level
- Relationship between 25(OH)D and iPTH levels described mathematically by quadratic model with plateau phase
- Typically 30 ng/mL 25(OH)D leads to iPTH suppression by reaching plateau phase

N = 1536 postmenopausal women on osteoporosis medication

Physiologically-based Endpoint: Impact of 25(OH)D on Bone Fracture Rate

- 8 high-quality trials with hip fracture endpoint included in meta analysis
  - N = 40,886 treated patients

- Relative risk of hip fracture decreased as 25(OH)D concentration increased

Current Agreed Upon Terminology & Cutoffs for Vitamin D Sufficiency

- Predominantly based on bone data
- Deficiency: <20 ng/mL
- Insufficiency: 21 – 29 ng/mL
- Sufficiency: >30 ng/mL
- Toxic level: 150 ng/mL
- Reference Range: 20 – 100 ng/mL
- Preferred Range: 30 – 60 ng/mL
- Variability across industry re definition of reportable reference range

Note: Levels of 1,25(OH)D$_2$ are misleading and should not be used for diagnosis or treatment of Vitamin D deficiency

Treatment Approaches

- Two forms: 25(OH)D$_3$ and 25(OH)D$_2$

- Maintenance dose (supplementation) – avoid deficiency
  - 400 to 2000 IU/ day
  - Most experts recommend 1000 to 2000 IU/ day

- Pharmacologic dose for established deficiency or insufficiency
  - 50,000 IU D$_2$ (ergocalciferol) weekly for 8 weeks – only FDA approved form of treatment in US
  - 100,000 IU D$_3$ (cholecalciferol) every 3 months
  - D$_3$ supplements (available OTC at 1000 to 10,000 IU)

- In short bowel syndrome/malabsorptive syndrome: UVB irradiation (tanning)

LC/MS/MS Procedure for Small Molecules

- Extraction and isotope dilution
- HPLC (high performance liquid chromatography)
- MS/MS (tandem mass spectrometry)
- Data reduction and reporting
- Measures both $25(OH)D_2$ and $25(OH)D_3$
Tandem Mass Spectrometry

- Triple quadrupole mass spectrometer
- 3 quadrupoles
- Q1 = mass filter (for precursor ions)
- Q2 = collision cell
- Q3 = mass filter (for productions)
Advantages of LC/MS/MS

- Deuterated internal standard
- Standard curve in every assay
- Limit of quantitation
- High specificity
- Reproducibility (low, medium, high)
## Assay Validation: Specificity

<table>
<thead>
<tr>
<th>Compound</th>
<th>Mass (Da)</th>
<th>Cross-reactivity (25OHD&lt;sub&gt;2&lt;/sub&gt;)</th>
<th>Cross-reactivity (25OHD&lt;sub&gt;3&lt;/sub&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25(OH)D&lt;sub&gt;2&lt;/sub&gt;</td>
<td>412</td>
<td>100%</td>
<td>ND</td>
</tr>
<tr>
<td>25(OH)D&lt;sub&gt;3&lt;/sub&gt;</td>
<td>400</td>
<td>ND</td>
<td>100%</td>
</tr>
<tr>
<td>&lt;sup&gt;6&lt;/sup&gt;D-25(OH)D&lt;sub&gt;3&lt;/sub&gt;</td>
<td>406</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Vitamin D&lt;sub&gt;2&lt;/sub&gt;</td>
<td>396</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Vitamin D&lt;sub&gt;3&lt;/sub&gt;</td>
<td>384</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>1α,25(OH)&lt;sub&gt;2&lt;/sub&gt;D&lt;sub&gt;2&lt;/sub&gt;</td>
<td>428</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>1α,25(OH)&lt;sub&gt;2&lt;/sub&gt;D&lt;sub&gt;3&lt;/sub&gt;</td>
<td>416</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>25,26(OH)&lt;sub&gt;2&lt;/sub&gt;D&lt;sub&gt;3&lt;/sub&gt;</td>
<td>416</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>1α(OH)D&lt;sub&gt;2&lt;/sub&gt;</td>
<td>412</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>1α(OH)D&lt;sub&gt;3&lt;/sub&gt;</td>
<td>400</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND, not detected.

Data on file at Quest Diagnostics Nichols Institute.
LC/MS/MS Chromatograms

Blank
0 ng/mL 25(OH)D₂
0 ng/mL 25(OH)D₃

High Standard
128 ng/mL 25(OH)D₂
128 ng/mL 25(OH)D₃

Data on file at Quest Diagnostics Nichols Institute.
“Normal” Subject
35 ng/mL 25(OH)D₃
<4 ng/mL 25(OH)D₂

Patient Taking Vitamin D₂
<4 ng/mL 25(OH)D₃
85 ng/mL 25(OH)D₂

Data on file at Quest Diagnostics Nichols Institute.
LC/MS/MS – Reference Method?

- Chromatographic methods (HPLC, LC/MS/MS) traditionally considered reference methods
- NIST and CDC trying to provide reference method and calibrators for several common immunoassays
  - Vitamin D: roadmap for NIST standardization
    - Reference sera (SRM 972) with values assigned by LC/MS/MS: done
    - Solvent-based calibrators (SRM 2972): done
## LC/MS/MS – Reference Method?

<table>
<thead>
<tr>
<th>SRM 972</th>
<th>NIST Target</th>
<th>Quest Diagnostics LC/MS/MS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Level I, 25(OH)D$_3$</td>
<td>12.3 ± 0.6</td>
<td>12.8 ± 1.7</td>
</tr>
<tr>
<td>Level II, 25(OH)D$_3$</td>
<td>23.9 ± 0.8</td>
<td>24.7 ± 2.7</td>
</tr>
<tr>
<td>Level III, 25(OH)D$_3$</td>
<td>18.5 ± 1.1</td>
<td>19.1 ± 2.1</td>
</tr>
<tr>
<td>Level III, 25(OH)D$_2$</td>
<td>26.4 ± 2.0</td>
<td>26.3 ± 2.2</td>
</tr>
</tbody>
</table>

Data (ng/mL) based results over 4 months
n = 486 measurements at each level

Data on file at Quest Diagnostics Nichols Institute.
Comparison of 25(OH)D₃ Levels Measured in 7 Methods

Bias in 25(OH)D$_2$ Immunoassay Measurements (CDC Data)

- N = 551 specimens
  - 38 with 25(OH)D$_2$
    - 5% to 92% of total 25(OH)D
  - LC/MS/MS concentrations average of 17% (95% CI: 8%-25%) higher than RIA per Bland-Altman analysis.

CDC substituted Diasorin 25(OH)D assay with their own LC/MS/MS in NHANES nutritional survey. Diasorin assay lot-to-lot shifts lead to wide and unacceptable QC targets.

**Table 3**

<table>
<thead>
<tr>
<th>Level</th>
<th>NIST certificate of analysis values from LC-MS/MS (nmol/L)</th>
<th>CDC DiaSorin RIA (nmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>25(OH)D3</td>
<td>25(OH)D2</td>
</tr>
<tr>
<td>1</td>
<td>59.6 ± 2.1</td>
<td>1.46 ± 0.49</td>
</tr>
<tr>
<td>2</td>
<td>30.8 ± 1.5</td>
<td>4.14 ± 0.19</td>
</tr>
<tr>
<td>3</td>
<td>46.2 ± 2.8</td>
<td>64.1 ± 4.8</td>
</tr>
</tbody>
</table>

Levels 1, 2, and 3 using the DiaSorin RIA compared with NIST-certified (blue) or reference (green) values. Means ± U95 (expanded uncertainty) for NIST data and mean ± SD for CDC data are displayed.

Proportional Measurement Bias
Post Treatment with 25(OH)D$_2$

Immunoassay results in 52 hip fracture patients (linear regression analysis)

\[
\text{DS} = 0.21 \text{ HPLC} + 8; \quad \text{IDS} = 0.1 \text{ HPLC} + 10.
\]

D$_2$ + D$_3$ Levels Higher in Quest

LC/MS/MS vs Diasorin Methods

Data on file at Quest Diagnostics Nichols Institute.
LC/MS/MS Data on 25(OH)D vs Plateau Phase of iPTH Suppression

Salameh et al. *Endocrine Reviews*. 2010;31(3)[Suppl 1]:S238.
• What defines the standard for vitamin D testing?
  • Analytical accuracy
  • Use by NIST
  • Professional society recommendations

• Is there a rational basis to adjust LC/MS/MS values to match immunoassays?
  • No
  • “Adjusting LC/MS/MS results to match those of any immunoassay, however well established, would no doubt be described as pragmatic, but I believe that most clinical chemists would share my belief that it is more akin to “cooking the books” (albeit with the best of intentions).”

• Is there a bias in ability to measure 25(OH)D$_3$ and 25(OH)D$_2$?
  • Yes; immunoassays overestimate the former and underestimate the latter

• Is separate measurement of 25(OH)D$_3$ and 25(OH)D$_2$ clinically relevant or is it confusing to the physician?
  • Separate measurements useful for both clinical and research purposes
Take Home Messages: Advantages of LC/MSMS Assays

- Accurate, as evidenced by ability to meet very narrow NIST targets
- Precise, as evidenced by low CV compared to immunoassay method mean in DEQAS surveys
- Minimal bias in 25(OH)D$_3$ measurements decreases inaccurate vitamin D deficiency/insufficiency diagnoses and unnecessary treatment
- Accurate measurement of 25(OH)D$_2$ prevents underestimation of impact of therapeutic intervention and possibility of inappropriate dosing
Take Home Messages: Advantages of LC/MSMS Assays

- Fast, high throughput method, with smaller volume requirements
- In subjects treated with ergocalciferol, ability to separately measure both 25(OH)D$_3$ and 25(OH)D$_2$ provides
  - Opportunity to research potential isoform differences in biological activity
  - Ability to follow patient compliance or evaluate possible malabsorptive syndrome after high dose D$_2$ therapy
Questions?

• Press *1 on your telephone keypad to speak live with the presenter

OR

• Use the chat feature to send your question to the program host
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