

## PUBLIC HEALTH AND CLINICAL DILEMMAS RESULTING FROM IMPRECISE VITAMIN D TESTS

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### ABSTRACT

**Objective:** To determine whether there are clinical and public health dilemmas resulting from the reproducibility of routine vitamin D assays. **Methods:** Blinded agreement studies were conducted in eight clinical laboratories using two commonly used assays to measure serum 25-hydroxyvitamin D (25(OH)D) levels in Australasia and Canada (DiaSorin Radioimmunoassay (RIA) and DiaSorin LIAISON<sup>®</sup> one). **Results:** Only one laboratory measured 25(OH)D with excellent precision. Replicate 25(OH)D measurements varied by up to 97% and 15% of paired results differed by more than 50%. Thirteen percent of subjects received one result indicating insufficiency [25-50 nmol/l] and another suggesting adequacy [ $>50$  nmol/l]. Agreement ranged from poor to excellent for laboratories using the manual RIA, while the precision of the semi-automated Liaison<sup>®</sup> system was consistently poor. **Conclusions:** Recent interest in the relevance of vitamin D to human health has increased demand for 25(OH)D testing and associated costs. Our results suggest clinicians and public health authorities are making decisions about treatment or changes to public health policy based on imprecise data. Clinicians, researchers and policy makers should be made aware of the imprecision of current 25(OH)D testing so that they exercise caution when using these assays for clinical practice, and when interpreting the findings of epidemiological studies based on vitamin D levels measured using these assays. Development of a rapid, reproducible, accurate and robust assay should be a priority due to interest in population-based screening programs and research to inform public health policy about the amount of sun exposure required for human health. In the interim, 25(OH)D results should routinely include a statement of measurement uncertainty.

**KEYWORDS:** Vitamin D assay; Vitamin D deficiency; Sun exposure; Skin cancer; Precision.

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### INTRODUCTION

Historically, low levels of circulating 25-hydroxyvitamin D (25(OH)D; the biomarker for an individual's vitamin D status) were of concern because they could result in the development of rickets among infants and osteomalacia among adults (Rajakumar, 2003). Thus "the sunshine vitamin" has been monitored among those lacking sun exposure because they are housebound or covered when outdoors for cultural or other reasons (Nowson and Margerison, 2002).

With the aging population in developed countries, osteoporosis and the need for adequate vitamin D have become a major focus (Diamond et al., 2005). There are now also suggestions that lower 25(OH)D levels are associated with various cancers, autoimmune diseases and cardiovascular disease (Holick 2004; Ponsonby et al., 2005), although this remains controversial (Young and Walker, 2005). Thus the recommended requirements for vitamin D have risen from the level required to prevent rickets (Fraser, 1990) to a higher level, which is considered necessary to prevent osteoporosis, with its associated risk of falls and fractures and potentially a diverse group of other diseases (Diamond et al., 2005; Holick, 2004; Ponsonby et al., 2005).

Recommending sun exposure to increase 25(OH)D has public health risks, particularly in sunny climates, since it is likely to

increase the risk of skin cancer (Armstrong, 2004). An increasing number of researchers, clinicians and public health authorities are using commercially-available serum 25(OH)D assays to determine background levels in their communities, the needs of their patients, or to guide public health policies. Therefore, reliable 25(OH)D tests are required, and several methods to measure 25(OH)D have been developed (Hollis, 2007) although consensus about which method should be used has not been reached (Glendenning, 2003).

Concerns have been expressed in the biochemical literature about inter-method bias (Wootton, 2005), inter-laboratory variability (Binkley et al., 2004), and issues of 25(OH)D assay standardization and performance (Glendenning, 2003). However, the reproducibility (i.e. precision) of 25(OH)D testing within laboratories has not been given the attention it deserves. Precision is the "closeness of repeated measurements of the same quantity to each other" (Sokal and Rohlf, 1995). Synonyms include reproducibility, reliability and consistency (Feinstein, 1985). Unfortunately, these terms are often used incorrectly.

We assessed the precision of the two most common commercial 25(OH)D assays used in Australasia and North America in 2006-7. Samples were collected in the standard manner, and presented to eight laboratories in three countries for testing as clinical samples for a Vitamin D project.

## METHODS

Ethical approval was granted by James Cook University (H2358). Eight cross-sectional blinded laboratory-based agreement studies were conducted in Australia, New Zealand and Canada between August 2006 and June 2007 to determine the precision of 25(OH)D measured using the non-chromatographic DiaSorin radioimmunoassay (RIA) (Hollis et al., 1993) and the newer semi-automated chemiluminescence DiaSorin LIAISON® one system (Ersfeld et al., 2004) (DiaSorin Corporation, Stillwater, MN). In one country, all laboratories used DiaSorin RIA to determine serum 25(OH)D levels. In each of the remaining two countries, at least one participating laboratory used DiaSorin RIA, and at least one other used DiaSorin LIAISON® one.

Groups of 8-19 consenting adult volunteers were recruited at each of eight study sites. Forty-six individuals were bled 1-5 times to provide 102 participant venepunctures. All participants except one were Australian residents who participated while at home or while travelling abroad, and all gave written informed consent before providing between two and six 5 ml vials of peripheral venous blood. Identical blood samples were labeled with a different name and date of birth to blind laboratory staff to the identity of the specimens. Serum was separated in the designated commercial pathology laboratory within three hours of collection.

Sera were processed according to each laboratory's routine serum 25(OH)D testing protocol. The samples were identified as research samples, but the laboratories were not told the study was investigating the precision of 25(OH)D testing. Four laboratories used the DiaSorin RIA method, and the other four laboratories used the DiaSorin LIAISON® method.

### Statistical Methods

Precision within each of the eight laboratories was assessed by comparing 25(OH)D results of the first two blinded replicates using concordance correlation coefficients (CCC) (I-Kuei Lin, 1989). The CCC adjusts Pearson's correlation coefficient ( $r$ ) for

scale shift (comparing standard deviations) and for location shift (comparing mean values relative to the standard deviations). CCCs are given together with approximate 95%-confidence intervals (95%-CI). The mean absolute difference between the first two replicates is provided for each laboratory together with the correlation between the average and the difference between measurements (Table 1). The mean coefficient of variation (CV) and standard deviation (SD) was calculated for each laboratory using all available replicates. CVs are presented together with the maximum difference between any two replicates (Table 2).

Vitamin D status was based on serum 25(OH)D and defined as:

- Deficient: <25nmol/l
- Insufficient: 25-50nmol/l
- Adequate: >50nmol/l (Diamond et al., 2005).

The proportion of replicates crossing these boundaries is reported (Table 2).

Results are not shown by country to preserve the anonymity of laboratories in small countries.

## RESULTS

The age of participants ranged from 21 to 84 years (mean  $45.7 \pm 15.7$  years, 60.9% female). The CCC varied between 0.49 (95%-CI=[0.32, 0.63]) and 0.97 (95%-CI=[0.81, 0.996]) for the four laboratories using DiaSorin RIA, while the CCC ranged between 0.57 (95%-CI=[0.15, 0.82]) and 0.68 (95%-CI=[0.42, 0.84]) for the four laboratories using DiaSorin LIAISON® (Table 1). One laboratory measured 25(OH)D with high precision (CCC=0.97) using DiaSorin RIA. The largest mean absolute difference was 21.1nmol/l (SD=16.2) for a laboratory using DiaSorin LIAISON® (Table 1). The lowest mean CV was 4.8% (SD=4.0) and was achieved by a laboratory using DiaSorin RIA (Table 2).

**Table 1: Precision of measurements of serum 25 hydroxyvitamin D performed by eight large clinical laboratories in three Commonwealth nations.**

Laboratory; sample size (subjects with more than 2 replicates); [assay]	Original measurements	Correlation between the average and difference	
	CCC (95%-CI)*	Mean absolute difference in nmol/l** (SD)†	r; p-value
Lab A n=14 (4) [LIAISON]	CCC = 0.57 (0.15, 0.82)	16.6 (14.4)	r = 0.02, p = 0.952
Lab B n=9 (8) [LIAISON]	CCC = 0.60 (-0.11, 0.90)	15.3 (12.3)	r = 0.45, p = 0.226
Lab C n=9 (8) [LIAISON]	CCC = 0.62 (-0.01, 0.90)	13.3 (8.1)	r = 0.30, p = 0.428
Lab D n=19 (0) [LIAISON]	CCC = 0.68 (0.42, 0.84)	21.1 (16.2)	r = 0.34, p = 0.150
Lab E n=8 (0) [RIA]	CCC = 0.49 (0.32, 0.63)	14.0 (13.7)	r = 0.97, p < 0.001
Lab F n=16 (4) [RIA]	CCC = 0.58 (0.24, 0.80)	10.9 (7.6)	r = -0.04, p = 0.878
Lab G n=19 (0) [RIA]	CCC = 0.81 (0.63, 0.91)	7.9 (5.3)	r = 0.34, p = 0.150
Lab H n=8 (0) [RIA]	CCC = 0.97 (0.81, 0.996)	5.5 (4.4)	r = -0.21, p = 0.620

RIA = DiaSorin Corporation (Stillwater, MN) Radioimmunoassay;

LIAISON = DiaSorin LIAISON® one semi-automated chemiluminescence method;

\*CCC (95%-CI) = Concordance correlation coefficient and 95%-confidence interval; \*\*absolute differences calculated using the first two replicates for each subject; †SD = Standard deviation, r = Pearson's correlation coefficient.

The greatest numerical difference in 25(OH)D results for an individual was 61nmol/l and was recorded by the laboratory with the highest mean CV (Table 2). The maximum difference between replicates was similar for the other laboratories using DiaSorin LIAISON®. In some instances, measurements of 25(OH)D performed on the same serum by the same laboratory varied by up to 97%, and replicates for at least one individual in all laboratories, except one, varied by more than 35% (Table 2).

Overall, 40% of the 102 participants had replicate samples which differed by 20% or more, 29% of participants had results which differed by 30% or more, and 15% of participants had results which differed by more than 50% and 13% differed by more than 60%. The proportion of subjects who were considered vitamin D insufficient (25-50nmol/l) based on the results of one replicate and adequate (>50nmol/l) on another was 13% (Diamond et al., 2005). Five of the eight laboratories had replicates which crossed the clinical cut-point of 50nmol/l (Table 2).

**Table 2: Coefficient of variation for blinded replicate measurements of serum 25(OH)D undertaken in eight large clinical laboratories in three Commonwealth countries in 2006-2007.**

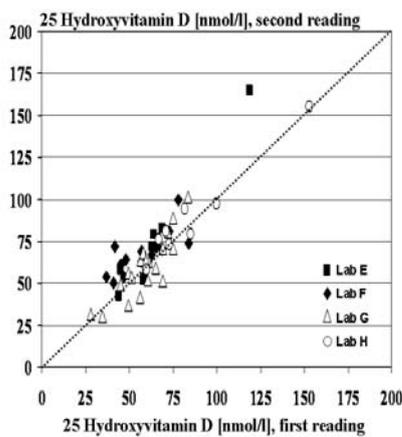
Laboratory; sample size (subjects with more than 2 replicates); [assay]	Coefficient of variation (%) and (SD)*	Min & Max difference between any two replicates	Max % difference between any two replicates	Proportion categorized differently+
Lab A n=14 (4) [LIAISON]	18.2 (14.0)	2 – 60 nmol/l	97%	3/14= 21.4%
Lab B n=9 (8) [LIAISON]	16.2 (10.3)	0 – 60 nmol/l	80%	0/9 = 0%
Lab C n=9 (8) [LIAISON]	8.3 (5.1)	2 – 47 nmol/l	47%	0/9=0%
Lab D n=19 (0) [LIAISON]	18.3 (12.5)	2 – 61 nmol/l	82%	2/19=10.5%
Lab E n=8 (0) [RIA]	12.1 (6.3)	2 – 46 nmol/l	39%	1/8=12.5%
Lab F n=16 (4) [RIA]	12.9 (9.5)	4 – 30 nmol/l	71%	5/16 = 31.3%
Lab G n=19 (0) [RIA]	9.6 (6.1)	1.6 – 17.9 nmol/l	36%	2/19=10.5%
Lab H n=8 (0) [RIA]	4.8 (4.0)	0 – 12 nmol/l	15%	0/8=0%

+Proportion of subjects who were considered insufficient based on the results of one replicate and adequate on another (vitamin D deficiency defined as serum 25(OH)D <25nmol/l; insufficiency as 25-50 nmol/l and adequacy >50 nmol/l); \*SD = standard deviation.

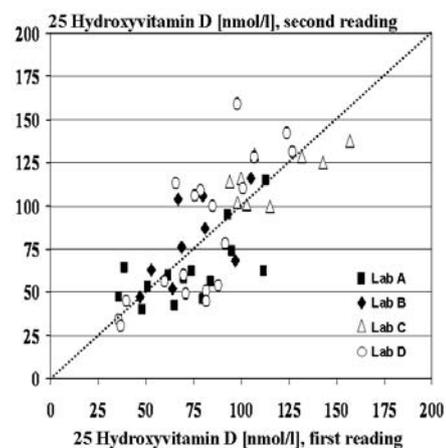
One laboratory using DiaSorin RIA produced a significant correlation between the averages and the absolute differences of the replicates ( $r=0.97$ ;  $p<0.001$ ) indicating larger differences between the replicates with larger values (Table 1). Replicates

tested using DiaSorin RIA were generally more similar and therefore closer to the line of equality on the scatter plot (Figure 1) than replicates tested using DiaSorin LIAISON® (Figure 2).

**Figure 1:** Comparison of first and second replicate serum samples (n=51 pairs) tested by four laboratories using the DiaSorin 25(OH)D Radioimmunoassay.



**Figure 2:** Comparison of first and second replicate serum samples (n=51 pairs) tested by four laboratories using the DiaSorin LIAISON® 25(OH)D assay.



## DISCUSSION

This study identified substantial imprecision in 25(OH)D testing conducted using the two assays most commonly used in Australasia and North America in 2006-2007, and still widely used today. Duplicate measurements in some laboratories differed by almost 100%, and only one of the eight participating laboratories measured 25(OH)D with high precision and a low CV. Our findings indicate that considerable caution is required both in using these assays for clinical practice, and in interpreting the findings of epidemiological studies based on measurements using these assays.

The laboratories conducted the testing on a commercial basis, were blinded to conceal duplicates and used routine methods of collection, storage and testing. However, as they were aware the samples were for research, they may have taken additional care when reporting results. Although there did not appear to be a difference in precision with geographic location, performance was generally worse in laboratories using DiaSorin LIAISON® (CCC 0.57-0.68; CV 8.3%-18.3%) than in laboratories using DiaSorin RIA (CCC 0.49-0.97; CV 4.8%-12.9%). This is a concern as several laboratories in Australia have recently switched from the manual RIA to the less precise semi-automated LIAISON® system to cope with the growing demand for clinical 25(OH)D testing.

While CVs are well known in the biochemical community (Morris, 2005), they are not intuitive and are often poorly understood by policy makers and clinicians (Nowak et al, unpublished data). For example a CV of 18% does not mean the error in a 25(OH)D result varies by 18% in either direction. The CV is a statistical measure calculated as the standard deviation divided by the mean of a group of readings (Sokal and Rohlf, 1995). It is used to compare the variability of different populations with vastly different mean values. In laboratory D the CV was 18.3% but the maximum difference between replicates was 82%, i.e. one divided blood draw (treated identically and tested at the same time) was reported as 45nmol/l (insufficient) and 82nmol/l (adequate): the lower value could result in treatment whereas the higher value would not. Overall, the doctors of 13% of participants would have received one result identifying their patient as vitamin D insufficient, and another classifying their level as adequate. Furthermore, the results from 40% of participants yielded duplicates which differed by 20% or more, while replicates from 15% of participants differed by more than 50%.

In a large population-based study, serum 25(OH)D was measured using DiaSorin RIA and CVs ranging from 10%-25% (depending on concentration) were reported without discussion (Looker et al., 2002). In analytical and clinical validation assessments, within-run CVs of up to 13% were reported for DiaSorin LIAISON® (Ersfeld et al., 2004), and CVs of less than 9% were reported for both DiaSorin RIA and another RIA method (Hollis, 2000), and even though one would expect these analyses to be conducted under ideal conditions (thus producing lower CVs (Hollis, 2000; Ersfeld et al., 2004) than the present study), our results demonstrate that DiaSorin LIAISON® tends to produce higher CVs than DiaSorin RIA.

In addition, non-automated 25(OH)D assays appear to be dependant on the skills of the operator (Carter et al., 2004). Our results support this finding, as one laboratory (H) using the manual RIA had considerably higher precision (CCC 0.97; CV 4.8%) than other laboratories using the same method. Thus, it is possible that the imprecision is partly due to the actual test,

and partly due to pre-analytical error associated with routine clinical testing, in contrast to the stricter conditions that may occur in research studies. Nevertheless, it is imperative that the tests are sufficiently robust under routine conditions.

Quality control of 25(OH)D testing is conducted by DEQAS internationally ([www.deqas.org](http://www.deqas.org)) (Carter et al., 2004a; Carter et al., 2004b) and the Royal College of Pathologists Australasia - Australasian Association of Clinical Biochemists Quality Assurance Program (RCPA-AACB QAP) within Australasia ([www.rcpaqap.com.au/chempath/endocrine.html](http://www.rcpaqap.com.au/chempath/endocrine.html)) (Glendenning, 2003). These quality assurance programs (QAPs) monitor the analytical performance of 25(OH)D assays by regularly distributing pooled serum samples to laboratories seeking accreditation. In 2003, only 59% of participating laboratories achieved the generous performance targets set by DEQAS (Carter et al, 2004b) and precision was similar to that reported in the present study, suggesting negligible improvement in the reproducibility of 25(OH)D testing over this period (Morris, 2005).

We know from discussions with laboratory staff that measurements of external QA samples are treated with greater care than specimens sent for routine testing and, that considerable effort is often put into recalibrating instruments prior to testing. In our study, sera were collected from individuals in the normal manner for routine testing (rather than pooled sera), and relabeled (for blinding) ensuring that laboratory staff were unaware of the purpose of the study or that there were replicates. Thus, our results should be more indicative of the usual level of precision (reproducibility) of the 25(OH)D results that clinicians receive to inform patient care, than data collected in QAPs.

Study limitations include the small number of laboratories and participants tested in each laboratory, and the assessment of only two of the available assay kits. The validity of these results should be confirmed using more participants, a larger number of laboratories, and all commercially available 25(OH)D assays.

As the focus of concern about vitamin D shifts from rickets and osteomalacia to the prevention of osteoporosis and potential relevance for non-osseous disease outcomes, the recommended level of serum 25(OH)D is moving from the levels required to prevent frank deficiency to "optimal levels", which are currently being debated (Dawson-Hughes et al., 2005). Assays that were originally intended to provide a clinical assessment of vitamin D deficiency to inform the management of patients with clinical bone disease (Hollis, 2000) are increasingly being used as screening tools in the general population, and indeed in Australia, requests for 25(OH)D testing have increased dramatically in recent years ([www.medicareaustralia.gov.au/statistics/mbs\\_item.shtml](http://www.medicareaustralia.gov.au/statistics/mbs_item.shtml) item 66608). The median and maximum 25(OH)D values encountered in the general population, especially in sunny Australia, are higher than in identified high-risk sub-groups for vitamin D deficiency (e.g. infirmed elderly). Although the performance of 25(OH)D assays may be quite good at lower 25(OH)D concentrations (i.e. levels where vitamin D supplementation is advised) (Carter et al., 2004b), there is some evidence to suggest that the precision of these assays decreases as concentrations increase (Hollis et al., 1993; Ersfeld et al., 2004; Carter et al., 2004b) which may explain why 13% of subjects in the present study with moderate 25(OH)D levels received contradictory results (one indicating insufficiency and another suggesting adequacy [ $>50$  nmol/l]). Clinical dilemmas such as this, resulting from imprecision in the

"adequate" 25(OH)D range, would probably have been rarer in previous decades when 25(OH)D was only usually measured in high-risk patients and recommended 25(OH)D levels were lower than the levels currently suggested to be "optimal" (Dawson-Hughes et al., 2005). Assay-specific decision limits also need to be established as 25(OH)D results can vary substantially with the assay used (Glendenning, 2003). However, neither "optimal levels" nor "decision limits" can be set without an accurate and precise assay.

The increased level of vitamin D testing involves a large financial burden which should be questioned by governments, which subsidize biochemical testing, as well as doctors and their patients, if current testing procedures are as imprecise as we report. Assay manufacturers should focus their efforts on developing a rapid, high volume throughput 25(OH)D assay that is sufficiently robust for non-specialist laboratories to produce accurate and precise measurements of 25(OH)D (Morris, 2005) across the entire spectrum of concentrations. Theoretically, such an assay could be used to inform clinical management of patients at high-risk of vitamin D deficiency, with the added versatility of being suitable for population-based screening programs and research to inform public health policy, than the two assays considered here. Furthermore, clinical validation studies should include sera collected in populations residing in tropical and sub-tropical areas, as well as temperate zones, particularly with the suggestion that vitamin D levels may have a diverse range of implications for human health (Holick, 2004; Ponsonby et al., 2005), with greater geographic relevance than previously thought (Nowson and Margerison, 2002).

Our results suggest that clinicians and public health authorities may be making decisions on the need for treatment or changes to public health policy based on imprecise data. If the clinical response is unnecessary vitamin D supplementation, this may not be a serious concern as long-term supplementation at "normal levels" appears to cause no harm (Autier and Gandini, 2007). However, if the response in Australasia includes intentional sun exposure, it may undermine many years of public health education about sun safe behaviour, and trigger a further rise in the incidence of skin cancer.

## CONCLUSION

Recent interest in the relevance of vitamin D to human health has increased demand for 25(OH)D testing and associated costs. Our results suggest clinicians and public health authorities are making decisions about treatment or changes to public health policy based on imprecise data. Development of a rapid, reproducible, accurate and robust assay should be a priority due to interest in population-based screening programs and research to inform public health policy about the amount of sun exposure required for human health. In the interim, it is essential that public health professionals and clinicians are made aware of the lack of precision in routine vitamin D testing and that laboratory results include a statement of measurement uncertainty.

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