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# Determination of Reference Intervals for Serum Total Vitamin D in Indian Population

**Dr. Gurmeet Singh[1], Dr. B. Rajsekhar[2], Dr Suchitra Reddy[3], Dr. Sabitha Devi. R.[4], Dr. K. A. Pasha[5]**

[1]MD, Department of Pathology, MMU, Medical College, Solan

[2]MD, Professor, Department of Pathology, P. K. Das Medical College, Kerala

[3]Prosthodontist Reader, BIDS, Banaglore

[4]Associate Professor, SJDC, Eluru

[5]Consultant Prosthodontist

**Abstract:** ***Widespread vitaminD in sufficiency raises concerns regarding their liability of reference intervals for serum vitaminD levels. We sought to determine the reference intervals for serum vitamin D[25-hydroxyvitamin D [25(OH)D]] deficiency [<20ng/mL(20nmol/L)by global definition]. This was a retrospective study of laboratory data obtained from all patients at P.K.Das medical college from April 2015 to June-2016, through 2000 subjects. Patients taking vitaminD supplement were excluded .Total serum 25(OH)D was determined by chemiluminescence method(CLIA) using AbbottimmunologyanalyzerArchitectI1000. We as curtained above 2000 (Male –999, Female – 1130) subjects who had a serum 25(OH) D <50ng/mL. Parametric analyses generated age-specific reference intervals for serum for each of several age groups (Males10yrs–40yrs,40yrs–90 yrs and females10 yrs– 40 yrs,40yrs– 90 yrs). A two-way ANOVA with Tukey's correction showed significant differences between the lower limits of normal (P<.001) and the normal range (P<.001) but not for the upper limit of normal for these subjects compared with unselected subjects. Sources of vitaminD: The major source of vitamin D for most humans is exposure to sunlight .Seasonal variation is found in the major circulating form of vitamin D, 25-hydroxy vitamin D [25(OH) D]. Few foods naturally contain vitamin D, including oily fish such as salmon, mackerel, and herring and oils from fish, including cod liver oil. Recent studies has observed that wild-caught salmon had on average 500–1000IUvitamin Din100g (3.5ounces), where as farmed salmon contained 100–250IU vitaminD per 100-gserving.The most likely reason is that vitamin D is plentiful in the food chain but is not plenty full in the pelleted diet fed to farmed salmon. In the United States, milk, some juice products, some breads, yogurts, and cheeses are fortified with vitamin D. Multivitamins that contain 400IUvitaminD and supplements containing vitamin D only are now available in various amounts including 400, 1000, 2000, 4000, 5000 and 50000IU vitamin D3.The pharmaceutical form of vitamin D in the United States is vitaminD 2 and is available as 50000IUvitaminD2 in a capsule or 8000 IU vitamin D 2/mL. In Canada, Europe, Japan, and India, vitaminD3 is available as a pharmaceutical. Causes of vitamin D deficiency: The major source of vitamin D for humans is exposure to sunlight .Anything that diminishes the transmission of solar UVB radiation to the earth's surface or anything that interferes with the penetration of UVB radiation in to the skin will affect the cutaneous synthesis of vitaminD3 Melanin is extremely efficient in absorbing UV B radiation, and, thus, increased skin pigmentation markedly reduces vitamin D 3synthesis. Similarly, a sunscreen with a sun protection of 15absorbs99% of the incident UVB radiation, and, thus, when topically applied properly will decrease the synthesis of vitaminD3 in the skin by99%. African Americans with very dark skin have an SPF of 15, and, thus, their ability to make vitamin D in their skin is reduced by as much as 99%. This along with decreased milk in take are the explanations for why most African Americans who live in a temperate climate are vitamin D deficient, where as Africans living in earth equator where vitamin D 3 synthesis is more efficient because of the higher flux of UVB photons are not.***

**Keywords:** 25 –Hydroxy vitamin D, Age specific reference intervals

**Definition:** Vitamin D, 25OHVit D are synonymous with Total Vit D3.

## 1. Introduction

These reference intervals refine previous normal ranges that likely included subjects with vitamin D deficiency. Reference intervals are conventionally determined using data sets comprised of test results obtained for a specific apparently healthy population and generating 95% confidence interval to define the normal range.

The assumption under laying this approach is that only a small proportion of a normal population. Will consist of subjects with an abnormal test result, and thus, the effect of these outliers will not influence the final reference interval. Here were ports an innovative approach to determination of age-adjusted reference intervals for vitamin D deficiency. Our results provider fined reference intervals for serum concentrations of 25(OH)D.

## 2. Materials & Methods

We measured total serum25OHVIT-D using chemiluminessence method (CLIA) using Abbott immunology analyzer Architecti1000.

This assay uses calibrators trace able to the certified National Institute of Standards and Technology reference material.

Retrospective data was analyzed after ensuring that QC was acceptable on each day of testing.

We reviewed serum concentrations25 (OH)D that had been determined during the calendar year2015-2016 through 2163 subjects from all age wise male and female (10-40,40-90) at P. K. Das medical college.

Vitamin D [25-hydroxyvitamin D(25[OH]D)] deficiency (25 [OH] D< 20ng/dL and insufficiency [25(OH)D <30 ng/dL needs to be evaluated in any given population to avoid unnecessary treatment in non-deficient subjects/ individuals.

Data from LIMS was collected for the retrospective study.

Certified reference material and external quality control samples were analyzed to meet the Standards out lined by the National Institute of Standards and Technology. Validation Step included recovery and both precision and accuracy under inter- and intraday variation limit of detection and analysis of the analyte over a line arrange as described in Clinical and Laboratory Standards Institute guidelines.

We excluded patients who had a serum 25(OH) D concentration more than 50 ng/mL. These criteria yielded unique vitamin-D values for 2163 (male 999, female 664subjects). For comparison, values for all unique vitamin-D measurements without any selection of patients were generated simultaneously. We used Medcalc version 9 (Data In-novations, Inc) in accordance with Clinical and Laboratory Standards Institute guidelines to generate age- specific normal reference intervals. Using these datasets, we determined reference intervals for serum calcium in the patients selected for 5-to95

## 3. Definitions

**Reference Interval**

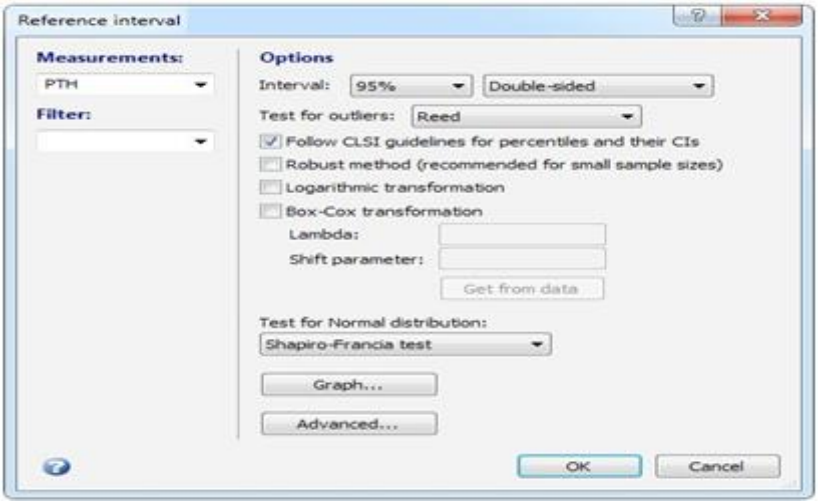
### 3.1 Description

A Reference interval (Reference range, Normal range)can be calculated using the following 3 methods: (a) using the Normal distribution, (b) using a non-parametrical percentile method, and (c)optionally "robust method" as described in the CLSI Guidelines C28-A3.

The program will give the90, 95 or 99% Reference interval, double sided or left or right sided only, as selected in the dialog box. The reference interval is calculated using3different methods: (a) using the Normal distribution (Bland,2000;CLSI 2008), (b)using a non- parametrical percentile method, and (c) optionally a "robust method" as described in the CLSI GuidelinesC28-A3.90%Confidence Intervals are given for the reference limits.

For the robust method the confidence intervals are estimated with the boot strap method(percentile interval method, Efron& Tibshirani,1993).When sample size is very small and/ or the sample contains too many equal values, it may be impossible to calculate the CIs.

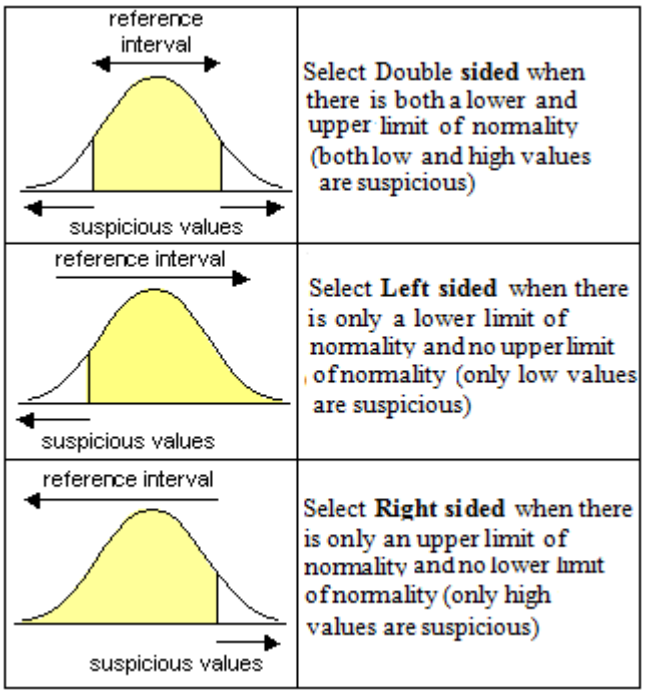
The results from the Normal distribution method are not appropriate when the Test for Normal distribution (see above) fails. If the sample size is large (120ormore) the CLSI C28-A3guideline recommends the percentile method and for smaller sample sizes the "robust method" is recommended.




The minimal sample size of 120 for the percentile method is the minimum number required to calculate 90% Confidence Intervals for the reference limits. A higher number of cases is required to achieve more reliable reference limits with more narrow 90 % Confidence Intervals.

### 3.2 Required input Options

- **Reference interval**: you can select a **90%, 95% or 99%** reference interval.A95% interval is the most usual and preferred setting.
- **Double sided, left or right sided**




**Test for outliers**: select the method based on Reedetal. (1971)or Tukey (1977)to automatically check the measurements for outliers(alternatively select *none* for no outlier testing).The method by Reedetal. Will test only the minimum and maximum observations; the Tu key test can identify more values as outliers. The tests will create a list of possible outlying observations, but these will not automatically be excluded from the analysis. The possible outliers should be inspected by the investigator who can decide to exclude the values (see Exclude &Include).

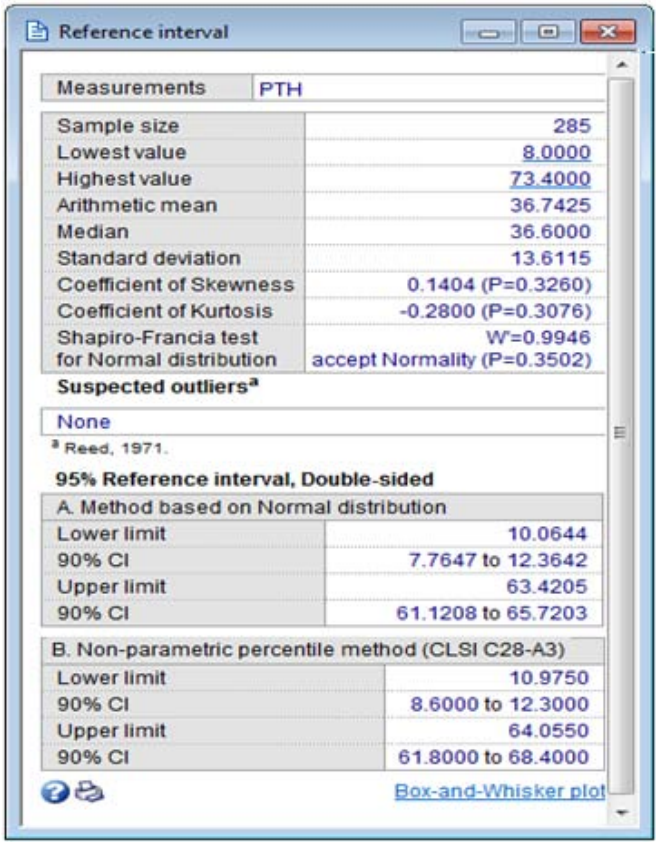
For other methods for outlier detection see Outlier detection.

**Follow CLSI guide lines for percentiles and their CIs**: select this option to follow the NCCLS and Clinical and Laboratory Standards Institute (CLSI) guidelines C28-A2 and C28-A3 for estimating percentiles and their90% confidence intervals. In these guide lines, percentiles are calculated as the observations or responding to rank r=p*(n+1).Also for the 90% confidence intervals of the reference limits the CLSI guidelines are followed and conservative confidence intervals are calculated using integer ranks (and therefore the confidence intervals are at least 90% wide). If you do not select this option, MedCalc calculates percentiles as the observations or responding to rank r=p*n+0.5 (Lentner, 1982; Schoonjansetal.,2011),and calculate sales conservative and more precise confidence interval using an iterative method.

**Robust Method**: Select this option to calculate the reference limits with the "robust method" (CLSI Guidelines C28-A3). Recommended for smaller sample sizes(lessthan120). With the Robust method, the confidence intervals for the reference limits are estimated using boot strapping (percentile interval method, Efron & Tibshirani,1993). Click the Advanced...button for boot strapping options such as number of replications an random-number seed.

- **Logarithmic transformation**: if the data require a logarithmic transformation(e.g. when the data are positively skewed),select the Logarithmic transformation option.
- **Box-Coxtrans formation**: This will allow to perform a Box-Coxtrans formation with the following parameters:
- **Lambda**: the power parameter λ
- **Shift parameter**: the shift parameter is a constant *c* that needs to beaded to the data when some of the data are negative.
- **Test for Normal distribution**: see Tests for Normal distribution.
- **Graph**: click this button for graph options(see below).
- **Advanced**: boot strapping options for the calculation of confidence intervals with the Robust method.

**Results-format**


Reference interval

| Measurements | PTH |
|---|---|
| Sample size | 285 |
| Lowest value | 8.0000 |
| Highest value | 73.4000 |
| Arithmetic mean | 36.7425 |
| Median | 36.6000 |
| Standard deviation | 13.6115 |
| Coefficient of Skewness | 0.1404 (P=0.3260) |
| Coefficient of Kurtosis | -0.2800 (P=0.3076) |
| Shapiro-Francia test for Normal distribution | W'=0.9946 accept Normality (P=0.3502) |

**Suspected outliers**[a]

None

[a] Reed, 1971.

**95% Reference interval, Double-sided**

| A. Method based on Normal distribution | |
|---|---|
| Lower limit | 10.0644 |
| 90% CI | 7.7647 to 12.3642 |
| Upper limit | 63.4205 |
| 90% CI | 61.1208 to 65.7203 |

| B. Non-parametric percentile method (CLSI C28-A3) | |
|---|---|
| Lower limit | 10.9750 |
| 90% CI | 8.6000 to 12.3000 |
| Upper limit | 64.0550 |
| 90% CI | 61.8000 to 68.4000 |

Box-and-Whisker plot

- **Standard Deviation**: the standard deviation is the square root of the variance. When the distribution of the observations is Normal, then95% of observations are located in the intervalMean±2SD.

- **Skewness**: the coefficient of Skewness is am ea sure forth e degree of symmetry in the variable distribution. If the corresponding P-value is low(P<0.05)then the variable symmetry is significantly different from that of a Normal distribution, which has a coefficient of Skewness equal to0(Sheskin, 2011) (see Skewness &Kurtosis).

**Kurtosis**: The coefficient of Kurtosis is a measure for the degree of peakedness/flatness in the variable distribution. If the corresponding P-value is low(P<0.05)then the variable peakedness is significantly different from that of a Normal distribution, which has a coefficient of Kurtosis equal to0 (Sheskin,2011) (see Skewness &Kurtosis).

**Test for Normal Distribution**: The result of this test is expressed as' accept Normality 'or' reject Normality', with P value. If P is higher than 0.05,it may be assumed that the data follow a Normal distribution and the conclusion 'accept Normality' is displayed. IfPislessthan0.05, then the hypothesis that the distribution of the observations in the sample is Normal, should be rejected, and the conclusion 'reject Normality 'is displayed.

***Log transformation***

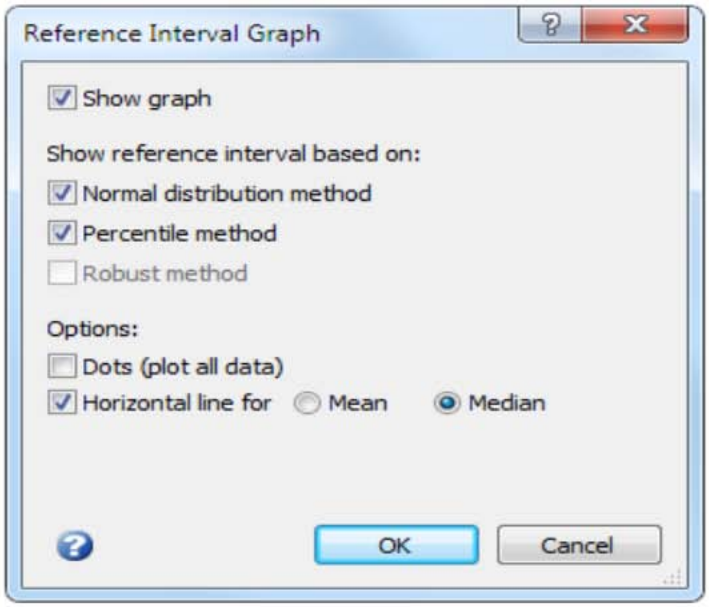
If the option Log transformation was selected, the program will display the back-transformed results. The back-transformed mean is named the Geometric mean. The Standard deviation cannot be back-transformed meaningfully and is not reported.

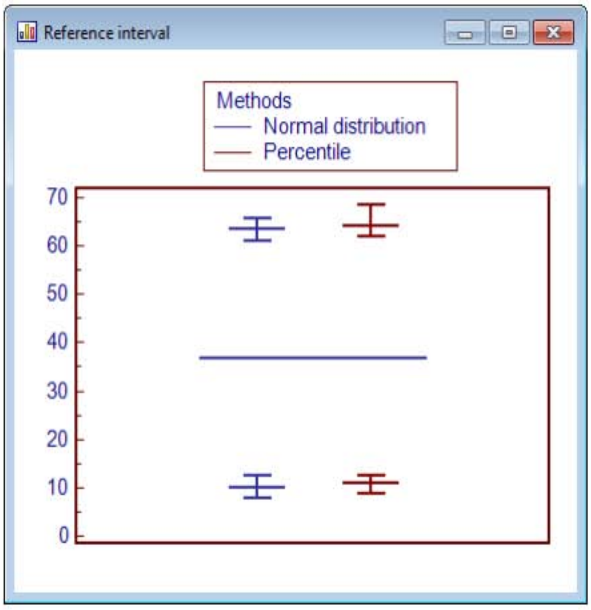
***Suspected outliers***

The program produces a list of possible outliers, detected by the methods based on Reedetal. (1971)or Tukey(1977).The method by Reedetal. tests only the minimum and maximum observations; the Tukey test can identify more values as outliers. Note that this does not automatically exclude any values from the analysis. The observations should be further inspected by the investigator who can decide to exclude the values. Click on the listed values (which are displayed as hyper links) to show the corresponding data in the spread sheet (see Exclude &Include).

***Graph***

Click the **Graph** but to ninthe dialog box shown above to obtain the following Reference Interval Graph box:

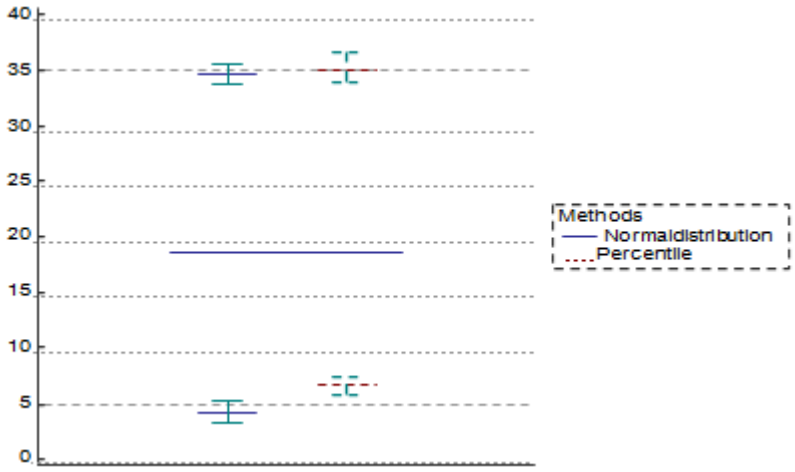



This results in the following graph:

## 4. Results

Male double sided ref. Intervals 10- 40 Yrs



| Measurements | Readings |
|---|---|
| Sample size | 500 |
| Lowest value | 4.5000 |
| Highest value | 39.8000 |
| Arithmetic mean | 19.4672 |
| Median | 18.6500 |
| Standard deviation | 7.7515 |
| Coefficient of Skewness | 0.3705(P=0.0009) |
| Coefficient of Kurtosis | -0.6402(P<0.0001) |
| Shapiro-Francia test for Normal distribution | W'=0.9769 reject Normality (P<0.0001) |

**Suspected outliers**[a]

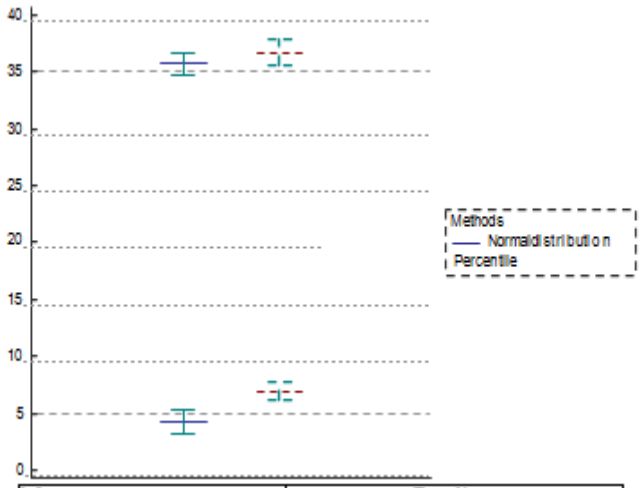
None

[a]Reed,1971

**95% Reference interval, Double-sided**

| A. Method based on Normal distribution | |
|---|---|
| Lower limit | 4.2745 |
| 90% CI | 3.2862to5.2627 |
| Upper limit | 34.6599 |
| 90% CI | 33.6717to35.6482 |
| B. Non-parametric percentile method (CLSI C28-A3) | |
| Lower limit | 6.6000 |
| 90% CI | 5.7000 to7.4000 |
| Upper limit | 35.0000 |
| 90%CI | 33.9000 to36.6000 |

Male Double Sided Ref. Intervals 40- 90 Yrs




| Measurements | Readings |
|---|---|
| Sample size | 499 |
| Lowest value | 4.0000 |
| Highest value | 39.4000 |
| Arithmetic mean | 20.0228 |
| Median | 19.5000 |
| Standard deviation | 7.9895 |
| Coefficient of Skewness | 0.3068(P=0.0056) |
| Coefficient of Kurtosis | -0.6656(P<0.0001) |
| Shapiro-Francia test for Normal distribution | W'=0.9795 reject Normality (P<0.0001) |

**Suspected outliers**[a]

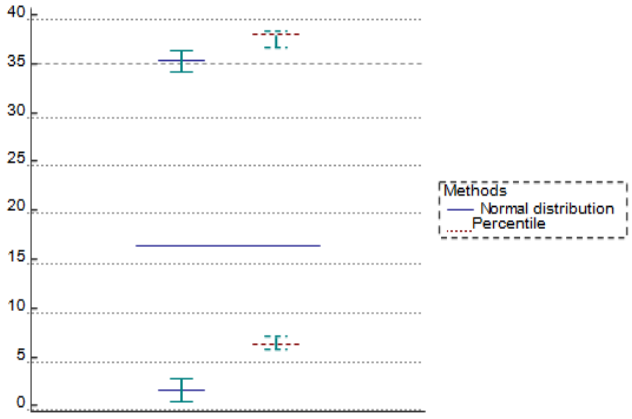
None

[a]Reed, 1971

**95% Reference interval, Double-sided**

| A. Method based on Normal distribution | |
|---|---|
| Lower limit | 4.3638 |
| 90%CI | 3.3442to5.3834 |
| Upper limit | 35.6819 |
| 90%CI | 34.6622to36.7015 |
| B. Non-parametric percentile method (CLSI C28-A3) | |
| Lower limit | 6.9000 |
| 90%CI | 6.3000to7.9000 |
| Upper limit | 36.7000 |
| 90%CI | 35.6000 to37.9000 |

Female double sided ref. Intervals 10- 40 Yrs




| Measurements | Readings |
|---|---|
| Sample size | 506 |
| Lowest value | 3.9 |
| Highest value | 43.1 |
| Arithmetic mean | 18.4417 |
| Median | 16.3 |
| Standard deviation | 8.6183 |
| Coefficient of Skewness | 0.6658(P<0.0001) |
| Coefficient of Kurtosis | -0.5019(P=0.0024) |
| Shapiro-Francia test for | W'=0.9412 reject |

**Suspected outliers**[a]

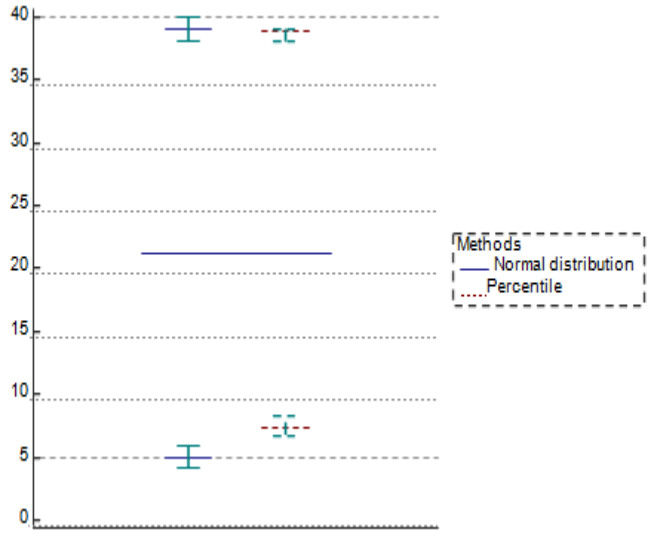
None

[a]Reed,1971.

**95% Reference interval, Double-sided**

| A. Method based on Normal distribution | |
|---|---|
| Lower limit | 1.5502 |
| 90% CI | 0.4579to2.6424 |
| Upper limit | 35.3332 |
| 90%CI | 34.2410to36.4255 |
| B. Non-parametric percentile method(CLSI C28-A3) | |
| Lower limit | 6.2675 |
| 90% CI | 5.7000to7.0000 |
| Upper limit | 38 |
| 90% CI | 36.6000to38.3000 |

Female double sided ref. Intervals 40- 90 Yrs




| Measurements | Readings |
|---|---|
| Sample size | 653 |
| Lowest value | 2.8 |
| Highest value | 47.8 |
| Arithmetic mean | 22.0187 |
| Median | 21.3 |
| Standard deviation | 8.658 |
| Coefficient of Skewness | 0.2422(P=0.0119) |
| Coefficient of Kurtosis | -0.7779(P<0.0001) |
| Shapiro-Francia test for Normal distribution | W'=0.9793 reject Normality(P<0.0001) |

**Suspected outliers**[a]

None

[a]Reed,1971.

**95% Reference interval, Double-sided**

| A. Method based on Normal distribution | |
|---|---|
| Lower limit | 5.0492 |
| 90% CI | 4.0835to6.0150 |
| Upper limit | 38.9881 |
| 90%CI | 38.0224to39.9539 |
| B.Non-parametric percentile method(CLSI C28-A3) | |
| Lower limit | 7.335 |
| 90% CI | 6.8000to8.4000 |
| Upper limit | 38.865 |
| 90% CI | 38.0000to39.1000 |

## 5. Discussion

VitaminD refers to a group of fat-solubles costeroids responsible for enhancing intestinal absorption of calcium, iron, magnesium, phosphate and zinc. In humans, the most important compounds in this group are vitaminD3 (also known as cholecalciferol) and vitaminD2 ergocalciferol).[1] Cholecalciferol and ergocalciferol can be ingested from the diet and from supplements. The body can also synthesize vitamin D (specifically cholecalciferol) in the skin, from cholesterol, when sun exposure is adequate (hence its nick name, the "sunshine vitamin").

Synthesis from exposure to sunlight and in take from the diet generally contributes to the maintenance of adequate serum concentrations. Evidence indicates the synthesis of vitaminD from sun exposure is regulated by a negative feedback loop that prevents toxicity, but because of uncertainty about the cancer risk from sunlight, no recommendations are issued by the Institute of Medicine, USA, for the amount of sun exposure required to meet vitaminD requirements .Accordingly, the Dietary Reference Intake for vitaminD assumes no synthesis occurs and all of a person's vitaminD is from food intake, although that will rarely occur in practice. As vitamin D is synthesized in adequate amounts by most mammals exposed to sunlight, it is not strictly a vitamin, and may be considered a hormone as its synthesis and activity occur in different locations. Its discovery was due to effort of in d the dietary substance lacking in rickets (the childhood form of osteomalacia).

Beyond its use to prevent osteomalacia or rickets, the evidence for other health effects of vitaminD supplementation in the general population is in consistent. The best evidence of benefit is for bone health. The effect of vitaminD supplementation on mortality is not clear, with one meta-analysis finding a decrease in mortality in elderly people, and another concluding no clear justification exists for recommending vitamin D. Because it found mounting evidence for a benefit to bone health, and though it had not found good evidence of other benefits, the FDA intends to begin requiring manufacturers to declare the amount of Vitamin D on Nutrition Facts labels, because they are now "nutrients of public health significance." It's also preparing to increase the minimum recommended Daily Value(DV) for Vitamin D from15 mcgto20 mcg.

In the liver, cholecalciferol(vitaminD3) is converted to calcidiol, which is also known as calcifediol (INN), 25-hydroxyl cholecalciferol, or 25-hydroxy vitaminD3—abbreviated25(OH)D3.Ergocalciferol(vitaminD2)is converted in the liver to 25-hydroxyergocalciferol,also known as 25-hydroxyvitaminD2 — abbreviated 25( OH) D2. These two specific vitamin D me tabolites are measured in serum to determine a person's vitaminD status. Part of the calcidiol is converted by the kidneys to calcitriol, the biologically active form of vitamin D. Calcitriol circulates as a hormone in the blood, regulating the concentration of calcium and phosphate in the blood stream and promoting the healthy growth and remodeling of bone. Calcitriol also affects neuromuscular and immune function.

## 6. Our Study

1) Double sided reference interval study of serum vitamin D for males between age group of 10to40years the sample size and category was 500 lowest value was 4.5 and highest value39.8.with standard deviation of 7.7 515. 1.1.95% confidence interval was generated and we got the lower limit of 4.2745 and upper limit of 34. 6599.
2) Double sided reference interval study of serum vitamin D for males between age group of 40 to 90years the sample size and category was 499 lowest value was 4.0and highest value39.4.with standard deviation of 7.9895. 2.1.95%confidence interval was generated and we got the lower limit of 4.3638 and upper limit of 35.6819.
3) Double sided reference interval study of serum vitaminD for females between age group of 10 to 40 years the sample size and category was 506 lowest value was 3.9 and highest value43.1. with standard deviation of 8.6183.

3.1.95%confidence interval was generated and we got the lower limit of 1.5502 and upper limit of 35.3332

4) Double sided reference interval study of serum vitaminD for females between age group of 40 to 90years the sample size and category was 653 lowest value was2.8 and highest value 47.8with standard deviation of 8.6580. 4.1.95% confidence interval was generated and we got the lower limit of 5.0492 and upper limit of 38.9881.

## 7. Conclusion

Possible limitations of the study:

1) Sample size was relatively low
2) History of patient taking any Vitamin D therapy was not available in retrospective data,
3) In females, there was absence of obstetric status,
4) Study was limited to patients who visited medical college.

The global reference range of the total 25(OH) Dlevelis20-100ng/mL, However, we concluded that the normal reference range of 25(OH) D in our population is between 1.5 to38.9 in different age groups. In current study we understand that the reference range in local population could be lower than global reference range. We also concluded that there is no much variation of vitamin D levels in different age groups in case of males.

The difference in females were not very significant, the slight variation in females may be related to reproductive age of females, antenatal values etc,

**Outcome**

1) Vitamin D treatment, obstetric case, interferes with calculation of exact population reference range for Vitamin D.
2) Ongoing study is mandatory for exact and accurate outcome of vitamin D reference ranges,
3) Separate directory of service is mandatory for patients taking treatment of Vitamin-D, so that bias can be avoided.
4) Vitamin D testing on antenatal samples should also have separate directory of service.

## 8. Interest of Conflict

Nil

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