

## **8 EVALUATION OF BIOLOGICAL REFERENCE INTERVAL OF 25 HYDROXY VITAMIN D IN WEST BENGAL**

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### **Abstract**

#### **Background**

The number of patients visiting pain clinic and being suggested for estimation of 25 hydroxy vitamin D(25OHD) have increased surprisingly. The 25OHD results being estimated by the laboratory have also shown values below the health reference range in majority number of cases. Such a large number of 25OHD deficient patient in adult population without any presentation of classical deficiency symptom except generalized pain and fatigue seems improbable and the author decided to evaluate the local health reference range. The manufacturer also suggests to evaluate the BRI in the laboratory in the inserts. The established Biological reference Interval (BRI)of 25 hydroxy Vitamin D(25OHD) which is popularly known as health reference range and BRI obtained from population based study by the kit manufacturer (Roche Diagnostics) were found out to be not in accordance. The local population results did not correlate with established BRI but nicely correlated with the population study result of the manufacturer.

#### **Method**

Healthy volunteers, male and female were chosen. Selection of healthy population was as per CLSI (Clinical & Laboratory Standards Institute) instruction. Reference range has been established from non parametric distribution. Both male and female patient population were simultaneously evaluated to find out whether 25OHD was the sole attributor of the patient complaints or health reference range should be replaced by population based reference range. Patients are of same age group with the volunteers with no history of addiction and any other history of chronic disease or medication except they are from pain clinic came with the history of joint pain, body ache, fatigue. 25OHD of both patient and healthy volunteer group was estimated in Cobas e 411 system by electro chemi luminescence immunoassay(ECLIA). The pain profile parameters were uric acid (UA), totalcalcium (Ca), C-Reactive protein(CRP) and rheumatoid arthritis factor(RF) were usual test requirements along with 25OHD. The parameters were tested for both patient and volunteer group. The tests were performed in Cobas Integra 400 plus. Number of male healthy volunteers was 120 and female 122 which satisfies the criteria of CLSI for reference range determination.

#### **Conclusion**

The BRI established by the laboratory is in accordance with the population study of Roche Diagnostics. The author mentions the evaluated reference range as "Laboratory Evaluated Reference range" in the test results.

**Key words:**

**BRI(Biological Reference Interval) ,25OHD,Percentile(p),Parametric distribution,Non parametric distribution**

**AAAA Abbreviations**

1. *UA*
2. *Ca*
3. *CRP*
4. *RF*
5. *BRI(Biological Reference Interval)*
6. *25 Hydroxy Vitamin D(25OHD)*

**Introduction**

25 Hydroxy Vitamin D(25OHD) is presently being considered as a popular health check up parameter. As India is a tropical country insufficient exposure to sunlight is supposed to be very uncommon. But in practice, the accumulated patient data when assessed at random ,showed a major population in West Bengal is suffering from 25OHD deficiency. There might be three possibilities:

1. *Present day life style ie., lack of exposure to sunlight , use of Sun blockers having direct effect on 25OHDconcentration.*
2. *Estimation of 25OHD was not popular earlier. So, either there was lack of awareness regarding 25OHD deficiency or evaluation of reference range is necessary.*
3. *In the manufacturers insert(Roche Diagnostics), though Biological Reference Interval(BRI) was stated to be  $\geq 30\text{ng/ml}$  , the range of consensus value for male and female population studied in North Germany reflected a different feature[1]. The patient population value obtained from the data collected from the laboratory resources of present author correlated well with the consensus range. The difference led to the need of evaluation of BRI.*

Extensive studies were made on prevention of Vitamin D deficiency. It was observed that exposure to sunlight and a diet rich in oily fish prevents Vitamin D deficiency[2-4]. The people living near to the equator who are exposed to sunlight without sun protection were stated to have robust level of 25OHD above 30 ng/ml. But in the sunniest areas like Saudi Arabia, United Arab Emirates , Turkey, India and Lebanon adults and children are having 25OHD levels  $<20\text{ng/ml}$  as most of the skins are shielded from the Sun[5,6]. In India a major population is not shielded from Sunlight neither user of Sun blockers. Hence, lack of exposure to sunlight may be ruled out for general population except a fraction of society having different life style as mentioned above. Moreover, the population of West Bengal are accustomed to have milk and oily fish in the diet and though 25OHD  $<20\text{ng/ml}$  have been defined by most experts as

deficiency level no consensus on optimal level were obtained except by Roche Global which is only a study of a fraction of North German population and a study by Bischoff et al [7] recommending optimal concentration of 25OHD 36-40ng/ml and supplementation limit up to 600IU [1, 8]. Unfortunately, there are some studies which were unable to show any positive outcome of 25OHD supplementation [8]. A meta-analysis of seven randomized clinical trials that evaluated the risk of fracture in older persons given 400IU of Vitamin D<sub>3</sub> per day revealed little benefit with respect to the risk of either non-vertebral or hip fracture. In studies using doses of 700-800IU of Vitamin D<sub>3</sub> per day, the relative risk of non vertebral fracture was reduced by 23% and hip fracture by 26% as compared with calcium or placebo[8]. A Women's health initiative study that compared the effects of 400IU of Vitamin D<sub>3</sub> plus 1000mg of calcium per day with placebo in more than 36,000 postmenopausal women confirmed these results and reported an increased risk of kidney stones but no benefit with respect to the risk of hip fracture[8]. Hence, the outcome of such studies raises the question to what extent and to whom Vitamin D supplementation is necessary? Vitamin D being fat soluble vitamin may create toxicity situation if it is not being utilized. So, the question is whether Vitamin D supplementation needs to be done on the basis of health reference range or BRI needs to be redefined.

Several studies have been done showing direct relation of muscle weakness and 25OHD deficiency [6], control of Vitamin D on more than 200 genes [9, 10], link of Vitamin D deficiency with schizophrenia and depression [11]. The dilemma of whom to be declared as deficient yet remained inconclusive. Neither it is easy to accept that 95% population of a State of India are 25OHD deficient and remained undetected for such a long time. Hence, it was felt that a group of volunteers would be selected and their concentration of 25OHD in serum would be estimated. Patient population data and healthy volunteer group data would be compared. Ideally, such study should be interstate and all over India but the author has only access to the local population. On the basis of accumulated data the author may mention the evaluated range as "laboratory defined range" in the test report along with textbook health reference range. But the author found out the reference range has been modified on the year 2008[1] and being implemented as BRI strengthening present study reports'

## **Materials and Methods**

### **Selection of healthy volunteers**

Adult healthy male and female volunteers were chosen. Age limit 25 – 60 years. As per Clinical and Laboratory Standards Institute(CLSI) guideline for BRI determination minimum number of volunteers should be 120[10]. In the present study 122 female and 120 male volunteers opted for the study. The volunteers were detailed about the project and they have given consent to give their blood sample for the study. The volunteers were having no previous history of addiction, no history of

chronic illness, use of any medicine prior 6 months of the study. Neither male nor female volunteers were user of sun blockers. The minimum exposure to sunlight is at least 2 hours in the midday. The volunteers were having no history of backache, joints pain, fatigue, general weakness.

### **Selection of patients**

Age group and history of addiction is same as healthy population. The patient population have shown history of joint pain, backache, general weakness, body ache and diminished energy. Out of 1000 data 120 male and 143 female patient data were selected on the basis of age group & similarity in nature of complaint. The patients were not supplemented Vitamin D prior estimation. Though the patients had history of body ache, joint pain and diminished energy the pain parameters like RF, Ca, UA, CRP were within normal reference range. The clinician never mentioned the patients as case of rheumatoid arthritis (RF were normal), lupus or fibromyalgia. Neither they were suggested to test the Bone Mineral Density(BMD). The patients were suggested to supplement 25OHD on the basis of low 25OHD value as per health reference range( $\geq 30$ ng/ml).

### **Methods**

The tests performed were 25OHD, Uric acid(UA), Total Calcium(Ca), C-Reactive Protein(CRP) and Rheumatoid Factor (RF). For the patient group the tests were suggested by the Clinician of Pain Clinic as the tests are in the standard pain clinic profile. For healthy volunteers also similar profile of tests were performed. 25OHD was estimated by electro-chemiluminescence immunoassay (ECLIA) in Cobas e 411 system. UA, Ca were estimated by Cobas Integra 400 plus system. CRP & RF were estimated in EC-5 batch analyser by Immuno turbidimetric method. Internal quality control results were satisfactory. UA estimated by uricase method and Ca by OrthoCresol phthalein complexone method using BAPTA buffer. All the parameters are under Proficiency testing program with z-score below 2. All the parameters are under the scope of accreditation by the National Accreditation Body of India. Test methods adopted were IFCC certified.

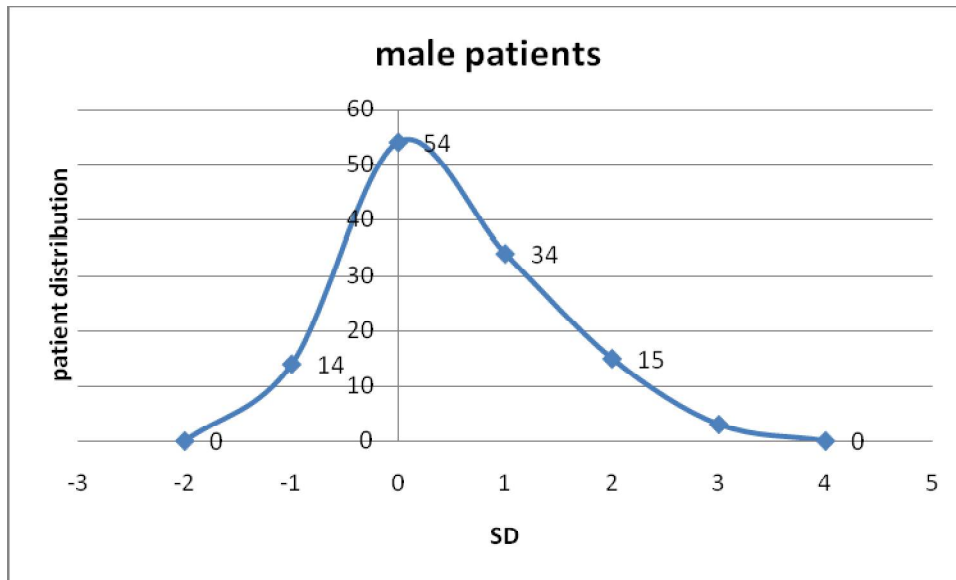
### **Statistical Calculation**

For BRI determination non parametric distribution analysis has been suggested by CLSI. But in the present study both parametric and non parametric distribution analysis were performed to evaluate the difference. In parametric statistical analysis only mean and SD for all the parameters tested were done. CV% determination is not applicable for population study due to wide variance in results. In non parametric statistical analysis 2.5percentile-97.5 percentile distribution was considered for reference range evaluation. For other parameters (UA, Ca, CRP, RF) mean and SD of the results were calculated. Textbook statistical guideline has been used for result analysis(11).

### **Results and Discussion**

The patient data would show why the determination of BRI was felt to be necessary. Almost 90% of the patient population were found out to be Vitamin D deficient as per manufacturer's range[ $\geq 30$  ng/ml]. The results & distribution of selected male patient population is given in Figure[1],Table [1]:

**Figure [1]: Parametric distribution of 25OHD of 120 male patients**



**Table [1]: Mean & SD of 25OHD,UA,Ca,CRP & RF120male**

**patients**

Statistical Parameter	25OHD (ng/ml)	UA(mg/dl)	Ca(mg/dl)	CRP(mg/L)	RF(IU/ml)
Mean	17.57	5.31	9.24	3.86	10
SD	7.53	1.12	0.404	0.79	5

**Y axis : number of patients in the range of -2SD to 4SD.**

**X axis: SD; 1SD is 7.53 ng/ml 25OHD .0:Mean value:17.57ng/ml[Table 1].**

- **On Y axis : exact number of patient within the SD range.**

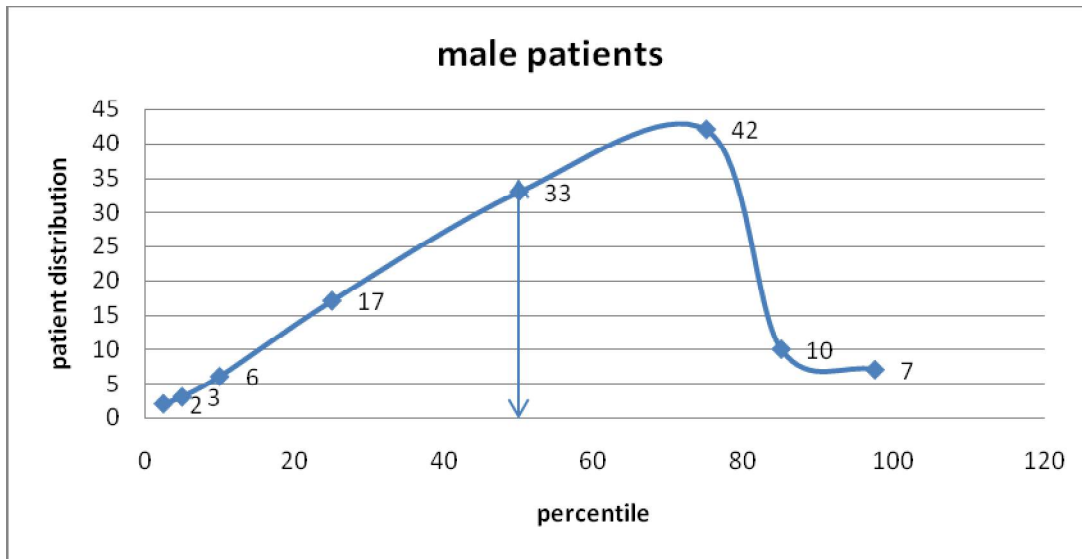
**\*Same graphical pattern for Figures [1,3,5,7],Tables[1,3,5,7].**

102 male patients were within  $\pm 1$ SD range(10.04-25.1ng/ml). Other parameters are within normal reference interval. Hence, non parametric distribution of 25OHD was done. For other parameters

BRI were already established. So, patient distribution analysis were not necessary.

The patient distribution in the parametric distribution curve suggests maximum population remains within -1SD to +2SD. The range is( 10.04 – 32.63 )ng/ml. The range covers 117 patients ie, 97.5% population.

**Figure[2]: Non parametric distribution of 25OHD of 120 male patients**



Median(similar pattern, Figure 2, 4, 6, 8)

**Table [2]: 25OHD result distribution of 120 male patients**

Value(ng/ml)	Percentile	Number of patients
5.5	2.5	2
6.5	5	3
8.3	10	6
12.6	25	17
16.5	50	33
22.8	75	42
25.5	85	10
39.7	97.5	7

**Y axis : number of patients in the range of 0 to 100 percentile.**

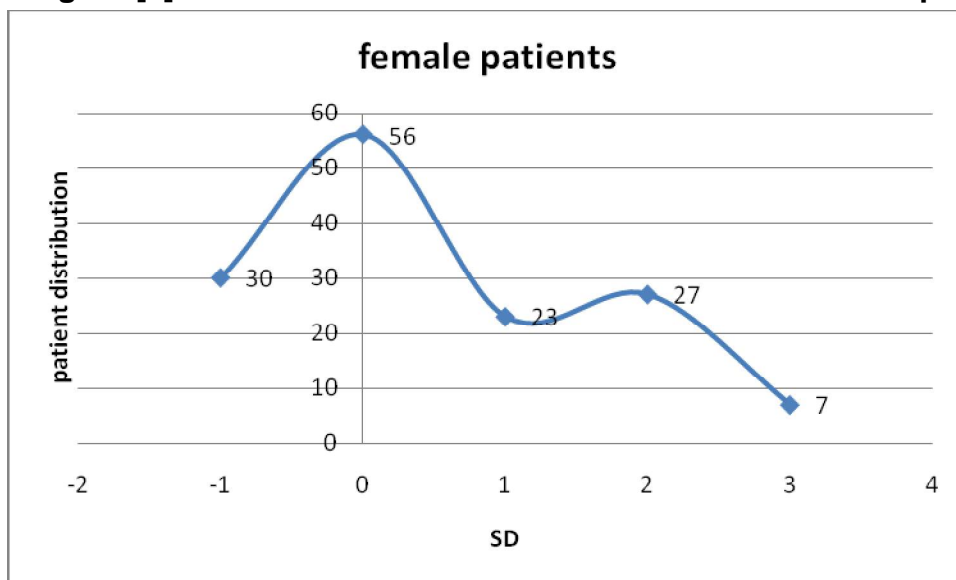
**X axis: percentile range. Distribution as per [Table 2].**

- **On Y axis : exact number of patient within the percentile range.**

**\*Same graphical pattern for Figures [2,4,6,8], Tables[2,4,6,8].**

10percentile to 97.5 percentile covers the total population. 95.8 percent of the male patient population are within the range of ( 8.3-39.7) ng/ml. The parametric and non parametric distribution data are very close. Non parametric distribution ranges are generally wider. The decision range for the male patient population is ( 8.3-39.7)ng/ml. Number of female patient data collected were 143. Number of collected data were more than 800 but the author eliminated other patients as per patient elimination criteria ie the patients priory supplemented with Vitamin D and age group not at par with healthy volunteers were eliminated.

**Figure [3]: Parametric distribution of 25OHD of 143 female patients**



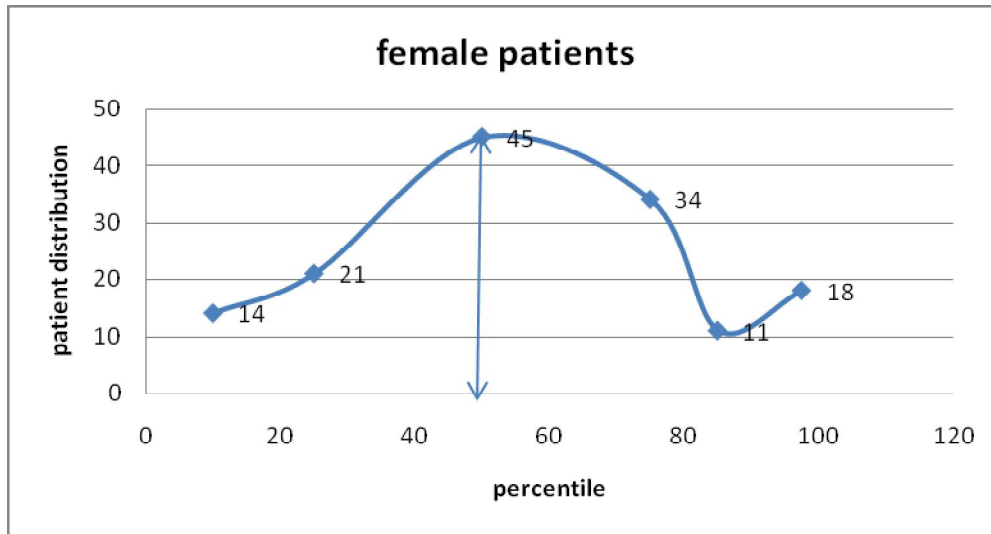
**Table [3]: Mean & SD of 25OHD,UA,Ca,CRP,RF female patient population (143 patients)**

Statistical Parameter	25OHD (ng/ml)	UA(mg/dl)	Ca(mg/dl)	CRP(mg/L)	RF(IU/ml)
Mean	14.09	4.44	9.51	4.37	16
SD	11.53	1.4	0.66	0.49	5

The parametric distribution is uneven. Lower limit is -1SD but upper limit may be extended up to 3SD. Moreover, - 1SD lower limit cannot be acceptable, as lowest limit becomes 3ng/ml which shows severe Vitamin D deficiency.

So, for female patient population only non parametric distribution study is preferred. However, the range as per graphical distribution is (3.65-48.68)ng/ml and optimal distribution range is 10.2- 39.7ng/ml, 75% of female patient population. The distribution is in accordance with Bischoff et al[7].

**Figure [4]: Non parametric distribution of 25OHD of 143 female patients**



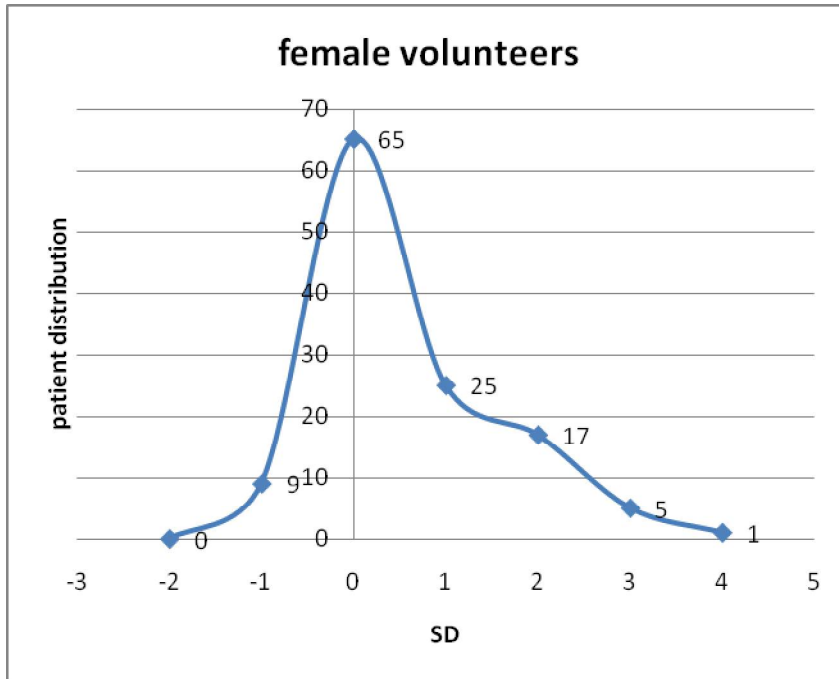
**Table[4]: 25OHD result distribution of 143 female patients**

Value(ng/ml)	Percentile	Number of patients
3	10	14
4.5	25	21
10.2	50	45
22.1	75	34
27.6	85	11
39.7	97.5	18

The pain profile data other than Vitamin D are well within normal range for both male and female patients & SD's are also having narrow range. The 25OHD of female patients are ranging from 3ng/ml to 39.7ng/ml and all the patients came with similar nature of complaint. The accumulated results raised the question about the extent of the contribution of 25OHD deficiency to such complaints and from which concentration of 25OHD the patients are to be considered as Vitamin D deficient. The joint pain, back ache and general weakness may be precipitation of so many factors but empirical correlation of such complaint with Vitamin D deficiency is likely to be a simplification process specifically when none of the patients were suggested to check the bone mineral density.

**Figure [5]: Parametric distribution of 25OHD of 122 female healthy volunteers**



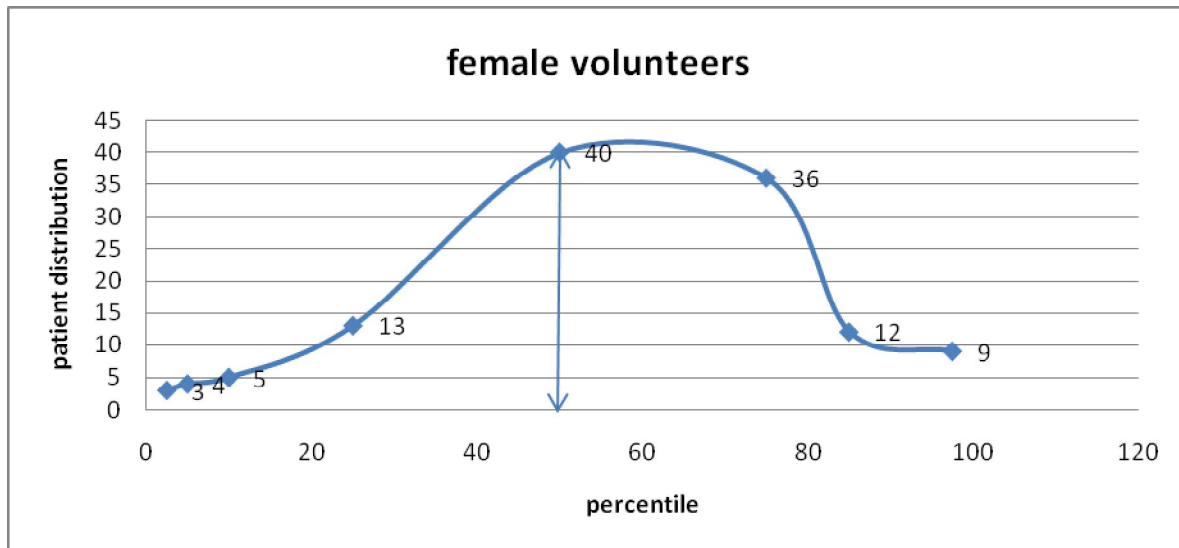


**Table [5] : Mean & SD of 25OHD,UA,Ca,CRP,RF of 122 female volunteers**

Statistical Parameter	25OHD (ng/ml)	UA(mg/dl)	Ca(mg/dl)	CRP(mg/L)	RF(IU/ml)
Mean	15.52	5.03	8.9	3.55	15
SD	5.78	0.98	0.204	0.35	4.65

The results of healthy volunteers are comparable to patients. The distribution pattern suggests to consider the range from -1SD to +2SD. The range is (9.74- 27.08)ng/ml. The range covers 116 healthy volunteers ie, 95% of healthy female population. So, the history of pain and fatigue may not solely be attributed to Vitamin D deficiency(Figure6,table 6).

**Figure [6]: Non parametric distribution of 25OHD of 122 female volunteers**



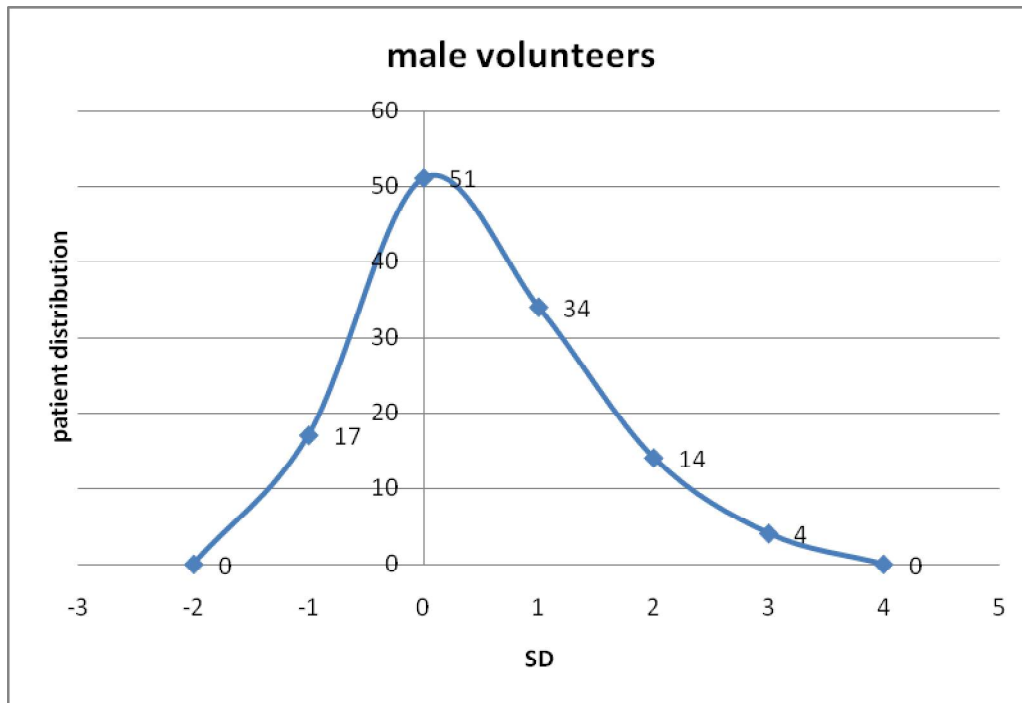
**Table [6]: 25OHD result distribution of 122 female volunteers**

Value(ng/ml)	Percentile	Number of patients
7.7	2.5	03
8.0	5	04
9.0	10	05
11.5	25	13
14.6	50	40
18.5	75	36
20.6	85	12
32.0	97.5	09

The distribution of 10 percentile to 97.5 percentile ranges from (9.0-32.0)ng/ml and covers 94% population. The 2.5-5 percentile value ie,(7.7-8.0)ng/ml was felt to be the controversial range. Whether this range would be incorporated within BRI or to be considered as deficiency range, multicentre study only can give satisfactory answer. The author also studied the 25OHD results of male volunteers. Male volunteers were chosen as per the protocol mentioned.

Total number of male volunteers were 120. The parametric distribution is being shown in figure[7] and table[7].

**Figure [7]: Parametric distribution of 25OHD of 120 male healthy volunteers**

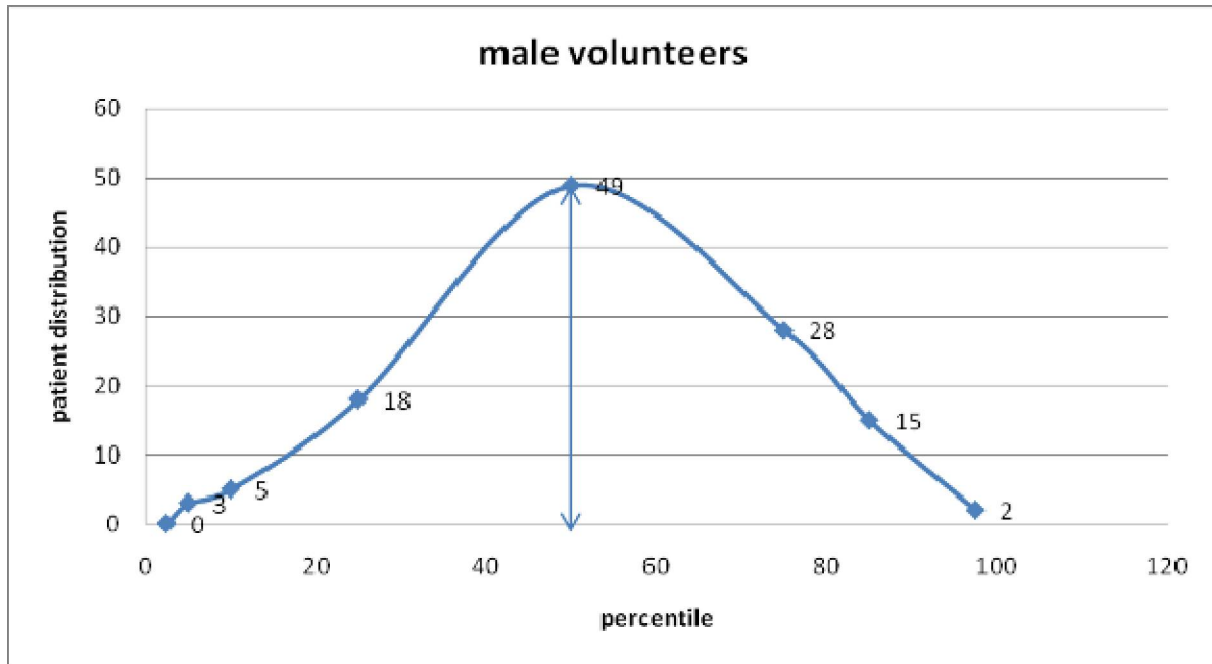


**Table [7]: Mean & SD of 25OHD,UA,Ca,CRP,RF of 120 male healthy volunteers**

Statistical Parameter	25OHD (ng/ml)	UA(mg/dl)	Ca(mg/dl)	CRP(mg/L)	RF(IU/ml)
Mean	15.85	3.95	9.9	3.88	21
SD	9.6	0.75	0.22	0.45	5.35

The parametric distribution of male volunteers shows a range of (6.26 – 44.65)ng/ml. The whole population is coming under this range. The statistical correlation is uncommon as no statistical calculation ideally does cover 100% population range. The sunlight exposure history of 3SD range volunteers were taken. Some of them are found out to remain exposed to direct sunlight for 5-6hrs per day. Another group gets direct exposure for 5-6 hrs and under indirect exposure for the rest of the day. The non parametric distribution has been shown in Figure[8] and table[8].

**Figure [8]: Non parametric distribution of 25OHD of 120 male healthy volunteers**



**Table [8]:25OHD result distribution of 120 male healthy**

**volunteers**

Value(ng/ml)	Percentile	Number of patients
3.0	2.5	00
6.25	5	03
8.5	10	05
10.05	25	18
14.85	50	49
25.7	75	28
33.43	85	15
40.25	97.5	02

The expected normal range is (8.5-40.25)ng/ml, keeping the 2.5percentile value under consideration. The references ranges are compared with the consensus values of North Germany (1), recently revised reference range(12) published by Roche Diagnostics( Table 9) .

**Table [9]: Comparison of population survey results**

**Parameter : 25OHD (ng/ml)**

Report obtained from	Age group	Number of Male	Reference Range	Number of Female	Reference Range
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	(years)	population	(ng/ml)	population	(ng/ml)
Consensus report, Roche, Northern Germany	20-77	201	6.23-49.9	252	4.92-42.7
Population study, Roche Diagnostics	Adults	Not available	11.1-42.9	Not available	11.1-42.9
Present study data (healthy volunteers)	25-60	120	8.5-40.25	122	9.0-32.0
Present study data (patient group)	25-60	120	5.5-39.7	143	3-39.7
Present study data (healthy volunteers) -Parametric distribution	25-60	120	6.26-44.65	122	9.74-27.08
Present study data (patient group) -Parametric distribution	25-60	120	10.04-32.63	143	3.65-48.68

The data obtained from the population study of West Bengal is correlating with the population study data of Roche Diagnostics. If health reference range is considered as optimal which is  $\geq 30$ ng/ml, 17% of male and 7% female volunteers satisfy the criteria. The lifestyle analysis of this specific volunteer group revealed that they are exposed to direct sunlight for more than 6 hrs (10am -4pm approximately) and indirectly exposed for the rest part of the day (8am-10am, 4pm-5-30pm approximately). It seems to obtain existing health reference range people need to get exposed for the whole day in a tropical country like India. Hence, it may be concluded that the whole patient group should not be considered as 25OHD deficiency group on the basis of the nature of complaints happened to be observed in 25OHD deficiency. Vitamin D is a fat soluble vitamin so use in excess is not advisable and the complaints of joint pain, body ache and general weakness may have several reasons other than Vitamin D deficiency. Current studies in India shown a table of UP (Uttar Pradesh, India) based data of 25OHD concentration in adults and children. The concentration range table [13] shown similarity in data presented by the author but the authors of the article concluded the situation as 25OHD hypovitaminosis and stated the situation as critical situation. Similar conclusion has been supported by Babu et al [14] and Gupta et al [15] suggesting Vitamin D fortification as remedial measure. But the conclusions were based on health reference range ie, considering the health reference range needs no re-evaluation. But sunlight exposure and dress code of West Bengal and UP are similar. To rule out deficiency /re-evaluation which one is the actual need of the situation the study to be taken up in Hilly & monsoon prone region where chance of exposure to sunlight is minimum.

The parametric distribution data analysis is generally not applicable for reference range determination. But in the present study the same has been done for all group and following observations were noted.

- *Two distribution analysis surprisingly resembles strengthening the conclusion.*
- *The empirical (Mean $\pm$ 2SD) calculation is not applicable for evaluation of BRI. The SD's are wide in population analysis. So, CV% calculation is not applicable. The graphical presentations show that mean and range to be determined as per patient distribution. Hence, reference range may be considered from -1SD to +3/4SD. In such situation mean to be calculated after taking decision about the range. As an example, the mean of male volunteers is 15.85ng/ml. But -1SD is the minimum range where as maximum value distributed upto 3SD. Evaluation as per the distribution analysis gives the range (6.26-44.65) ng/ml. Similar calculations has been applied for both patient and healthy population group. Close resemblance of reference range which strengthened the conclusion.*

### **Conclusion**

- The health reference range should be re evaluated for any parameter in situation/ situations of confusion.
- The reference range of the parameter felt to be re-evaluated as the result analysis of the patients shown negative bias. The reference range ideally should have no bias. The laboratory medicine practitioners are expected to observe such bias and take necessary action.
- It is being mentioned as mandatory note that the laboratories should evaluate the local reference range as and when necessary. So, such evaluation study helps cross verification of existing health reference range also.
- In the present situation the re-evaluation was need of the situation. The laboratory is presently mentioning the evaluated reference range as "Laboratory Defined Reference Range" in the test results.
- Whether India should start fortification/ evaluate reference range? Considering fortification is essential the hypovitaminosis could have been reported by this time. Till date, articles gave situations of 25OHD hypovitaminosis and requirement of fortification but no presentation of data on number of affected population due to such hypovitaminosis.

### **Limitations of the study**

- *The study should have been multicentric study. A study in Hilly and monsoon affected region is essential.*
- *Exposure of sunlight varies with season. Whether seasonal and difference in clothing affects the reference range yet to be studied.*
- *Bone Mineral Density study results of the patients and volunteers would have added to a concrete conclusion. But the test is very expensive , the project had no fund support and suggestion of bone mineral density test is Clinicians option.*

## Consent

The author obtained consent from patients. Volunteers were informed about the study .

## Ethical issue:

The results are test results. Patient identity has not been disclosed. The author obtained consent of the patients and volunteers also. So, no ethical issue would be raised or was raised during the study.

## Competing Interest:

Not applicable.

## Acknowledgements:

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2. JMD Diagnostics Private Limited – For allowing the author to perform the laboratory work.

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