Research Article

Reassessing Vitamin D Deficiency: Data Driven Indirect Reference Intervals for 25-Hydroxyvitamin D in Pakistan via refineR Algorithm

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Keywords

Vitamin D, Reference interval, Pakistan Classification Description, Metabolism, Endocrinology, Vitamin D

Abstract

Objectives: To establish population-specific reference intervals (RIs) for serum 25-hydroxyvitamin D [25(OH) D] in the Pakistani population using an indirect big data approach, and to evaluate the prevalence of vitamin D deficiency across age and sex strata.

Methods: This retrospective study analyzed serum 25(OH) D levels from 201,625 individuals aged ≥18 years who underwent testing at a tertiary care center over five years. Data were extracted from electronic medical records. Serum 25(OH)D was measured via chemiluminescent immunoassay (DiaSorin Liaison), with internal and external quality controls. The refineR algorithm with modBoxCox transformation was used to derive RIs. Statistical analysis was conducted using R software.

Results: The median serum 25(OH)D level was 17.2 ng/mL, with 25.7% of individuals classified as deficient (≤20 ng/mL), and 33% severely deficient (≤12 ng/mL). Vitamin D deficiency was more pronounced in men and younger adults. Reference intervals derived using modBoxCox transformation were 10.3–51.5 ng/mL for females and 6.1–50.6 ng/mL for males. Notably, females demonstrated higher vitamin D levels across all age groups compared to males.

Conclusion: This study presents large-scale, data-driven estimated reference intervals for 25(OH)D in Pakistan using an indirect approach. The findings highlight a high burden of vitamin D deficiency, particularly among men and younger adults, and reinforce the need for localized reference values to improve diagnostic accuracy. These insights support the development of targeted screening and supplementation strategies, and the reevaluation of clinical thresholds based on population specific data.

Introduction

Vitamin D is fat soluble vitamin that essential for maintaining physiological homeostasis, particularly in calcium and phosphorus metabolism, and bone health through the regulation of bone mineralization. Beyond its classical role in skeletal function, vitamin D exerts significant immunomodulatory effects by influencing the activity of T and B lymphocytes. These actions suggest broader function of vitamin D in managing both innate and adaptive immune mechanisms. A growing body of evidence suggests that insufficient levels of vitamin D may contribute to the development of several autoimmune conditions such as multiple sclerosis and type 1 diabetes by affecting immune regulation and promoting inflammatory responses [1].

Vitamin D is primarily found in two variants: ergocalciferol (D2) and cholecalciferol (D3). The D2 form is made when ultraviolet B (UVB) radiation interacts with ergosterol, a compound present in fungi, yeast, and certain plants. It can also be obtained from plant-derived sources such as mushrooms. In contrast, vitamin D3 is formed in the skin when 7-dehydrocholesterol is exposed to UVB radiation, and it is also present in animal-derived foods such as cod liver oil. After entering the body, whether obtained from the diet or produced by the skin, vitamin D2 and D3 (collectively termed calciol) attach to a transport protein known as vitamin D-binding protein (VDBP) in the bloodstream. This complex then travels to the liver, where vitamin D undergoes its first hydroxylation step, converting it into 25-hydroxyvitamin D, the primary circulating form found in blood. Subsequently, a second hydroxylation occurs in the kidneys, resulting in the formation of calcitriol, or 1,25-dihydroxyvitamin D the biologically active version which then binds to vitamin D receptors to carry out its functions [2].

Different health authorities have issued varying recommendations for vitamin D, but a common consensus is that most people maintain adequate levels when their serum 25-hydroxyvitamin D [25(OH)D] concentration reaches at least 50 nmol/L (equivalent to 20 ng/mL). According to the National Academies of Sciences, Engineering, and Medicine, vitamin D levels under 12 ng/mL (30 nmol/L) are classified as deficient, while concentrations ranging from 12 to 20 ng/mL (30–50 nmol/L) may not be sufficient for certain individuals [3]. The European Food Safety Authority (EFSA) and the Nordic Nutrition Recommendations also support a threshold of 50 nmol/L as adequate for the general population [4]. The Endocrine Society previously categorized vitamin D deficiency in 2011 as having serum 25(OH)D levels below 20 ng/mL (50 nmol/L). However, in its 2024 revision, the organization shifted away from using a universal threshold, instead promoting a more personalized evaluation approach [5].

Research shows that low 25(OH)D are prevalent across the world. Approximately 40.4% of European individuals have serum 25(OH)D concentrations under 50 nmol/L, and roughly 13% experience more severe deficiency, with levels falling below 30 nmol/L (equivalent to 12 ng/mL) [6]. In the United States, around 5.9% of the population experiences severe 25(OH)D deficiency, while in Canada, the figure is approximately 7.4% [7,8]. In Pakistan, inadequate 25(OH) D levels are a major public health issue, influenced by factors such as insufficient sunlight exposure, cultural norms, and poor dietary intake across various segments of the population. Pakistan has the highest incidence of adult vitamin D deficiency in South Asia (73%), with a mean level of 17.93 ng/mL [9].

Clinicians depend on established reference intervals (RIs) to accurately interpret laboratory test results. Traditionally, these RIs have been determined using direct methods outlined by the Clinical and Laboratory Standards Institute (CLSI), which involve selecting a well-defined group of healthy individuals who meet strict criteria. The reference range is typically defined by the values between the 2.5th and 97.5th percentiles [10]. However, more recent advances favor indirect methods that analyze large datasets often exceeding 50,000 samples drawn from routine clinical data stored in laboratory information systems. These approaches have proven to be more precise than traditional methods that rely on just 120 samples. An innovative approach, known as the refineR algorithm, applies inverse modeling techniques to more precisely calculate reference intervals (RIs), even when data distributions are skewed or normal. Research indicates that refineR is capable of generating RIs that closely align with those obtained through traditional direct methods, making it a useful and efficient option for clinical laboratories [11].

There is a lack of population-specific reference intervals that accurately reflect the 25(OH)D status of healthy individuals in Pakistan. Most available reference values are based on direct sampling of limited, often non-representative populations, or extrapolated from Western data, which may not be appropriate for local demographics due to ethnic, environmental, and lifestyle differences. To address this gap, our study aims to leverage the power of big data and apply the RefineR algorithm, an indirect statistical approach, to establish more accurate and representative reference intervals for serum 25(OH)D in the Pakistani population. By analyzing a large dataset from routine laboratory results, this study seeks to establish evidence-based reference intervals that more accurately represent the overall health status of the general population.

Methods

Study design

This retrospective, observational study examined serum samples from individuals who had received inpatient or outpatient care at a tertiary hospital over the preceding five-year period from 01/01/2018 to 31/12/2022. This study was exempted by the institutional ethical committee of Aga Khan University (2023-8678-25064) and the study was performed using anonymized data set in accordance with the Declaration of Helsinki.

Data collection

Information including patient sex, age, date of testing, and clinical department was retrieved from electronic health records and systematically categorized. Initially, data from 273,240 individuals over a five-year period were considered. To avoid duplicate entries, if multiple 25(OH)D measurements were recorded for a patient on the same day, only the earliest result was retained, and subsequent ones were excluded. Before conducting statistical analysis, the dataset was refined by removing patients aged <18 and any entries lacking key demographic or clinical information. After removal of duplicates and data of patients less than 18, the remaining data included in the final analysis was 201,625 patients. As this was a retrospective study based on routine laboratory data, no explicit clinical exclusion criteria (e.g., chronic kidney disease, liver disease, malabsorption syndromes, or vitamin D supplementation) could be applied due to limitations in available metadata. Therefore, the dataset likely includes both healthy individuals and patients with underlying conditions affecting vitamin D status. However, the refineR indirect method is specifically designed to analyze mixed populations and estimate the underlying non-pathological distribution by minimizing the influence of extreme values.

Serum vitamin-D analysis

Serum 25(OH)D concentrations were measured using the Liaison analyzer (DiaSorin Inc., USA), which employs a chemiluminescent immunoassay method. The assay featured an analytical range of 4.0 to 150 ng/mL. Internal quality control was maintained by including two levels of control materials with each sample run. External quality assurance was ensured through participation in the College of American Pathologists (CAP) proficiency testing, conducted three times annually over the course of the study. All results from these external assessments were within acceptable performance criteria.

Data management and statistical analysis

Following data validation, statistical analyses were conducted using R software (version 4.4.1). The refineR package was utilized to estimate reference intervals (RIs) for serum 25(OH) D. Continuous data were described using either the mean and standard deviation (SD) or the median with interquartile range (IQR) depending on the distribution. Categorical variables were reported as counts and corresponding percentages.
The refineR algorithm accessible as a free R package is designed to derive RIs from real-world datasets that include both healthy and abnormal results. For datasets with skewed values, it uses the modified Box-Cox (modBoxCox) transformation, which incorporates an additional parameter, utilized to better normalize the data and improve RI accuracy [11].

Results

The mean age of the individuals in this study was approximately 55.5 years. Among the total population, females accounted for 68.51% (138,155 individuals), while males made up 31.49% (63,470 individuals) Table 1. The median serum level of 25(OH)D was measured at 17.2 ng/mL, with an interquartile range of 9.94 to 26.7 ng/mL. The mean concentration was calculated to be 21.58 ng/mL, with a standard deviation of ±17.28 ng/mL. According to the Endocrine Society's 2011 criteria, 25.7% (51,896 individuals) exhibited vitamin D deficiency (≤20 ng/mL or ≤50 nmol/L), 20% (40,393 individuals) were categorized as insufficient (21–29 ng/mL or 52.5–72.5 nmol/L), and 21.2% (42,725 individuals) had adequate levels (≥30 ng/mL or ≥75 nmol/L). Furthermore, a severe deficiency (≤12 ng/mL or ≤30 nmol/L) was observed in 33.03% (66,611 individuals) of the cohort (Table 2).

Table 1: Stratification of data according to age group and gender.

Age group	Female	Male	Total
18-44	76,619	27,939	104,558
45-65	47,627	25,820	73,448
>65	13,909	9,711	23,620
Total	**138,155**	**63,470**	**201,625**

Table 2: Percentage of serum [25(OH)D] results indicating severe deficiency, and insufficiency.

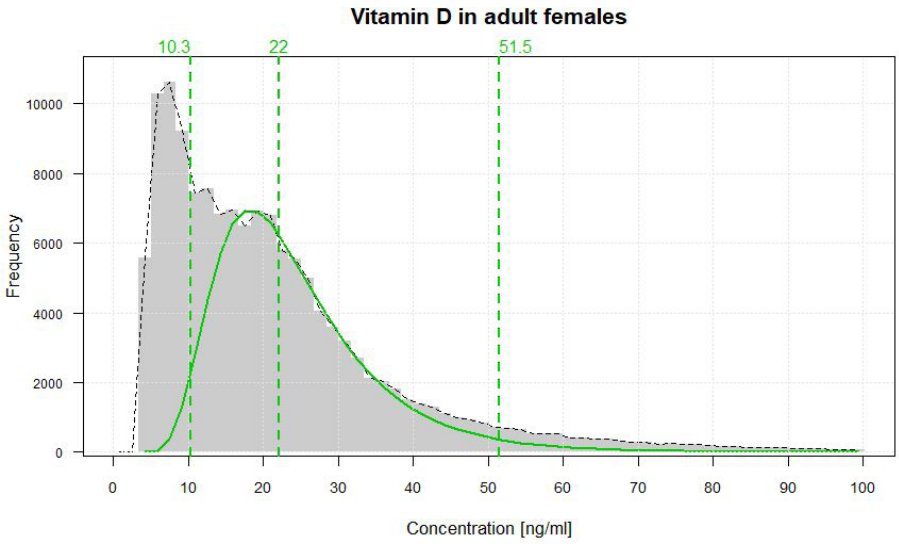
Age group	Vitamin D status	Females (76,619)	Males
18-44	Severe deficiency	29,959 (39.1%)	11,603(41.5%)
	Deficiency	18,319 (23.9%)	8,382 (30%)
	Insufficiency	13,409 (17.5%)	4,080 (14.6%)
	Sufficiencyw	14,932 (18.7%)	3,874 (13.6)
45-65	Severe deficiency	12,555 (26.4%)	7,937 (30.7%)
	Deficiency	11,800 (24.8%)	7,926 (30.69%)
	Insufficiency	11,547 (24.2%)	5,093 (19.7%)
	Sufficiency	11,725 (24.6%)	4,864 (18.9%)
>65	Severe deficiency	2,498 (18%)	2,059 (21.2%)
	Deficiency	2,911(20.9%)	2,558(26.3%)
	Insufficiency	3,763 (27.1%)	2,501 (25.8%)
	Sufficiency	4,737 (34%)	2,593 (26.7%)

A total of 201,625 serum 25(OH)D samples were analyzed using the refineR approach to determine optimal reference intervals (RIs). Due to the right-skewed nature of the dataset, standard statistical techniques were less appropriate. Therefore, a modified Box-Cox (modBoxCox) transformation was applied to our study cohort. The modBoxCox method adjusts for this imbalance by transforming the data into a shape that is closer to normal distribution, allowing for more precise calculation of reference intervals and better representation of the population's 25(OH)D status.

Females

For females (n = 138,155), the reference intervals derived from the overall dataset after applying the modBoxCox transformation showed a lower limit (LL) of 10.3 ng/mL (95% CI: 7.37–11.7 ng/mL) and an upper limit (UL) of 51.5 ng/mL (95% CI: 44.9–66.9 ng/mL). Distribution of 25(OH)D in females is depicted in Figure 1.

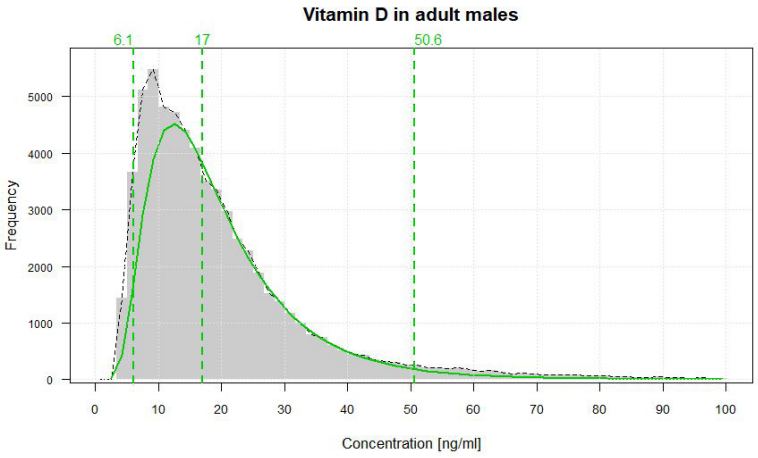
Table 1: Distribution of test results in females.



Males

For the male subgroup (n = 53,024), the estimated reference interval for serum 25(OH)D levels ranged from a lower limit of 6.1 ng/mL (95% CI: 2.36–7.36 ng/mL) to an upper limit of 50.6 ng/mL (95% CI 41.8–54.7ng/mL). Distribution of 25(OH) D in males is depicted in Figure 2.

Figure 2: Distribution of test results in Males.

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Reference Intervals
-------------------------------------------------
lower limit [ 2.5% perc]: 6.1 (2.36; 7.36)
upper limit [97.5% perc]: 50.6 (41.8; 54.7)

Model Parameters
-------------------------------------------------
     method: refineR (v1.6.2)
      model: modBoxCox
     N data: 63470
N bootstrap: 200
    rounded: yes (base: 0.01)
  point est: fullDataEst
     lambda: 0.049
         mu: 2.92
      sigma: 0.696
      shift: 1.61
       cost: -22.9
NP fraction: 0.845
```

Discussion

Vitamin D deficiency is widely acknowledged as a major global health issue, affecting not only bone strength but also the immune system and the risk of developing long-term illnesses. In regions like South Asia, particularly Pakistan, deficiency rates remain alarmingly high across all age groups, despite abundant sunlight [9,12]. This paradox is often linked to several contributing factors, including insufficient exposure to sunlight, cultural norms involving modest clothing, inadequate dietary sources of vitamin D, and darker skin tones [13]. Although many studies have documented prevalence of vitamin D deficiency in Pakistan, there remains a clear gap in establishing standardized reference intervals tailored to the local population. Currently, most clinical evaluations are based on ranges derived from Western cohorts, which may not adequately account for the unique environmental and physiological characteristics of Pakistani population. This gap hampers accurate diagnosis, risk stratification, and the formulation of effective public health strategies.

This study presents a large-scale population-based analysis generating indirectly derived, data-driven reference interval estimates for 25(OH)D in Pakistan, utilizing data from over 200,000 individuals stratified by sex and age. The findings offer critical insights into the national burden of vitamin D deficiency, highlight notable sex- and age-based differences, and underscore the urgent need for targeted public health interventions.

When stratified by age, the data reveals that younger adults (18–44 years) constitute the largest proportion of the tested population. Among them, severe deficiency (<12 ng/mL) was alarmingly common, 39.1% of females and 41.5% of males. This is consistent with the available literature reporting that young adults have high prevalence of vitamin D deficiency [14]. These findings are concerning given that this age group represents the most economically productive segment of the population and suggests that early-life vitamin D supplementation or dietary interventions may be lacking.

In the 45–65 age group, the prevalence of severe deficiency slightly declined, possibly due to increased health awareness or supplementation with age. However, a significant proportion still remained either deficient or insufficient. The elderly group (>65 years) showed the lowest rates of severe deficiency, though this might reflect survivorship bias or greater medical attention and supplementation in this age group. Studies show that prevalence of vitamin D deficiency is less among the age group above 65 years [15,16]. Notably, sufficiency was most prevalent in this age group among females (34%). The vitamin D status reported in this Pakistani cohort is indicative of a widespread deficiency, with patterns similar to those observed in other South Asian countries [9]. However, the higher rates of severe deficiency among men is particularly striking and contrast with trends in other populations, where women typically show higher rates of deficiency [17]. These differences may stem from lifestyle, socioeconomic, and cultural factors unique to Pakistan, such as dietary patterns low in vitamin D-rich foods, limited sun exposure, and a lack of fortification policies.

To derive epidemiologically sound, population-based reference intervals (RIs) for serum 25(OH)D, we employed the refineR indirect method, modified Box-Cox (modBoxCox) transformations. This approach allows for modeling of non-pathological distributions within large clinical datasets by identifying the main distribution peak and estimating the

parameters of a transformed normal distribution. In our dataset, which included over 200,000 individuals, the distribution of vitamin D levels was right-skewed, warranting the application of a two-parameter modBoxCox transformation, in line with recommendations by Ammer et al. [18].

Using this approach, we obtained refined reference intervals for 25(OH)D across different age and sex strata. For the entire population, modBoxCox transformation yielded a lower reference limit (LRL) closer to the clinically significant deficiency threshold of 12 ng/mL, strengthening the clinical relevance of the calculated RIs. Specifically, as seen in our histogram-based plots (Figure 2), the modBoxCox-derived LRLs aligned with early signs of hypovitaminosis D, confirming its suitability for Pakistani populations where right-skewed distributions are common due to high deficiency prevalence. The calculated reference intervals demonstrate a clear sex-based disparity. Women (n = 138,155) exhibited higher lower limits (LL = 10.3 ng/mL) and upper limits (UL = 51.5 ng/mL) compared to men (n = 63,470), who showed an LL of 6.1 ng/mL and UL of 50.6 ng/mL. These findings are consistent with previous studies indicating gender-related differences in vitamin D metabolism [19]. The wider range and lower LRL in men suggest a greater prevalence of suboptimal vitamin D status among males, which is further supported by the distribution patterns observed in the histograms.

Notably, the reference intervals derived via modBoxCox transformation closely matched those obtained via standard transformation methods, but offered greater alignment with biological plausibility and clinical thresholds. For example, among females, the modBoxCox-derived LRL was 10.3 ng/mL, while for males it was 6.1 ng/mL, values that mirror the levels below which physiological symptoms may occur and are closer to the sever deficiency cut off (<12ng/ml). This reinforces the applicability and transferability of modBoxCox-derived RIs to clinical settings, especially in low-resource contexts where direct recruitment of healthy reference populations may not be feasible. A recent cross sectional study by Usama et al. (2024) focused on determining reference ranges for serum 25(OH) D levels tailored to the population of Peshawar, Pakistan. The researchers determined a reference range of 6.43–45.0 ng/mL (2.5th–97.5th percentile) for 25(OH)D. This study however utilized data from only 200 adults aged 18-54 from Peshawar, limiting the applicability of the data [20]. A study by Peralas-Afan et al. underscored the importance of seasonal differences in the vitamin D levels whereby the median vitamin d levels were higher in summer than winter. One of the reason ins possible low penetrance of UV light in winter season [21,22]. The authors suggest that 25(OH)D levels above 12.0 ng/mL may be sufficient for a healthy population, challenging the prevailing notion of a widespread vitamin D deficiency pandemic. This perspective advocates for a more refined interpretation of vitamin D sufficiency, potentially reducing unnecessary supplementation [22].

The findings have major implications for clinical practice and public health policy. First, the establishment of locally derived RIs provides a more accurate benchmark for diagnosing deficiency in Pakistani populations. Second, the high prevalence of deficiency and insufficiency in all age groups, particularly among men and younger adults, supports the need for routine screening, community education, and national supplementation programs. Moreover, these results call for a re-evaluation of current diagnostic thresholds used in clinical labs across Pakistan, which are often based on Western reference standards. A locally adapted approach to diagnosing and managing vitamin D deficiency could help mitigate long-term health outcomes, including bone disorders, immune dysfunction, and cardiometabolic diseases.

One of the key strengths of this research lies in its extensive sample size and the detailed analysis stratified by age and gender, enhancing the reliability and applicability of the results. The application of the modBoxCox transformation contributed to the accurate determination of reference intervals. Nonetheless, there are certain limitations to consider. This study has several limitations. First, the dataset was derived from a tertiary care center and likely includes symptomatic individuals and patients undergoing testing due to suspected vitamin D deficiency, introducing potential selection bias. As a result, the assumption of a strictly healthy reference population is not fully met. Second, due to the retrospective design and lack of detailed clinical metadata, we were unable to exclude individuals with conditions known to affect vitamin D levels, such as chronic kidney disease, liver disease, malabsorption syndromes, or use of vitamin D supplementation. These factors may have influenced the distribution of 25(OH)D concentrations. Although the refineR algorithm is designed to account for mixed populations by estimating the central non-pathological distribution, the derived intervals should be interpreted as statistical estimates rather than true physiological reference intervals. Lastly, seasonal variation in vitamin D levels was not accounted for and may represent an additional confounder. Since the study relied on retrospective data, there is a possibility of selection bias, particularly because individuals tested for vitamin D levels may have already been suspected of having deficiencies. Moreover, the influence of seasonal changes on vitamin D concentrations was not accounted for, indicating a potential area for investigation in future studies.

Conclusion

This study provides large-scale, data-driven estimated reference intervals for serum 25(OH)D in a Pakistani population using an indirect approach. While these findings offer important insights into population-level vitamin D status and highlight a high burden of deficiency, the intervals should be interpreted as indirectly derived statistical estimates rather than definitive physiological reference intervals. Further prospective studies with well-characterized healthy populations are required for external validation before clinical implementation

Ethical Consideration
This study was exempted by the institutional ethical committee of Aga Khan University (2023-8678-25064).

Consent
Not applicable as anonymized data was analyzed.

Conflicts of Interests
All authors declare no conflicts of interests.

Funding
None.

Data Availability
Anonymized data is available on reasonable requests from corresponding author.

Author contribution
Sibtain Ahmed contributed to study conceptualization and manuscript writing. Amal Mahmood contributed to the literature review and manuscript writing. Ashishkumar Agravatt performed data analysis and contributed to manuscript writing. Imran Siddiqui contributed to manuscript writing, table preparation, and figure development. Aysha Habib Khan contributed to manuscript writing and critical intellectual review.

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