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BIOCHEMICAL CHARACTERISTICS OF VITAMIN D DEFICIENT AND INSUFFICIENT PATIENTS IN DISTRICT NORTH WAZIRISTAN

Dawood Khan[1], Ammara Ishaq[2], Najm Ud Din[*3], Qaisar Ud Din[4], Nabila Iqbal[5], Syeda Nadra Mukhtiar[6]

[1,2, *3,5,6]*Institute of Biological Sciences, Gomal University, D.I Khan, Pakistan*
[4]*The First Clinical Medical College of Chongqing Medical University, China*

[1]daud54567@gmail.com, [2]ch.ammaraishaq@gmail.com,[*3]najmuddindawar20@gmail.com, [4]qaisaruddin55@gmail.com, [5]captnia7@gmail.com [6]nadriii345@gmail.com

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Corresponding Author: *
Najm Ud Din

Abstract

Vitamin D is one of the important vitamin rendering services of regulating metabolism of formation bones and growth. There are a lot of people suffering from the deficiency of vitamin D resulting in the degenerative disorders, bone and muscles issues. In this study individuals from healthcare centers having signs and symptoms were included. The subjects were primarily checked for leading clinical indications, vitamin D, calcium and C peptide concentrations. The leading clinical condition was observed as legs pain and low BMI in all age groups with almost 25 % incidence. Among the males the calcium level was observed as 60% decrease with P value of 0.867 (P> 0.05). 35% of the female individuals of the study showed 55% decrease in their Ca level with significant P value of 0.678. Among the males the vitamin D level was observed as 20% decrease with P value of 0.282 (P> 0.05). 35% of the female individuals of the study showed 30 % decrease in their vitamin D level with significant P <0.05. The estimation of C peptide showed that 44 % decrease in the C peptide concentration in males with P value of 0.808 and in female's C peptide concentration was between 4-5 mg/dl with P <0.05. This research successfully delved in determining the threshold limit of blood calcium level, vitamin D and C peptide concentration.

INTRODUCTION

Vitamin D is a lipid-soluble vitamin. Organ meats, liver, mushrooms, salmon, and egg yolks provide as natural sources of vitamin D [1]. Ergocalciferol, or vitamin D2, is sourced from plants, whereas cholecalciferol, or vitamin D3, is produced from animal sources [2]. Upon exposure to ultraviolet (UV) light (wavelength range of 290–315 nm), epidermal cells in the skin transform cholesterol precursors into provitamin, which then undergoes isomerisation to become vitamin D3. Vitamin D2 and D3 possess similar biological properties and need particular enzymatic processes to attain active form. Vitamin D3 is activated by many regulatory factors, including parathyroid hormone (PTH) and particular mediators such as growth hormone and hypophosphatemia. These factors interact to affect and complete the activation transformation process [3]. Calcium homeostasis is regulated via enterocyte differentiation, facilitated by vitamin D's primary function in promoting intestinal calcium absorption. When blood calcium levels decline, specialised calcium receptors in the parathyroid gland diminish plasma concentrations of ionised calcium [4]. The parathyroid gland's secretion of parathyroid hormone subsequently prompts the kidneys to produce increased amounts of $1,25(OH)_2D$ from

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circulating 25(OH)D [5]. Elevated 1,25(OH)$_2$D influences the activity of bone cells, including osteoblasts and osteoclasts, as well as calcium absorption in the kidneys, intestines, and bones. PTH production continues until plasma calcium levels stabilise. This cycle of vitamin D and calcium homeostasis illustrates the significance of 25(OH)D in maintaining the synthesis of 1,25(OH)$_2$D and regulating blood calcium levels. In 2023, van Driel and van Leeuwen published their work. Vitamin D insufficiency may result in diminished blood concentrations of 25(OH)D, leading to elevated PTH levels and reduced synthesis of 1,25(OH)$_2$D and calcium absorption [6]. Dietitians assert that clinical vitamin D testing should concentrate on plasma 25(OH)D concentrations. Moreover, vitamin D receptors in the colon, osteoblasts, T and B lymphocytes, mononuclear cells, beta islet cells, and vital organs like the heart, brain, gonads, skin, and breast have been stimulated. Vitamin D insufficiency also has extraskeletal consequences [7]. The significance of vitamin D has markedly increased in recent decades, particularly due to its association with many diseases and the apparent global deficiency of vitamin D Currently, several physiological and pathological processes associated with different organs and systems in the human body have been correlated with vitamin D's function as a hormone [8].

Despite substantial evidence indicating that the vitamin D hormone influences skeletal health across all ages, there are contentious discussions surrounding the possible extra-skeletal benefits of vitamin D supplementation and the associated risks of elevated dosages [9]. Most studies agree that people with a vitamin D deficiency or insufficiency need therapy to maintain bone health and overall well-being. This is particularly applicable to persons at elevated risk of deficiency, such as those with diabetes, chronic kidney disease (CKD), malabsorption issues, and the elderly, particularly those living in long-term care facilities [10].

2. Martial & Methods

2.1Study Area

North Waziristan is situated in the Khyber Pakhtunkhwa province of Pakistan, in the northwestern region of the country, bordering Afghanistan. North Waziristan, once part of the Federally Administered Tribal Areas (FATA), has been integrated into Khyber Pakhtunkhwa since 2018. It has a robust topography characterised by steep terrains, valleys, and rivers, particularly the famous Kurram River. The extensive border with Afghanistan has established the area as a center for cross-border immigration and conflicts. The population of North Waziristan mostly consists of Pashtuns, namely the Wazir and Dawar tribes. These tribes possess a profound oral history and are recognized for their constant commitment to old rites and the Pashtunwali code of conduct. The population is mostly rural, and there are small towns and villages scattered across the region.

2.2. Obtaining the sample:

Samples were collected from 34 individuals, from DHQ Miran Shah hospital. Their basic data was recorded. 5 ml blood was collected and serum was extracted.

2.3 Serum Extraction

2.3.1 Materials Needed.

The blood collection tubes (vacuum tubes with or without anticoagulants); the needles and syringes or vacuum systems for blood collection and centrifugation; then the serum separation tubes or plain tubes; pipettes; and microcentrifuge tubes (if processing small volumes) Refrigerator or freezer for storing serum

2.3.2. Procedure:

Preparation:

material was sterilizse and cleaned to avoid any type of contamination. Serum tubes were labeled

2.3.3 Blood Collection:

Depending on the size of the animal, collect blood using either needles and syringes or a vacuum system. The choice of anticoagulant depends on the downstream applications. Common anticoagulants include heparin and EDTA. Collect the blood into the appropriate blood collection tubes. Mix gently to ensure proper anticoagulant mixing if used.

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2.3.4 Centrifugation:

Allow the blood to clot by leaving the collection tubes undisturbed at room temperature for about 20-30 minutes. After clotting, centrifuge the blood collection tubes at a moderate speed (typically 1000-1500 g) for around 10-15 minutes. The blood is separated into three layers by centrifugation: the red blood cells are in the bottom layer, the buffy coat (white blood cells and platelets) is in the middle, and the serum is on top.

2.3.5 Serum Separation:

Carefully transfer the serum from the blood collection tube to a clean serum separation tube or plain tube using a sterile pipette. Take care not to disturb the clot or the underlying layers. Avoid transferring any red blood cells or fibrin clots into the serum tube. Store the serum tubes in a refrigerator at around 4°C, or in a freezer at -20°C or lower, depending on the storage duration. If you plan to store serum for extended periods, consider liquating it into smaller volumes to avoid repeated freeze-thaw cycles. Always follow safety guidelines and procedures when working with blood and biological samples. Wear appropriate personal protective equipment (PPE) to prevent potential exposure to blood borne pathogens.

2.4 Quantitative Analysis of C protein

The quantitative measurement of C-reactive protein (CRP) using the Enzyme-Linked Immunosorbent Assay (ELISA) approach involves a series of processes to detect the amount of CRP contained in a sample. In response to inflammation or infection, the liver generates CRP, an acute-phase protein. ELISA is a highly sensitive and specific technique commonly used for protein quantification. Here's a general procedure for quantitative CRP analysis using ELISA:

2.4.1 Materials Needed.

Microplate coated with anti-CRP antibodies and CRP standards of known concentrations and Sample(s) containing CRP. An enzyme coupled to a secondary antibody (e.g., horseradish peroxidase) and Substrate solution. Wash buffer (usually a phosphate-buffered saline with detergent), Blocking buffer (e.g., BSA or milk) and Micropipettes and tips and Plate reader

Procedure.

Prepare Reagents:

Reconstitute and dilute the CRP standards to create a series of known concentrations. Prepare the secondary antibody-enzyme conjugate according to the manufacturer's instructions. Prepare the wash buffer and blocking buffer. Add a volume of the anti-CRP antibody solution to each well of the microplate. Incubate the plate for a specified time (usually overnight) at a specified temperature (often 4°C). To get rid of any unbound antibody, wash the plate multiple times with the wash buffer after incubation. Add blocking buffer to the wells to prevent non-specific binding of proteins. Incubate for a specified time at a specified temperature. Plates were washed to remove excess blocking buffer.

Sample and Standard Addition:

Add diluted CRP standards and your sample(s) to the appropriate wells in duplicate or triplicate. Incubate the plate for a specified time at a specified temperature. Wash the plate again to remove unbound CRP or other substances, Add the secondary antibody-enzyme conjugate to each well. Incubate the plate for a specified time at a specified temperature. To get rid of the secondary antibody-enzyme conjugate that is unbound, wash the plate. Incorporate a substrate solution, which combines with the enzyme to generate a discernible signal. Incubate for a specified time, typically in the dark. Add a stop solution to halt the enzymatic reaction. The **read the plate** using a microplate reader, determine each well's absorbance (optical density) at the proper wavelength. Plot the known concentrations of CRP standards against their corresponding absorbance values to construct a standard curve using the absorbance data. **Calculate CRP concentration u**se the standard curve to interpolate the CRP concentration in your samples based on their absorbance values.

4.5 Data Analysis.

An analysis was conducted on the data's average, variance, and standard deviation. The risk factors were examined using the Chi-Square test. The

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significance level was less than 0.05, and the analysis was done with SPSS (statistics 22).

3 Results

3.1Gender and Age wise Distribution.

In the first age group from one year to fifteen years three male and five females were included in this study. From the second age group from ages sixteen to thirty years fourteen participants were selected equally from both genders. In the third age group from the ages between thirty-one to forty-five years has the similar subjects' structure as discussed in first age group. The forth age group (46-60 years) comprised a male and two females. The last age group (60+ years) has a female only. Their standard deviation, variance and P-values are significant.

Table No 3.1 Gender and Age wise Distribution.

Age groups	Gender		Total	Std. Deviation		Variance		P Value
	Male	Female		Age	Gender	Age	Gender	
I (1-15)	3	5	8	1.0242	0.4995	1.0490	0.249554	0.858744
II (16-30)	7	7	14					
III (31-45)	3	5	8					
IV (46-60)	1	2	3					
V (>60)	0	1	1					
Total	14	20	34					

Clinical findings of the patients

The second considerable thing is clinical sign and symptoms which have to be possessed by the vitamin D deficient individual. The first age group contained the individuals of low weight, muscles weakness, leg pain, seizures and chest pain. The second age group between sixteen to thirty years contained the same subjects including obesity in greater proportions. Same is the situation of third age group which has the makeup of participants similar to the first age group. The forth age group has muscle weakness and leg pain while the fifth age group has only participant of legs pain.

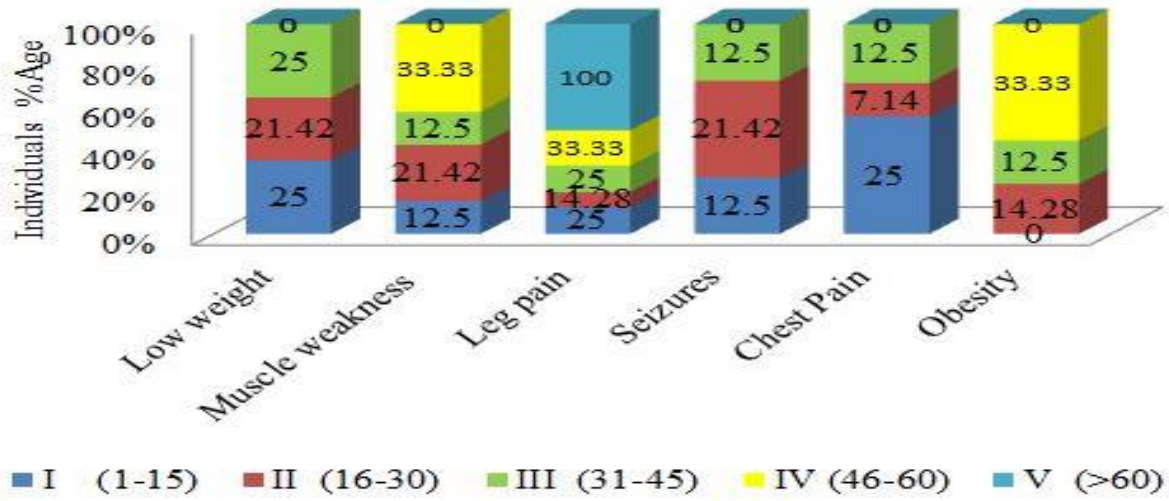


Figure 3.4 Clinical finding total age wise %age.

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Studies on variation of Ca level in Vit-D deficient individuals among males.

Now come to the laboratory tests performed by technician. We estimated for the calcium level. Among the males we measured extremely low (2-3mg/dl) and moderate low (3-4 mg/dl) levels of calcium. In the first age group two individuals had extremely low Ca level while one patient had moderate level of Calcium. In the second age group 4 individuals have extremely low and 3 individuals have moderate level of Calcium. The third age group has similar to the fist age group. The forth age group has 1 patient with extremely low level of calcium. In the last age group on participant reported with severe calcium deficiency. Their standard deviation, variance and P-values are significant.

Table: 3.3 Studies on variation of Ca level in Vit-D deficient individuals among males

Age Group	Calcium normal range 8.3-10.2(mg/dl)		Total	%age	Std. deviation		Variance		P-value
	2.1- 3	3.1- 4			Age	Ca	Age	Ca	
I (1-15)	2	1	3	21.43	0.864438	0.747253	0.497245	0.247253	0.867090
II (16-30)	4	3	7	50					
III (31-45)	2	1	3	21.43					
IV (46-60)	1	0	1	7.14					
V (>60)	0	0	0	0					
Total	9	5	14	100					

3.4 Studies on variation of Ca level in Vit-D deficient individuals among female.

Now come to the laboratory tests performed by technician. We estimated for the calcium level. Among the females we measured extremely low (2-3mg/dl) and moderate low (3-4 mg/dl) levels of calcium. In the first age group two individuals had extremely low Ca level while three patients had moderate level of Calcium. In the second age group 3 individuals have extremely low and 4 individuals have moderate level of Calcium. The third age group has one patient with extremely low level of calcium and four with moderate low levels of calcium. The forth age group has 2 patient with moderate low level of calcium. In the last age group on participant reported with moderate level of calcium deficiency. Their standard deviation, variance and P-values are significant.

Table: 3.4 studies on variation of Ca level in Vit-D deficient individuals among female

Age Group	Calcium normal range 8.3-10.2(mg/dl)		Total	%age	Std. deviation		Variance		P-value
	2.1- 3	3.1- 4			Age	Ca	Age	Ca	
I (1-15)	2	3	5	25	1.136708	0.470162	1.292105	0.221053	0.678417
II (16-30)	3	4	7	35					
III (31-45)	1	4	5	25					
IV (46-60)	0	2	2	10					
V (>60)	0	1	1	5					
Total	6	14	14	100					

3.5 Studies on variation of vitamin D level in Vit-D deficient individuals among male.

Next important thing to the laboratory tests performed by technician is the vitamin D level. Among the males we measured severe low (14-15 ng/dl) and moderate low (15-16 ng/dl) and low (16-17ng/dl) levels of vitamin D. The first age group contained 2,1 with moderate low and low levels of vitamin D respectively. The second age group contained 1,2 and 4 with severe low, moderate low and low levels of vitamin D respectively. The third age group contained 1,2 with moderate low and low

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levels of vitamin D respectively. The forth age group has one patient with severe low level of vitamin D and the fifth group no one was reported with vitamin D deficiency. Their standard deviation, variance and P-values are significant.

Table: 3.5 Studies on variation of vitamin D level in Vit-D deficient individuals among male

Age Group	Vitamin D normal range 20-50 (ng/dl)			Total	%age	Std. deviation		Variance		P-value
	14-15	15.1-16	16.1-17			Age	Vit-D	Age	Vit-D	
I (1-15)	0	2	1	3	21.43	0.864438	0.744946	0.554945	0.747253	0.282623
II (16-30)	1	2	4	7	50					
III (31-45)	0	1	2	3	21.43					
IV (46-60)	1	0	0	1	7.14					
V (>60)	0	0	0	0	0					
Total	2	5	7	14	100					

3.6 Studies on variation of vitamin D level in Vit-D deficient individuals among females

Moving on to another important thing to the laboratory tests performed by technician is the vitamin D level. Among the females we measured severe low (14-15 ng/dl) and moderate low (15-16 ng/dl). The first age group contained 2,3 with severe low and moderate low levels of vitamin D respectively. The second age group contained 2,5 with severe low and moderate low levels of vitamin D respectively. The third age group contained 3,2 with severe low and moderate low levels of vitamin D respectively. The forth age group has 2 patient with severe low level of vitamin D and the fifth group one was reported with severe vitamin D deficiency. Their standard deviation, variance and P-values are significant.

Table No 3.6 Studies on variation of vitamin D level in Vit-D deficient individuals among females

Age Group	Vitamin D normal range 20-50 (ng/dl)		Total	%age	Std. deviation		Variance		P-value
	V-D(14-15)	V-D(15.1-16)			Age	V-D	Age	V-D	
I (1-15)	2	3	5	25	1.136708	0.512989	1.292105	0.263158	0.321091
II (16-30)	2	5	7	35					
III (31-45)	3	2	5	25					
IV (46-60)	2	0	2	10					
V (>60)	1	0	1	5					
Total	10	10	20	100					

3.6 Studies on variation of C-peptide level in Vit-D deficient individuals among male

Measurement of C peptide among males is also a crucial factor in determining the Vitamin D deficient level in individuals. It was estimated in very low (3-4), low (4-5), and moderate low (5-6) mgs/dl. In the first age group 2,1 and 0 individuals reported in aforementioned order. In the second age group 3,3,1 people was in categories as discussed earlier. In the third age group 1,2 and 0 people possessed the C peptide level corresponding to very low, low and moderate low respectively. Discussing the forth age group which contain 1,0 and 0 people respectively and the fifth age group has not reported any one for variable C peptide estimation. Their standard deviation, variance and P values are significant.

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Table: 3.7 studies on variation of C-peptide level in Vit-D deficient individuals among male

Age Group	C-peptide normal range 0.9- 0.7 (mg/dl)			Total	%age	Std. deviation		Variance		P- value
	C-P (3-4)	C-P (4.1-5)	C-P (5.1-6)			Age	C-P	Age	C-P	
I (1-15)	2	1	0	3	21.43	0.864438	0.646206	0.747253	0.417582	0.808847
II (16-30)	3	3	1	7	50					
III (31-45)	1	2	0	3	21.43					
IV (46-60)	1	0	0	1	7.14					
V (>60)	0	0	0	0	0					
Total	7	6	1	14	100					

3.8 Studies on variation of C-peptide level in Vit-D deficient individuals among female.

Measurement of C peptide among Females is also a crucial factor in determining the Vitamin D deficient level in individuals. It was estimated in very low (3-4), low (4-5), and moderate low (5-6) mgs/dl. In the first age group 3,2 and 0 individuals reported in aforementioned order. In the second age group 2,3,2 people was in categories as discussed earlier. In the third age group 1,2 and 2 people possessed the C peptide level corresponding to very low, low and moderate low respectively. Discussing the forth age group which contain 0,2 and 0 people respectively and the fifth age group which contain 0,1,0 reported any one for variable C peptide estimation. Their standard deviation, variance and P values are significant.

Table: 3.8 Studies on variation of C-peptide level in Vit-D deficient individuals among female

Age Group	C-peptide normal range 0.9- 0.7 (mg/dl)			Total	%age	Std. deviation		Variance		P- value
	C-P (3-4)	C-P (4.1-5)	C-P (5.1-6)			Age	C-P	Age	C-P	
I (1-15)	3	2	0	5	25	1.136708	0.718185	1.292105	0.515789	0.515216
II (16-30)	2	3	2	7	35					
III (31-45)	1	2	2	5	25					
IV (46-60)	0	2	0	2	10					
V (>60)	0	1	0	1	5					
Total	6	10	4	14	100					

3.9 Gender wise Vit Deficiency

Among males participants 9 people had mild level of vitamin D deficiency, 4 people had moderate level and 1 had severe level of vitamin deficiency. Among females participants 0 people had mild level of vitamin D deficiency, 12 people had moderate level and 8 had severe level of vitamin deficiency Their standard deviation, variance and P values are significant.

Table No 3.9 Gender wise Vit Deficiency

Gender	Mild	Moderate	Severe	Total	%age	Std. deviation		Variance		P-
						Gender	D-Def	Gender	D-Def	
Male	9	4	1	14	41.2	0.499554	0.738549	0.249554	0.545455	0.00012
Female	0	12	8	20	58.8					
Total	9	16	9	34	100					

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3.10 Age wise Vit-D deficiency.

Discussing the age wise distribution of vitamin D deficiency showed that the first age group has 2,4,2 subjects in mild, moderate and severe vitamin D deficiency respectively. The second age group has 5,9,0 subjects in mild, moderate and severe vitamin D deficiency respectively. The third age group has 2,3,3 subjects in mild, moderate and severe vitamin D deficiency respectively. The forth age group has 0,0,3 subjects in mild, moderate and severe vitamin D deficiency respectively. The fifth age group has 0,0, and 1 subjects in mild, moderate and severe vitamin D deficiency respectively. Their standard deviation, variance and P values are significant.

Table 3.10 Age wise Vit-D deficiency.

Age Group	Mild	Moderate	Severe	Total	%age	Std. Dv		Variance		P-valu
						Age	D-Def	Age	D-Def	
I (1-15)	2	4	2	8	23.5	1.024217	0.738549	1.049020	0.545455	0.033285
II (16-30)	5	9	0	14	41.2					
III (31-45)	2	3	3	8	23.5					
IV (46-60)	0	0	3	3	8.8					
V (>60)	0	0	1	1	2.9					
Total	9	16	9	34	100					

4 Discussion

The current research examined the biochemical characteristics of patients with insufficient or inadequate vitamin D in the North Waziristan region. Blood vitamin D deficiency was shown to be rather frequent (25%) in American and Canadian people (36%), as well as in European populations (40%) and South Asian populations (61%), according to earlier study spanning a variety of age groups [11.12.13]. The largest cohort of male and female participants was included in the current investigation, as far as we are aware, ranging in age from birth to over fifty years, who were inhabitants of North Waziristan. The demographic under investigation for the statistical analysis had a higher female to male ratio (2:1). It was shown that 56% of individuals had low blood vitamin D levels. Women were found to have greater amounts than males. Serum vitamin D deficiency affected around 20% of people overall, with a larger female-to-male ratio. As a result, women are more likely than men to have insufficient or limited vitamin D. These results might be explained by a larger proportion of girls than males in the cohort. Hussain et al. discovered that 59% of Pakistani housewives in Quetta lacked enough vitamin D in previous study. Women are more likely to suffer from vitamin D deficiency when they are less educated, spend fewer than 15 minutes in the sun on weekdays, avoid the sun or cover up when it's sunny, or consume less eggs, milk, or fish—either once a week or never at all [11]. These findings also align with those of earlier research conducted in Khyber Pakhtunkhwa and Punjab, which shown that 31% of asymptomatic individuals had blood vitamin D levels that were inadequate [14]. Male 12-year-olds seemed to have a greater percentage of vitamin D deficiency (57%), and insufficiency (60%) than female 12-year-olds. The fact that there were more male children than female children in the cohort of children with vitamin D insufficiency can account for the higher percentage of male children. Children and adolescents between the ages of 6 and 12 exhibited lower blood vitamin D levels than adults, although having smaller cohorts. The rising frequency of vitamin D insufficiency may be attributed to indoor lifestyles, a lack of knowledge on the amount of time individuals need to spend in the sun to obtain the necessary daily intake of vitamin D, and foods enhanced with vitamin D [15]. Since this study only reported the vitamin D levels of patients who were given laboratory testing and did not evaluate the levels of non-symptomatic patients, it may have some selection bias. The study's strength lies in its large sample size, which allows the results to be applied to the study population. This allows for the best estimation of serum vitamin D deficiency and insufficiency status among children, adolescents, adults, and elders in Pakistan, a developing country. Since children and adolescents appear to be the most impacted age group, managing blood vitamin D

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levels necessitates the creation of awareness campaigns addressing the causes, prevention measures, and treatment methods

Consolation

I recommend that these research findings be disseminated widely across relevant academic and medical communities, as well as to policymakers and healthcare practitioners. The implications of this research extend beyond the academic sphere, reaching into the realms of public health policy and clinical practice. Implementing the identified strategies has the potential to not only improve individual health but also reduce the burden on healthcare systems.

In conclusion, the research on Vitamin D deficiency reduction, resulting in a 50% decrease in deficient individuals, is a significant milestone in the quest for improved public health outcomes. I urge you to consider the importance of these findings and support their dissemination to ensure that the broader community can benefit from the knowledge gained through this impactful research.

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