Vitamin D [25(OH)D] and 1,25$(OH)_2$D serum concentrations in patients tested at the Charlotte Maxeke Johannesburg Academic Hospital

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A research report submitted to the Faculty of Health Sciences, University of Witwatersrand, in partial fulfilment of the requirements for the degree of Master of Medicine.

Johannesburg, 2020

Declaration

I Sunet Botha declare that:

a) This Research Report is my own work.

b) It is being submitted for the Degree of Master of Medicine in the branch of Clinical Pathology, under the department of Chemical Pathology at University of the Witwatersrand, Johannesburg.

c) It has not been submitted before for any degree or examination at any other university.

d) This research report does not contain other people's data, pictures, graphs or information, unless specifically acknowledged, and sources are listed in the referenced section.

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(Signature of candidate)

15th day of December 2020

Abstract

Introduction: There has been a significant increase in vitamin D [25(OH)D] testing worldwide in recent years. However, interpretation is confounded by the lack of data to determine appropriate cut-off levels to define Vitamin D deficiency, insufficiency and sufficiency in the South African population.

Aims: To describe the number of tests, concentrations for 25(OH)D and $1,25(OH)_2D$ in adults (≥ 18 years), characteristics of those tested and to determine the 25(OH)D concentration at which parathyroid hormone increases (PTH threshold).

Methods: Data was extracted for 25(OH)D and $1,25(OH)_2D$ tests, from the National Health Laboratory Services data warehouse for Charlotte Maxeke Johannesburg Academic Hospital from 2015 to 2017. Results were categorized by age, sex and race. Vitamin D status was described using National Academy of Medicine guidelines. The PTH threshold was determined by LOWESS plots.

Results: 25(OH)D and $1,25(OH)_2D$ tests increased, with no change in median concentrations over time. Black Africans (6.7%) had the highest prevalence of VDD. Males had significantly lower 25(OH)D values ($p < 0.001$) and a higher proportion of VDD ($p = 0.009$). Younger patients (<30 years; 7.9%) and elderly (>74 years; 10.5%) Black Africans had highest prevalence of VDD. The PTH threshold differed by race group.

Conclusions: Clear testing guidelines are needed to curb test overutilization. Further work is required to understand the appropriate cut-off levels to define VDD in the populations.

Acknowledgement

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Note on the Contents and the Format of this Research Report

This research report forms part of an article that has been submitted for review in the Journal of Endocrinology, Metabolism and Diabetes of South Africa (JEMDSA).

Before this research project was undertaken, a research protocol was written describing the methods to be used in this study and also including a review of the appropriate literature. This protocol was submitted to an assessor panel from the School of Pathology at the University of the Witwatersrand Faculty of Health Sciences, where it was reviewed and approved. A copy of this protocol is included in the appendices of this research report.

The appendices also contain a copy of the ethics approval form for this project. The project was approved by the Ethics Committee (Human Research) of the University of the Witwatersrand.

The format of this research report is as stipulated by the Graduate Studies Committee of the University of the Witwatersrand Faculty of Health Sciences.

Introduction

There has been increasing interest in the medical community on the role of vitamin D [25(OH)D] in bone and cardiovascular health, cancers, autoimmune diseases, diabetes mellitus as well as infectious diseases such as tuberculosis.[1, 2] The possible 25(OH)D health benefits with regards to these diseases may explain the marked increase in 25(OH)D analysis noted in laboratories worldwide over the last decade.[3, 4]

The cut-off values used to define vitamin D deficiency (VDD), insufficiency and sufficiency remain controversial. Currently there are two main diagnostic guidelines issued by the United States Endocrine Society and the National Academy of Medicine (NAM), formerly known as the Institute of Medicine (IOM).[1, 5, 6] The latter guidelines use the following cut-offs to define vitamin D deficiency, insufficiency and sufficiency: < 30nmol/L, 30-50nmol/L and > 50nmol/L[1, 5], respectively. These are different to the cut-off levels as defined by the United States Endocrine Society.[1, 6] Using the NAM cut-offs, a recent systematic review and meta-analysis of VDD in Africa, noted that one in five people have a 25(OH)D level < 30nmol/L despite yearlong sunlight exposure.[7] The overall pooled prevalence was reported as 18.46% (95% CI 10.66 – 27.78), with South Africa and northern African countries reported to have the lowest vitamin D levels.[7]

The parathyroid hormone (PTH) threshold, a plateau point for 25(OH)D below which there is a rise in PTH (secondary hyperparathyroidism), has been considered an indication of VDD.[8] There is limited data on the appropriate cut-off levels to use in Africans. A systematic literature review in 2006 of studies analysing the association between PTH and 25(OH)D levels, revealed a putative threshold between 25 to 122 nmol/L[8], while a study carried out in African American and White women showed different cut-offs for both races with a threshold of 37 nmol/L in

Black women and 59 nmol/L in White women.[8] This threshold appears to be dependent on race, age, body composition, renal function and geographical location. [5, 8]

Currently, 25(OH)D (comprising D_2 and D_3) is used to assess vitamin D related disorders and the body's vitamin D stores.[1, 6] Analysis of 1,25$(OH)_2$D is only required in limited circumstances, which include vitamin D-dependent rickets type 1, autosomal-dominant hypophosphataemic rickets and X-linked hypophosphataemic rickets (mutations that result in elevated levels of FGF-23).[6] The monitoring of 1,25$(OH)_2$D in patients with chronic kidney disease (CKD) is not currently advocated by the 2017 Kidney Disease Improving Global Outcomes (KDIGO) guidelines on bone mineral disorders.[9, 10] The testing of 25(OH)D is costly and the current consensus among experts, is that testing should only be performed on those at increased risk for VDD.[3, 6, 11]

The objectives of the current study were to (i) describe test numbers for both 25(OH)D and 1,25$(OH)_2$D from our laboratory over a three-year period, (ii) to describe the vitamin D profiles for 25(OH)D according to NAM guidelines by age, sex and race, (iii) to determine the PTH threshold for VDD by race for those with normal renal status and (iv) to assess the percentage of 25(OH)D and 1,25$(OH)_2$D requests in patients with CKD.

Methods

Study design and setting

This was a retrospective study using laboratory data extracted from the corporate data warehouse (CDW) of the National Health Laboratory Services (NHLS) for the period 1 January 2015 to 31 December 2017. The extracted data included all total 25(OH)D, $25(OH)D_2$, $25(OH)D_3$ and $1,25(OH)_2D$ results carried out in the NHLS, Department of Chemical Pathology laboratory based at Charlotte Maxeke Johannesburg Academic Hospital (CMJAH). This laboratory received vitamin D requests from across the South African public sector. This study was approved by the Ethics Committee of the University of Witwatersrand (M180808).

Study population selection

The sample population included adults (≥ 18 years), who had either 25(OH)D and/ or $1,25(OH)_2D$ tests done during the study period. Additionally, the accompanying results (total serum calcium, inorganic phosphate, creatinine, estimated glomerular filtration rate (eGFR), alkaline phosphatase (ALP) and PTH) taken within a week of the vitamin D result were included to obtain a complete profile.

Race was categorised into five groups: White, Black African, Indian, mixed ancestry and unknown. The 'unknown' category was used when race could not be assigned and is described in the supplementary material. The 25(OH)D results were separated into $25(OH)D_2$ and $25(OH)D_3$ by race and subdivided by sex. The population was categorised according to the NAM's criteria.[1, 5]

To determine test numbers, samples from patients < 18 years and quality assurance (QA) were excluded. Repeat tests, whether 25(OH)D or $1,25(OH)_2D$, done from the same patient during the study period, were identified by ways of a unique patient identifier. The unique patient identifier, implemented at the CDW, uses a probabilistic matching record linkage algorithm. The number of samples over the three-year period was reported after which repeat tests were excluded from the detailed descriptive analysis to determine the distribution of 25(OH)D concentrations, patient characteristics and season of testing.

Laboratory methods

25(OH)D was measured by high-performance liquid chromatography (Shimadzu Corporation, Long Beach, CA, USA) with separation into $25(OH)D_2$ and $25(OH)D_3$ and $1,25(OH)_2D$ was measured by competitive radioimmunoassay (Immunodiagnostics Systems Holdings PLC, Tyne & Wear, UK). The other methods used were immunoassay (PTH), colorimetric (serum total calcium, serum inorganic phosphate, ALP) and enzymatic (creatinine) assays performed on the Roche Cobas 8100 (Roche Diagnostics Ltd, Mannheim, Germany). The eGFR was calculated using the Modification of Diet in Renal Disease (MDRD) formula.[12] CKD was defined as a consistent eGFR < $60ml/min/1.73^2$ for more than three consecutive months.

Statistical analysis

Data analysis was done using Statistical Analysis Software (SAS) 9.4 (Cary, NC, USA) and Microsoft Excel 2016 (Redmond, CA, USA). The mean with standard deviation (SD) was reported for normally distributed data and the median with interquartile ranges for skewed data. Descriptive data analysis was used to describe the 25(OH)D profiles. ANOVA analysis was used to compare continuous parametric data by race, Kruskal Wallis for non-parametric

291 continuous data and chi squared test for categorical data, with statistical significance set at p
292 <0.05.
293
294 As race group was specified for very few patients, we used the hot deck imputation algorithm
295 to impute missing values using well populated cancer registry data with patient-reported race
296 group data.[13] The National Cancer Registry (NCR) data was used to construct the imputation
297 reference panel (approximately 1.2 million values).[14] Locally weighted scatter plot smoothing
298 (LOWESS) was used to determine the relationship between 25(OH)D and PTH across race
299 groups in patients with normal renal status.

Results

Over the three-year period, 15 407 tests were identified of which 3283 were excluded, giving a final number of 12 124 tests. After exclusion of repeat testing, a cohort of 10 008 patients were used for the descriptive data analysis as shown in Figure 1. The 25(OH)D and 1,25$(OH)_2$D tests comprised 7 383 and 2 600 patients respectively, with 25 patients having both tests.

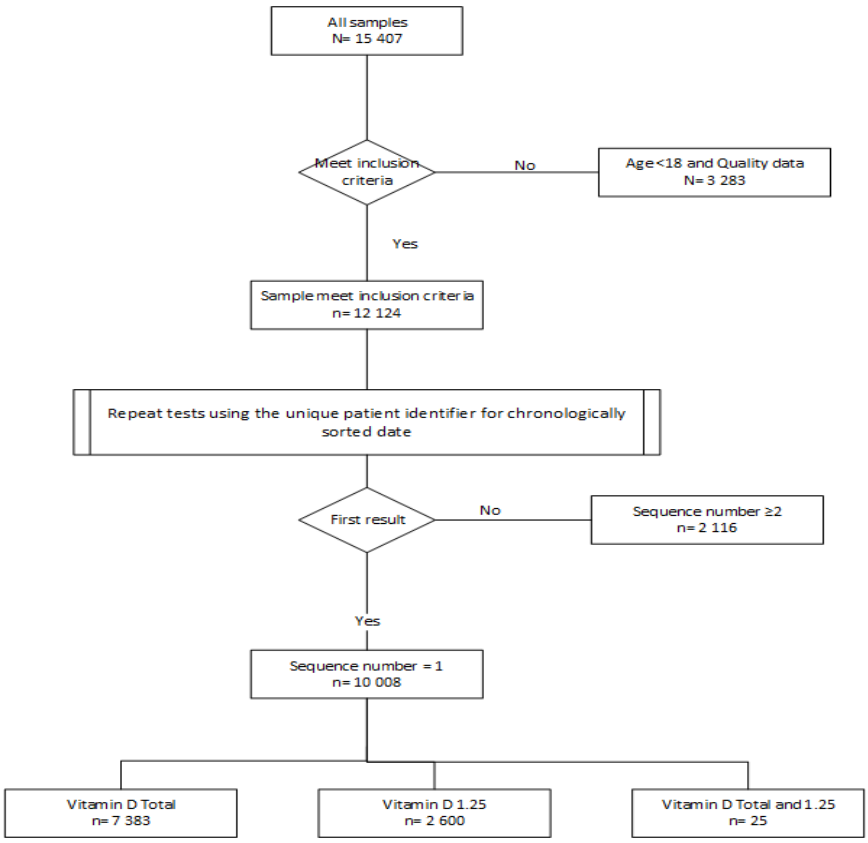

Figure 1: Study population selection. Exclusion criteria applied to reach the final 25(OH)D and 1,25$(OH)_2$D results used for data analysis.

An increase in test numbers across the three-year period was noted as indicated in Figure 2A. The 25(OH)D test volumes increased from 1982 in 2015 to 2786 in 2017. This was a 53% and 18% increase between 2015-16 and 2016-17, respectively. The increase in $1,25(OH)_2D$ test volumes shown, represented a 22% and 13% increase between 2015-16 and 2016-17, respectively. There was no significant change in median 25(OH)D and $1,25(OH)_2D$ levels over this time period. The greatest increases in testing were seen in Black African males and females (Figure 2B). This represented a 75.70% increase in testing of Black African males and an 88.10% increase in Black African females over the three-year period. The majority of tests were requested from the Gauteng (73.10%), KwaZulu-Natal (10.70%) and the Eastern Cape (6.30%) provinces (data not shown).

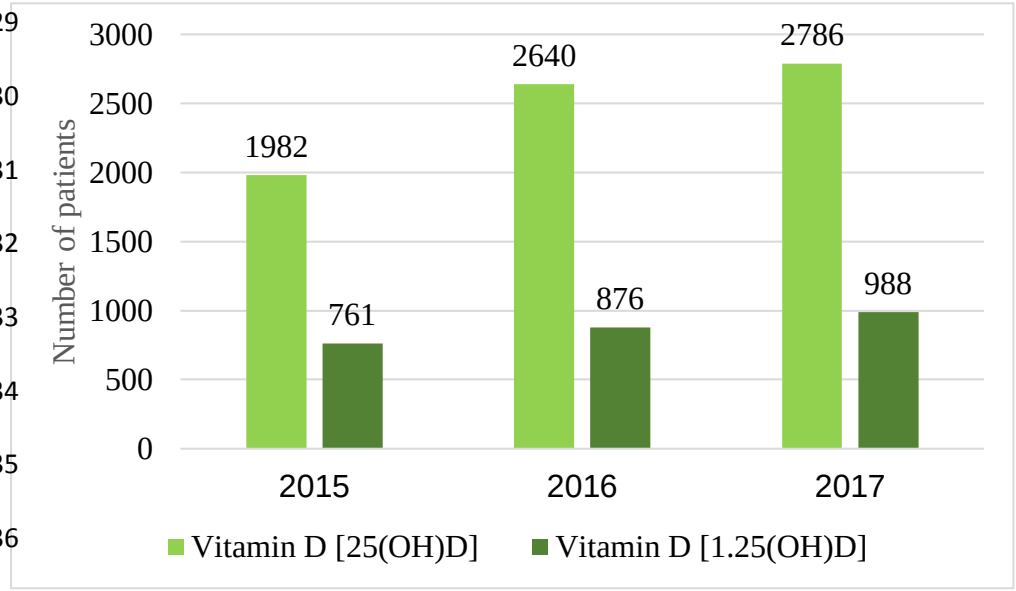

Figure 2A: Volume trends for 25(OH)D and $1,25(OH)_2D$.

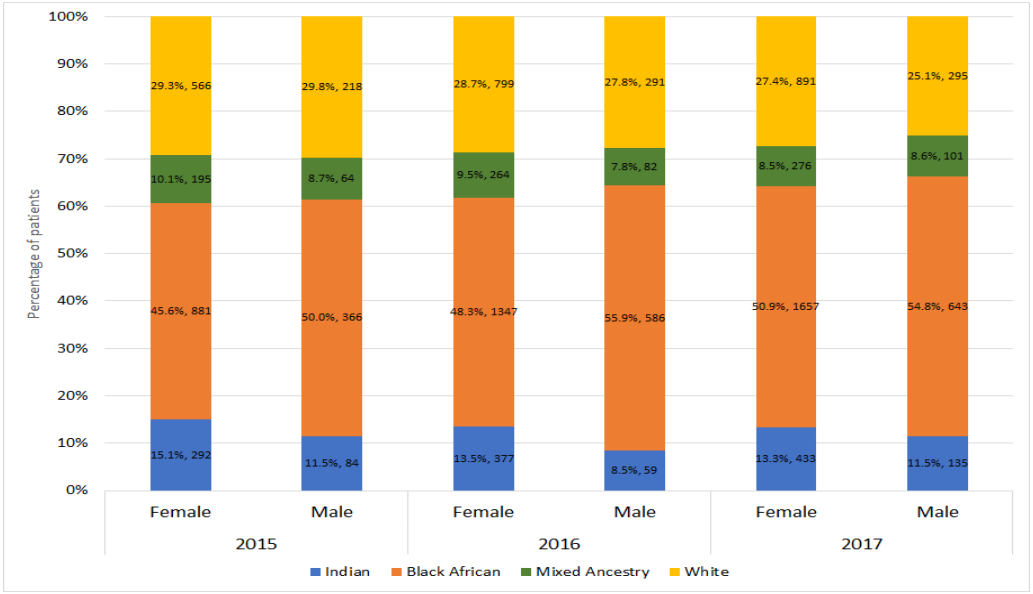

Figure 2B: Test percentages per race and sex for 25(OH)D.

Within the total cohort, 3 884 (45.40%) were Black African, 2 387 (27.90%) White, 875 (10.20%) Indian, 765 (8.90%) mix ancestry and 638 (7.60%) of unknown ethnicity (see Supplementary Table I). Overall, Black Africans were significantly younger than all other racial groups (p <0.0001, 50.65 ±16.88 years). Black Africans had the lowest levels of 25(OH)D (69.00 (47.20-95.20) nmol/L), which was significantly lower when compared to the other racial groups (p <0.0001). Additionally, Black Africans also had the lowest D_2 levels (7.00 (7.00-34.41) nmol/L) (Table I).

Table I: Overall descriptive characteristics of study population.

Race	**White**		**Black African**		**Indian**		**Mixed Ancestry**	
N (%)	2387 (27.90)		3884 (45.40)		875 (10.20)		765 (8.90)	
Sex	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**
N (%)	1778 (22.40)	609 (7.60)	2782 (35.10)	1102 (13.90)	698 (8.80)	177 (2.20)	573 (7.20)	192 (2.80)
Age (years)	58.08 ± 16.89		50.65 ± 16.88[1]		56.26 ± 15.46[2]		55.37 ± 16.51[3]	
Total 25(OH)D (nmol/L)	83.80 (59.20-112.00)		69.00 (47.20-95.20)[4]		69.20 (48.50-103.00)		79.50 (54.90-109.00)	
D_2 (nmol/L)	11.70 (7.00-57.35)		7.00 (7.00-34.41)[4]		20.50 (7.00-65.20)		11.95 (7.00-57.10)	
D_3 (nmol/L)	46.80 (27.30-72.20)		42.60 (26.70-64.00)		33.90 (20.95-51.45)		44.15 (27.35-66.30)	
$1,25(OH)_2D$ (pmol/L)	134 (91-181)		133 (66-194)		125 (87-166)		137 (89-193)	

25(OH)D = hydroxyvitamin D, D2 = ergocalciferol, D3 = cholecalciferol, 1,25(OH)2D = di-hydroxyvitamin D, data expressed as median and interquartile ranges except age, which is expressed as mean ± SD. [1]p <0.0001 vs all other race groups, [2]p <0.05 vs mix ancestry and white, [3]p <0.05 vs. white, [4]p <0.0001 across all other racial groups

In the total cohort by race and sex, the majority of patients were females (73.50%). The male mean age was lower as compared to females for all racial groups ($p < 0.001$). Black African and mix ancestry males were younger than White and Indians (46.90±15.70 and 50.90±16.80 vs. 56.20±17.00 and 53.20±15.90 years) males, respectively (Table II).

The total 25(OH)D levels for males across all racial groups were found to be lower than females ($p < 0.05$), with the lowest values in Indian (63.70 (44.10-86.10) nmol/L) followed by Black African (69.90 (48.40-96.80) nmol/L). Females had higher $25(OH)D_2$ levels when compared to males across all racial groups ($p < 0.0001$), and males had higher $25(OH)D_3$ compared to females across all racial groups ($p < 0.0005$) as seen in Table II.

Analysis of $1,25(OH)_2D$ levels showed that median concentrations were all within the laboratory defined reference range of 43-168 pmol/L, with no statistically significant differences between racial groups. Black African males had the highest median PTH (11.2 pmol/L). Additionally, Black African females and males had the highest proportion (40.00% and 23.10%) of CKD as compared to other racial groups (Table II).

Table II: Descriptive characteristics of study population by race and sex

Race N (%)	**White 2387 (27.90)**		**Black African 3884 (45.40)**		**Indian 875 (10.20)**		**Mixed Ancestry 765 (8.90)**	
Sex N (%)	**F 1778 (22.40)**	**M 609 (7.60)**	**F 2782 (35.10)**	**M 1102 (13.90)**	**F 698 (8.80)**	**M 177 (2.20)**	**F 573 (7.20)**	**M 192 (2.80)**
Age (years)	60.20 ±16.60	56.20±17.00[1]	52.80±16.90	46.90±15.70[1]	58.70±14.90	53.20±15.90[1]	57.20±16.30	50.90±16.80[1]
Total 25(OH)D (nmol/L)	89.20 (62.90-123.50)	79.00[1] (57.30-100.40)	73.50 (50.50-101.50)	69.90[2] (48.40-96.80)	85.60 (56.80-118.70)	63.70[1] (44.10-86.10)	85.30 (58.70-121.50)	78.70[1] (52.50-107.45)
D2 (nmol/L)	26.80 (7.00-84.60)	7.00[1] (7.00-25.40)	15.00 (7.00-52.60)	7.00[1] (7.00-26.40)	42.10 (7.00-95.40)	7.00[1] (7.00-31.80)	25.10 (7.00-87.00)	7.00[1] (7.00-34.80)
D3 (nmol/L)	39.60 (23.60-65.60)	56.70[3] (31.20-77.80)	39.10 (23.90-60.60)	48.20[3] (30.30-69.70)	29.00 (17.30-46.40)	37.50[3] (22.80-56.40)	38.90 (24.60-61.90)	48.90[3] (30.50-73.40)
1,25(OH)$_2$D (pmol/L)	128 (67-154)	113 (56-192)	141 (54-182)	81.0 (27-134)	104 (79-121)	128 (95-142)	113 (90-142)	-
PTH (pmol/L)	7.0 (4.8-11.7)	6.6 (4.1-22.6)	8.4 (4.7-17.8)	11.2 (3.6-35.9)	7.1 (4.1-10.3)	6.8 (3.3-29.1)	8.4 (5.1-17.0)	9.6 (4.5-33.9)
ALP (U/L)	82 (64-106)	97[2] (73-140)	96 (74-135)	97 (73-156)	79 (62-97)	90 (65-102)	83 (66-104)	85 (59-104)
With CKD N (%)	117 (15.00)	44.00 (5.60)	311 (40.00)	180 (23.10)	29.00 (3.70)	13.00 (1.60)	59.00 (7.60)	23.00 (3.40)
Without CKD N (%)	156 (23.60)	34.00 (5.10)	271 (41.10)	85.00 (12.80)	57.00 (8.60)	9.00 (1.30)	38.00 (5.70)	9.00 (1.80)

F = female, M = male, 25(OH)D = hydroxyvitamin D, D2 = ergocalciferol, D3 = cholecalciferol, 1,25(OH)2D = di-hydroxyvitamin D, PTH = parathyroid hormone, ALP = Alkaline Phosphatase, - = no tests in this category, data expressed as median and interquartile ranges except age, which is expressed as mean ± SD. [1] $p < 0.0001$ vs females across race, [2] $p < 0.05$ vs females across race, 3 $p < 0.0005$ vs females across race

The majority of 25(OH)D results were categorised as sufficient (Table III, Figure 3), with the highest proportion of sufficiency in White (83.70%) and mix ancestry (80.10%) patients. The overall prevalence of VDD was 5.50%, with proportionately more Black Africans and Indians deficient (6.70% and 6.60%) and insufficient (20.40% and 18.30%) ($p < 0.001$). Vitamin D insufficient and deficient patients were younger than those that

were sufficient ($p < 0.05$ and $p = 0.0001$, respectively). More males as compared to females ($p < 0.009$) were found to be deficient (6.10% vs 5.20%) and insufficient (19.40% vs 16.90%). By age category, the highest proportion of VDD (7.90%) and insufficiency (20.60%) was seen in the < 30 years category (Table III). In patients with CKD, the prevalence of VDD was 5.80% across all racial groups.

The median serum calcium level was found to be significantly lower in the deficient group (2.06 (1.26-2.29) mmol/L) as compared to 2.33 (1.86 - 2.44) mmol/L in the sufficient group ($p = 0.001$). Median inorganic phosphate and ALP were within the normal reference ranges across all three categories. Median PTH levels were elevated across all categories (reference range of 1.6-6.0 pmol/L), with a significantly higher level found in the deficient category (11.5 (1.3-32.2) pmol/L) as compared to the sufficient category ($p = 0.0001$) (Table III).

Table III: Descriptive characteristics according to NAM categories.

	Deficient	Insufficient	Sufficient
Overall N (%)	408 (5.50)	1314 (17.70)	5670 (76.70)
Race Group N (%)			
White	62 (3.20)	255 (13.10)	1617 (83.70)
Black African	235 (6.70)[1]	712 (20.40)[1]	2531 (72.90)
Indian	48 (6.60)[1]	133 (18.30)[1]	544 (75.10)
Mixed Ancestry	23 (3.60)	104 (16.30)	511 (80.10)
Sex N (%)			
Female	276 (5.20)	899 (16.90)	4087 (77.90)
Male	127 (6.10)[4]	400 (19.40)[4]	1525 (74.50)
Age Category (%)	51.31(±17.92)	53.30(17.16)	54.94(17.34)[2,3]
< 30	7.90	20.60	71.50
30 -59	5.70	19.30	75.00
60 -74	4.20	16.80	78.80
> 74	5.60	17.20	77.00
CKD	5.80	19.00	75.10
Calcium (mmol/L)	2.06 (1.26 - 2.29)	2.22 (1.39 - 2.39)	2.33 (1.86 - 2.44)[3]
Inorganic phosphate (mmol/L)	1.08 (0.43 - 1.41)	1.13 (0.59 - 1.38)	1.09 (0.70 - 1.28)
PTH (pmol/L)	11.5 (1.3 - 32.2)	9.9 (0.9 - 25.7)	7.4 (1.7 - 14.0)[3]
ALP (U/L)	116 (48 - 203)	97 (46 - 138)	86 (47 - 118)

CKD = chronic kidney disease, PTH = parathyroid hormone, ALP = alkaline phosphatase. Data expressed as percentages, median and interquartile ranges except age, which is expressed as mean ± SD.

[1] $p < 0.0001$ for Black African and Indian, [2] $p < 0.05$ vs insufficient, [3] $p = 0.0001$ vs deficient, [4] $p < 0.009$ vs deficient and insufficient females

Further analysis by race and age (Figure 3) showed that VDD varied across racial groups. Among those over the age of 74, the prevalence of VDD was highest in Black Africans (10.50%).

The highest proportion of VDD were from the Free State (9.1%), Limpopo (6.3%) and the Eastern Cape (6.2%) provinces. Provinces with the least VDD were North West (1.2%), Mpumalanga (4.9%) and the Western Cape (3.7%) (data not shown). The study looked at

seasonal variation in VDD and noted that it was highest in the winter (7.78%), and the lowest in late autumn (7.78% vs 3.43%, p =0.0001) (data not shown).

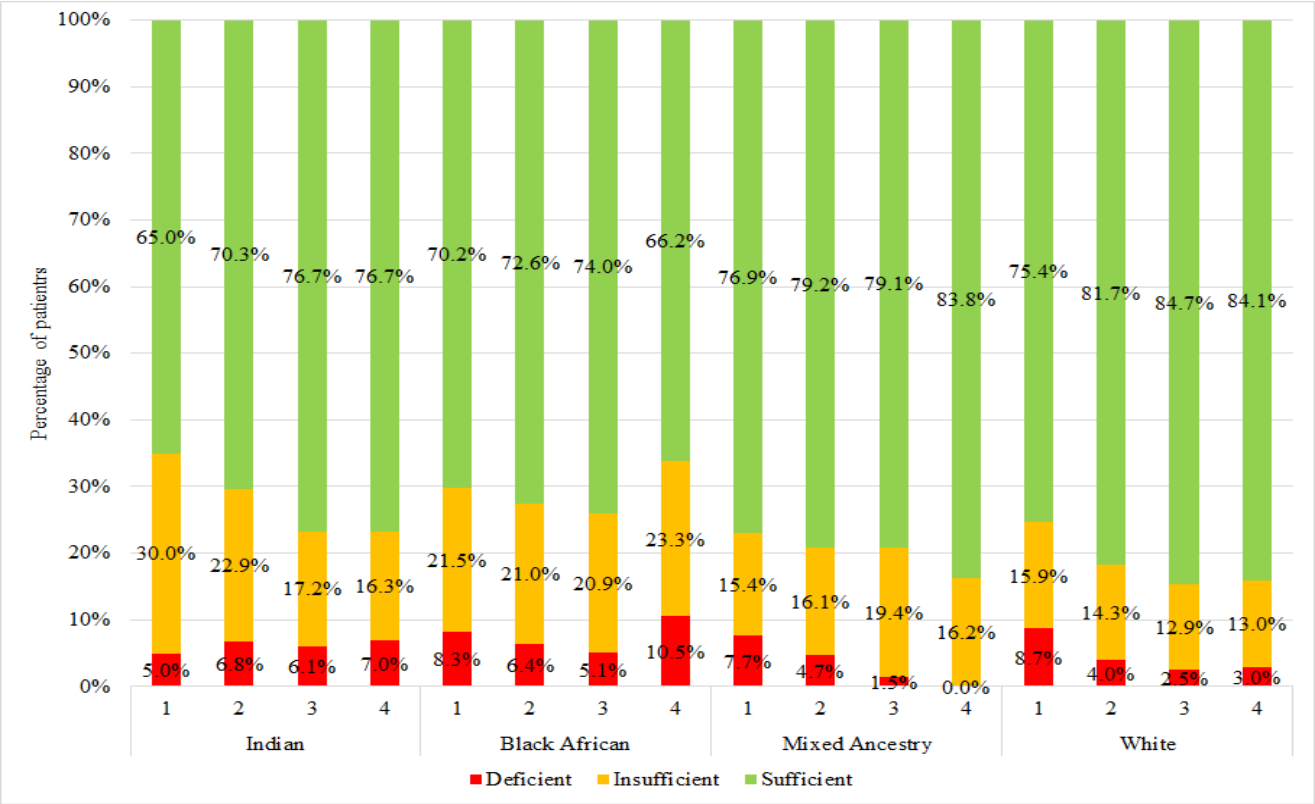

Figure 3: NAM categories by race and age.

Data expressed as percentages, 1 = <30 years, 2 = 30-59 years, 3 = 60-74 years and 4 = >74 years.

Creatinine results were available for 1588 patients, of which 844 (53.15%) had CKD. In the CKD group, 822 (97.39%) and 22 (2.61%) of requests were identified as 25(OH)D and 1,25$(OH)_2$D, respectively. The prevalence of VDD was 6.50% and 7.52%, in males and females with CKD respectively (Supplementary Table II).

The LOWESS plots (Figure 4 and Supplementary Figures 1-2) showed an inverse relationship between the levels of 25(OH)D and the PTH. The results indicated that the point at which circulating PTH concentration was maximally suppressed differed by race group. It was lowest in Black Africans and highest in Indians. The threshold could not be estimated in the mixed

ancestry group due to a small sample size.

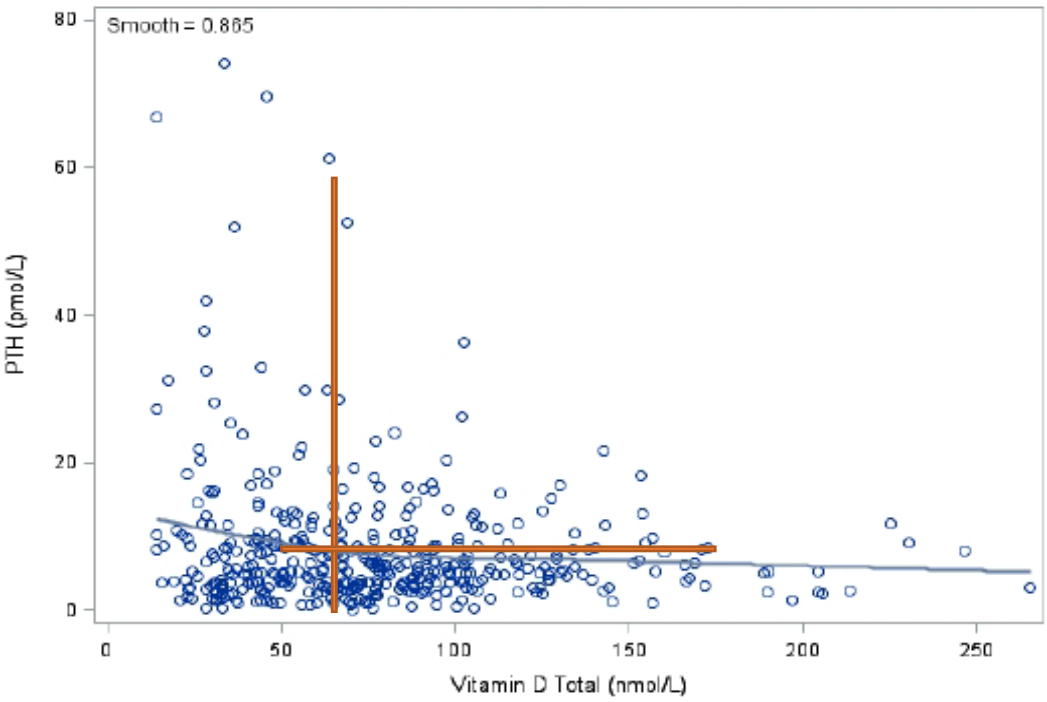

Figure 4: LOWESS plot for the Black African group. The linear line (—) is indicative of the 25(OH)D value at which PTH is maximally suppressed.

Discussion

The number of 25(OH)D and 1,25$(OH)_2$D tests received from public sector hospitals and clinics from across South Africa over a three-year period were evaluated. An increase in test requests for both 25(OH)D and 1,25$(OH)_2$D, especially for samples from Black African patients were noted. There was no change in median 25(OH)D levels over the period. Overall, the Black African and Indian groups had the highest percentages of VDD and males were found to have significant lower levels of 25(OH)D as compared to females. The highest percentage of deficient and insufficient levels were found in patients less than 30 years of age and Black Africans > 74 years. A provincial variation in the prevalence of deficiency, with more VDD found in the Free State was noted.

Both 25(OH)D and 1,25$(OH)_2$D tests numbers demonstrated yearly increases, even though there was no significant change in median concentrations. Although there are no definitive testing criteria, routine widespread testing is not recommended in the general population.[6, 11, 15] The National Osteoporosis Foundation of South Africa (NOFSA) recommends that testing should be aimed at high-risk groups.[15] These include older adults, especially the frail, institutionalised elderly, osteoporosis, limited or ineffective sun exposure due to dark skin, clothing or hospitalisation, malabsorptive diseases, medication that interferes with vitamin D metabolism and lastly, pregnancy with any of the risk factors as mentioned.[15] Increased research on the health benefits of vitamin D, both in the musculoskeletal system and other non-skeletal systems and frequent media attention over the past years, may have contributed to the volume trends.[3, 4] Similar volume trends have been reported from countries across the globe, although the observed increases in this study was far lower.[3, 4] Recent studies suggest a low 25(OH)D level is an independent risk factor for COVID-19 infection and hospitalisation.[16, 17]

We anticipate that this may lead to further increases in test requests. In SA, 25(OH)D and $1,25(OH)_2D$ are not subject to gatekeeping which has been shown to curb overutilization.[3, 18]

There is wide variation in 25(OH)D concentrations reported from studies across SA, depending on the geographical location and the age.[1] This study is one of the few that have reported concentrations by age and sex. Limited data is available for Black African males as most studies included female participants only.[1, 2] Contrary to literature, a lower prevalence of VDD in females was found.[2, 7] Females across all racial groups had a higher 25(OH)D level as compared to males. This was due to higher D_2 levels, suggesting possible supplementation. Additionally, an increased prevalence of VDD was found in patients less than 30 years which is different to other local studies carried out in community dwelling adults.[1,15] South Africa is known to have a high burden of infectious diseases such as HIV and tuberculosis as well as non-communicable diseases, and lower concentrations of 25(OH)D have been reported in patients with chronic diseases.[2] It is likely that underlying diseases may partly explain the increased prevalence in the younger age groups. The observation that 10.5% of Black Africans above the age of 74 are deficient, is significant. A number of SA studies have reported VDD in the elderly, though not by race group.[1, 19, 20] The elderly are prone to VDD for a number of reasons including reduced cutaneous synthesis and daily sun exposure, as well as chronic diseases, and VDD may contribute to frailty and increased fracture risk.[1, 21] This data suggest that this group may benefit from testing as maintaining sufficient 25(OH)D levels have been shown to promote healthy ageing and reduction in frailty.[21]

The results show that Black African males and females had a higher proportion of CKD, as compared to other racial groups. Patients with CKD are prone to VDD, with early diagnosis and intervention leading to a reduction in CKD complications.[10, 22] Using laboratory data, we

were unable to determine the reason for most requests, but it appears that in Black African patients, monitoring of vitamin D levels in those with CKD may be one of the reasons. According to the 2017 KDIGO guidelines, in CKD, monitoring with 25(OH)D levels and not $1,25(OH)_2D$, is recommended.[9, 10] Repeat testing should be individualised according to the baseline values and interventions.[10] These guidelines possibly also explains why only 25 patients had both 25(OH)D and $1,25(OH)_2D$ tests requested.

The PTH threshold was found to differ by race group. Aloai *et al.* have demonstrated that Black females had a lower PTH threshold compared to White females (37 nmol/L vs. 59 nmol/L, respectively).[8] While this study was unable to determine a specific cut-off, the observed threshold in Black Africans was lower than that of White patients. Thus, a single cut-off to define deficiency may not be appropriate in our setting as higher circulating PTH, secondary to deficiency, may have negative extra-skeletal effects.[8, 22]

Overutilization of 25(OH)D and/or $1,25(OH)_2D$ testing significantly increases healthcare costs.[3, 11] Countries such as Canada have introduced specific indications for 25(OH)D testing which resulted in an 80% reduction in test requests.[3, 18] It is imperative that vitamin D testing, whether 25(OH)D and/or $1,25(OH)_2D$, be based on evidence-based guidelines that offer testing for specific populations at higher risk of VDD. While routine testing to monitor 25(OH)D levels in patients taking supplementation for VDD is not recommended we need to determine what is appropriate locally.[11, 23]

The strengths of this study include a large sample size and a data collection period of three-years. However, our study had a number of limitations. This study did not have clinical data and information, specifically regarding bone health, making it difficult to assess the reason for

testing requests, patient profiling and the establishment of appropriate cut-off levels by race. The majority of the data is from patients seeking hospital care in Gauteng, and we did not include 25(OH)D and $1,25(OH)_2D$ tests that were done at all NHLS laboratories or the private sector. Therefore, the yearly volume increases for the country may be different from those reported here. The findings are not applicable to the entire population and are not representative of a healthy population. The data is from 2015-17 and current trends may have changed. This study was unable to further describe the vitamin D profiles in CKD patients due to a small sample size. Finally, racial groups were determined by imputation which has some limitations.

Conclusion:

The findings of this study suggest that VDD varies with age and race group, with proportionally more hospitalised Black Africans and Indians, deficient and insufficient. Those at risk of VDD include young patients and elderly Black Africans attending hospital. Further work is required to understand the appropriate cut-off levels to define VDD in our populations. Clear testing guidelines are needed to curb test overutilization. It might be of value to consider implementing test gatekeeping.

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Supplementary material

Supplementary Table I: Descriptive characteristics of the unknown category.

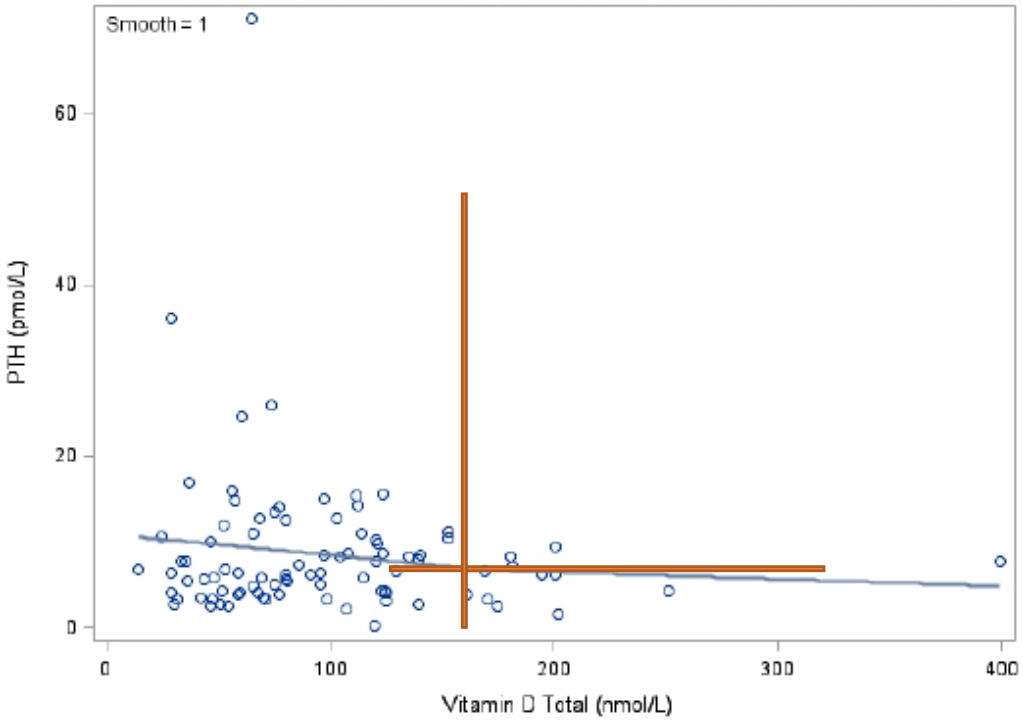
Race N (%)	**Unknown 638 (7.60)**	
Sex	**Female**	**Male**
N (%)	460 (72.10)	178 (27.90)
Age (years)	54.1 ± 16.9	48.7 ± 17.5
Total 25(OH)D (nmol/L)	79.40 (52.90-109.70)	69.80 (51.80-95.40)
D2 (nmol/L)	18.20 (7.00-63.70)	7.00 (7.00-27.80)
D3 (nmol/L)	39.50 (23.10-61.20)	50.70 (29.40-69.60)
1,25(OH)$_2$D (pmol/L)	93 (64-167)	39 (39-39)
PTH (pmol/L)	7.9 (4.9-12.3)	6.2 (3.0-11.5)
ALP (U/L)	82 (60-122)	102 (72-133)

25(OH)D = hydroxyvitamin D, D2 = ergocalciferol, D3 = cholecalciferol, 1,25(OH)2D = di-hydroxyvitamin D, PTH = parathyroid hormone, ALP = Alkaline Phosphatase, data expressed as median and interquartile ranges except age, which is expressed as mean ± SD.

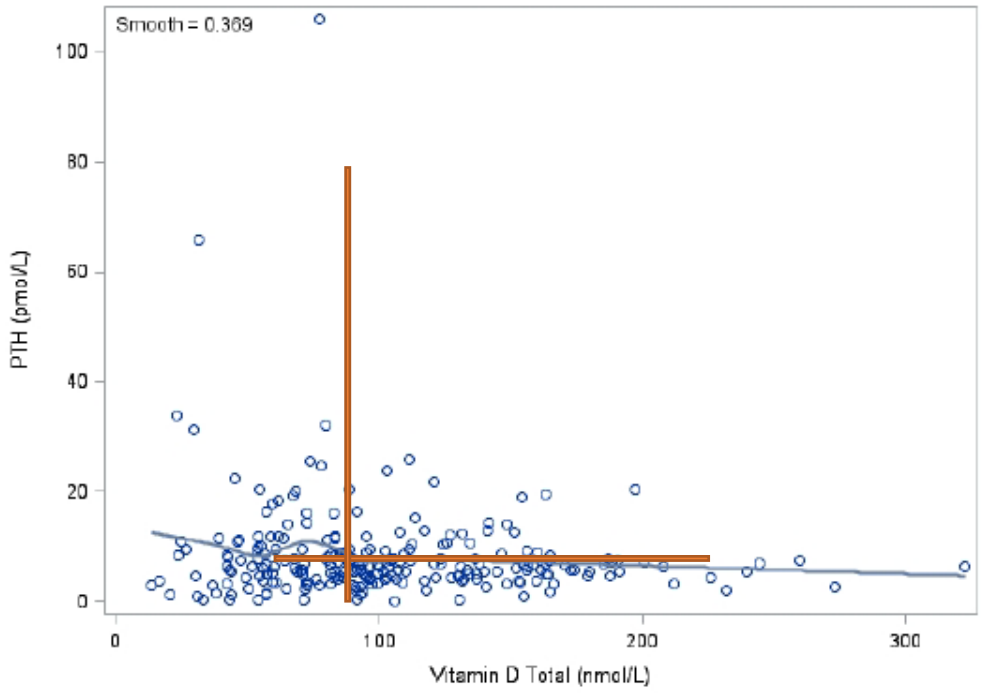
Supplementary Table II: Descriptive characteristics for patients with CKD.

	CKD (n=844)	
	Males	**Females**
N (%)	281 (17.65)	563 (35.36)
Age (years)	48.9 ±15.6	55.9 ±17.6
25(OH)D (nmol/L)	66.70 (45.40-98.70)	69.70 (44.50-106)
D2 (nmol/L)	7.00 (7.00-22.90)	7.00 (7.00-52.90)
D3 (nmol/L)	45.25 (30.05-65.80)	38.10 (25.50-59.00)
Sufficient (%)	67.87	68.44
Insufficient (%)	25.63	24.04
Deficient (%)	6.50	7.52
1,25(OH)D (pmol/L)	34.00 (21.00-47.00)	112 (76.00-171)
Ca (mmol/L)	2.19 (2.09-2.35)	2.27 (2.03-2.43)
PO4 (mmol/L)	1.33 (1.03-1.75)	1.18 (0.97-1.50)
ALP (IU/L)	103 (74-150)	101 (76-143)
PTH (pmol/L)	19.8 (7.5-47.5)	13.5 (6.1-33.0)

25(OH)D = hydroxyvitamin D, D2 = ergocalciferol, D3 = cholecalciferol, 1,25(OH)2D = di-hydroxyvitamin D, PTH = parathyroid hormone, ALP = Alkaline Phosphatase, data expressed as median and interquartile ranges except age, which is expressed as mean ± SD.



Supplementary Figure 1: LOWESS plot for the Indian group. The linear line (—) is indicative of the 25(OH)D value at which PTH is maximally suppressed.



Supplementary Figure 2: LOWESS plot for the White group. The linear line (—) is indicative of the 25(OH)D value at which PTH is maximally suppressed.

Research Protocol

25(OH) D and 1,25(OH)$_2$D concentrations in patients tested at the Charlotte Maxeke Johannesburg Academic Hospital.

Dr Sunet Botha

Student number: 1807945

Introduction

Vitamin D is important in maintaining health and its biological effects can be divided into a classical and alternative/ non-classical pathway.(1) The classical pathway is essential for bone health and maintaining the calcium and phosphate homeostasis in healthy individuals. The alternative pathway include a wide variety of effects ranging from the inhibition of cellular proliferation, induction of apoptosis/ terminal differentiation, stimulation of insulin to its immunosuppressive effects.(1-3) Different cut offs exist to define vitamin D deficiency and insufficiency.(2, 4, 5)

Vitamin D deficiency is regarded as a worldwide epidemic.(6, 7) Globally it is estimated that approximately 1 billion people are affected by vitamin D deficiency and insufficiency.(8) It affects all age groups ranging from infants, school children, young men and women, pregnant women, their neonates and the elderly in rural as well as urban areas.(6) In childhood it is associated with growth retardation and skeletal abnormalities, most commonly rickets. In adults it is associated with muscle weakness, fractures and osteomalacia.(6) The classical biochemical profile due to bone involvement caused by vitamin D deficiency is: hypocalcaemia, hypophosphataemia, elevation in alkaline phosphatase (ALP) and a raise in parathyroid hormone (PTH) termed secondary hyperparathyroidism. The secondary hyperparathyroidism is a physiological response to a decrease in intestinal calcium and phosphate absorption due to deficient/ low vitamin D levels in an attempt to correct the imbalance created by deficient/ low vitamin D levels. (4) Thus, an inverse relationship exists between vitamin D [25(OH) D] and PTH levels with a rise in PTH as a consequence of a fall in 25(OH) D levels.

Currently 25-hydroxyvitamin D [25(OH)D] is the metabolite used to assess vitamin D related disorders and the body's vitamin D stores as it has the longest half-life (approximately 15days) in vivo compared to all the significant metabolites and as its metabolism is not subjected to significant regulation.(4) Currently the United States Endocrine Society and the Institute of Medicine (IOM) define vitamin D deficiency for adults based on the serum 25-hydroxyvitamin D [25(OH)D] as follows:

	Endocrine Society	IOM	References
Vitamin D deficient	< 50nmol/L	< 30nmol/L	(1, 3, 9)
Vitamin D insufficient	52.5-72.5nmol/L	30-49nmol/L	(8)
Vitamin D sufficient	> 72.5nmol/L	> 50nmol/L	

Table 1

The IOM concluded the Endocrine Society cut off was based on the PTH level elevation below 50nmol/L, an increase in calcium absorption above 50nmol/L and a reduction in fall rates among the elderly when supplemented with 800IU of vitamin D. The IOM also concluded that PTH reaches a plateau point at a 25(OH)D level of 72.5nmol/L separating sufficiency from insufficiency.(10) The concept is that there is a threshold/ plateau point for 25(OH) vitamin D below which there is a rise in PTH (secondary hyperparathyroidism occurs) termed the PTH threshold.(11) This plateau point is still under debate as age, race, renal function, body composition and geographical locations seem to influence this point.(10) There is limited data on the PTH threshold in Africans. A systemic literature review in 2006 revealed a putative threshold between 25 to125nmol/L while a study carried out in African Americans and whites showed different cut offs for both races.(9, 11, 12) Different cut offs are used to define vitamin D deficiency and insufficiency. In our laboratory we use the United States Endocrine Society reference ranges.

Studies conducted in Africa suggest that vitamin D deficiency and insufficiency seems to be common despite yearlong exposure to sunlight.(13) In South Africa, black African children were shown to have approximately 24% lower levels compared to age-matched white children. (9) Children of Asian/ Indian descent were found to have insufficient 25(OH)D levels which were proposed to be due to cultural preference to clothe almost the entire body.(9) Studies among the elderly population yielded contradictory results with some studies suggesting a high prevalence of vitamin D deficiency and others stating adequate levels.(9) A very high level of deficiency has also been reported in Asian men and women compared to African men and women.(14)

Vitamin D (calciferol) is recognized as a pro-hormone and comprises of a group of fat-soluble secosterols.(15) Vitamin D exists in two forms, vitamin D_2 (ergocalciferol) and vitamin D_3 (cholecalciferol). Sources include endogenous synthesis in the skin (vitamin D_3), food products (vitamin D_2) and supplementation (vitamin D_2 or D_3).(2) Vitamin D_2 and vitamin D_3 circulates bound to vitamin D-binding protein (VDBP) with the result that there is only a small circulating free fraction. It undergoes hydroxylation in the liver to 25(OH)D, which is the storage form.(4) In the kidney 25(OH)D undergoes hydroxylation to form 1,25-dihydroxyvitamin D/ 1,25$(OH)_2$D (calcitriol), the biological active form of vitamin D.(4) This step is regulated by PTH, fibroblast growth factor 23 (FGF-23) and 1,25$(OH)_2$D.(16) It is estimated that more than 90% of the body's vitamin D production comes from vitamin D_3 synthesis when skin is exposed to ultraviolet B wavelength sunlight. Therefore, factors including season, latitude, sun exposure duration, clothing, sunscreen usage and skin phototype may influence vitamin D_3 synthesis.(2, 17) Vitamin D_2 is estimated to contribute a mere 10% to the total 25(OH)D levels due to low dietary intake. The production of 1,25$(OH)_2$D is very tightly regulated which results in little fluctuation of levels, except in

cases of extreme vitamin-D deficiency or toxicity. 1,25$(OH)_2$D serum concentrations typically remain between 22–65 pg/ ml. Analysis of 1,25$(OH)_2$D, is only required in limited circumstances such as in the follow up of patients with chronic kidney disease (CKD) who receive vitamin D supplementation and in cases like vitamin D–dependent rickets type 1 (inactivating mutation in the 1-hydroxylase gene), autosomal-dominant hypophosphatemic rickets (mutation of the gene coding for FGF-23, which prevents its breakdown), and X-linked hypophosphatemic rickets (mutations that elevate levels of FGF-23).

We are receiving increasing volumes of 25(OH)D and 1,25 $(OH)_2$D requests at the Charlotte Maxeke Johannesburg Academic Hospital laboratory. We do not know how many of these actually have vitamin D deficiency. There is limited literature on which groups of people are most likely to have vitamin D deficiency. We also do not know how many of the 1,25$(OH)_2$D requests are associated with CKD or are possibly inappropriately requested.

Aim

This study will use secondary laboratory data to describe the populations being tested for both 25(OH)D and 1,25$(OH)_2$D in terms of the IOM guidelines, race, age, gender and renal function. Additionally, the data will be used to describe the trend of vitamin D profiles over the three-year period and to determine a possible PTH threshold indicative of vitamin D deficiency by race. 1,25$(OH)_2$D test utilization as a ratio to 25(OH)D will be described using benchmarking.

Objectives

1. To describe vitamin D profiles [both 25(OH)D and 1,25$(OH)_2$D] according to IOM guidelines by age, gender and race.

2. To describe the trend in vitamin D profiles [both 25(OH)D and $1,25(OH)_2D$] over the last three years with regards to the number of vitamin D requests received and the number of vitamin D deficient patients.
3. To determine a PTH threshold indicative of vitamin D deficiency by race for those patients who have normal renal function.
4. To describe vitamin D concentrations [both 25(OH)D and $1,25(OH)_2D$] in patients with chronic kidney disease as these patients are expected to receive vitamin D supplementation either in the form of 25(OH)D or $1,25(OH)_2D$.
5. To describe $1,25(OH)_2D$ test utilization as a ratio to 25(OH)D to assess appropriate testing.

Methodology

Study design

The retrospective study will be used to assess vitamin D levels for samples tested by the Department of Chemical Pathology at the National Health Laboratory Service at CMJAH.

1. 25(OH)D and $1,25(OH)_2D$ results (from 1 January 2015 to 31 December 2017) will be retrieved from the Laboratory Information System (LIS).
2. We will retrieve any associated calcium, PTH, creatinine, phosphate and alkaline phosphate results.

Sample population

Vitamin D profiles for adults (> 18 years of age) will be assessed looking at the proportions in the different groups described by race, renal function tests: chronic kidney disease (CKD) and non-chronic kidney disease and vitamin D status: deficient, insufficient and sufficient according to IOM guidelines. Seasons of the year will be categorized as winter (June to August), spring (September to November), summer (December to February) and autumn

(March to May). A pilot study conducted on October 2017 vitamin D data from the National Health Laboratory at CMJAH revealed approximately 56 complete vitamin D profile requests of which 14 had chronic kidney disease.

Method of sample selection

Convenience sampling will be used to select all patients that received a Vitamin D test [25 (OH)D]. If there is an accompanying 1,25$(OH)_2$D test that will be requested as well.

Inclusion/ Exclusion criteria

Inclusion criteria: all complete vitamin D profiles, [as defined by a request for 25(OH)D, PTH and CMP with or without an accompanying creatinine/ eGFR and 1, 25$(OH)_2$D] from 1 January 2015 to 31 December 2017. Accompanying results that are taken within a week of the 25 (OH) D results will still be utilized.

Exclusion criteria: incomplete vitamin D profiles [defined as lacking an accompanying PTH or CMP request], any sample from subjects < 18 years or with no age recorded and duplicate samples.

Methodology for lab tests

These are in the Appendices.

We will have two groups of patients: adults with and without CKD.

Data Analysis

Data analysis will take place from 1 December 2018 till 31 March 2019.

Analysis will be conducted using the Stata software package version 12 (College Station, TX, USA). The Shapiro-Wilk's test will be used to test for normality. The results of non-normally distributed data will be reported as medians and interquartile ranges (IQR) and for normally distributed data as means and standard deviations (SD). Non-normally distributed

data will be transformed to achieve normality (log transformed or square root). Contingency tables (2x2) will be used to define renal function and vitamin D status for adults, see Appendix 2. The PTH threshold will be determined by means of a locally weighted regression smoothing scatterplot (LOESS). This is a non-parametric method for fitting a smooth curve that best characterizes the relationship between 25(OH)D and PTH. Multivariate regression analysis using season, age, gender, race and creatinine will be used in a model to assess the determinants of 25(OH)D status. The correlations between serum calcium, ALP and 25(OH)D as well as 1, $25(OH)_2D$ will also be assessed using either linear correlation or Spearman correlation, depending on if the data is parametric or not. We will also look at concentrations of 25(OH)D over the last three years and compare differences by ANOVA and by the linear trend test.

We will compare proportions of subjects with CKD and 25(OH)D deficiency to those without CKD but with 25(OH)D deficiency using the chi squared test.

We will compare ratios of 1,25(OH)D2 to 25(OH)D by using benchmarking comparison.(18)

Outputs

MMEd for Dr S Botha

Publication

Possible recommendations on appropriate 25(OH)D concentrations based on PTH levels that may be followed up by a detailed study that involves the body mass index (BMI).

Provide recommendations on the current policy for 1,25 $(OH)_2$ D requests received at the CMJAH.

Ethics

We have submitted an application to the Human Research Ethics Committee of the University of the Witwatersrand on the 7th of August 2018.

Funding

No funding is required as vitamin D results will be traced by the LIS system.

Timing

	Task Name	2017				2018				2019			
		Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4
1	Watch how-to video on this template (4:16)												
2													
3	**Planning**					**Planning**							
4	Literature review		Literature review										
5	Protocol write-up					Protocol write-up							
6	Departmental presentation							Departmental presentation					
7	Ethics submission							Ethics submission					
8	Protocol submission							Protocol submission					
9	**Data collection**								**Data collection**				
10	**Data analysis**								**Data analysis**				
11	Thesis write-up										Thesis		

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R14/49 Dr Sunet Botha

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M180808

NAME: (Principal Investigator)	Dr Sunet Botha
DEPARTMENT:	Chemical Pathology Charlotte Maxeke Johannesburg Academic Hospital National Health Laboratories Service
PROJECT TITLE:	25-hydroxyvitamin D and 1,25-dihydroxyvitamin D concentrations in patients tested at the Charlotte Maxeke Johannesburg Academic Hospital
DATE CONSIDERED:	31/08/2018
DECISION:	Approved unconditionally
CONDITIONS:	
SUPERVISOR:	Prof Jaya George
APPROVED BY:	Dr CB Penny, Chairperson, HREC (Medical)
DATE OF APPROVAL:	02/10/2018

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office Secretary on the Third Floor, Faculty of Health Sciences, Phillip Tobias Building, 29 Princess of Wales Terrace, Parktown, 2193, University of the Witwatersrand. I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit the application to the Committee. **I agree to submit a yearly progress report**. The date for annual re-certification will be one year after the date of convened meeting where the study was initially reviewed. In this case, the study was initially reviewed in August and will therefore be due in the month of August each year. Unreported changes to the application may invalidate the clearance given by the HREC (Medical).

Principal Investigator Signature

Date 09/10/18

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

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