The association between 25-hydroxyvitamin D and parathyroid hormone in adolescents living with HIV in southern Africa: a cross sectional study

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Abbreviations

$1,25(OH)_2D$: 1,25-dihydroxyvitamin-D

25(OH)D: 25-hydroxyvitamin-D

AIC: Akaike Information Criterion

ART: antiretroviral therapy

CDC: Centre for Disease Control and Prevention

CV: Coefficient of variation

DEQAS: Vitamin D external quality assessment

EDTA: Ethylenediaminetetraacetic acid

HAZ: Height for age z-score

HIV: Human immunodeficiency virus

IM: Institute of Medicine

IQR: Interquartile range

LC-MS/MS: Liquid chromatography Tandem Mass Spectrometry

NIST: National Institute of Science and Technology

PTH: Parathyroid hormone

RMP: Reference measurement procedure

SD: Standard deviation

TDF: Tenofovir disoproxil fumarate

VITALITY: Vitamin D for adolescents with HIV to reduce musculoskeletal morbidity and immunopathology

WAZ: Weight for age z-score

Abstract

Low vitamin D associated with high parathyroid hormone (PTH) is commonly reported in the context of HIV infection. We determined the association between total 25-hydroxyvitamin-D [25(OH)D] and PTH in adolescents living with HIV, in Zambia and Zimbabwe. Adolescents (11-19 years) perinatally-infected with HIV and established on antiretroviral therapy (ART) for ≥6 months were recruited into a cross-sectional study. Socio-demographic and clinical characteristics were recorded, anthropometry measured, and fasted serum concentrations of 1,25-dihydroxyvitamin-D ($1,25(OH)_2D$), total 25(OH)D and intact PTH measured. The association between total 25(OH)D and PTH was examined using natural cubic spline regression modelling. 842 participants (female: 53.2%) with median age 15.5 (IQR:13.2-17.9) years were enrolled. Median ART duration was 9.8 [IQR:6.3-12.3] years and 165/841 (19.6%) had an HIV viral load >60copies/ml. Stunting (HAZ score<-2) and underweight (WAZ score<-2) were observed in 29.9% and 30.0% respectively. Three quarters (n=639) reported daily calcium intakes <150mg/day. The mean (SD) concentrations of total 25(OH)D and $1,25(OH)_2D$ were 66.1(16.5) nmol/L and 210.6(70.4) pmol/L respectively, and median PTH level was 4.3 (IQR:3.3-5.5) pmol/L. There was an inverse non-linear relationship between total 25(OH)D and PTH, 25(OH)D levelling-off at 74.6nmol/L (95%CI: 74.5-75.2). Results were consistent in those taking tenofovir disoproxil fumarate (81.7%) and in those who were virally unsuppressed. In this population with extremely low habitual calcium intakes, the lack of association between 25(OH)D and PTH when 25(OH)D exceeded 75nmol/L, potentially suggests levels of 25(OH)D >75nmol/l may need to be achieved to improve bone health; investigation is needed in future research studies.

1. Introduction

What constitutes 'adequate' vitamin D is debated around the world [1–3]. There is no global consensus on the definition of 25-hydroxyvitamin D [25(OH)D] deficiency, with suggested values ranging between 30 and 100 nmol/L [4–6]. 25(OH)D regulates skeletal mineralisation during growth and also thought to play an important role in facilitating macrophage and T-cell function and maintaining a healthy gut microbiome [7–9]. In children and adolescents, vitamin D deficiency is associated with secondary hyperparathyroidism, rickets, osteomalacia and poor bone growth [10].

Fifty three percent of individuals with HIV live in Eastern and Southern Africa [11], a region characterized by a predominantly temperate climate, providing ample sunshine throughout the year [12,13]. Low vitamin D is commonly reported among people living with HIV [14,15] in part, it is thought, due to certain anti-retroviral drugs [16]. Tenofovir disoproxil fumarate (TDF) is associated with lower 25(OH)D, thought to result from the upregulation of 24-hydroxylase, leading to lower circulating 25(OH)D and 1,25-dihydroxyvitamin D ($1,25(OH)_2D$) concentrations [16,17]. To date, studies on the prevalence of vitamin D deficiency in children and adolescents with HIV have generated variable estimates, mainly due to varying thresholds for insufficiency [18–20].

In 2018, a global systematic review of vitamin D deficiency in children and adolescents living with HIV concluded that the literature comprised multiple small, underpowered and heterogenous vitamin D studies from which it was not possible to draw a firm conclusion on what constitutes an adequate concentration of 25(OH)D for optimising bone health, lowering the risk of secondary hyperparathyroidism and prevention of rickets and osteomalacia [21].

Methods of modelling the relationship between 25(OH)D and PTH have so far been limited to linear spline models [22–24] and non-linear locally weighted regression smoothing (loess) [25–27]. However, the use of linear spline models is questionable since the relationship between 25(OH)D and PTH is non-linear, regardless of the method of segmentation. Similarly, besides its flexibility and ability to show a pattern of association between two variables, a loess function requires dense data to give a smoothed estimate such that they lack tail precision when data are sparse [28,29].

This study aimed to determine the concentration of 25(OH)D at which the association with PTH changes, in children and adolescents living with HIV in Zimbabwe and Zambia. Understanding of such relationships may provide insights into what might constitute 'adequate' vitamin D in the context of HIV infection, chronically low habitual calcium

intakes, and in turn an understanding of musculoskeletal development in peripubertal adolescents growing up with HIV

2. Methods

2.1 Study design, setting and population

We conducted a cross-sectional study nested within a phase III individually randomised, double-blinded, placebo-controlled trial of vitamin D_3/calcium carbonate or placebo [Vitamin D for adolescents with HIV to reduce musculoskeletal morbidity and immunopathology (VITALITY): Pan African Clinical Trials Registry PACTR202009897660 29] [30].

The trial enrolled 842 [$n = \frac{p(1-p)\times\alpha^2}{d^2}$, where p: prevalence vitamin D deficiency (<30nmol/L) [31], α: 95% confidential level; 1.96, d: error; 3%] adolescents aged 11-19 years living with HIV recruited from public sector HIV outpatient clinics in Harare, Zimbabwe and Lusaka, Zambia between January and December 2021. Lusaka and Harare have relatively similar latitude of -15.4^o and -17.8^o respectively suggesting comparable sunlight exposure [32]. The inclusion criteria were perinatally-acquired HIV, taking antiretroviral therapy (ART) for at least six months, and willing to give blood samples. Exclusion criteria included: being acutely unwell, taking tuberculosis treatment, currently pregnant or breastfeeding and a history of either thyrotoxicosis, chronic renal disease, hypercalcemia, phosphate metabolism disorder or osteomalacia. Baseline data were used for this analysis.

2.2 Data collection

An interview-administered questionnaire pre-programmed using Open Data Kit on to electronic tablets, was used to collect socio-demographic and clinical data including HIV history. Socioeconomic status (SES) quintiles were derived from a principal component analysis of the participant's household assets. Dietary calcium intake was assessed using a dietary diversity questionnaire adopted on that of the Food and Agriculture Organization of the United Nations (FAO) questionnaire adapted to Zimbabwe and Zambia, and focussing on multi-micronutrients rich foods (e.g., dairy, eggs, fish, legumes) [33]. Using the FAO food composition tables, estimated dietary calcium intake was then calculated based on the International Osteoporosis Foundation (IOF) frequency of consumption, serving size and calcium quantity per portion size (Supplementary table 1) [34–36].

Participants underwent height and weight measurements and Tanner pubertal staging measurement; Z-scores were calculated using UK reference data [37–39]. Stunting and underweight were classified as height- and weight-for-age Z-scores <-2 respectively [38]. Venous blood was collected into EDTA tubes (BD Vacutainer) for 1,25$(OH)_2$D, total

25(OH)D and intact PTH measurements. Blood tubes were promptly centrifuged, and aliquots stored at -20°C, with all analyses performed on the first thaw. HIV viral load testing was performed using the Qiagen rotor gene Q, Hologic Panther, or GeneXpert machines in Zambia and the Roche COBAS Ampliprep/COBAS Taqman48 in Zimbabwe. The classification of HIV viral load suppression (<60 vs ≥ 60 copies/ml) was based on the assay limit of detection.

2.3 Total 25(OH)D, 1,25(OH)$_2$D, 24,25(OH)$_2$D and intact PTH measurements

25(OH)D, 1,25(OH)$_2$D and intact PTH concentrations were analysed at the Bioanalytical Facility, University of East Anglia (Norwich, UK). Liquid chromatography Tandem Mass Spectrometry (LC-MS/MS) methods were used for 25(OH)D and 1,25(OH)$_2$D as previously described [40,41]. The 25(OH)D3 and 25(OH)D2 assays were calibrated using the National Institute of Science and Technology (NIST) standard reference material SRM972a. Inter-assay coefficient of variation (CV) were <8.4% across the assay working range of 0.1 to 200.0 nmol/L. 1,25(OH)$_2$D3, 1,25(OH)$_2$D2, 24,25(OH)$_2$D3 and 24,25(OH)$_2$D2 were analysed by LC-MS/MS following immunoaffinity sample pre-treatment and derivatisation. The assays were calibrated using certified pure internal standards (Cerilliant, LGC). Inter-assay CV were <9.8% across the assay working range of 20.0 to 800.0 pmol/L. All vitamin D metabolite assays met the requirements specified by vitamin D external quality assessment (DEQAS) scheme (http://www.deqas.org/; accessed on 30th Oct 2023). The 25(OH)D3 and 25(OH)D2 assays showed <6% accuracy bias against the Centre for Disease Control and Prevention (CDC) reference measurement (RMP) target values on the DEQAS scheme. Intact PTH was analysed by electrochemiluminescence immunoassay (ECLIA) on the COBAS (Roche Diagnostics, Mannheim, Germany) platform. The inter-assay CV was ≤ 3.8% across the analytical range of 0.1-530.0 pmoL.

2.4 Ethical approval

This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures were approved by the Biomedical Research and Training Institute Institutional Review Board (reference AP158/2020); Harare Central Hospital Ethics committee on 18 May 2020 (reference HCHEC030320/12); London School of Hygiene and Tropical Medicine Ethics Committee (reference 22030); Medical Research Council of Zimbabwe (reference A/2626) and University of Zambia Biomedical Research Ethics Committee (reference 1116-2020). Written informed consent in the local vernacular was obtained from all subjects/patients.

2.5 Statistical analysis

Data were cleaned, checked and analysed using RStudio (2023: v.421; Integrated Development for R. Boston, MA) [42]. All quantitative variables were summarised using mean ± standard deviation (SD) if normally distributed, or otherwise as median with an interquartile range (IQR). Categorical variables were summarised as frequencies with percentages. Participant age, dietary calcium intake, PTH and $1,25(OH)_2D$ distributions were summarised by Tanner stage and sex. Monthly variation in 25(OH)D concentrations over the data collection period was investigated using box (median and IQR) plots. Correlations between $1,25(OH)_2D$ and PTH, 25(OH)D, and dietary calcium intakes were determined using scatter plots.

The analysis was conducted sequentially: (i) explored the relationship between total 25(OH)D and PTH using a scatter plot with a non-parametric, loess line fitted. As the association exhibited different slopes in different ranges of the data, piecewise regression was used to model the relationship between total 25(OH)D and PTH; (ii) considered different univariable piecewise regression models ranging from linear to order-six polynomial functions for total 25(OH)D and PTH to best fit the data and determine the slope pattern; (iii) used the likelihood ratio test to identify the best fitting univariable piecewise regression model; (iv) identified a natural cubic spline regression as the best model fitting the relationship between total 25(OH)D and natural log transformed PTH (see supplementary methods) [43]; (v) further used the Akaike Information Criterion (AIC) to determine the optimal degrees of freedom for the natural cubic spline regression curve [range of degrees of freedom (2-6)]; (vi) assessed the slope pattern by plotting the piece-wise natural cubic spline regression coefficient against total 25(OH)D to determine an inflection point (a point where the relationship between 25(OH)D and PTH differed before and after that point [44]); (vii) identified an inflection point in the cubic spline regression curve where the association between 25(OH)D and PTH levelled-off, as informed by the 95% confidence interval of the regression coefficient.

In sensitivity analyses, the study first assessed the natural cubic spline model stratified by (i) ART regimen: those taking TDF vs. those not, and (ii) HIV viral load: (those < and > 60 copies/mL) to determine the consistency of inflection points. Secondly, a natural cubic spline model was fitted for the relationship between the vitamin D metabolic ratio (VMR) [$25(OH)D/24,25(OH)_2D$] and 25(OH)D to confirm the consistency of the inflection point where the association between 25(OH)D and the VMR levelled-off [27].

3. Results

3.1 Participant characteristics

We enrolled 842 participants, with median age of 15.5 (IQR: 13.2-17.9) years, and 53.2% female (n=448) (Table 1). Most participants were in Tanner stages IV (n=207; 24.6%) and V (n=261; 31.1%) with a higher proportion of girls in the latter category (Tanner stage V: 38% vs 23.2%) (Supplementary table 2). Stunting was common, occurring in 29.9% of participants (n=251/840), as was being underweight (n=253/842; 30%). Three quarters (n=639; 75.9%) reported consuming no more than 150 mg of dietary calcium per day. The median duration of ART was 9.8 (IQR: 6.3-12.3) years: 81.7% (n=688) were taking a TDF containing ART regimen. Overall, 164/841 (19.5%) were virally unsuppressed with an HIV viral load ≥ 60 copies/ml.

3.2 Serum 1,25(OH)$_2$D 24,25(OH)$_2$D total 25(OH)D and PTH

The mean 25(OH)D was 66.1 (SD:16.5) nmol/L; 25(OH)D was comparable between Zimbabwean [mean: 61.3 (SD: 14.2) nmol/L] and Zambian [mean: 70.8 (SD: 17.3) nmol/L] participants. The mean 1,25(OH)$_2$D was 210.6 (SD: 70.4) pmol/L and likewise the distribution was similar in the two countries [Zimbabwe: 213.1 (SD: 74.9) pmol/L vs Zambia: 208.1 (65.5) pmol/L]. In contrast, serum 24,25(OH)$_2$D was higher in Zimbabwe [4.5 (SD:1.8) nmol/L] than Zambia [3.7 (SD:1.3) nmol/L] with the overall mean of 4.1 (SD:1.6) nmol/L. The distribution of PTH was right skewed, with median 4.3 (IQR: 3.3-5.6) pmol/L, with no differences by country (Table 1 and Supplementary figure 1a). No evidence of seasonal variation in 25(OH)D concentrations was seen as medians (IQR) were similar (Supplementary figure 2a). The study determined moderate positive (r= 0.274), very weak positive (r= 0.013), and very weak negative (r= -0.065) correlations between 1,25(OH)$_2$D and PTH, 25(OH)D and dietary calcium intake respectively (Supplementary figure 3a). Dietary calcium intakes and PTH concentrations were similar by Tanner stage in males and females. Marginally higher 1,25(OH)$_2$D concentrations were seen in participants in Tanner stage III (Supplementary table 2).

3.3 The association between total 25(OH)D and PTH

Figure 1 illustrates the relationship between total 25(OH)D and PTH in all participants. The scatterplot (with loess smoother) indicated an inverse non-linear relationship. Notably, the loess line also indicated no discernible change in the concentration of PTH for higher values of 25(OH)D.

3.4 PTH-associated total 25(OH)D inflection points

The relationship between total 25(OH)D and log transformed PTH, modelled using a natural cubic spline (Figure 2a), showed a visually similar association pattern to the scatter plot in Figure 1. Figure 2b shows the regression coefficient for the natural cubic spline model [total 25(OH)D vs PTH] at different values of 25(OH)D. The model showed a rapid change in the regression coefficient for the natural cubic spline model for values of 25(OH)D from 59.6 nmol/L (95% CI: 59.4-59.6) to 74.6 nmol/L (95% CI: 74.5-75.2). Figure 2b also shows that the association of total 25(OH)D and log-transformed PTH levels off (inflection point) at 74.6 nmol/L (95% CI: 74.5-75.2), which is also the point at which the 95% confidence interval of the regression coefficient crosses the null (Figure 2b). In sensitivity analyses, stratifying by (i) TDF containing ART regimen (ii) HIV viral load (≥60 copies/ml), we identified consistent evidence of an inflection point (at approximately 75nmol/L) from the natural cubic spline models (Supplementary figures 4a and 5a). Furthermore, a similar association between 25(OH)D and the VMR was observed with an inflection point at 72.4 nmol/L (95% CI: 67.1-78.7) (Supplementary 8a).

4. Discussion

There is no global consensus for the clinical threshold value for defining vitamin D [25(OH)D] insufficiency or deficiency [45,46]. This study confirms the established non-linear association between total 25(OH)D and PTH, and determines for the first time, using natural cubic spline modelling, a clear inflection point in the relationship between serum total 25(OH)D and PTH among children and adolescents living with HIV in southern Africa. This inflection point suggests that, in this population, a plasma total 25(OH)D of at least 75 nmol/L is required to see the 25(OH)D – PTH association levelling off. The inverse relationship between total 25(OH)D and PTH is strongest, at serum concentrations of 25(OH)D levels <60 nmol/L. These thresholds appeared robust to ART regimen and HIV viral load.

A non-linear relationship between total 25(OH)D and PTH has been widely observed [23,47], indicative of the role of low 25(OH)D contributing to increasing PTH concentrations in this population [48]. Unlike linear spline models [22,49,50], which assume linearity leading to underfitting, the use of a natural cubic spline is more valid as (i) it allows for a non-linear association between 25(OH)D and PTH which better fits the true relationship, (ii) can handle outliers for the association between 25(OH)D and PTH, by modelling the points with an

additional constraint of linearity [51], and (iii) can provide smoother flexible patterns showing different variations in the relationship between total 25(OH)D and PTH.

The current study suggests that vitamin D [25(OH)D] concentrations of at least 75nmol/L may be required for PTH to be at its lowest among adolescents with HIV in southern Africa, in a setting with habitually low dietary calcium intakes. This aligns with the findings from other studies [1,31] although many studies among HIV-negative populations have used lower 25(OH)D concentration to define vitamin D insufficiency (e.g., 50nmol/L) [31,52–55]. Similar findings showing levels of 25(OH)D > 75nmol/L are required to lower PTH levels have also been reported in a healthy adult Kenyan population (n=253) [56], a country with a comparable climate to Zambia and Zimbabwe, although levels were determined using a quadratic model.

Using the second polynomial function showed a less realistic symmetrical inverse relationship between 25(OH)D and PTH with the minimum point of the quadratic model as the inflection point. Despite the well-established non-linear relationship between total 25(OH)D and PTH [56,57], some studies conducted in young people continue to use, arguably the less accurate, linear models to define 25(OH)D adequacy, and hence results vary considerably [22,56–60]. As such, the Institute of Medicine's (IOM) recommendation to use 25(OH)D ≥50 nmol/L (20 ng/mL) is widely supported as an acceptable approach to prevent musculoskeletal disease among children and adolescents [1,53]. Studies of African populations, regardless of health status, have generally shown higher mean total 25(OH)D concentrations than in other regions globally [20,61,62], as well as low dietary calcium intakes, as demonstrated in this analysis. Our findings that vitamin D [25(OH)D] concentrations of at least 75nmol/L may be required for PTH to be at its lowest among adolescents with HIV in southern Africa raises the possibility that the IM's recommended 50 nmol/L threshold to promote bone health might be too low in this setting.

The normal reference range for the LC-MS/MS assay used to measure $1,25(OH)_2D$ has been reported to be 108 – 246 pmol/L based on a population of Caucasian adolescents [63]. However, local $1,25(OH)_2D$ reference values for African adolescents are limited, and not available in literature. In this study, 73% of participants had a value that fell within this reference range, though the generalisability of this estimate is limited due to differences in sunlight exposure, dietary intake, genetic factors and skin pigmentation between the reference and study population.

This is the first study in East or Southern Africa to determine the association between 25(OH)D and PTH in adolescents living with HIV. Strengths include a large sample size, the use of a robust non-linear model and use of a common PTH assay with low levels of

laboratory variation. However, the cross-sectional nature of the study prevents inference on causality between total 25(OH)D and PTH concentrations. Participants with secondary hyperparathyroidism were excluded as it would have been unethical to randomise them to the placebo-controlled trial. Heterogeneity in the data arises as a result of inclusion of participants: (i) with different calcium intakes, (ii) at different pubertal stages, (iii) attending during different seasons, and (iv) by combining boys and girls (as do all vitamin D clinical guideline recommendations), such that interpretation should be made bearing the population in mind. Dietary calcium intake was only semi-quantitatively assessed using a diet diversity questionnaire without direct validation against quantified portions, which may have underestimated intake. The relationship between total 25(OH)D and PTH is affected by multiple factors like ethnicity, pubertal status, renal function, dietary calcium intake which was beyond the scope of this study to explore. Larger sample sizes, generating narrower confidence intervals, may identify an upper infection point > 75nmol/L. The lack of an HIV-negative control group limited the generalizability of findings although it should be noted that in the IMVASK study in the Harare adolescent population[64], calcium intakes were similarly low in HIV negative control children.

In conclusion, this study reports an inverse relationship between total 25(OH)D and PTH in adolescents living with HIV, and identifies inflection points at which the association changes; the association weakened when 25(OH)D exceeded 75nmol/L. These results may be used to inform the epidemiology of vitamin D insufficiency in Southern Africa among individuals living with HIV. To what extent our findings are explained by the very low dietary calcium intake reported in this population, during a critical period of growth, merits further investigation. Ultimately, understanding the 25(OH)D-PTH relationship in greater detail is intended to help healthcare providers tailor appropriate supplementation strategies to improve bone health during a period of rapid growth and mineral accumulation in a nutritionally vulnerable population.

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5.2 Declaration of interests

None to declare

5.3 Authorship

The study was conceived by TM, KAW, CLG. Design: TM, NVD, VS, LK, RAF, CLG. Data acquisition: TM, TB, VS, NVD, HBM, MC . Analysis: TM, AM, NIM, VS, KAW, CLG. Interpretation: TM, AM, KAW, NIM, RAF, CLG. Manuscript drafting: TM, AM, NIM, KAW, RAF, CLG. Manuscript revision: TM, JCYT, WDF, NVD,RAF, CLG. All authors take responsibility for their contributions outlined above and have read and approved the final manuscript.

5.4 Data availability

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request through the London School of Hygiene and Tropical Medicine (LSHTM) DataCompass.

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Titles and legends for Figures and Tables

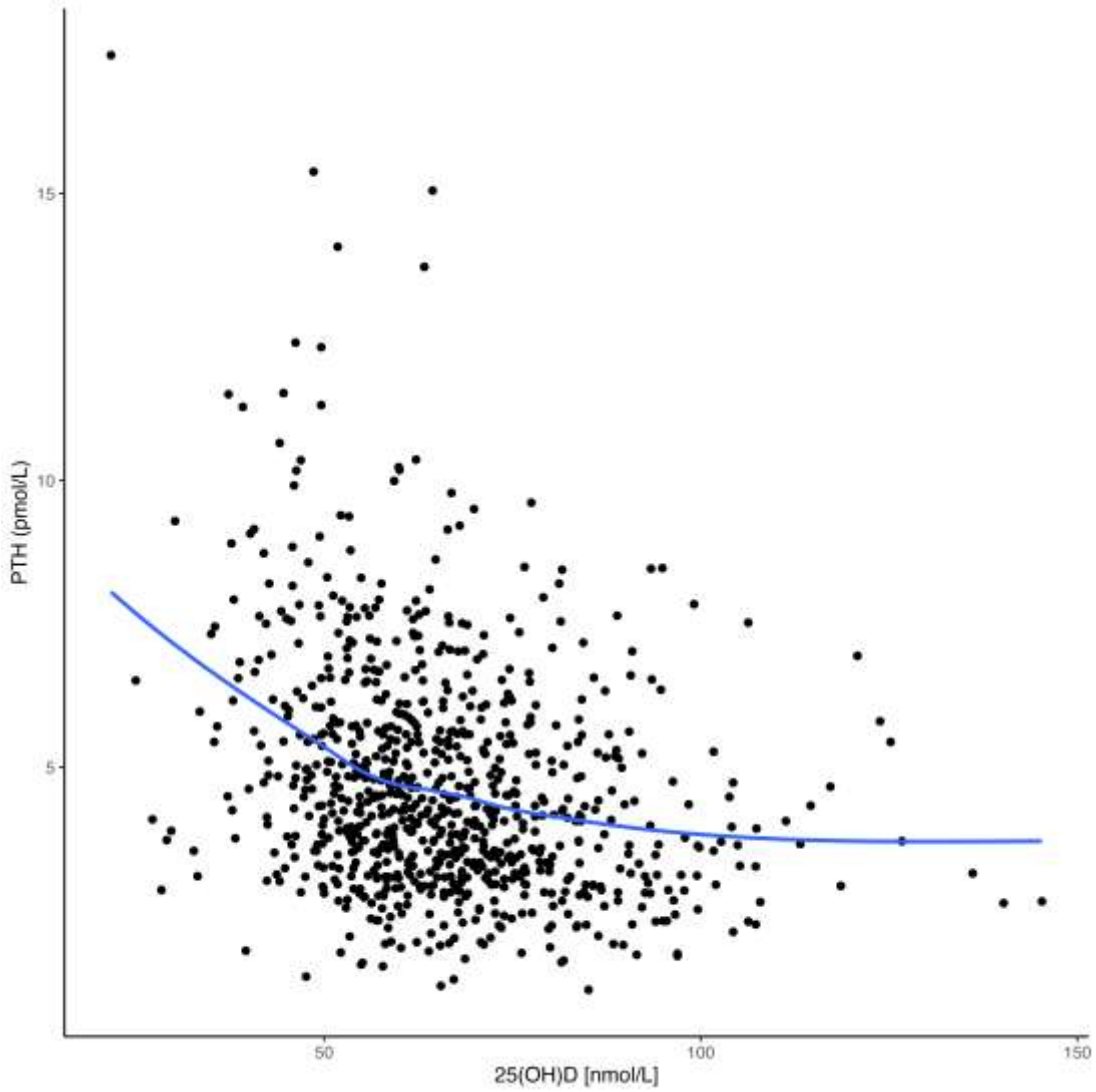


Figure 1a: Scatter plot and a non-parametric locally weighted smoothing (loess) fitted line, illustrating the relationship between total 25(OH)D and PTH.

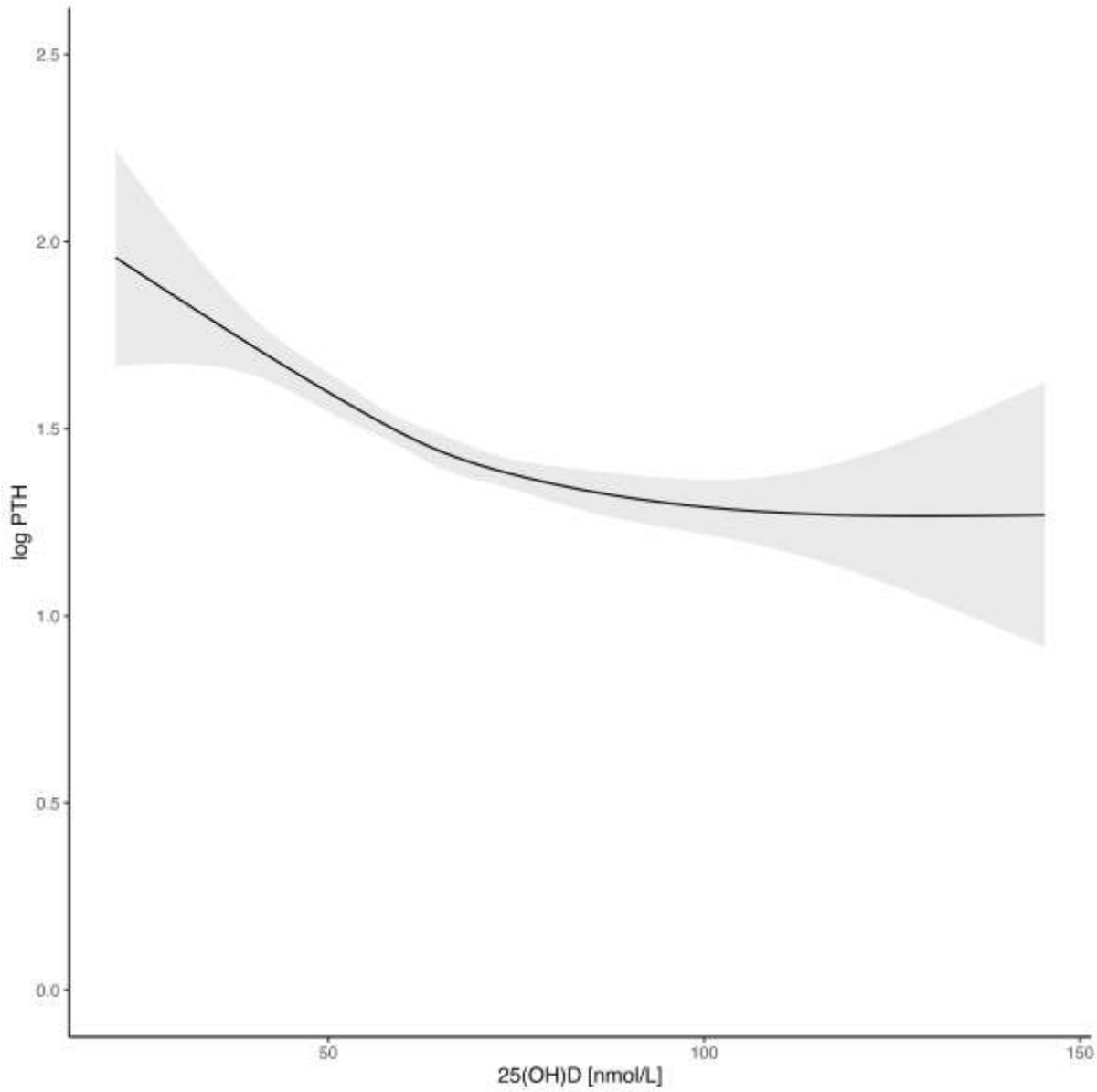


Figure 2a: The relationship between total 25(OH)D and PTH (log transformed), modelled using a non-parametric natural cubic spline curve. The regression curve is fitted with a 95% confidence interval showing the variation of the natural cubic spline coefficient.

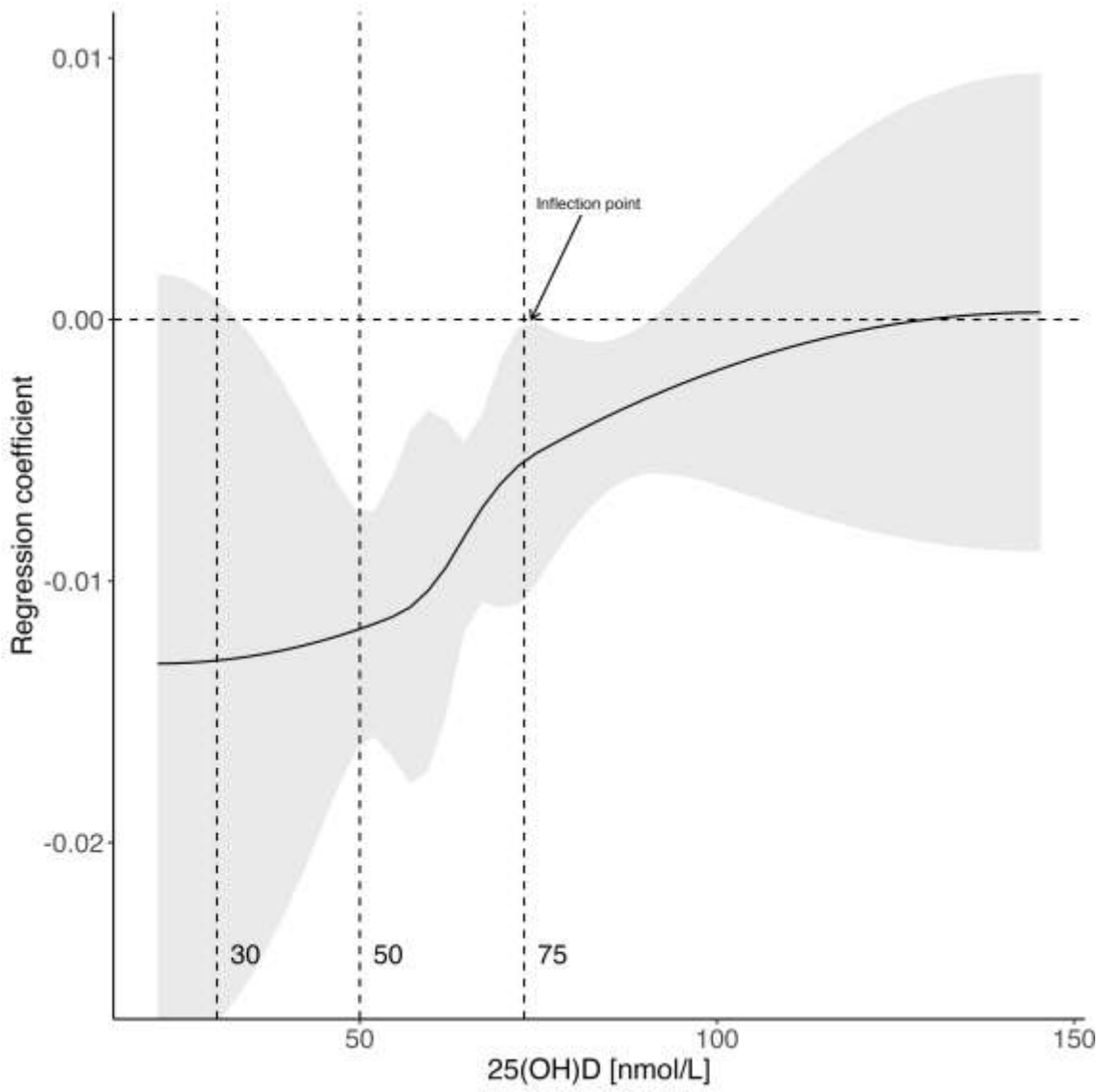
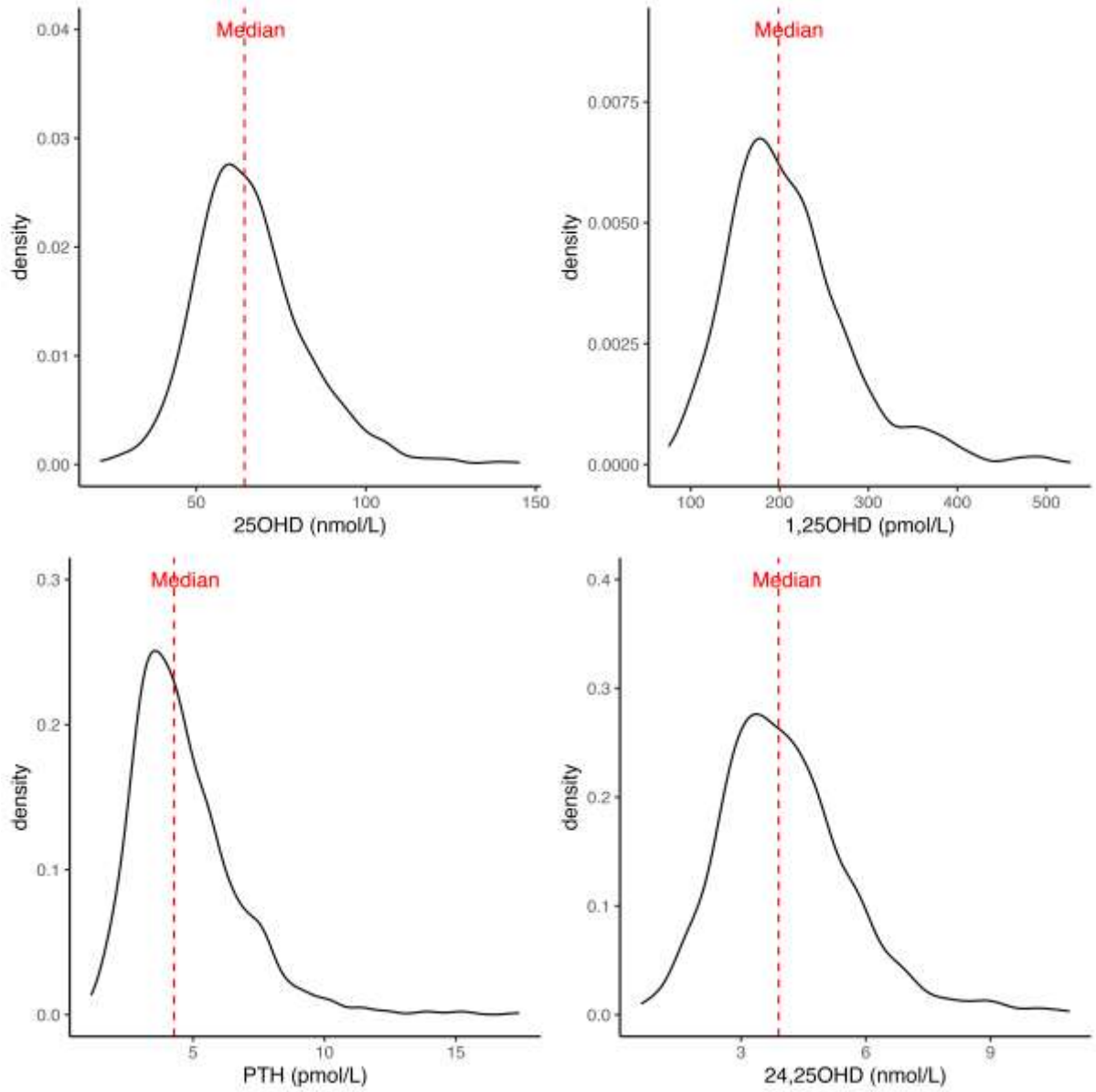
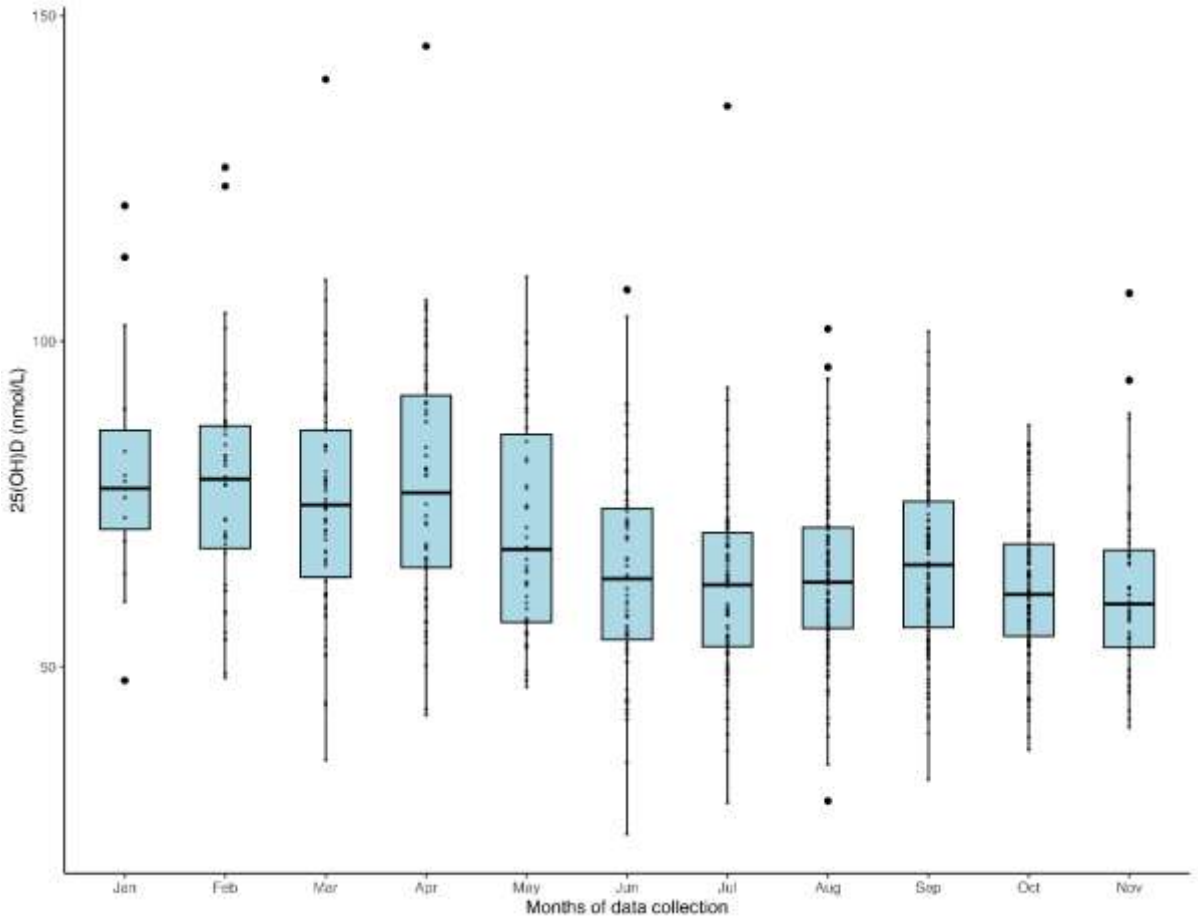


Figure 2b: Identification of inflection points for the relationship between total 25(OH)D and (log transformed) PTH at 59.6 and 74.6 nmol/l. The natural cubic spline regression coefficient with a 95% confidence interval is plotted against total 25(OH)D. Since, natural cubic splines involves appropriate partitioning of the curve such that the regression coefficient changes at different values of 25(OH)D, this helps in identification of inflection points. Commonly used definitions of 25(OH)D deficiency (<30 nmol/l) and insufficiency (<50 nmol/l) are shown for illustrative purposes.

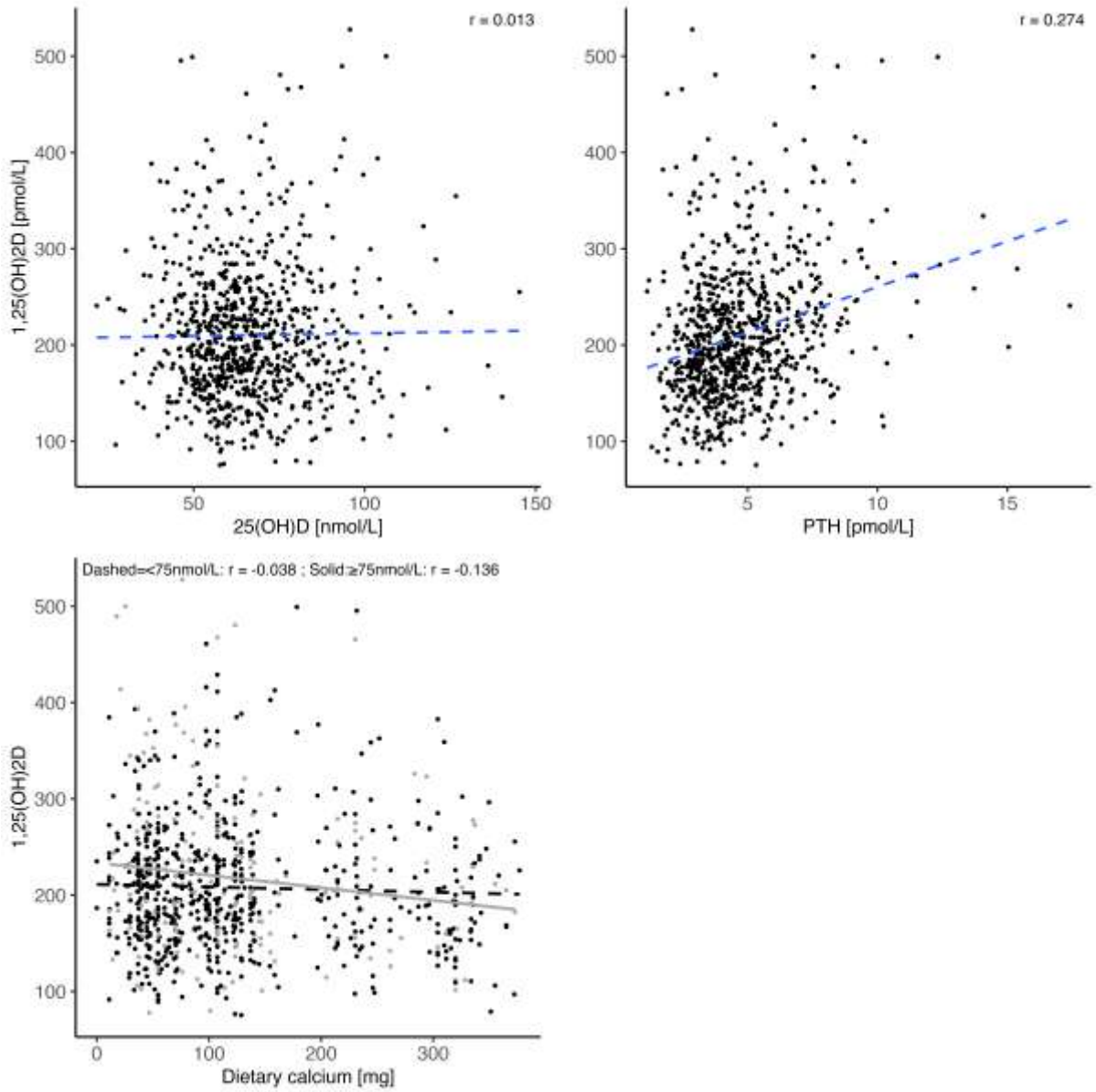


Supplementary figure 1a: Normal density functions showing the distributions for 25(OH)D, 1,25(OH)$_2$D, 24,25(OH)$_2$D and PTH with median values.

Normal density plots for total 25(OH)D, 1,25(OH)$_2$D, 24,25(OH)$_2$D and PTH showing their respective median values.

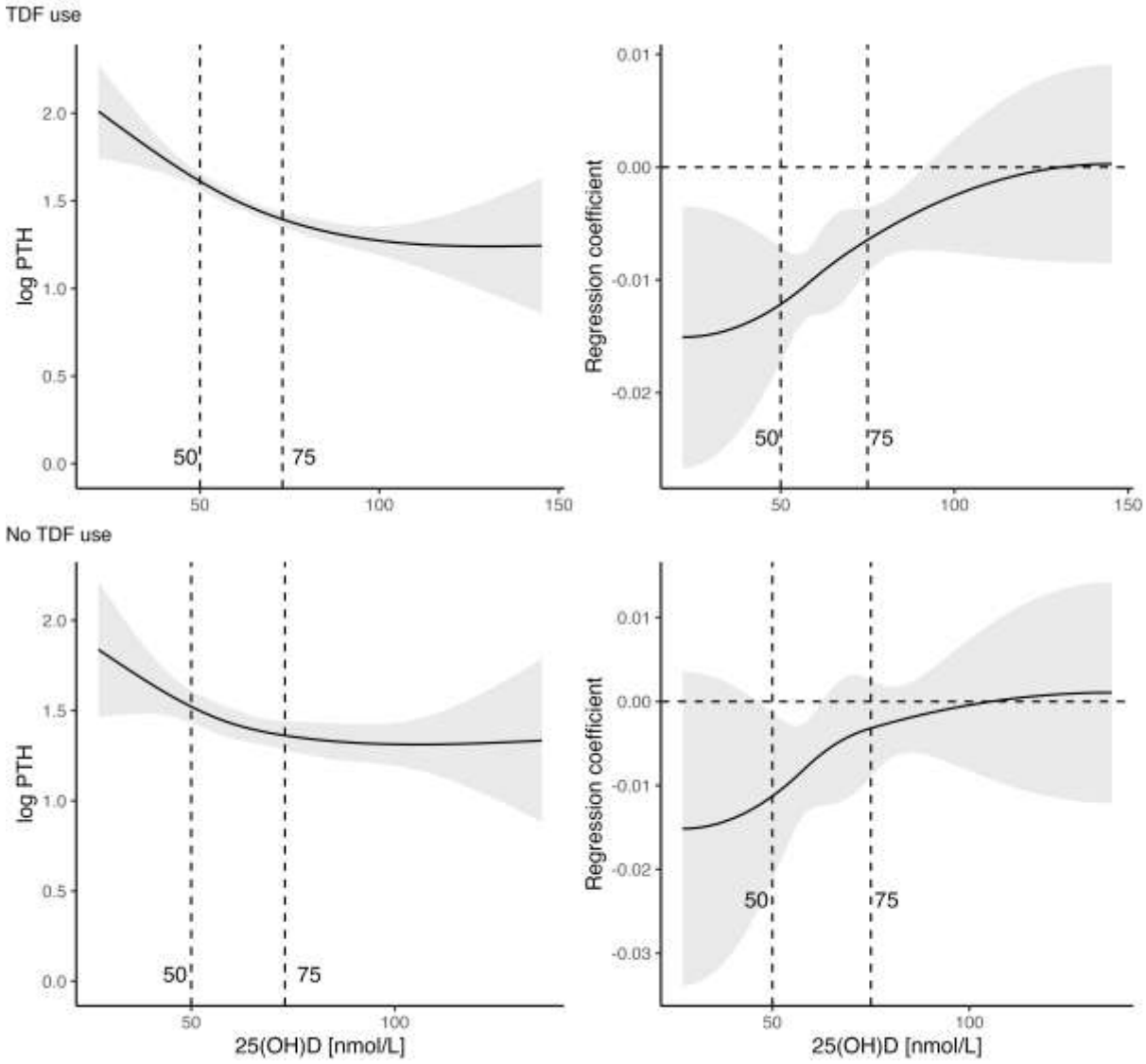


Supplementary figure 2a: Monthly variations in serum 25(OH)D concentrations. Box and whisker plots showing monthly median (horizontal line) and interquartile range (IQR) for 25(OH)D levels over the data collection period.

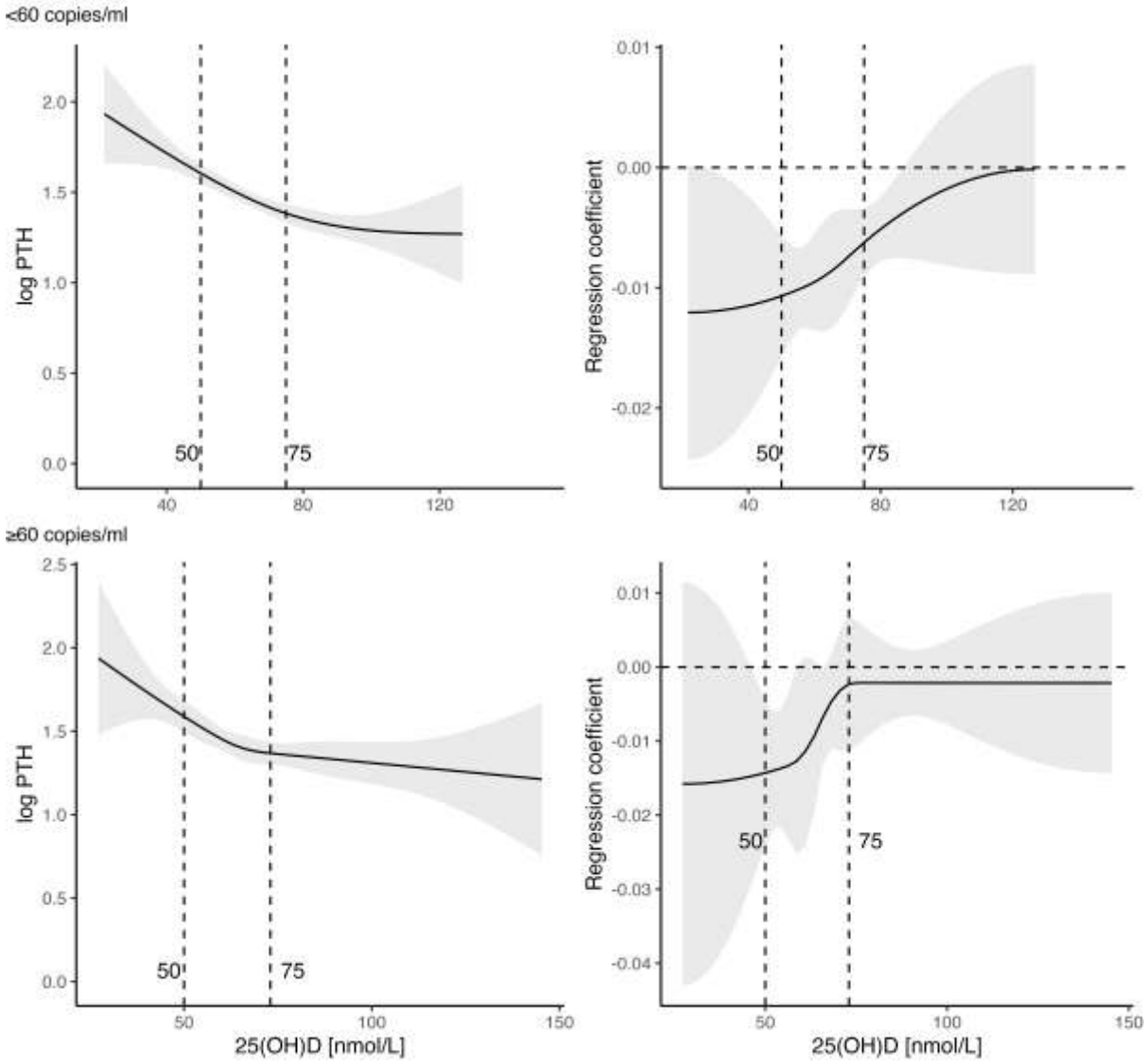


Supplementary 3a: Correlations between 1,25(OH)$_2$D and 25(OH)D, PTH and dietary calcium.

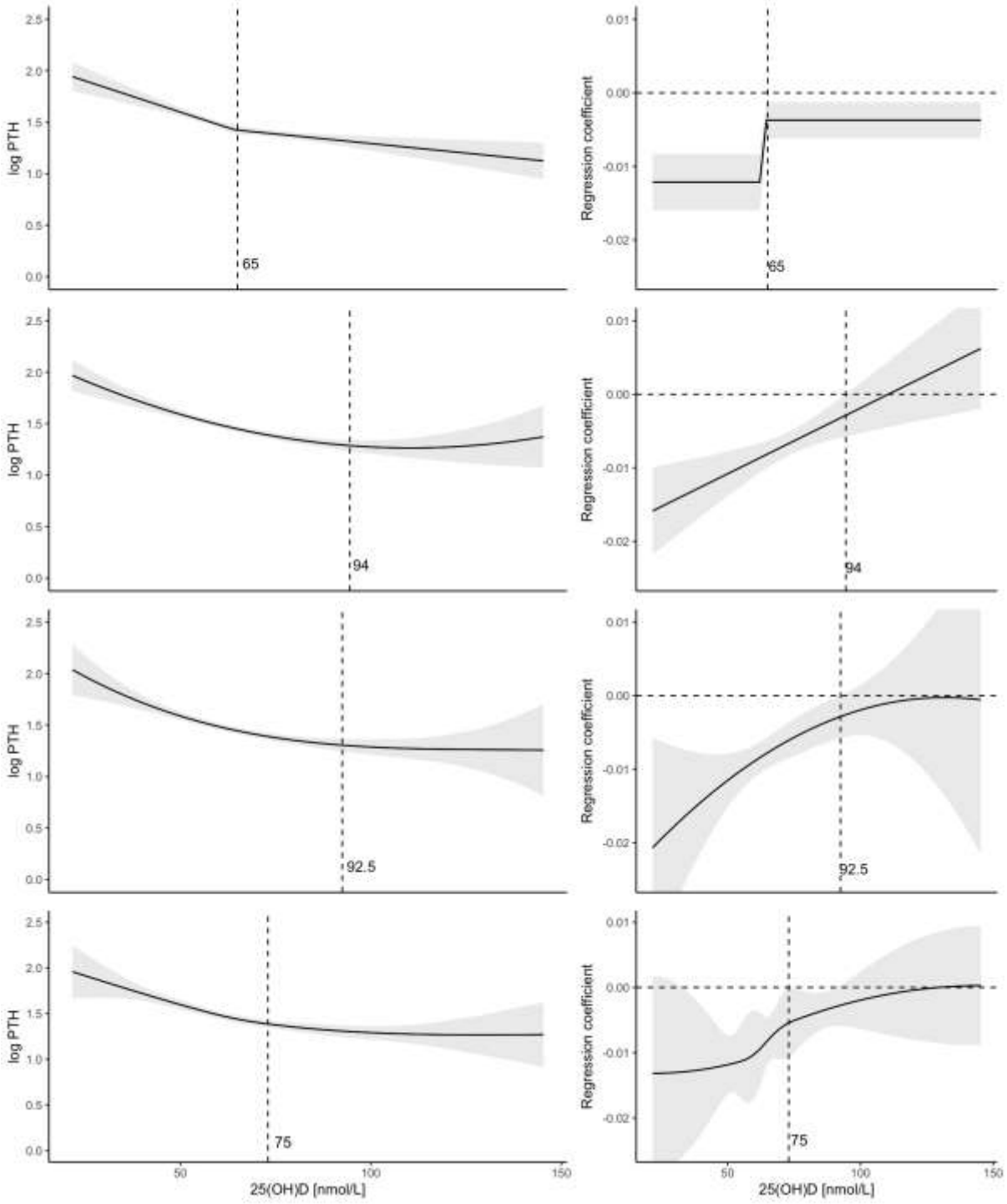
Scatter plots (black dots) showing linear associations between 1,25(OH)$_2$D with 25(OH)D, PTH and dietary calcium. The blue dashed line indicates the line of best fit for the linear correlation. The correlation between dietary calcium and 1,25(OH)$_2$D is stratified by 25(OH)D < 75 nmol/L (black dashed line) vs 25(OH)D ≥ 75 nmol/L (grey solid line). Correlation coefficients (r) are presented in each scatter plot.



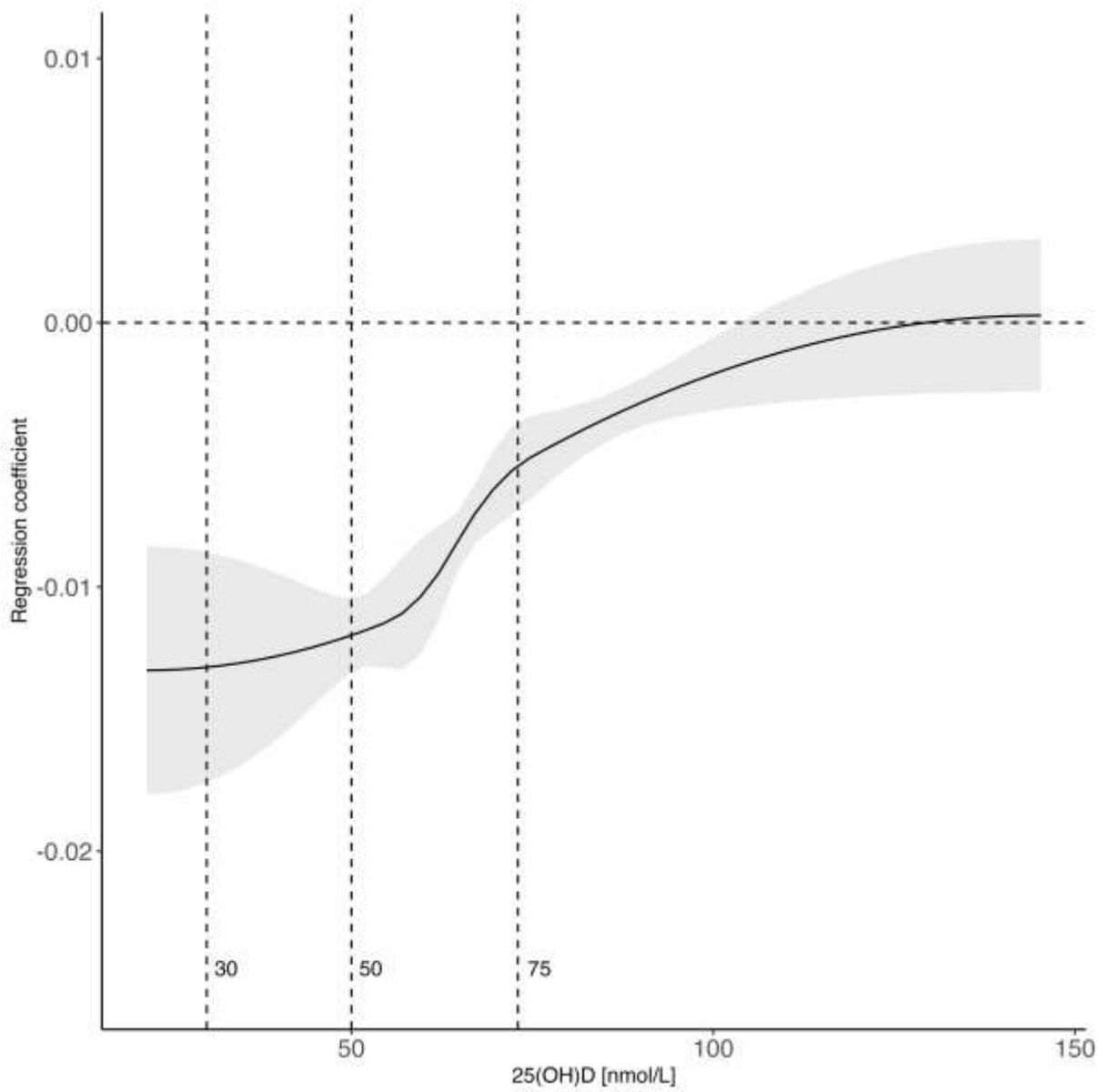
Supplementary figure 4a: Identification of inflection points for the relationship between total 25(OH)D and (log transformed) PTH by ART regimen. Natural cubic spline curves for the relationship between total 25(OH)D and log-transformed PTH and their respective regression coefficients plotted against total 25(OH)D with a 95% confidence interval. The plots are among participants on TDF-containing ART regimen vs those on other HIV treatment regimens showing dotted vertical lines at values 25(OH)D values of 50 and 75nmol/L.



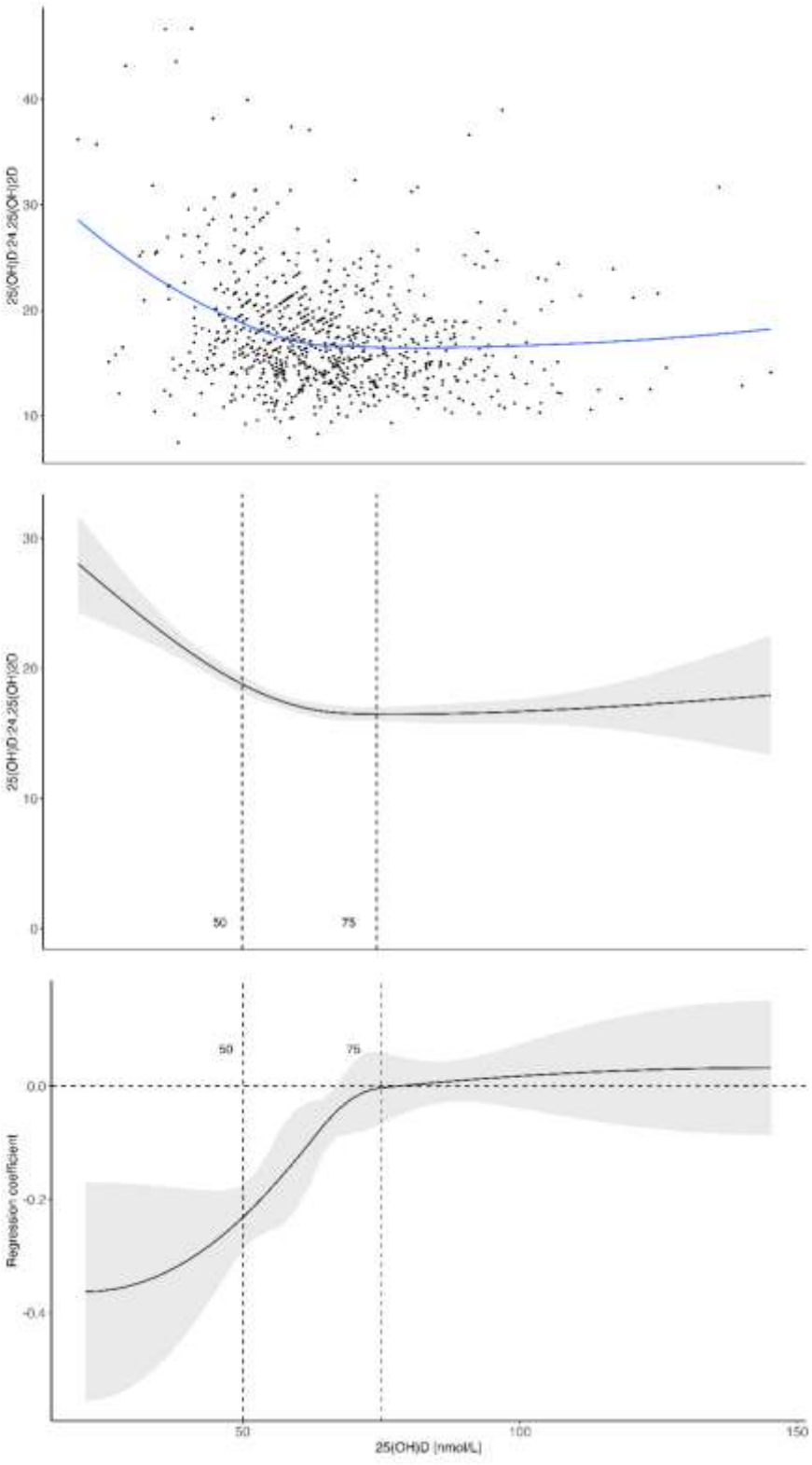
Supplementary figure 5a: Identification of inflection points for the relationship between total 25(OH)D and (log transformed) PTH by viral load. Natural cubic spline curves for the relationship between total 25(OH)D and log-transformed PTH and their respective regression coefficients plotted against total 25(OH)D with a 95% confidence interval. The plots are among participants who are virally suppressed (<60copies/ml) vs virally unsuppressed participants (≥60 copies/ml) showing dotted vertical lines at values 25(OH)D values of 50 and 75nmol/L.



Supplementary figure 6a: The different models evaluated in determining the best fitting model between total 25(OH)D and PTH. Comparison of different models for the relationship between total 25(OH)D and PTH showing the regression coefficient pattern (with a 95% CI) of association in relation to total 25(OH)D. We also show the Akaike Information Criterion (AIC) to estimate the prediction error of the models. AIC values for spline models: Linear (top row: 804.15); Quadratic (second row: 803.7); Cubic spline (third row: 801.4); Natural cubic spline (fourth row: 756.1).



Supplementary figure 7a: Identification of inflection points for the relationship between total 25(OH)D and (log transformed) PTH using simulated data [10 times more data points]. A plot for the natural cubic spline regression coefficient with a 95% confidence interval for the relationship between total 25(OH)D and log-transformed PTH using ten times more (simulated) data points plotted against total 25(OH)D. The non-linear curve also shows total 25(OH)D threshold values of 30 and 50 nmol/L.



Supplementary figure 8a: Identification of inflection points for the relationship between 25(OH)D and the vitamin D metabolic ratio [25(OH)D/24,25(OH)$_2$D]

A scatter plot (top) showing the relationship between 25(OH)D and VMR. The middle and bottom plots show the natural cubic spline coefficient and its 95% confidence interval for the relationship between 25(OH)D and VMR with vertical dotted lines at 50 and 75 nmol/L.

Supplementary methods

Cubic Spline Models

A natural cubic spline (S) which interpolates x values is defined by:

$S_i(x) = a_i + b_i(x - x_i) + c_i(x - x_i)^2 + d_i(x - x_i)^3$, where:

(i) $a_i = y_i$ (y-coordinate of the ith data point), (ii) b_i is the slope of the spline at the ith point and (iii) c_i and d_i are coefficients that depend on the curvature of the spline in the interval $[x_i, x_{i+1}]$. Additionally, the natural cubic spline imposes the condition that the second derivatives at the endpoints x_0 and x_n are both zero: $S''(x_0) = 0$ and $S''(x_n) = 0$ enabling calculations of the coefficients by solving the set equations

Table 1: Baseline descriptive characteristics

	Total subjects (n=842)	**Zambia (n=420)**	**Zimbabwe (n=422)**
Socio-demographic characteristics			
Sex (female), *n (%)*	448 (53.2)	230 (54.8)	218 (51.7)
Age, *median (IQR)*	15.5 (13.2-17.9)	15.0 (13.0-17.4)	16.2 (13.6-18.1)
Socio-economic status quintiles, *n (%)*			
Q_1	170 (20.2)	80 (19.0)	90 (21.3)
Q_2	167 (19.8)	86 (20.5)	81 (19.2)
Q_3	175 (20.8)	101 (24.0)	74 (17.5)
Q_4	162 (19.2)	77 (18.3)	85 (20.1)
Q_5	168 (19.9)	76 (18.1)	92 (21.8)
Daily dietary calcium intake/day (mg), *n (%)*	639 (75.9)	321 (76.4)	318 (75.4)
<150	129 (15.3)	66 (15.7)	63 (14.9)
150-299	74 (8.8)	33 (7.9)	41 (9.7)
300+			
Growth characteristics			
Tanner stage, *n (%)*			
I	77 (9.2)	31 (7.4)	46 (11.0)
II	129 (15.4)	72 (17.1)	57 (13.6)
III	166 (19.8)	90 (21.4)	76 (18.1)
IV	207 (24.6)	102 (24.3)	105 (25.0)
V	261 (31.1)	125 (29.8)	136 (32.4)
Height for age z-score <-2, *n (%)*	251/840 (29.9)	126/420 (30.0)	126/420 (30.0)
Weight for age z-score <-2, *n (%)*	253 (30.0)	135 (32.1)	118 (28.0)
BMI for age z-score <-2, *n (%)*	104/838 (12.4)	60/420 (14.3)	44/418 (10.5)
HIV characteristics			
Viral load (≥ *60*copies/ml), *n (%)*	164/841 (19.5)	64/419 (15.3)	100/422 (23.7)
ART duration, *median (IQR)*	9.8 (6.3-12.3)	8.3 (4.6-12.2)	10.3 (7.7-12.3)
TDF containing ART regimen, *n (%)*	688 (81.7)	360 (85.7)	328 (77.7)
Efavirenz containing ART regimen, n (%)	54 (6.4)	18 (4.3)	36 (8.5)
Vitamin D metabolites			
Total 25(OH)D (nmol/L), *mean (SD)*	66.1 (16.5)	61.3 (14.2)	70.8 (17.3)

1,25$(OH)_2$D (pmol/L), *mean (SD)*	210.6 (70.4)	208.1 (65.5)	213.1 (75.0)
24,25$(OH)_2$D (nmol/L), *mean (SD)*	4.1 (1.6)	3.7 (1.3)	4.5 (1.8)
PTH (pmol/L), *median (IQR)*	4.3 (3.3-5.6)	4.2 (3.3-5.4)	4.4 (3.3-5.8)

TDF: Tenofovir disoproxil fumarate; ART: Antiretroviral therapy; PTH: intact Parathyroid hormone`