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Jonathan C.Y. Tang, Holly Nicholls, Isabelle Piec, Christopher J. Washbourne, John J. Dutton, Sarah Jackson, Julie Greeves, William D. Fraser

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REFERENCE INTERVALS FOR SERUM 24,25-DIHYDROXYVITAMIN D AND THE RATIO WITH 25-HYDROXYVITAMIN D ESTABLISHED USING A NEWLY DEVELOPED LC-MS/MS METHOD.

Jonathan C. Y. Tang[1], Holly Nicholls[1], Isabelle Piec[1], Christopher J. Washbourne[1], John J. Dutton[1], Sarah Jackson[2], Julie Greeves[2], William D. Fraser[1,3]

[1]Bioanalytical Facility, Norwich Medical School, University of East Anglia, Norwich Research Park, Norwich, UK

[2]HQ Army, Andover, UK.

[3]Departments of Diabetes, Endocrinology and Clinical Biochemistry, Norfolk and Norwich University Hospital NHS Foundation Trust, Colney Lane, Norwich, UK

Abstract

$24,25(OH)_2D$ is the product of 25(OH)D catabolism by CYP24A1.The measurement of serum $24,25(OH)_2D$ concentration may serve as an indicator of vitamin D catabolic status and the relative ratio with 25(OH)D can be used to identify patients with inactivating mutations in *CYP24A1*. We describe a LC-MS/MS method to determine: 1) the relationships between serum $24,25(OH)_2D$ and 25(OH)D; 2) serum reference intervals in healthy individuals; 3) the diagnostic accuracy of $24,25(OH)_2D$ measurement as an indicator for vitamin D status; 4) $24,25(OH)_2D$ cut-off value for clinically significant change between inadequate and sufficient 25(OH)D status. Serum samples of healthy participants (n=1996) from Army recruits and patients (n=294) were analysed. The LC-MS/MS assay satisfied industry standards for method validation. We found a positive, concentration-dependent relationship between serum $24,25(OH)_2D$ and $25(OH)_2D$ concentrations. The 25(OH)D:$24,25(OH)_2D$ ratio was significantly higher (p<0.001) at 25(OH)D <50 nmol/L. The reference interval for 25(OH)D:$24,25(OH)_2D$ ratio in healthy subjects was 7-23. Measurement of serum $24,25(OH)_2D$ can be used as predictor of vitamin D status, a concentration of >4.2 nmol/L was identified as a diagnostic cut-off for 25(OH)D replete status. One patient sample with an elevated 25(OH)D:$24,25(OH)_2D$ ratio of 32 and hypercalcaemia who on genetic testing confirmed to have a biallelic mutation of *CYP24A1*. Our study demonstrated the feasibility of a combined $24,25(OH)_2D$ and 25(OH)D assessment profile. Our established cut-off value for $24,25(OH)_2D$ and ratio reference ranges can be useful to clinicians in the investigation of patients with an impaired calcium/phosphate metabolism and may point towards the existence of *CYP24A1* gene abnormalities.

1.0 Introduction

The vitamin D endocrine system plays a major role in human biological functions. The vitamin D pathway produces a large number of metabolites including 25-hydroxyvitamin D (25(OH)D), and 1,25-dihydroxyvitamin D (1,25(OH)D). Each vitamin D metabolites exists in two major forms: cholecalciferol (D_3) is derived from sunlight (UVB) exposure on the skin and meat products; and ergocalciferol (D_2) is derived from dietary plant sources. The synthesis of the metabolites are controlled by 25-hydroxylase in the liver and 1-hydroxylase in the kidney. Severe deficiency of serum 25(OH)D results in decreased production of $1,25(OH)_2D$ which is strongly associated with bone and neuromuscular diseases[1]. Epidemiological studies have shown an inverse relationship between serum 25(OH)D concentrations with a wide spectrum of disease states, including cardiovascular, cancer, diabetes, immunological and psychiatric disorders[2-8]. However, evidence from vitamin D intervention trials supporting beneficial effects of vitamin D supplementation on disease outcome are largely inconclusive[9-12]. The contradictory evidence indicates that the catabolism of 25(OH)D is a multistep process. Recent attention has centred on an alternative pathway of 25(OH)D metabolism driven by 24-hydroxylase producing 24,25-dihydroxyvitamin D ($24,25(OH)_2D$). Studies have found limited biological activity of $24,25(OH)_2D$ in humans, and similarly the metabolite 1,24,25-trihydroxyvitamin D ($1,24,25(OH)_3D$) which was considered to be an inactive excretory product of the vitamin D pathway[13]. The revival of interest in $24,25(OH)_2D$ is partly as a result of altered vitamin D catabolism discovered in patients with chronic kidney disease (CKD)[14], where impaired renal production of $1,25(OH)_2D$ was found to be associated with decreased CYP24A1 (cytochrome P450, family 24, subfamily A, polypeptide 1) activity. The *CYP24A1* gene is expressed in most tissues in the body[15, 16], it encodes the 24-hydroxylase enzyme that catalyses the conversion of 25(OH)D and $1,25(OH)_2D$ into 24-hydroxylated products[13]. The transcription of *CYP24A1* gene is stimulated by the phosphate regulating hormone fibroblast growth factor-23 (FGF23) and suppressed by PTH[14, 17]. As $24,25(OH)_2D$ is the main product of 25(OH)D catabolism by CYP24A1, the measurement of serum $24,25(OH)_2D$ concentration can be useful in identifying patients with loss-of-function mutations in *CYP24A1*. The relative ratio of serum 25(OH)D to $24,25(OH)_2D$ concentration may serve as an indicator of vitamin D catabolic status.

Recent advances in methods for measurement of vitamin D metabolites, in particular with assays for measurement 25(OH)D, have led to the development of a range of semi- and fully automated assays capable of performing measurement of large numbers of samples. Competitive protein binding assays

and enzyme immunoassays using chemiluminescent detection-based systems in fully automated platforms appeal to hospital laboratories due to their ease of use, speed and efficiency[18]. However, immunoassays have variable cross-reactivity with other vitamin D metabolites and are unable to distinguish the two major forms of vitamin D (D_3 and D_2). This results in some immunoassays significantly underestimated an individual's true vitamin D status[19]. Liquid chromatography tandem mass spectrometry (LC-MS/MS) methods quantify vitamin D metabolites based on the compound specific precursor to product ion mass-to-charge (m/z) transitions. They are able to distinguish each hydroxylation metabolite and their respective D_3 and D_2 forms. Proficiency testing bodies such as the International Vitamin D External Quality Control Scheme (DEQAS) have reported that measurement of 25(OH)D by LC-MS/MS methods using traceable calibration standards from the National Institute of Standards and Technology (NIST) exhibit the lowest inter-laboratory imprecision in comparison with immunoassays[20]. LC-MS/MS is now considered to be the reference method for measurement of 25(OH)D.

In this study, our aim was to develop a robust and sensitive assay to measure 25(OH)D and 24,25$(OH)_2$D simultaneously in human serum samples. Our objective was to apply the assay to measure vitamin D metabolites in a large cohort of healthy individuals and patients to examine the clinical correlation of 24,25$(OH)_2$D with 25(OH)D. Our goal was to establish from our cohort of healthy individuals: 1) reference intervals for serum 24,25$(OH)_2$D and 25(OH)D:24,25$(OH)_2$D ratio; 2) to determine the diagnostic accuracy of 24,25$(OH)_2$D measurement as an indicator for vitamin D status; and 3) to identify a 24,25$(OH)_2$D cut-off threshold when vitamin D status is sufficient; i.e. serum 25$(OH)_2$D concentration ≥50 nmol/L, as defined by the U.S Institute of Medicine (IOM) and the UK National Osteoporosis Society (NOS)[21, 22]. Recent publications have reported reference intervals of 25(OH)D:24,25$(OH)_2$D ratio on smaller cohorts [23-25], or post-menopausal women supplemented with vitamin D[26]. In this paper, we established the reference intervals from a large cohort of unsupplemented individuals using a statistical approach supported by the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) working group[27].

2.0 Materials and methods

2.1 Serum sample collection

The characteristics of the subjects included in the study are shown in Table 1. Serum samples used in the healthy cohort (n=1996) were obtained from young British Army recruits during initial training (ClinicalTrials.gov Identifier

NCT02416895). The study was approved by the UK Ministry of Defence research ethics committee (MODREC). Written informed consent was obtained from all study participants. The participants were from Caucasian population and each required to complete details in a health questionnaire, including the use of medications and supplements. Individuals taking calcium and vitamin D supplements were excluded from the study. Samples were collected between June 2014 and March 2016.

Serum from patients (n=294) were collected from residual samples following their course of routine care in accordance with generic ethical approval[28]. The patient samples collected were routine requests for 25(OH)D measurement received by the Department of Laboratory Medicine at the Norfolk and Norwich University Hospital between Feburary 2015 to March 2016. From the clinical information available, the cohort contained patients from Caucasian population presented with fatigue/wellness-check (42.6%), osteoporosis (23.9%), primary hyperparathyroidism (19.9%), liver disease (5.7%), chronic renal failure (6.2%) and unknown (1.7%). Use of vitamin D supplements or drugs known to affect mineral metabolism were reported in 32% of the patients. All samples were anonymised to the researchers at point of access. Blood samples were collected into serum gel separator tube (BD Vacutainer) and centrifuged immediately. After a 10 minute centrifugation at 3,000 x g, serum layer was aliquoted into a separate polystyrene tube and stored at -20°C until analysis.

2.2 Materials, calibration standards and controls

SRM972 traceable 25(OH)D_3 and 25(OH)D_2 serum based calibrators (Chromsystems, München, Germany) and internal quality controls (IQC) (UTAK Laboratories, CA, USA) were analysed in each run. Certified pure standards for 24,25$(OH)_2D_3$, 24,25$(OH)_2D_2$ were used for preparation of spiked standards and deuterated standards 25(OH)D_3-[$^2H_6$] and 24,25$(OH)_2D_3$-[$^2H_3$] (IsoSciences, King of Prussia, PA, USA) were used as internal standards. Deionised water, methanol, acetonitrile and formic acid were LCMS grade, n-heptane and isopropanol were analytical grade (Fisher Scientific, Loughborough, UK). 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD) and methylamine (Sigma-Aldrich, Dorset, UK) were used for derivatization and adduct formation.

2.3 Sample preparation procedure for LC-MS/MS

Sample preparation and extraction were processed using the Extrahera™ automation system (Biotage, Uppsala, Sweden), under positive pressure supplied from a nitrogen generator (Peak, Scotland, UK) at flow rate of 30L/min. In a 96 position 2mL deep well plate, 100 μL of calibration standards, IQC

materials or serum samples were diluted with 200 μL of pre-treatment solution consisting of deuterated internal standards in isopropanol:water 50:50 (v/v). After mixing, the samples were loaded onto ISOLUTE® supported liquid extraction (SLE+) 400 μL plate (Biotage). Elution was carried out by adding two cycles of 750 μL of n-heptane, both cycle of eluents were collected into corresponding deep well plate. Positive pressure was applied at each stage to remove residual solvent. Samples were then dried under a gentle stream of nitrogen gas heated to 45°C. Derivatization took place by adding 50 μL of 1.1 mmol/L PTAD in acetonitrile, into all wells. The plate was vortexed and allowed to incubate for 30 minutes at room temperature in the dark. 50 μL of water was then added and mixed to stop the reaction. 20 μL of the derivatized extracts was injected into the LC-MS/MS. Using this sample preparation procedure, a batch of 96 samples can be processed in one hour.

2.4 Liquid chromatography

Extracted samples were injected into LC-MS/MS by Waters® 2777 Sample manager (Waters Corp., Milford, MA, USA) equipped with 3-drawer cooler stack regulated at 10˚C. Chromatographic separation was achieved using a core-shell C18 50 × 2.1mm, 2.6 μm, reversed-phase (Restek, Bellefonte, PA, USA) column heated at 55˚C. An in-line 2μm, 6.35mm × 24mm guard filter was used to protect the column. A gradient elution profile was set up using a binary UPLC pump (Flux Instruments, Switzerland) to deliver mobile phase at flow rate of 0.4 mL/min. At the start of the gradient the mobile phase consisted of 50:50 (v/v) of (A) water containing 0.2 mM methylamine in 0.1% formic acid and (B) methanol containing 0.2 mM methylamine in 0.1% formic acid. The gradient was gradually increased to 99% of methanol mobile phase (B) then returned to starting gradient at 4 minutes. Solvent divert was employed to divert ion suppression regions of the separation to waste in order to minimise contamination to the source of the mass spectrometer.

2.5 Tandem mass spectrophotometry analysis

LC-MS/MS analysis of vitamin D metabolites was performed using Micromass Quattro Ultima Pt electrospray ionisation (ESI) tandem mass spectrometer (Waters Corp., Milford, MA, USA). MassLynx version 4.1 and QuanLynx software (Waters Corp., Milford, MA, USA) were used for system control, data acquisition, baseline integration and peak quantification. Optimisation of MS/MS parameters was accomplished by direct infusion of derivatized standards (Fig.1a). Capillary voltage was set at 3.0 kV and RF lenses 1 and 2 were set at 0.1. Source temperature was maintained at 90˚C. Nitrogen was used as both nebuliser gas at flow rate of 30 L/hr and as desolvation gas at

flow rate of 850 L/hr at 120˚C. Sample cone voltage and collision energy for all vitamin D metabolites were 35kV and 25kV respectively. Argon gas was applied to the collision cell during the Collision Induced Dissociation (CID) process. The precursor to product ion transitions for each of the compounds were ascertained based on the molecular weight of the methylamine adduct of PTAD derived products (Fig.1b and 1c).

2.6 Method validation

We followed assay validation guidance from the 2013 U.S Food and Drug Administration (FDA)[29] and the 2012 European Medicines Agency (EMA)[30]. Human sera based calibration standards for $25(OH)D_3$ and $25(OH)D_2$ (Chromsystems) were used to ensure traceability to SRM972. Where commercial calibration material was not available for $24,25(OH)_2D_3$ and $24,25(OH)_2D_2$, certified pure standards were spiked gravimetrically into vitamin D depleted human serum, representing the same biological matrix as samples. IQC materials were incorporated into each batch, the concentrations of IQCs were spread across the analytical range; from base level at three times the concentration of lower limit of quantification (LLoQ) to top level at the upper assay limit[29]. IQC provided the basis of accepting or rejecting the batch, our assay acceptance criteria require over half of the IQC samples analysed in each batch produce results within 15% from their respective target value, and 20% at LLoQ[30].

2.7 Linearity

Serum standards with known concentrations ranged from 0-180 nmol/L for $25(OH)D_3$ and $25(OH)D_2$, and 0-19.3 nmol/L for $24,25(OH)_2D_3$ and $24,25(OH)_2D_2$ were analysed at the start and end of every batch. A standard curve was generated by plotting the ratio of analyte peak area to internal standard peak on the y-axis against the weighted ($1/x$) concentration of their respective standards on the x-axis. The goodness-of-fit of a standard curve is justified by linear regression that produced a correlation coefficient (r^2) value of >0.980.

2.8 Accuracy, precision and recovery

The accuracy of an analytical method can be influenced by sample matrix[31, 32], endogenous components such as phospholipids in biological fluids are a major source of ion suppression in LC-MS/MS analysis[33]. Spike and recovery experiments were performed at low, medium and high concentrations across the analytical range to test the effects of biological matrix and sample types (serum and EDTA plasma) on efficiency of sample extraction. A multiple reaction monitoring (MRM) transition using the 184 Da product ion of

phospholipids was included into the acquisition method to monitor the level of phospholipids present in the sample matrix. Imprecision of the assay was assessed by ten consecutive measurements of IQC materials within a single run (intra-assay) and repeatedly (n = 53) over a three month period (inter-assay). Precision is expressed as coefficient of variation (CV), our acceptance criteria defined the intra-assay CV limit of <10% and cumulative inter-assay CV limit <15%.

2.9 Lower limit of quantification and detection

A precision profile was carried out to determine the lower limit of quantification (LLoQ) and detection (LLoD) of the assay. Samples containing $25(OH)D_3$, $25(OH)D_2$, $24,25(OH)_2D_3$ and $24,25(OH)_2D_2$ at concentrations of 0.05, 0.12, 0.19, 0.41, 0.81 and 1.24 nmol/L were each analysed six times, the CVs of each sample were plotted against their respective concentration. The analyte peak must produce a signal-to-noise (s/n) ratio of 10:1, the LLoQ was defined as the concentration at which the CV $\leq$ 22%.

2.10 Method comparison

Method comparison of $25(OH)D_3$ was conducted against our published LC-MS/MS method[34]. Serum samples (n=630) that were previously analysed for $25(OH)D_3$ and $25(OH)D_2$ were retested by the current method. Samples from the vitamin D external quality assurance scheme (DEQAS)[35] were analysed and results compared against the LC/MS method mean. $24,25(OH)_2D_3$ measurements from samples distributed between April 2015 to January 2016 (n = 20) were submitted to the proficiency testing scheme for variability assessment. Passing-Bablock regression was used to assess comparability of results between methods, a correlation is deemed satisfactory if the linear regression produced a correlation coefficient (r^2) value of >0.90.

2.11 Statistical data analysis

Scatterplots, residual plots, box-whisker graphs and Loess curve were constructed and analysed by Statistical Package for the Social Science (SPSS) version 22.0.0.1 (IBM, New York, USA). Passing-Bablock regression analysis and one-way ANOVA were used to establish inter-quartile range and compare variables. Loess curve fitting was employed to explore nonlinear relationships between variables and identify inflection points. Statistical significance was defined as p <0.05. Frequency distribution histograms of the data were visually examined and checked for transcriptional, pre/post analytical errors before exclusion for statistical analysis. Outliers were identified as outside the 2.5% and 97.5% of the population and removed to establish the Trimmed Mean.

2.12 Partitioning for gender

The mean and standard deviation of the data were divided according to gender. Harris and Boyd's standard deviate test[36] was used to determine the need for gender partition.

Deviate test: $Z = \bar{x}2 - \bar{x}1/[(\sigma 1^2/n1) + (\sigma 2^2/n2)]^{1/2}$

$$Z(critical) = 3[(n1 + n2/240)]^{1/2}$$

($\bar{x}$: mean, σ: standard deviation, n: sample size, 1: male, 2: female)

Partitioning is required if the statistical $Z \geq Z(critical)$. In this study, the $Z(critical)$ value is 8.65.

2.13 Establishing the reference intervals and cut-off value for clinically significant change

For the determination of reference intervals we followed the approach supported by the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) working group[27]. Statistical analysis was performed using SPSS Statistical package, based on non-parametric method, which does not assume Gaussian distribution. Assessment of the lower and upper reference limits were estimated at 2.5th and 97.5th percentiles of the reference distribution. Determination of the cut-off value for clinically significant change was estimated using the Jacobson and Truax method of analysis[37, 38]. By categorising the total 24,25(OH)$_2$D concentrations obtained from our cohort of healthy subjects into two subgroups of serum total 25(OH)D concentration 1) less than 50 nmol/L (vitamin D deficient/insufficient) or 2) greater than 50 nmol/L (vitamin D sufficient), the decision cut-off value was calculated as follows:

$$decision\ threshold = \frac{(\bar{x}1 \times \sigma 2) + (\bar{x}2 \times \sigma 1)}{\sigma 2 + \sigma 1}$$

($\bar{x}$: mean, σ: standard deviation, 1: vitamin D deficient/insufficient , 2: vitamin D sufficient group)

The diagnostic sensitivity and specificity of the cut-off was assessed by using Receiver Operating Characteristics (ROC) curve. In this method, the total 24,25(OH)$_2$D concentrations obtained from the healthy subjects were grouped according total 25(OH)D concentration of 1: greater (true negatives), or 0: less than 50 nmol/L (true positives). The ROC curve was generated by plotting sensitivity (true positive rate) against 1 – specificity (false-positive rate). The

accuracy of the test is measured by the area under the ROC curve (AUC), an AUC value of >0.9 is classified as a highly accurate test.

3.0 Results

Chromatographic peaks for 24,25$(OH)_2D_3$, 24,25$(OH)_2D_2$, 25$(OH)D_3$ and 25$(OH)D_2$ were eluted at 1.39, 1.42, 1.68 and 1.73 minutes respectively (Fig.1d). Total injection-to-injection run time was achieved in 4 minutes. An ion suppression test was conducted by post column infusion of an aqueous mixture of vitamin D metabolites with a simultaneous injection of an extracted pooled serum. Baseline signals were found to be depressed during 0 - 0.8 minutes of the chromatographic run and elevated between 2.8 – 3.2 minutes. A switch valve was employed to divert parts of the sample where ion suppression and ion enhancement occurred to waste.

3.1 Method validation, recovery efficiency and removal of phospholipids

Assay validation is summarised in Table 2. The assay achieved adequate linearity (Fig.1e), sensitivity, precision, and demonstrated reproducible recovery to satisfy method validation criteria. Injections of aqueous blank after running samples of spiked standards containing 1 μmol/L of 24,25$(OH)_2D_3$, 24,25$(OH)_2D_2$, 25$(OH)D_3$ and 25$(OH)D_2$ affirmed the absence of carry-over of analytes. Injection of a single analyte standard confirmed no cross interference in the other analyte transitions. Levels of phospholipids in sample matrix were monitored by the acquisition of m/z transitions of the 16 most abundant phospholipid precursor ions. However, due to the impracticality of monitoring all 16 ion transitions, a single lysophospholipid transition m/z 522>184 that co-eluted with the vitamin D metabolites was chosen in sample analysis (Fig.1f). We found complete elimination of lysophospholipid peaks in samples extracted using the supported liquid extraction method. This resulted in a ten-fold increase in peak signal-to-noise ratio observed in all analytes.

3.2 Method comparisons

To assess the comparability of the new method, we retested 630 serum samples previously analysed for 25$(OH)D_3/D_2$ using our existing published LC-MS/MS method. Passing-Bablock regression analysis (Fig.1g) showed a strong correlation with the existing method, the line of best fit generated a slope of 1.04 (r^2 = 0.962, p<0.001). Residual plot (Fig.1h) showed no systematic bias. Evaluation of variability in measurements of 24,25$(OH)_2D_3$ was performed by comparing our results produced from DEQAS samples against mean concentrations submitted by other participants. Passing-Bablock Regression (Fig.2a) and residual plots (Fig.2b) illustrated the correlation and difference in

values measured by our method with other registrants of DEQAS. A significant linear association (r^2 = 0.926, p<0.001) was found and, the residual plot showed no systematic bias or concentration dependent variance between the methods.

3.3 Partitioning for gender

The healthy male and healthy female groups were subjected to the Harris and Boyd deviate test. The Z values determined for total 25(OH)D, total 24,25$(OH)_2$D, and 25OHD:24,25$(OH)_2$D ratio were 1.61, 4.0 and 5.86, respectively. Each calculated Z value is ≤ $Z(critical)$ value of 8.65. Therefore, partitioning for gender is not required in establishing the reference intervals.

3.4 Reference intervals in healthy population

Samples from healthy individuals with plasma intact parathyroid hormone (PTH) or adjusted serum calcium concentration (ACa) of greater or less than the healthy reference range (PTH: 1.6-6.9 pmol/L, ACa 2.2-2.6 mmol/L) were excluded. Based on our exclusion criteria, 1996 samples (1492 male, 504 female, age 18-32yrs, mean BMD T-score 1.2±0.1) were included in the statistical study. The upper 97.5th and lower 2.5th reference limits for total (sum of D_3 + D_2) 25(OH)D, 24,25$(OH)_2$D, and 25OHD:24,25$(OH)_2$D ratio were established according to the non-parametric method of analysis (Table 3). No outlier was found. The ROC curve (Fig.3) produced an AUC value of 0.943, which classified 24,25$(OH)_2$D as an excellent test to discriminate individuals with vitamin D insufficiency from adequate status.

3.5 24,25$(OH)_2$D cut-off value for clinically significant change in vitamin D status.

Determination of the cut-off value for clinically significant change using the Jacobson and Truax[37, 38] method approach have estimated total 24,25$(OH)_2$D of >4.2 nmol/L to be the threshold value for vitamin D replete status, i.e. serum total 25(OH)D concentration ≥50 nmol/L. The decision cut-off has a true positive rate (sensitivity) of 88.8%, and false positive rate (specificity) of 86.3%, as indicated by the ROC curve (Fig.3).

3.6 Relationship between serum 25(OH)D, 24,25$(OH)_2$D and 25(OH)D:24,25$(OH)_2$D ratio among the healthy and patient population

In total, 2290 samples collected from healthy volunteers (n = 1996) and patients (n = 294) were analysed for serum vitamin D metabolites and included in the statistical analysis. 25$(OH)D_3$ was quantified in all samples with a concentration range (mean±SD) between 3.1 – 349.8 nmol/L (62±33.2 nmol/L),

from which we were able to quantify $24,25(OH)_2D_3$ in 99.8% of samples. $24,25(OH)_2D_3$ concentrations ranged between 0.2 – 51.1 nmol/L (5.5±3.7 nmol/L). $25(OH)D_2$ was present in 12.9% of samples, with a range between 0.1-74.5 nmol/L (5.4 ±9.5 nmol/L), of which 1.1% contained $24,25(OH)_2D_2$ concentrations between 1.3 – 5.0 nmol/L (2.4 ±1.1 nmol/L). Statistical analyses were performed on total (sum of $D_3 + D_2$) values, the distributions were untrimmed and no outlier was removed. A concentration-dependent relationship was observed between serum $24,25(OH)_2D$ and 25(OH)D (Fig.4a), linear regression analysis indicated $[24,25(OH)_2D] = 0.1 \times [25(OH)D] - 0.65$; r^2 value of 0.750. In contrast, the ratio of $25(OH)D:24,25(OH)_2D$ (Fig.4b) showed an indirect relationship with 25(OH)D; Loess fitting showed a downward trend in the ratio of $25(OH)D:24,25(OH)_2D$ as serum 25(OH)D concentrations increased. Analysis of variance between the subgroups showed 25(OH)D and $24,25(OH)_2D$ concentrations in patients were significantly lower than healthy individuals ($p<0.001$), whereas the relative ratio of $25(OH)D:24,25(OH)_2D$ was significantly higher in patients ($p<0.001$). The Box-whisker plots of total $25(OH)D:24,25(OH)_2D$ ratio against serum total 25(OH)D grouped into eight concentration categories with an equal number of cases (Fig.5) revealed a highly significant increase ($p<0.001$) in ratio occurred at total 25(OH)D concentration below 50 nmol/L.

3.7 CYP24A1 mutation on patients with elevated 25(OH)D:24,25(OH)$_2$D ratio.

Using our established reference intervals, we have identified a sample from the patient group with an elevated $25(OH)D:24,25(OH)_2D$ ratio of 32; clinical records revealed the patient presented with hypercalcaemia and elevated serum 1,25(OH)D of 293 pmol/L (reference range 43-144 pmol/L). Retrospective genetic analysis confirmed bialleic pathogenic mutations in *CYP24A1* (c.823T>C, p.W275R and c.1315C>T p.R439C).

4.0 Discussion

We describe a robust LC-MS/MS assay for simultaneous measurement of $24,25(OH)_2D_3$, $24,25(OH)_2D_2$, $25(OH)D_3$ and $25(OH)D_2$. Sample clean up using supported liquid extraction (SLE) is highly effective for removal of major proteins and phospholipids, which are often the cause of instrument loss of sensitivity and downtime. Derivatization with PTAD is essential, vitamin D metabolites have low ionisation efficiency and readily dissociate into water loss product ions at low collision energy. The advantage of the PTAD derivitization is that it adds proton-affinitive oxygen and nitrogen molecules (Fig.1c) to the structure of vitamin D, thus increasing ionisation potential. Derivatization shifts the compound to a higher mass range, where background interference from low molecular weight species is relatively low. In our hands, SLE with PTAD derivatization produced

peak signal response in the $x10^4$-$x10^6$ region; increase in an order of magnitude compared to our previously published method[34]. Assay sensitivity is paramount in order to achieve the detection limit required to measure $24,25(OH)_2D$; particularly in CYP24A1 deficient patients with low total 25(OH)D. Our method has demonstrated good recovery, intra/inter assay precision and linearity. Accuracy of $24,25(OH)_2D_3$ and $24,25(OH)_2D_2$ can be difficult to assess as there is currently no international standard material available. We used certified standards spiked into vitamin D depleted human sera in order to maintain matrix composition between calibration standards and samples. We evaluated our DEQAS $24,25(OH)_2D_3$ returns from the four most recent quarterly cycles and compared our values against the mean values from all participants. Our assay showed good agreement with other methods; all of our returned values were within ±2SD of the mean concentration from other laboratories. We must stress that the small number of participants currently in the DEQAS $24,25(OH)_2D_3$ scheme and the disparity in results between laboratories undermine the reliability of the statistics. The availability of a gold reference standard and traceable materials would help improve accuracy of measurements and reduce inter-laboratory variation in future.

The strong association between serum concentrations of 25(OH)D and $24,25(OH)_2D$ demonstrated in this study echo the findings from previous reports[23, 25, 26, 39, 40]. The concentrations of $24,25(OH)_2D$ exhibited a positive, concentration-dependent relationship, which were on average 10% of the concentrations of 25(OH)D. We observed a significant increase in 25(OH)D:$24,25(OH)_2D$ ratio at serum 25(OH)D concentration below 50 nmol/L. The decrease in relative production of 24,25(OH)2D may result from down regulation of CYP24A1 in response to the decline in circulating $25(OH)D_3$, in order to facilitate catabolism to 1,25(OH)D. The reverse is observed in individuals with high concentrations of serum 25(OH)D; where production favours 24,25(OH)D at the expense of $1,25(OH)_2D$ which may act to prevent toxicity.

We have investigated the usefulness of $24,25(OH)_2D$ measurement as a surrogate marker of vitamin D status and demonstrated its high diagnostic accuracy in our healthy cohort. The $24,25(OH)_2D$ cut-off value of 4.2 nmol/L can predict an individual's vitamin D status with good diagnostic sensitivity and specificity; a value below the cut-off indicating insufficiency and the likelihood of metabolism favouring $1,25(OH)_2D$; whereas a value above the cut-off indicates sufficient vitamin D status with 25(OH)D catabolism likely to favour $24,25(OH)_2D$ production.

The clinical utility of dual measurements to obtain absolute values as well as a ratio of 25(OH)D to $24,25(OH)_2D$ is valuable not only in the assessment of

vitamin D catabolic status, but also to provide an assessment of CYP24A1 activity. Based on our cohort of healthy young adult subjects, we defined the normal reference interval for total 25(OH)D:24,25$(OH)_2$D ratio between 7 to 23. In comparison with current publications, our upper limit is lower than those observed by others[23-25], which may be reflective of the subject population, selection criteria, and analytical method employed in our study. The reference interval provides an indication of an individual's vitamin D catabolism with a ratio value below 7 indicating elevated activity of 24-hydroxylase. Such individuals may be at risk of developing resistance to vitamin D treatment. A ratio above 23 indicates enzyme under activity and possible genetic abnormalities of *CYP24A1*. We detected one patient in our cohort with an elevated ratio (32) and hypercalcaemia who on genetic testing turned out to have a biallelic mutation of *CYP24A1.* Molin et al.[41] reported patients with heterozygous or biallelic mutations of *CYP24A1* are associated with 25(OH)D:24,25$(OH)_2$D ratios >80; whereas patients with a single mutant allele or no *CYP24A1* mutation have ratio values <25. Moderate elevation of 25(OH)D:24,25$(OH)_2$D ratio may be a result of substrate depletion from underlying diseases caused by renal impairment[40], bone disorders[42] or pathological conditions associated with an increased concentration of FGF23[43], all of which affect calcium and phosphate homeostasis resulting in an imbalance state of vitamin D catabolism. Detection of an elevated 25(OH)D:24,25$(OH)_2$D ratio, especially in patients with nephrocalcinosis or renal stones, may indicate a requirement to restrict or avoid vitamin D supplementation in order to prevent hypercalcaemia and further stone formation.

In conclusion, we have developed a robust LC-MS/MS method for the simultaneous measurement of 25(OH)D and its metabolite 24,25$(OH)_2$D. The reference intervals of 24,25$(OH)_2$D and 25(OH)D:24,25$(OH)_2$D ratio were determined from serum analysis of a large cohort of healthy young adults. We found remarkable consistency in the 25(OH)D:24,25$(OH)_2$D ratio across the entire range of 25(OH)D concentrations, and observed a significant increase in 25(OH)D:24,25$(OH)_2$D ratio in individuals with insufficient 25(OH)D. The reference intervals we have established for 25(OH)D:24,25$(OH)_2$D ratio can help clinician in the investigation of patients presenting with diseases and conditions resulted from loss of CYP24A1 activity, and help optimise supplementation therapy based on individual differences in serum vitamin D metabolite concentration ratio in order to achieve the best response.

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Author contributions

All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.

Declaration of conflicting interest

The authors declare no conflict of interest.

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Tables and figures

Table 1. Characteristics of the subjects included in the study.

Figure 1. **(a)** Mass spectrometer parameter settings and MRM precursor to product ion transitions for $25(OH)D_3/D_2$ and $24,25(OH)_2D_3/D_2$. **(b)** Collision-induced dissociation spectra of PTAD-derivatized $24,25(OH)_2D_3$ ($M+CH_3NH_3^+$ at *m*/*z* 623). The precursor to product ion transition *m*/*z* 623>298 was utilised for MRM. **(c)** Dienophilic reaction of $24,25(OH)_2D_3$ with PTAD and formation of methylamine adduct. **(d)** Chromatogram from an extracted serum sample containing 64.7 nmol/L of $25(OH)D_3$, 46.1 nmol/L of $25(OH\ D_2$, 6.4 nmol/L of $24,25(OH)_2D_3$ and 4.5 nmol/L of $24,25(OH)_2D_2$. **(e)** Typical standard

curves constructed by plotting the response of each standard on the *y*-axis against their respective concentrations (nmol/L) on the *x*-axis. Response of each compound were determined by the ratio of the peak area of the standards to the peak area of the deuterated internal standards. Regression analysis showed a typical correlation coefficient $r^2 > 0.99$. **(f)** Chromatographic traces showing co-elution of lysophospholipid transition m/z 522>184 with $25(OH)D_3/D_2$. **(g)** Comparison of $25(OH)D_3$ of the new method (n=630) showing a strong correlation ($r^2 = 0.962$) with existing published method. **(h)** Standardised residual plot showing the variability of $25(OH)D_3$ measurements between new and existing method. The dashed lines represents ±SD limits of agreement.

Table 2. LC-MS/MS assay characteristics. Recovery efficiency was determined by spiking known quantities of each compound into vitamin D depleted serum and into a base pool of EDTA plasma containing endogenous vitamin D metabolites. Each spiked sample was aliquoted and analysed over ten separate runs.

Figure 2. **(a)** Comparison of $24,25(OH)_2D_3$ returns (n=21) with DEQAS all laboratory mean. Solid line in the graph represents the fitted regression line. **(b)** Standardised residual plot showing the variability of $24,25(OH)_2D_3$ measurements. The dashed lines represents 95% limits of agreement. All values were within ±2SD.

Table 3. Summary of reference intervals and cut-off values for adequate vitamin D status.

Figure 3. Diagnostic performance of $24,25(OH)_2D$ in assessment of vitamin D status. Receiver Operating Characteristic (ROC) curve depicts diagnostic performance sensitivity and specificity levels. (O) represents the decision threshold for vitamin D replete at $24,25(OH)_2D$ concentration of 4.2 nmol/L (sensitivity = 88.8%, specificity = 86.3%). Diagonal line is the line of no discrimination.

Figure 4. The relationship of serum total 25(OH)D with **(a)** serum total $24,25(OH)_2D$, **(b)** total $25(OH)D:24,25(OH)_2D$ ratio. Sample from a cohort of mixed patients and healthy individuals (n = 2290). Solid line in (a) represents linear regression line, with 95% confidence intervals (dashed lines). Loess fitted curve in (b) depicted in solid line (99% point fit), dashed lines represent the lower and upper reference intervals (7-23).

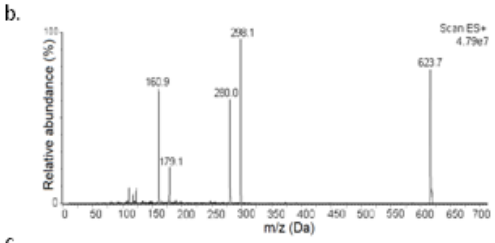
Figure 5. **(a)** $25(OH)D:24,25(OH)_2D$ ratio profile of 1996 healthy subjects against 25(OH)D bins. Each bin contains equal number of subjects to illustrate the significantly elevated ratio found in those with serum 25(OH)D <50 nmol/L.

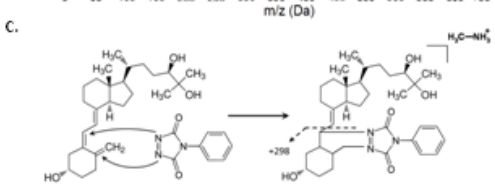
Box and whiskers represent the median, interquartile range and 95% population intervals.

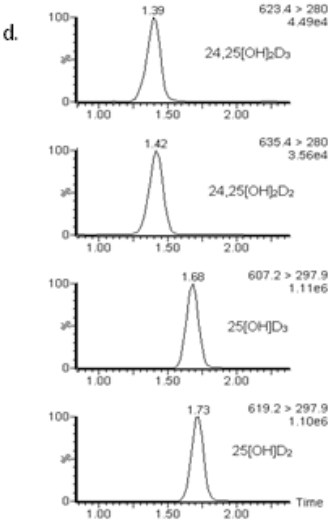
Figure 1

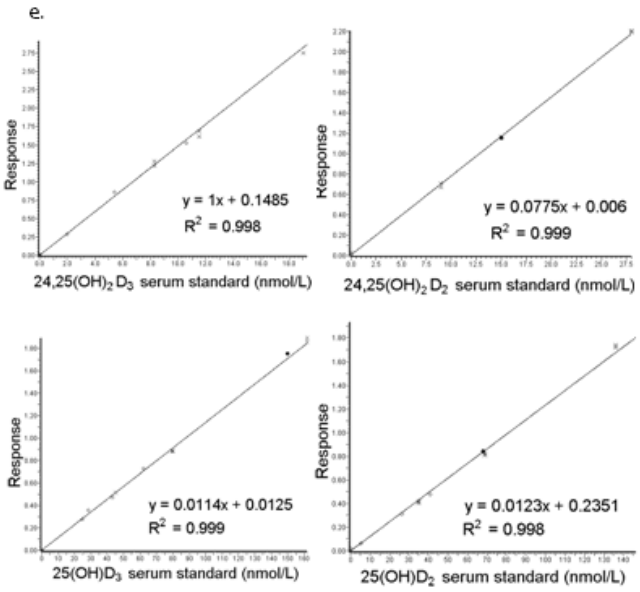
a.

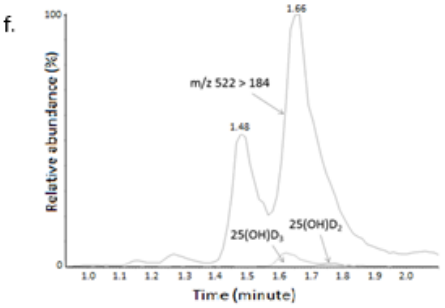
Parameter	Setting
Ion source	Electrospray positive
Capillary voltage	3.0 kV
Cone energy	35 kV
Collision energy	20kV
Nebuliser gas flow rate	30 L/hr
Desolvation gas flow rate	850 L/hr
Source temperature	85°C
Desolvation gas temperature	250°C
m/z transitions	
$25(OH)D_3$	607>298
$25(OH)D_2$	619>298
$24,25(OH)_2D_3$	623>298
$24,25(OH)_2D_2$	635>298
$25(OH)D_3$-[$^2H_6$]	613>298
$24R,25(OH)_2D_3$-[$^2H_3$]	626>316

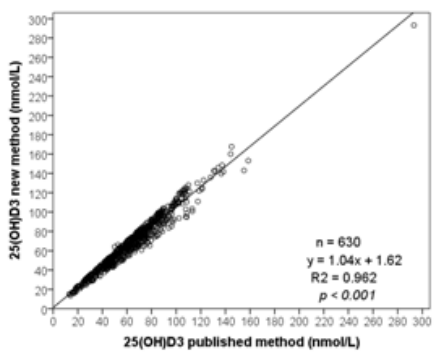












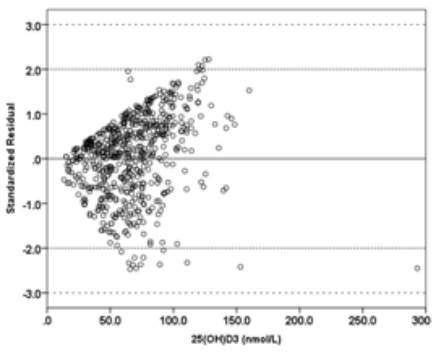
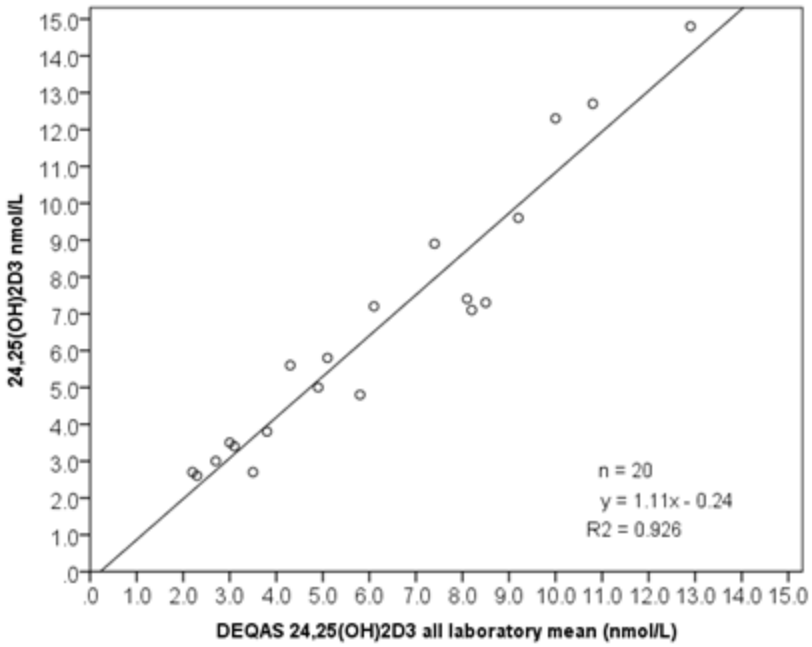


Figure 2

a.



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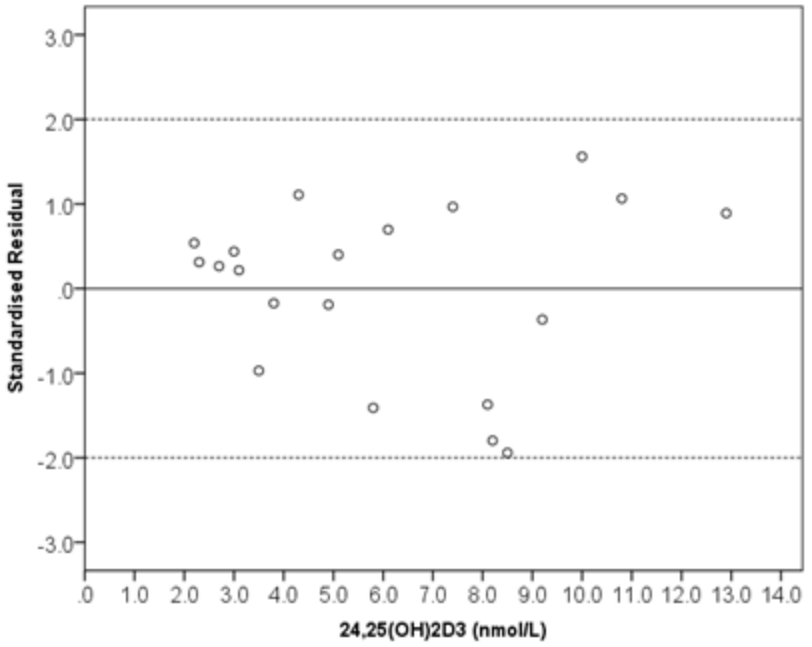


Figure 3

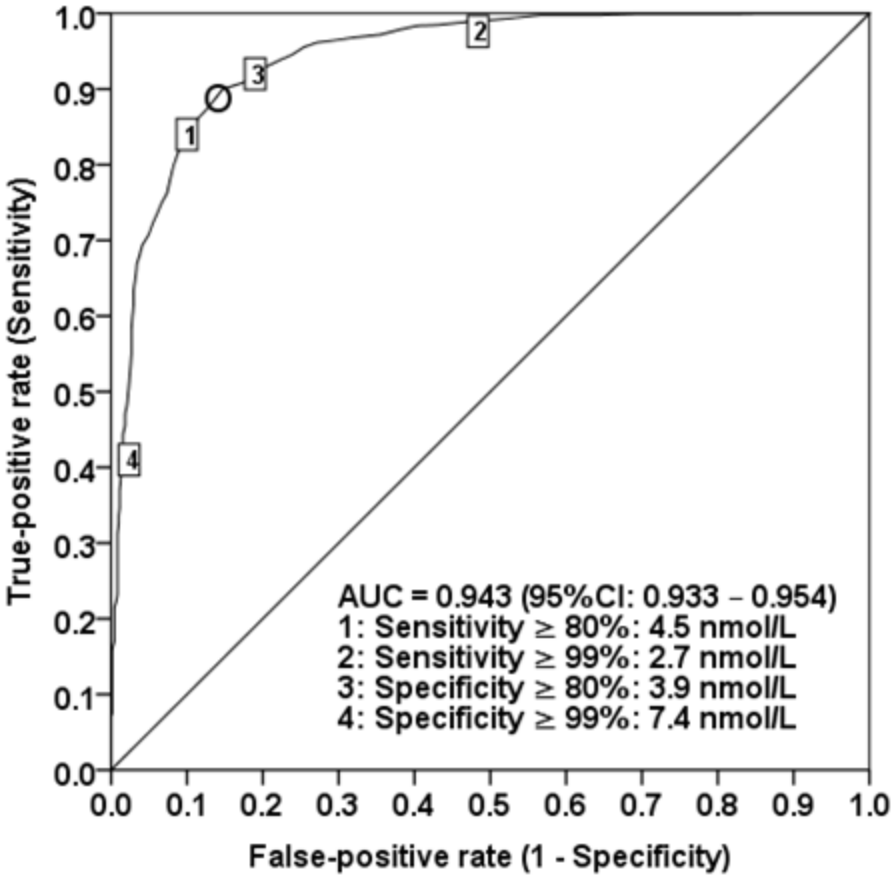
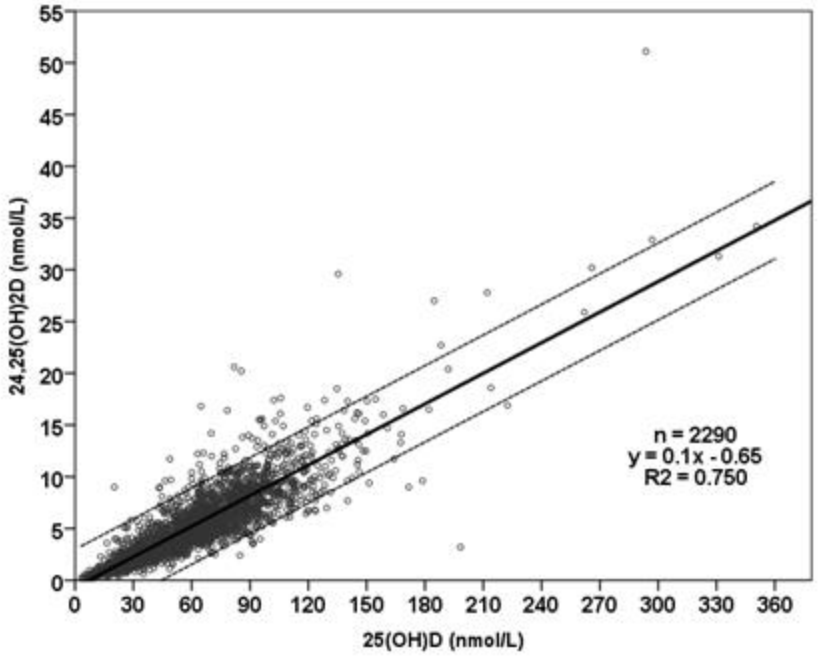


Figure 4

a.



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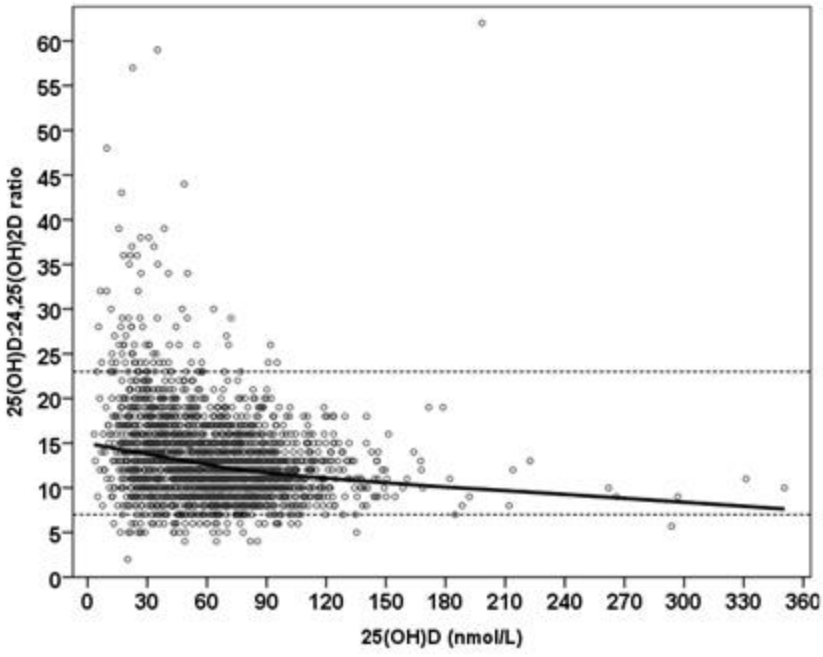

Figure 5

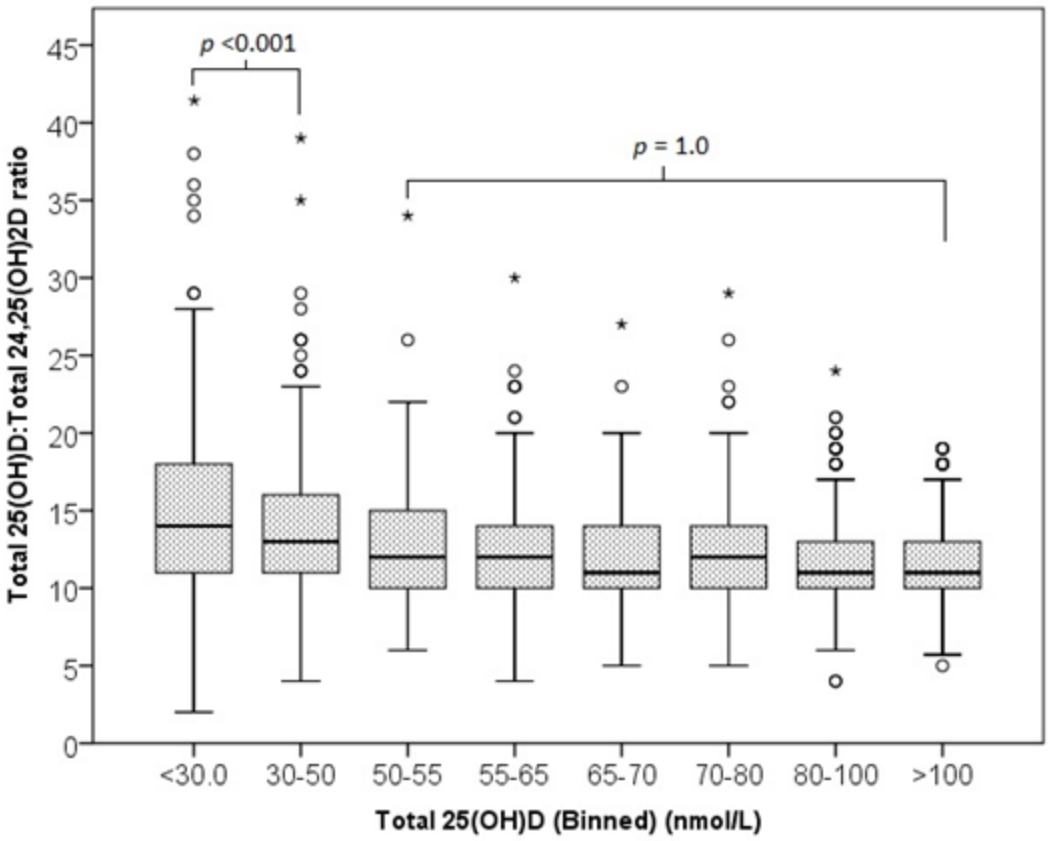

Table 1

		Healthy subjects	Patients	ρ-value
n		1996	294	0.001
Gender	**Male**	1492	177	0.001
	Female	504	117	0.001
Age, years (range)		23 (18-32)	52 (2-95)	0.001
25(OH)D_3, nmol/L		63.7±29.4	50.4±50.7	0.001
25(OH)D_2, nmol/L		ND	5.4±9.5	
24,25$(OH)_2D_3$, nmol/L		5.7±3.4	4.1±5.2	0.001
24,25$(OH)_2D_2$, nmol/L		ND	2.4±1.1	
25(OH)D:24,25$(OH)_2$D		13±4.3	17±7.4	0.001

*Data shown in mean±SD, ND: none detected

Table 2

	$24,25(OH)_2D_3$	$24,25(OH)_2D_2$	$25(OH)D_3$	$25(OH)D_2$
Linearity, nmol/L	0-25	0-25	0-250	0-250
Typical r^2	0.985	0.983	0.997	0.995
Intra assay imprecision mean (±SD) in nmol/L, %CV	1.9 (±0.19) 10.3% 5.8 (±0.44) 7.6%	2.1 (±0.16) 7.6% 6.5 (±0.5) 7.7%	32.5 (±3.0) 9.3% 88.1 (±5.7) 6.5% 180.4 (±16.5) 9.1%	24.8 (±1.6) 6.6% 64.9 (±3.2) 4.9% 140.6 (±4.7) 3.3%
Inter assay imprecision mean (±SD) in nmol/L, %CV	2.0 (±0.21) 10.5% 6.1 (±0.51) 8.3% 14.8 (±1.16) 7.9%	1.8 (±0.20) 11% 5.7 (±0.61) 10.7% 19.4 (±1.95) 10.0%	27.1 (±2.9) 10.9% 66.2 (±6.0) 9.1% 169.7 (±15.2) 8.9%	25.0 (±2.0) 8.0% 72.3 (±5.7) 7.9% 148.9 (±9.4) 6.3%
LLoQ, nmol/L	0.1	0.8	0.1	0.1
Sample matrix + concentration spiked in nmol/L	**Mean % recovery (±SD)**			
Depleted serum + 1.2	97% (±4)	108% (±3)	96% (±2)	102% (±4)
Depleted serum + 12.2	94% (±4)	97% (±2)	101% (±4)	107% (±2)
Depleted serum + 250	-	-	103% (±1)	95% (±4)
Pooled EDTA plasma + 1.2	96% (±5)	107% (±3)	99% (±2)	94% (±3)
Pooled EDTA plasma + 12.2	103% (±3)	99% (±2)	91% (±2)	104% (±2)
Pooled EDTA plasma + 250	-	-	98% (±2)	101% (±3)

* concentrations of pooled EDTA plasma: 14.3 nmol/L $25(OH)D_3$, 10.2 nmol/L $25(OH)D_2$, 1.3 nmol/L $24,25(OH)_2D_3$, 1.2 nmol/L $24,25(OH)_2D_3D_2$

Table 3

Parameters	Total cohort (n = 1996)	Reference interval (2.5-97.5 percentile)	Diagnostic cut-off for vitamin D replete
$24,25(OH)_2$ D, nmol/L	0.4 - 51.1	1.1 - 13.5	>4.2*
25(OH)D, nmol/L	6.9 – 293.7	18.9 – 126.4	
25(OH)D: $24,25(OH)_2$ D ratio	2 - 59	7 - 23	

*At sensitivity = 88.8%, specificity 86.3%

Highlights

- Our LC-MS/MS method for simultaneous measurement of 25(OH)D and 24,25$(OH)_2$D was validated against industry standard criteria for method validation, results were comparable with external proficiency scheme.
- The reference intervals of 24,25$(OH)_2$D and 25(OH)D:24,25$(OH)_2$D ratio were established a large cohort of healthy individuals and patients samples.
- Serum concentrations of 24,25$(OH)_2$D showed a positive, concentration-dependent relationship with 25(OH)D.
- 25(OH)D:24,25$(OH)_2$D ratio showed a negative relationship with 25$(OH)D_3$ that was significantly higher at 25(OH)D concentration <50 nmol/L.
- Elevated 25(OH)D:24,25$(OH)_2$D ratio may indicate possible genetic abnormalities of *CYP24A1*.