The metabolism of 1,25(OH)₂D₃ in clinical and experimental kidney disease

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Chronic kidney disease (CKD) results in calcitriol deficiency and altered vitamin D metabolism. The objective of this study was to assess the 24-hydroxylation-mediated metabolism of 25(OH)D₃ and 1,25(OH)₂D₃ in a cross-sectional analysis of participants with a range of kidney function assessed by precise measured GFR (mGFR) (N = 143) and in rats with the induction and progression of experimental kidney disease. Vitamin D metabolites were assessed with LC–MS/MS. Circulating measures of 24-hydroxylation of 25(OH)D₃ (24,25(OH)₂D₃:25(OH)D₃) precisely decreased according to mGFR in humans and progressively in rats with developing CKD. In contrast, the 1,24,25(OH)₃D₃:1,25(OH)₂D₃ vitamin D metabolite ratio increased in humans as the mGFR decreased and in rats with the induction and progression of CKD. Human participants taking cholecalciferol had higher circulating 1,24,25(OH)₃D₃, despite no increase of 1,25(OH)₂D₃. This first report of circulating 1,24,25(OH)₃D₃ in the setting of CKD provides novel insight into the uniquely altered vitamin D metabolism in this setting. A better understanding of the uniquely dysfunctional catabolic vitamin D profile in CKD may guide more effective treatment strategies. The potential that 24-hydroxylated products have biological activity of is an important area of future research.

Abbreviations

1,25-VMR 1,24,25(OH)₃D₃-to-1,25(OH)₂D₃ vitamin D metabolite ratio
25-VMR 24,25(OH)₂D₃-to-25(OH)D₃ vitamin D metabolite ratio

The 2017 KDIGO guidelines for the management of chronic kidney disease-mineral bone disorder (CKD-MBD) suggest that patients with rising PTH values be investigated for modifiable factors, including vitamin D deficiency¹. The clinical assessment of vitamin D deficiency is a measurement of 25-hydroxyvitamin D (25(OH)D), which captures dietary intake of precursor molecules and endogenous ultraviolet light-mediated generation². 25(OH)D₃ is converted to 1,25(OH)₂D₃, by the cytochrome P450 enzyme, CYP27B1, which is up-regulated by PTH and down-regulated by fibroblast growth factor 23 (FGF-23) and 1,25(OH)₂D₃. In contrast to the production of 25(OH)D, the catabolism of 25(OH)D is tightly regulated and an essential step is 24-hydroxylation, catalyzed by CYP24A1. CYP24A1, found in tissues where the VDR is expressed, controls local levels of 1,25(OH)₂D₃. Catabolism of 1,25(OH)₂D₃ to 1,24,25(OH)₃D₃ is also performed by CYP24A1, but whether this pathway is altered in CKD has not yet been studied.

Using liquid chromatography tandem mass spectroscopy (LC–MS/MS), the vitamin D metabolome assessed in this study consists of the main circulating form, 25(OH)D₃, the hormonally active product of CYP27B1 (1,25(OH)₂D₃), and the initial hydroxylation products of CYP24A1 (24,25(OH)₂D₃ and 1,24,25(OH)₃D₃). An estimate of the catabolic equilibrium of 25(OH)D₃ and 1,25(OH)₂D₃ was assessed by calculating the 25- and 1,25-vitamin D metabolite ratios (VMRs): 24,25(OH)₂D₃:25(OH)D₃ and 1,24,25(OH)₃D₃:1,25(OH)₂D₃.

The objective of this study was to evaluate the vitamin D metabolome across a range of kidney function, in (1) a cross-sectional study of participants with measured GFR (mGFR) and (2) longitudinally in rats with induction and progression of CKD.

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Methods

Vitamin D metabolite measurement. Serum vitamin D metabolites were measured by LC–MS/MS (Acquity LC/Xevo, TQ-S system; Waters Corp.) as previously described. For measurement of 1,25(OH)\(_2\)D\(_3\) and 1,24,25(OH)\(_3\)D\(_3\), 150 µL of serum was equilibrated with 200 pg/mL α-1,25(OH)\(_2\)D\(_3\) and 12.5 pg/mL α-1,24,25(OH)\(_3\)D\(_3\) internal standard. The sample was incubated with 100 µL of anti-1,25(OH)\(_2\)D\(_3\) antibody slurry (Immundiagnostik) for 2 h at RT with orbital shaking at 1200 RPM. The slurry was isolated by vacuum filtration and rinsed with 4 × 400 µL of water, and vitamin D metabolites were eluted with 2 × 200 µL ethanol. The eluate was dried and derivatized with DMEQ-TAD as previously described. The sample was re-dissolved in 50 µL 50/50 (% by vol) methanol/water and 35 µL was injected into the LC–MS/MS system as previously described. MRM transitions used for analysis of 1,25(OH)\(_2\)D\(_3\) and 1,24,25(OH)\(_3\)D\(_3\) were m/z 762 → 468 + 762 → 484, and m/z 778 → 468 + 778 → 484. Quantification was based on a 6-point calibrator generated in-house containing 5–300 pg/mL 1,25(OH)\(_2\)D\(_3\) and 1–25 pg/mL 1,24,25(OH)\(_3\)D\(_3\). The LoQ for 1,24,25(OH)\(_3\)D\(_3\) was 5 pg/mL with a coefficient of variation of 10–15%. Both humans and animal models were measured using this method.

Human study. We measured the vitamin D metabolome in participants of 2 cohort studies (Supplementary Fig. 1). All subjects were aged >18 and had been recruited from Kingston Health Sciences Centre and the general population of Kingston, Ontario Canada. All participants gave informed consent according to the Declaration of Helsinki and both study protocols were approved by Queen's University and Associated Teaching Hospitals Research Ethics Boards. Demographic data as well as medications and supplement use were obtained by chart review and participant interview at the time of sample collection. The GFR Measurement Study included a convenience sample of 98 community dwelling participants ≥ 18 years of age. We excluded kidney transplant recipients (n = 14), those without biological sample (n = 9), participants taking calcitriol (n = 6) and those with substantial circulating vitamin D\(_2\) (n = 3), as defined by >15% of circulating total 25(OH)D, indicating consumption of plant-based vitamin D supplements. The metabolic products of vitamin D\(_2\) are not captured by the assay used in this study. The second study was the Healthy Aging Study and included 78 healthy participants between the ages of 40 and 80 with targeted recruitment of 10 males and 10 females within each decade of life. We excluded 1 participant from the Healthy Aging Study with substantial circulating vitamin D\(_2\). Participants from two studies were pooled for a total convenience sample size of 143 participants.

For measurement of the serum vitamin D metabolites was identical to the procedures outlined above. A Brochner-Mortensen correction was used to account for the initial clearance phase. GFR was corrected for body surface area (BSA). Nine participants had technical errors with iohexol and inulin measurement and creatinine-based GFR approximations are more accurate at lower levels of GFR. Serum creatinine, phosphate and calcium were measured in the serum vitamin D metabolome in participants of 2 cohort studies (Supplementary Fig. 1). All subjects were aged >18 and had been recruited from Kingston Health Sciences Centre and the general population of Kingston, Ontario Canada. All participants gave informed consent according to the Declaration of Helsinki and both study protocols were approved by Queen's University and Associated Teaching Hospitals Research Ethics Boards. Demographic data as well as medications and supplement use were obtained by chart review and participant interview at the time of sample collection. The GFR Measurement Study included a convenience sample of 98 community dwelling participants ≥ 18 years of age. We excluded kidney transplant recipients (n = 14), those without biological sample (n = 9), participants taking calcitriol (n = 6) and those with substantial circulating vitamin D\(_2\) (n = 3), as defined by >15% of circulating total 25(OH)D, indicating consumption of plant-based vitamin D supplements. The metabolic products of vitamin D\(_2\) are not captured by the assay used in this study. The second study was the Healthy Aging Study and included 78 healthy participants between the ages of 40 and 80 with targeted recruitment of 10 males and 10 females within each decade of life. We excluded 1 participant from the Healthy Aging Study with substantial circulating vitamin D\(_2\). Participants from two studies were pooled for a total convenience sample size of 143 participants.

Rat study. Animal procedures were in accordance with the Canadian Council on Animal Care and approved by Queen's University Animal Care Committee. Male Sprague–Dawley rats (n = 18, Hilltop Lab Animals Inc, PA, USA) were 15 weeks of age at the time of induction of CKD. Prior to intervention, animals were acclimatized for a week whilst housed individually and maintained on a 12-h light/dark cycle. All animals were provided with a diet containing 0.5% phosphate, 1% calcium, 0.05% magnesium, 0.2 mg/kg vitamin K, 1 IU cholecalciferol and 6% protein (Harlen Tekland WI, USA TD 150555). CKD was induced in 10 animals with the addition of 0.25% adenine to the diet weeks whilst the parallel group of non-CKD controls (n = 6) remained on the same non-ade- nine diet. If animals did not eat all administered food or reached 10% weight loss, they were supplemented with a high calorie supplement (Boost, Clear H2O, USA). Animals were blood sampled from the saphenous vein.

Serum creatinine and serum and urine calcium and phosphate were evaluated spectrophotometrically (Synergy HT Microplate Reader; BioTek Instruments, Winooski, VT). Creatinine was evaluated using the Jaffe method (QuantiChrom™ Creatinine Assay Kit, Bioassay Systems). Calcium was measured using the o-cresolphthalein complexone method at 540 nm, as previously described. Free phosphate was measured using the malachite green (Sigma–Aldrich, CAN) method as described by Hereszty and Nicholson at 650 nm. Plasma levels of intact PTH and intact FGF-23 were measured using ELISAs (Immunotopics Inc., USA). The measurement of the serum vitamin D metabolites was identical to the procedures outlined above.

Statistical analysis. Data were analyzed using SAS software (V9.4), SAS/STAT software (V14.2) and GraphPad Prism (V8.4.2). The Shapiro–Wilk test was used to assess normality and several variables were log-transformed as a result. Participants were stratified into 15 mL/min/1.73 m\(^2\) mGFR intervals. Groups were compared using a Mann–Whitney U-test or ANOVA. Pairwise comparisons were completed between mGFR groups using Fisher’s LSD test in variables for which the global test was significant. Spearman’s correlation coefficient was used to evaluate the association between vitamin D metabolites and demographic and laboratory measures, with subsequent partial associations consecutively controlling for age and mGFR. The threshold for significance for all p-values was 0.05. Vitamin D metabolites in rats were compared using repeated measures one-way ANOVA with post hoc test for differences between adjacent time points.
Table 1. Demographics of participants stratified by measured GFR. Data represented by mean ± SD for normally distributed measures or median [IQR] for non-normally distributed. P-value indicates significance of one-way ANOVA or non-parametric equivalent. Differences between groups evaluated using Fisher LSD test if global test has p < 0.05. uACR urinary albumin-to-creatinine ratio, PTH parathyroid hormone, FGF-23 fibroblast growth factor 23. *p < 0.05, **p < 0.01, ***p < 0.001 indicates significance compared to > 90, †60–89, ‡45–59, &30–44, $ < 15. Significant values are given in bold.

| mGFR (mL/min/1.73 m²) | GFR categories | ALL (n = 143) | > 90 (n = 12) | 60–89 (n = 70) | 45–59 (n = 13) | 30–44 (n = 12) | 15–29 (n = 26) | < 15 (n = 10) | P-value |
|----------------------|----------------|--------------|--------------|--------------|--------------|--------------|--------------|------------|---------|
| **Demographics**     |                |              |              |              |              |              |              |           |         |
| Age                  | 59.71 ± 12.37  | 45.83 ± 11.41| 60.8 ± 11.74 | 63.00 ± 9.68 | 66.92 ± 9.62 | 65.73 ± 11.32| 60.80 ± 13.36| < 0.001    |         |
| Race (N, % white)    | 139 (97.2%)    | 12 (100.0%)  | 69 (98.6%)   | 13 (100.0%)  | 12 (100.0%)  | 24 (92.3%)  | 9 (90.0%)    | 0.332      |         |
| Sex (N, % female)    | 67 (46.9%)     | 4 (33.3%)    | 38 (54.3%)   | 5 (38.5%)    | 5 (41.7%)    | 11 (42.3%) | 4 (40.0%)    | 0.648      |         |
| Diabetic (N, % yes)  | 23 (16.1%)     | 0 (0.0%)     | 2 (2.9%)     | 1 (7.7%)     | 11 (42.3%)   | 12 (46.2%) | 4 (40.0%)    | < 0.001    |         |

**CKD biomarkers**

| uACR (n = 105)       | 2.03 [0.93, 16.3] | 2.07 [0.93, 16.3] | 1.04 [0.71, 2.26] | 1.40 [1.00, 8.25] | 8.05 [1.20, 16.20] | 160.3 [81.10, 340.2] | < 0.001 |
| Serum Ca (mM) (n = 133) | 2.29 ± 0.11     | 2.32 ± 0.06   | 2.30 ± 0.10   | 2.33 ± 0.14   | 2.32 ± 0.13   | 2.27 ± 0.1       | 2.20 ± 0.16 | 0.066   |
| Serum P04 (mM) (n = 132) | 1.19 ± 0.25     | 1.11 ± 0.11   | 1.13 ± 0.15   | 0.99 ± 0.16   | 1.11 ± 0.29   | 1.34 ± 0.22      | 1.52 ± 0.46 | < 0.001 |
| PTH (pmol/mL) (n = 140) | 6.35 [4.70, 10.03] | 6.75 [4.40, 7.99] | 5.50 [4.30, 6.50] | 7.40 [5.30, 9.80] | 6.0 [3.92, 7.34] | 10.46 [7.66, 15.74] | 23.31 [16.70, 29.80] | < 0.001 |
| FGF-23 (pmol/L) (n = 56) | 10.75 [7.11, 18.24] | 3.79 [3.09, 4.48] | 4.23 [2.35, 8.11] | 6.42 [3.67, 7.22] | 10.83 [8.03, 15.06] | 12.42 [8.71, 24.80] | 36.26 [23.37, 46.85] | < 0.001 |

Results

Participant characteristics. The 143 human participants had a range in mGFR from 8.4 to 128 mL/min/1.73 m² and were predominantly white (97.2%), with mean age of 59.7 ± 12.4 years, body mass index (BMI) of 28.4 ± 5.9 kg/m², and a prevalence of diabetes of 16.1%. Table 1 stratifies the participants by mGFR categories of 15 mL/min/1.73 m². Participants with lower mGFR were older, had slightly higher BMI but similar BSA, and were more likely to be diabetic. Consistent with CKD progression, there was an increase in albuminuria, phosphate, PTH, and FGF-23 as mGFR declined.

Vitamin D metabolites and ratios delineated by kidney function. The level of 25(OH)D3 was similar across the range of kidney function (Fig. 1A). As kidney function declined, 24,25(OH)2D3 (Fig. 1B) and the 25-VMR (24,25(OH)2D3:25(OH)D3) decreased (Fig. 1C). 1,25(OH)2D3 and 1,24,25(OH)3D3 both decreased as kidney function declined (Fig. 1D,E) however, the 1,25-VMR (1,24,25(OH)3D3:1,25(OH)2D3) showed an increasing trend (Fig. 1F). Both with and without adjustment for mGFR and age, a higher level of PTH was associated with a lower 24,25(OH)2D3:25(OH)D3 ratio. In contrast, FGF-23 was not associated with the 25-VMR but increasing trend (Fig. 1F). Both with and without adjustment for mGFR and age, a higher level of PTH was associated with a lower 24,25(OH)2D3:25(OH)D3 ratio. In contrast, FGF-23 was not associated with the 25-VMR but increasing trend (Fig. 1F).

Animal study. To evaluate the progression of metabolite changes longitudinally with the development of CKD, we measured the same vitamin D metabolite profile in rats with adenine-induced CKD and time-control healthy rats, as confirmed by elevations in serum creatinine (Fig. 3A). The longitudinal data demonstrated unchanged levels of 25(OH)D with progression of CKD (Fig. 3B) and a progressive decline in 24,25(OH)2D3 (Fig. 3C) and the 25-VMR (Fig. 3D). The level of 1,25(OH)2D3 and 1,24,25(OH)3D3 decreased progressively with declining kidney function (Fig. 3E,F). However, aligned with our observation in humans, the ratio between 1,25(OH)2D3 and 1,25(OH)D3 increased with the induction and progression of CKD (Fig. 3G). As expected, blood levels of phosphate, PTH and FGF-23 increased as CKD progressed (Supplementary Fig. 1).

Discussion

This study reports 1,24,25(OH)3D3 levels in humans across a spectrum of measured GFR. To address the limitations of a cross-sectional study, we evaluated the same parameters longitudinally in the circulation of rats with the induction and progression of CKD. 1,25(OH)2D3 catabolism reflected by the 1,25-VMR increased whereas...
25(OH)D₃ catabolism (25-VMR) decreased as mGFR declined, a finding that we also observed longitudinally in rats. The progressive reduction in the 25-VMR with decreasing mGFR in humans, and with the induction and progression of CKD in rats, supports the work of others and indicates stagnation of 25(OH)D₃ catabolism. The biological significance of 24-hydroxylated metabolites in the circulation is unclear; however, there is evidence that 1,24,25(OH)₃D₃ is biologically active and 24,25(OH)₂D₃ facilitates fracture repair. Global reduction of 24-hydroxylation in CKD has been proposed to explain low levels of 24,25(OH)₂D₃, however, this theory requires careful further evaluation given that the 1,25-VMR does not trend similarly to the 25-VMR. The kidneys are the main site of origin of 1,25(OH)₂D₃ in the circulation, yet increased or unchanged expression of CYP24A1 in kidney tissue has been reported. While those finding are not consistent with the reduction in the 25-VMR, they are consistent with the 1,25-VMR.

Participants taking cholecalciferol had higher levels of 1,24,25(OH)₃D₃. One possibility is that cholecalciferol may have increased 1,25(OH)₂D₃ production, and subsequent catabolism, despite low kidney function. If this hypothesis were true, this could support the use of cholecalciferol in patients with kidney disease. Alternatively, it could also suggest the presence of pathways that convert 25(OH)D₃ to 1,24,25(OH)₃D₃ independently of a 1,25(OH)₂D₃ intermediary as suggested by Martineau et al. In a study of participants with moderate to severe CKD, cholecalciferol supplementation increased levels of 25(OH)D₃ substantially, but the change in 24,25(OH)₂D₃ was more than proportional to the increase in 25(OH)D suggesting that supplementation increased delivery of 25(OH)D to CYP24A1 and/or increased CYP24A1 activity. Levels of 1,25(OH)₂D₃ did
Table 2. Spearman correlations of vitamin D metabolites with demographics and serum measures in participants unadjusted and partially adjusted controlling for age and mGFR. Spearman r value indicated. Colour delineates p-value: Red < 0.001, Yellow < 0.01, Green < 0.05.

| Variable | 25D3 | 24,25D3 | 1,25D3 | 1,24,25D3 | 24,25D3 to 25D3 | 1,24,25D3 to 1,25D3 |
|----------|------|---------|--------|-----------|-----------------|---------------------|
| Unadjusted correlations |      |         |        |           |                 |                     |
| Age      | 0.20 | 0.05    | -0.26  | 0.04      | -0.08           | 0.29                |
| Sex      | -0.17| -0.16   | -0.20  | -0.04     | -0.11           | 0.16                |
| mGFR     | 0.48 | 0.65    | 0.32   | 0.64      | -0.37           |                     |
| Diabetic | -0.17| -0.27   | -0.38  | -0.07     | -0.26           | 0.31                |
| Serum Ca | 0.11 | 0.19    | 0.25   | 0.09      | 0.17            | -0.17               |
| Serum PO4| -0.08| 0.09    | -0.33  | -0.12     | -0.21           | 0.24                |
| PTH      | -0.26| -0.45   | -0.36  | -0.22     | -0.46           | 0.14                |
| FGF-23 (N = 56) | -0.18| -0.36   | -0.43  | -0.1      | -0.36           | 0.36                |
| Partial correlations controlling for age and mGFR |      |         |        |           |                 |                     |
| Sex      | -0.16| -0.18   | -0.28  | -0.03     | -0.15           | 0.19                |
| Diabetic | -0.20| -0.11   | -0.17  | 0.06      | 0.00            | 0.19                |
| Serum Ca | 0.08 | 0.10    | 0.22   | 0.02      | 0.07            | -0.16               |
| Serum PO4| -0.07| -0.01   | -0.12  | -0.01     | 0.06            | 0.13                |
| PTH      | -0.27| -0.25   | -0.05  | -0.04     | -0.18           | -0.02               |
| FGF-23 (N = 56) | -0.32| -0.36   | -0.30  | 0.02      | -0.24           | 0.29                |

Figure 2. Vitamin D metabolites separated by cholecalciferol use delineated by measured GFR. (A) 25(OH)D3, (B) 24,25(OH)2D3, (C) 25-VMR: 24,25(OH)2D3:25(OH)D3, (D) 1,25(OH)2D3, (E) 1,24,25(OH)3D3, (F) 1,25-VMR: 1,24,25(OH)3D3:1,25(OH)2D3. Two-way ANOVA. Mean SD. P-value indicates whether cholecalciferol use was a significant source of variation. *p < 0.05, **p < 0.01, ***p < 0.001.
not change. In a study involving participants of the Multi-Ethnic Study of Atherosclerosis, lower 25(OH)D and a lower 25-VMR was associated with a greater treatment response to cholecalciferol supplementation, as assessed by a change in PTH. Similar to the previous study, there was no change in 1,25(OH)2D3 in the cholecalciferol supplemented participants.

Study strengths include the LC–MS/MS assessment of vitamin D metabolites which allows for accurate assessment of these structurally very similar metabolites, a limitation of other methods as well as the measurement of GFR, as opposed to estimation based on endogenous markers. The cross-sectional design of the human study limits interpretation, however the longitudinal rat data demonstrated the identical evolution of changes in vitamin D metabolites with progressing CKD. The participants were predominantly white limiting generalizability. Future studies should consider acute challenges of different metabolites to assess specificity and kinetics of transformation. We acknowledge that variability in metabolite-to-parent ratios may not solely reflect true enzymatic activity. For example, Hsu et al. demonstrated higher 25(OH)D clearance in Black individuals, despite lower a 25-VMR. Further, given that 1α-hydroxylated metabolites circulate at ~ 1000× lower concentration than their non-1α-hydroxylated counterparts, differences unrelated to 24-hydroxylation, for example intra-individual variation or assay variability, may meaningfully alter the 1,25-VMR, without altering other metabolites circulating at a higher-levels, and thus the circadian rhythm and the metabolism of 1,24,25(OH)3D3 is an important area of future research before use as a potential diagnostic tool. The development of a vitamin D profile that includes VMRs and evaluates the response over time to supplementation may better define adequacy in this population and guide future therapy as suggested by Melamed et al.

In summary, the circulating 24-hydroxylated 1,25(OH)2D ratio does not decrease like the 25-VMR as kidney function declines. The clinical implications of the divergence between catabolism of 25(OH)D3 versus 1,25(OH)2D3 requires further study. Understanding vitamin D catabolism and the potential that 24-hydroxylation products may have biological activity may help inform treatment strategies in the future.

Data availability
Data lies with the Principal Investigators and can be made available upon request.

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Author contributions

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Competing interests

The authors declare no competing interests.

Additional information

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