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The Vitamin D metabolite diagnostic ratio associates with phenotypic traits of idiopathic hypercalciuria

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Running headline: Vitamin D ratio and idiopathic hypercalciuria

Abstract

Introduction: Underlying mechanisms for hypercalciuria remain unknown in most cases, hence the designation "idiopathic". We hypothesized that the Vitamin D-inactivating enzyme CYP24A1 contributes to the pathogenesis of hypercalciuria in kidney stone formers.

Methods: We conducted association analyses between CYP24A1 activity, estimated by the Vitamin D metabolite diagnostic ratio (25(OH) Vitamin D_3/total 24,25 $(OH)_2$ Vitamin D ratio; VMDR), and the phenotype of participants in two observational cohorts of kidney stone formers, the Swiss Kidney Stone Cohort and the Bern Kidney Stone Registry. Circulating 25(OH)- and 24,25 $(OH)_2$ Vitamin D were quantified using a validated LC-MS/MS assay.

Results: 974 participants were included in the analysis. We found a positive association of VMDR (and hence negative association of CYP24A1 activity) with total (β 0.009 mmol/L; 95% CI 0.002, 0.016; p = 0.02) and ionized plasma calcium (β 0.005 mmol/L; 95% CI 0.002, 0.008; p < 0.01), absolute and fractional excretion of urinary calcium (β 0.054 mmol/24h; 95% CI 0.010, 0.097; p = 0.02 and β 0.046 %; 95% CI 0.018, 0.074; p < 0.01, respectively). Further, VMDR was associated with an increased likelihood of forming calcium oxalate dihydrate stones (Odds ratio 1.64; 95% CI 1.22, 2.35; p < 0.01) and reduced bone mineral density at the femoral neck (β -0.005 g/cm^2; 95% CI -0.010, -0.001; p = 0.04). The described associations became stronger when the analysis was confined to idiopathic calcium stone formers.

Conclusion: Our study reveals that CYP24A1 activity, estimated by VMDR, is associated with clinical traits previously linked to idiopathic hypercalciuria.

Keywords: Kidney stones, nephrolithiasis, CYP24A1, Vitamin D, Vitamin D metabolite diagnostic ratio, calcium, calcium oxalate, bone mineral density.

Introduction

Kidney stone formation is complex and depends on dietary, environmental, and genetic factors [1]. Eighty to 90% of stones are composed of calcium oxalate, calcium phosphate, or a mixture of both [2,3]. Hypercalciuria, defined as increased urinary calcium excretion, is the most frequent pro-lithogenic abnormality encountered in patients with kidney stones and is present in up to 80% of affected patients [3,4]. Metabolic work-up of hypercalciuric stone formers rarely reveals an underlying systemic cause, and hence in most cases, hypercalciuria is defined as "idiopathic" [5]. The phenotype of idiopathic hypercalciuria can be replicated by exogenous administration of 1,25$(OH)_2$ Vitamin D_3 to healthy individuals [6]. In contrast, the P450 inhibitor ketoconazole, which attenuates 1,25$(OH)_2$ Vitamin D_3 synthesis, normalized the clinical features of patients with idiopathic hypercalciuria [7]. Circulating 1,25$(OH)_2$ Vitamin D_3 is determined by the rate of synthesis from its substrate, 25(OH) Vitamin D_3, through the activity of 25(OH)D-1-hydroxylase (CYP27B1), and its degradation by 1,25$(OH)_2$D-24-hydroxylase (CYP24A1) [8]. CYP24A1 is a P450 enzyme expressed in target tissues and is strongly up-regulated by its substrate 1,25$(OH)_2$ Vitamin D_3. In addition to 1,25$(OH)_2$ Vitamin D_3, CYP24A1 also inactivates its precursor 25(OH) Vitamin D. Homozygous pathogenic variants in *CYP24A1* cause idiopathic infantile hypercalcemia, a condition first described in children undergoing Vitamin D supplementation characterized by failure to thrive, dehydration, hypercalcemia and nephrocalcinosis [9]. Subsequently, bi-allelic pathogenic variants in *CYP24A1* were also discovered in adults with elevated Vitamin D, suppressed PTH, hypercalcemia, hypercalciuria, osteopenia, and recurrent calcium nephrolithiasis [10,11]. Individuals with heterozygous pathogenic *CYP24A1* variants exhibit an intermediary phenotype characterized by recurrent calcium nephrolithiasis, normocalcemic hypercalciuria, high-normal 1,25$(OH)_2$ Vitamin D_3 and low-normal PTH, a constellation typically encountered in idiopathic hypercalciuria, suggesting a gene dosage effect for pathogenic *CYP24A1* variants [10,12,13].

The ratio between 25(OH)- and 24,25(OH)$_2$ Vitamin D (Vitamin D metabolite diagnostic ratio, VMDR) is considered as a proxy of CYP24A1-mediated Vitamin D clearance [13–15]. In a cohort of 153 first-time calcium stone formers, increased serum calcium and 1,25(OH)$_2$ vitamin D$_3$, a lower serum 24,25(OH)$_2$ vitamin D / 25(OH) vitamin D ratio but no difference in urine calcium was observed compared to non-stone formers [16].

The association of CYP24A1 activity, estimated by the VMDR, with stone composition and comprehensive clinical traits linked to kidney stone disease, has not been studied yet. Hence, the diagnostic utility of the VMDR in kidney stone formers remains unclear. Given the high phenotypic similarity between carriers of pathogenic *CYP24A1* variants and kidney stone formers with idiopathic hypercalciuria, we hypothesized that reduced CYP24A1 activity may be an important cause of idiopathic hypercalciuria. To this end, we assessed the association of VMDR with the clinical phenotype of participants in two large prospective observational Swiss cohorts of kidney stone formers.

Materials and Methods

Study population

The Swiss Kidney Stone Cohort (SKSC) is an investigator-initiated prospective, multicentric, observational study of patients with kidney stones recruited at the nephrology outpatient clinics of six tertiary care nephrology centers in Switzerland (Aarau, Basel, Bern, Geneva, Lausanne, and Zürich) [17]. The Bern Kidney Stone Registry (BKSR) includes kidney stone formers recruited at the nephrology outpatient clinic of the Department of Nephrology and Hypertension at the Bern University Hospital, Bern, Switzerland [18]. SKSC and BKSR adhered to the Declaration of Helsinki and were approved by the responsible cantonal ethical committees (approval #BE 173/13 and #BE 95/06, respectively). Inclusion criteria for both cohorts are written informed consent, age ≥ 18 years, ≥ 2 kidney past stone events or 1 past stone event combined with additional risk factors for stone recurrence, such as first stone episode < 25 years, positive family history, stones other than calcium oxalate, bilateral or multiple stones or nephrocalcinosis detected by imaging, single kidney or chronic kidney disease (eGFR <60 mL/min), metabolic syndrome, gout, or osteoporosis. As stone event was defined as a symptomatic stone event with visible passage of a stone (with or without accompanying typical symptoms), or urological intervention of a symptomatic or asymptomatic stone. At the baseline visit, demographic and anthropometric data, comorbidities and stone composition analysis results were collected, and a comprehensive blood and 24-h urine metabolic workup performed. In addition, biobank plasma samples were collected at the baseline visit, immediately frozen and stored at – 80° C. Stone formers with end stage kidney disease were excluded from this study. A total of 974 participants (560 SKSC participants and 414 BKSR participants) met the eligibility criteria and had biobank plasma available for the analysis of Vitamin D metabolites.

Measurements and definitions

Plasma 25(OH) Vitamin D_2 and D_3, and total plasma 24,25$(OH)_2$ Vitamin D were measured by an established, highly sensitive and specific LC-MS/MS method at the Mayo Clinic Central Laboratories, 3050 Superior Drive NW, Rochester, MN 55901 [19,20]. 25(OH) Vitamin D_2 was not detected in any of the participants, thus the VMDR was calculated as 25(OH) Vitamin D_3 / total 24,25$(OH)_2$ Vitamin D. All other blood and urinary parameters of BKSR and SKSC participants were measured centrally by standard clinical laboratory methods at the Central Laboratory of the Bern University Hospital, Bern, Switzerland. Assay characteristics for the measurements of PTH, C-terminal fibroblast growth factor 23 (cFGF23) and 1,25$(OH)_2$ Vitamin D_3 were previously described [21]. Estimated glomerular filtration rate (eGFR) was determined with the CKD-EPI 2009 equation [22]. The mean value of two 24h-urine collections on consecutive days was used to calculate mean 24h urinary calcium excretion. Fractional excretion of calcium was determined from blood and urine samples collected on the same day. Osteodensitometry at the lumbar spine and the femoral neck was performed in all BKSR participants at the time point of metabolic work-up at the Department of Osteoporosis of the Bern University Hospital, Bern, Switzerland, by dual-energy X-ray absorptiometry (DEXA; Hologic QDR 4500A, Hologic, Bedford, MA, USA) [23]. Kidney stone composition was determined by Fourier-transform infrared spectroscopy. In participants with several stone composition analyses available, the most recent analysis prior to metabolic work-up was included in the analysis. Urine relative supersaturations (RSS) for calcium oxalate and brushite (calcium phosphate) were calculated using the EQUIL2 program [24]. Data related to loop and thiazide diuretics, and medications that could potentially influence plasma 25(OH) Vitamin D_3 concentration were collected. These included Vitamin D supplementation (cholecalciferol, ergocalciferol, calcifediol, alfacalcidol), carbamazepine, oxcarbazepine, clonazepam, St. John's wort, ritonavir, efavirenz, tenofovir, emtricitabine, glucocorticoids, rifampin, ketoconazole and calcium antagonists (felodipine, amlodipine, nifedipine, lercanidipine). Non-

calcium stones were defined as stone composition containing ≥ 50% uric acid, struvite or cystine.

Statistical analyses

Continuous variables were reported as medians ± interquartile ranges (IQR) or means ± and standard deviations (SD). Categorical variables were reported as counts and percentages, as appropriate. All values were first analysed by descriptive statistics. All statistical tests were two-sided, and a *p*-value <0.05 was considered statistically significant. Unadjusted and adjusted linear and logistic regression analyses of CYP24A1 activity, measured by the VMDR, were conducted as predictor variables with appropriately transformed outcome variables. Age, sex, body mass index (BMI), eGFR and plasma 25(OH) Vitamin D_3 were considered potential confounders and included in the multivariable model. These variables were selected for their known interaction with the outcome variables and total plasma 24,25$(OH)_2$ Vitamin D concentration by affecting 1,25$(OH)_2$ vitamin D_3 synthesis. To further assess the association between clinical traits of idiopathic hypercalciuria and the VMDR in patients with or without low 25(OH) Vitamin D_3, we stratified the study population in 2 subgroups (< or ≥20 ng/mL). We additionally performed sensitivity analyses for medications (drugs modulating plasma 25(OH) Vitamin D_3 concentration, loop and thiazide diuretics), urinary sodium excretion, which can directly affect urinary calcium excretion and total calcium balance [25], and for month of VMDR measurement, to account for fluctuations related to seasonality. A fully-adjusted subgroup analysis was conducted specifically on idiopathic calcium stone formers. This excluded patients without available stone composition analysis, those with non-calcium stones, and patients with secondary forms of calcium stones [26]. None of the participants included in this study had an established diagnosis of infantile hypercalcemia. All continuous predictor and outcome variables were scaled to Z-scores. The standardized regression coefficients (β) were subsequently multiplied by the standard deviation of the corresponding outcome variable.

Therefore, the resulting β coefficients are interpreted as the expected change, in absolute values, of the outcome variable per each standard deviation increase in the predictor variable (VMDR), which corresponds to 0.47. All regression models were tested for residuals' normality and homoscedasticity using visual inspection and for highly influential observations by plotting the Cook's distance for each data point. Residual plots were generated and carefully examined for normality through histograms along with Q-Q plots, while for homoscedasticity, a scatter plot of the predicted values against the residuals was inspected. All plots displayed expected patterns, suggesting that the assumptions of normality and equal variances were reasonably met for our models. Further, all analyses were checked for incomplete or over-collected 24h urine samples using reference values for urinary 24-hour creatinine excretion based on the adult Swiss population [27]. None of the results reported were biased by collection adequacy. Statistical analyses were conducted using the R software, version 4.2.1 [28].

Results

Characteristics of the study population

A total of 974 kidney stone formers met the predefined eligibility criteria and were included in the analysis. Baseline characteristics of the study population are shown in Table 1. Overall, 69.5% of participants were men (n = 677). Mean age±SD and age at the first kidney stone event±SD were 47.5±14.4 and 36.9±14.2 years, respectively. The majority of participants (n = 762, 81%) were recurrent stone formers. A stone composition analysis was available in 77% (n = 751) of participants. The most abundant stone type in our cohort (defined as ≥ 50% of total stone content) was calcium oxalate monohydrate (54%) – as reported previously [29], followed by calcium oxalate dihydrate (18%), total calcium phosphate (12%), and uric acid (8%).

Median values (interquartile ranges, IQR) for plasma 25(OH) Vitamin D_3, total 24,25$(OH)_2$ Vitamin D, and the VMDR (plasma 25(OH) Vitamin D_3 / total 24,25$(OH)_2$ Vitamin D ratio) were 21.0 (14.0, 29.0) ng/mL, 1.46 (0.73, 2.30) ng/mL, and 14.8 (11.5, 19.7), respectively (Table 1). There was a strong correlation between 25(OH)- and 24,25$(OH)_2$ Vitamin D (Pearson's correlation coefficient = 0.83, $p < 0.001$) (Figure 1A). The VMDR was not normally distributed (Supplementary Figure 1).

A VMDR < 25, considered normal (https://www.mayocliniclabs.com/test-catalog/Overview/63416#Clinical-and-Interpretive), was present in 87.1% (n = 848) of participants [19,20]. A VMDR between 25 and 80, previously reported in monoallelic carriers of pathogenic *CYP24A1* variants, was found in 12.1% (n = 118) of participants [19]. A VMDR > 80, previously reported in biallelic carriers of pathogenic *CYP24A1* variants, was found in 0.8% (n=8) participants [19]. Baseline characteristics stratified by these VMDR cut-offs are shown in Table 2. A replete 25(OH) Vitamin D_3 status (≥ 20 ng/mL) was found in 56.2% (n = 547) of participants. Within this subgroup, 94.9% (n = 519) had a VMDR < 25, 4.6% (n = 25) between 25-80, and 0.5% (n = 3) > 80.

Stone formers with low 25(OH) Vitamin D_3 (< 20 ng/mL) had significantly higher VMDR compared to the normal range (Figure 1B), in line with previous studies [19,20]. As expected, a negative relationship was observed between 24,25$(OH)_2$ Vitamin D and VMDR (Figure 1C). The VMDR exhibited seasonal variability, with a median peak value in February, suggesting the lowest CYP24A1 activity, and with a nadir reached in September, indicating the highest enzyme activity (Supplementary Figure 2, Supplementary Table S8).

Association analyses

The unadjusted analysis revealed a significant direct association of VMDR with total and ionized calcium, cFGF23, fractional excretion of calcium, and an inverse correlation with bone mineral density at the femoral neck (Table 3). After adjusting for multiple confounders, including age, sex, BMI, eGFR, and plasma 25(OH) Vitamin D_3 (Table 3), the results remained broadly consistent, confirming the association between VMDR and total (β 0.009 mmol/L; 95% CI 0.002, 0.016; p = 0.02) and ionized calcium (β 0.005 mmol/L; 95% CI 0.002, 0.008; p < 0.01) , cFGF23 (β 0.045 RU/mL; 95% CI 0.004, 0.086; p = 0.03), fractional excretion of calcium (β 0.046%; 95% CI 0.018, 0.074; p < 0.01) and BMD at the femoral neck (β -0.005 g/cm^2; 95% CI -0.010, -0.001; p = 0.04). In addition, absolute urinary calcium excretion became directly correlated with VMDR (β 0.054 mmol/24h; 95% CI 0.010, 0.097; p = 0.02). No association was found between VMDR and plasma 1,25$(OH)_2$ Vitamin D_3 or parathyroid hormone. Only after accounting for cFGF23 as an additional confounder, a trend toward a direct association between 1,25$(OH)_2$ Vitamin D_3 and VMDR was observed (β 0.131 pmol/L; 95% CI 0.000, 0.267; p = 0.05).

Further, our analysis also revealed an inverse relationship between VMDR and the odds ratio (OR) to develop calcium oxalate monohydrate-containing stones (Table 4), while the likelihood of forming calcium oxalate stones in general was not associated with VMDR. Specifically, a lower CYP24A1 activity, as reflected by a higher VMDR, was associated with a decreased odds

of forming stones composed of ≥ 50% calcium oxalate monohydrate (adjusted OR 0.56; 95% CI 0.38, 0.79; p < 0.01). In contrast, VMDR was associated with an increased odds of stones with a calcium oxalate dihydrate component ≥ 50% (adjusted OR 1.64; 95% CI 1.22, 2.35; p < 0.01). No correlations between VMDR and RSS calcium oxalate or RSS brushite were observed.

Sensitivity analyses

Sodium intake is an important determinant of urinary calcium excretion [25], and medications that modulate plasma 25(OH) Vitamin D_3 or diuretics may potentially affect the VMDR or urine calcium, respectively. Additionally, we found a mild seasonal variability of VMDR. To address these issues, we performed sensitivity analyses that incorporated the month of VMDR measurement, 24-h urinary sodium excretion, an established proxy of sodium intake, along with the aforementioned medications. All the associations found remained robust in these sensitivity analyses (Supplementary Tables S1-S6).

Further sensitivity analyses were conducted to examine the persistence of associations in subgroups of participants with varying plasma 25(OH) Vitamin D_3 levels. In stone formers with normal plasma 25(OH) Vitamin D_3 (≥ 20 ng/mL), the aforementioned adjusted associations remained significant (Supplementary Table S7). On the opposite, in patients with low 25(OH) Vitamin D_3 (< 20 ng/mL), adjusted associations remained valid only for ionized calcium (β 0.01 mmol/L; 95% CI 0.00 , 0.02; p = 0.04) and its fractional excretion (β 0.08 %; 95% CI 0.02, 0.14; p < 0.01).

Confirmatory analysis for idiopathic calcium stone formers

To confirm these results in the selected population of interest, we conducted a full-adjusted analysis on idiopathic calcium stone formers (Tables 5 and 6). In this analysis, we excluded non-calcium stone formers, patients without available stone analysis, and those with secondary

causes of calcium stones. Consistent with our main analysis, this subgroup exhibited significant direct associations of VMDR with total plasma calcium (β 0.014 mmol/L; 95% CI 0.003, 0.025; p = 0.011), ionized calcium (β 0.009 mmol/L; 95% CI 0.001, 0.016; p = 0.025), absolute (β 0.077 mmol/24h; 95% CI 0.018, 0.136; p = 0.011), and fractional urinary excretion of calcium (β 0.051 %; 95% CI 0.01, 0.093; p = 0.016) . The inverse association with femoral BMD was also confirmed (β -0.018 g/cm2; 95% CI -0.030, -0.007; p = 0.002) .

In terms of stone composition, the VMDR was inversely associated with the odds of calcium oxalate monohydrate stones formation (adjusted OR 0.40; 95% CI 0.26, 0.63; p < 0.001), and positively with calcium oxalate dihydrate stones (adjusted OR 1.62; 95% CI 1.15, 2.29; p = 0.006), in line with our results obtained in the full cohort.

Discussion

High urinary calcium excretion ("hypercalciuria"), is a risk factor for both kidney stone formation and low bone mass. However, the underlying mechanisms remain elusive in most patients, and hence the condition is labelled "idiopathic hypercalciuria" [3,4].

Our study conducted in two large and deeply phenotyped Swiss cohorts of kidney stone formers now reveals that the VMDR (and hence the activity of CYP24A1 inversely) is directly associated with urinary calcium, the key prolithogenic abnormality in kidney stone formers. In support of this finding, CYP24A1 activity was associated with kidney stone composition: we observed an direct association of VMDR (and hence inverse association with CYP24A1 activity) with calcium oxalate dihydrate stones, the classical stone type of patients with a high urine calcium/oxalate ratio [29]. In contrast, there was an inverse association with VMDR (and hence direct association with CYP24A1 activity) with calcium oxalate monohydrate stones, typically encountered in patients with a high urinary oxalate/calcium ratio. Consonant with the function of CYP24A1 as Vitamin D-inactivating enzyme, we observed a direct association of VMDR with both total plasma calcium and ionized calcium.

Reduced BMD and an increased fracture risk has been repeatedly observed in kidney stone formers, especially in patients with idiopathic hypercalciuria [30,31]. Intriguingly, we also observed that reduced CYP24A1 activity is associated with a lower BMD at the femoral neck. In contrast, VMDR was not associated with BMD at the lumbar spine, indicating that CYP24A1 activity rather affects cortical than trabecular bone. Given the cross-sectional nature of our analysis, we can only speculate on the underlying mechanisms. Despite a Vitamin D-mediated increase in intestinal calcium absorption in patients with low CYP24A1 activity, urinary calcium losses may prevail and hence patients are overall in a negative calcium balance, as previously reported in patients with idiopathic hypercalciuria [32]. Indeed, urine calcium excretion was found to be inversely correlated with BMD changes at the femoral neck but not at the lumbar spine in patients with idiopathic hypercalciuria [32]. Alternatively, given the known

function of 24,25 $(OH)_2$ Vitamin D in bone remodeling, cell migration and proliferation [33–35], low levels of 24,25 $(OH)_2$ Vitamin D may directly be responsible for reduced BMD observed at the femoral neck [36].

Results of our analyses in kidney stone formers reveal that CYP24A1 activity, estimated by the VMDR, correlates with clinical traits characteristic for idiopathic hypercalciuria after adjustment for multiple confounders, including plasma 25(OH) Vitamin D_3 and drugs that modulate its concentration, loop and thiazide diuretics, and urinary sodium excretion. Notably, the aforementioned associations became even stronger when only idiopathic calcium stone formers were analyzed. However, these observations appear only valid in patients which are 25(OH) Vitamin D_3 replete, whereas with 25(OH) Vitamin D_3 deficiency (< 20 ng/mL), the VMDR will be falsely elevated.

Surprisingly, we observed a significant correlation between CYP24A1 activity and cFGF23, but no association was evident with plasma PTH and 1,25 $(OH)_2$ Vitamin D_3. Of note, only 13 participants exhibited increased serum ionized calcium, and the majority of our cohort did not demonstrate overt hypercalcemia, which may have further weakened the association between PTH and reduced CYP24A1 activity. Furthermore, there was only a very weak correlation between 1,25 $(OH)_2$ Vitamin D_3 and urinary calcium excretion in our study population (Spearman's rho 0.11; $p < 0.01$). FGF23 plays a pivotal role in the regulation of Vitamin D metabolism by decreasing circulating 1,25 $(OH)_2$ Vitamin D_3 through CYP27B1 inhibition and CYP24A1 induction in the kidney [37]. Individuals with infantile hypercalcemia type 1 and very low CYP24A1 activity have high FGF23 levels [38,39]. In our analysis, a trend toward an association between lower CYP24A1 activity and higher 1,25 $(OH)_2$ Vitamin D_3 was observed after adjusting for FGF23. Thus, we speculate that reduced CYP24A1 activity might lead to higher FGF23, which in turn reduces 1,25 $(OH)_2$ Vitamin D_3 synthesis through inhibition of CYP27B1. However, 1,25 $(OH)_2$ Vitamin D_3 is also regulated by PTH as part of a complex

interplay between calcium, 25(OH) Vitamin D_3, and PTH. In an observational study, such intricate relationships may be challenging to assess, and reverse causality or alternative explanations, such as tissue-specific non-systemic effects of CYP24A1 or a 1,25(OH)2 Vitamin D3–independent regulation of renal calcium handling by CYP24A1 cannot be excluded. Consonant with this, kidney stone formers were recently shown to exhibit higher CYP24A1 expression in circulating monocytes compared to non-stone formers when individual 1,25(OH)2 Vitamin D_3 concentrations were taken into account (i.e. higher circulating 1,25 $(OH)_2$ Vitamin D_3 /monocyte CYP24A1 expression ratios) [40].

According to previous reports, a VMDR >80 suggests bi-allelic pathogenic *CYP24A1* variants, whereas values between 25 and 80 have been reported in heterozygous carriers or in patients with low plasma 25(OH) Vitamin D_3 [10,19]. The prevalence of pathogenic variants in *CYP24A1* in the general population has been estimated between 420 to 1960 per 100,000 individuals (0.4 to 2%) [11]. The observation that 28 of 547 (5 %) of Vitamin D-replete kidney stone formers in our cohort had a VMDR >25 indicates that other factors may play an important role in the regulation of CYP24A1 activity. Indeed, CYP24A1 expression is regulated by a myriad of endocrine factors including PTH, FGF23, 1,25 $(OH)_2$ Vitamin D_3, estrogens, and retinoid receptor ligands as well as inflammatory cytokines such as IL-6 and TNF-α [41–46]. Furthermore, epigenetic modifications at the *CYP24A1* promoter and tissue-specific intronic enhancer modulate transcription of the *CYP24A1* gene [47,48]. Thus, the VMDR employed in this study integrates both genetic and non-genetic factors influencing CYP24A1 activity. As such, our measurements of the VMDR in both idiopathic calcium- and unselected kidney stone formers therefore offer a comprehensive view of biochemical and clinical traits associated with CYP24A1 activity. Additional strengths of our study include the large sample size, the multicentric study design and a very detailed phenotype including stone analysis and BMD measurements. Our study has also several limitations, including the observational design, the

exploratory nature of the analyses, an almost exclusive Caucasian study population, the lack of genetic data and absence of low risk first-time kidney stone formers in the study population. Despite these limitations, our analyses significantly extend current knowledge on the role of the Vitamin D-inactivating enzyme CYP24A1 in nephrolithiasis. Our results demonstrate that CYP24A1 activity, estimated by VMDR,is directly linked to urine calcium excretion, kidney stone composition and BMD at the femoral neck. Future studies are now needed to confirm the validity of VMDR as a diagnostic tool in the metabolic work-up of kidney stone formers and to determine if VMDR status prospectively predicts risk of recurrence and is associated with the response to dietary and pharmacologic preventive strategies. If validated, VMDR measurements could offer clinicians valuable insights for tailored treatment recommendations in kidney stone formers.

Disclosures

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Data Availability Statement

The data underlying this article will be shared on reasonable request to the corresponding author.

Supplementary Material (PDF)

Supplementary information is available at KI Report's website.

Supplementary Methods

Formulas

Supplementary Table S1: Sensitivity linear regression analysis for urinary sodium excretion.

Supplementary Table S2: Sensitivity logistic regression analysis for urinary sodium excretion.

Supplementary Table S3: Sensitivity linear regression analysis for medications.

Supplementary Table S4: Sensitivity logistic regression analysis for medications.

Supplementary Table S5: Sensitivity linear regression analysis for seasonality of VMDR measurement.

Supplementary Table S6: Sensitivity logistic regression analysis for seasonality of VMDR measurement.

Supplementary Table S7: Multivariable linear regression analysis stratified by plasma 25(OH) Vitamin D3.

Supplementary Table S8: Seasonal variability of VMDR.

Supplementary Table S9: Missing variables report.

Supplementary Figure 1: Distribution of VMDR.

Supplementary Figure 2: Seasonal variation of 25(OH) Vitamin D3, 24,25(OH)2 Vitamin D3, and their ratio.

STROBE Statement

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Tables

Table 1 | Baseline characteristics of study population. Characteristics are indicated for all participants enrolled from the BKSR and the SKSC**.** Categorical variables are described by number of participants N (%), continuous variables are described by their mean (SD) or median (25^{th}-75^{th} percentile). BSA, body surface area; eGFR, estimated glomerular filtration rate; DEXA, dual-energy X-ray absorptiometry; PTH, parathyroid hormone; BMD, bone mineral density; VMDR, Vitamin D metabolite diagnostic ratio; SD, standard deviation.

Characteristics	Study participants (N=974)
Males	677 (69.5%)
Age, years	47.5 (14.4)
Age at first self-reported stone event	36.9 (14.2)
Age at first stone composition analysis	45.8 (14.6)
Body mass index, kg/m^2	27.1 (4.9)
eGFR creatinine Equation CKD-EPI 2009, mL/min per 1.73 m^2 BSA	95.0 (19.7)
Hypertension medication usage	268 (27.6%)
Diabetes	114 (11.7%)
Stone recurrence (≥ 2 stone events)	793 (81.3%)
Medications affecting plasma 25(OH) Vitamin D3	71 (7.3%)
Loop diuretics	14 (1.4%)
Thiazide diuretics	127 (13.0%)
DEXA parameters	
Femoral neck BMD, g/cm^2	0.83 (0.75, 0.93)
Femoral neck T-score, SD	-0.60 (-1.30, 0.00)
Lumbar spine BMD, g/cm^2	1.01 (0.93, 1.11)
Lumbar spine T-score, SD	-0.60 (-1.40, 0.30)
Blood parameters	
Total plasma calcium, mmol/L	2.34 (2.28, 2.39)
Corrected plasma calcium, mmol/L	2.36 (2.30, 2.42)
Ionized calcium, mmol/L	1.20 (1.17, 1.22)
Phosphate, mmol/L	0.99 (0.87, 1.11)
25(OH) Vitamin D_3, ng/mL	21.00 (14.00, 29.00)
1,25(OH)$_2$ Vitamin D_3, pmol/L	99.00 (74.79, 127.00)
Total 24,25(OH)$_2$ Vitamin D ng/mL	1.46 (0.73, 2.30)
VMDR	14.78 (11.51, 19.67)
Intact PTH, ng/L	39.00 (30.60, 50.00)
cFGF23, RU/mL	69.50 (54.35, 96.00)
Urinary parameters	
Urinary calcium, mmol/24h	5.14 (3.33, 7.48)
Fractional excretion of calcium, %	2.72 (1.79, 3.57)
RSS for calcium oxalate	4.08 (2.05, 7.62)
RSS for brushite	0.74 (0.27, 2.06)
Relative kidney stone composition ≥ 50%	
Available stone composition analysis	751 (77.1%)
Total calcium oxalate	595 (79.2%)
Calcium oxalate monohydrate	399 (54.0%)
Calcium oxalate dihydrate	132 (17.9%)
Total calcium phosphate	91 (12.1%)
Apatite	63 (8.4%)
Brushite	15 (2.0%)
Uric acid	60 (8.0%)
Cystine	9 (1.2%)

Table 2 | Blood and urine mineral metabolism parameters and DEXA parameters stratified by subgroups of VMDR. Categorical variables are described by number of participants N (%), continuous variables are described by their mean (SD) or median (25th-75th percentile). BSA, body surface area; eGFR, estimated glomerular filtration rate; DEXA, dual-energy X-ray absorptiometry; PTH, parathyroid hormone; BMD, bone mineral density; VMDR, Vitamin D metabolite diagnostic ratio; SD, standard deviation.

	Vitamin D metabolite diagnostic ratio		
Characteristics	**VMDR < 25 (N=848)**	**VMDR 25-80 (N=118)**	**VMDR > 80 (N=8)**
Males	604 (71.2%)	68 (57.6%)	5 (62.5%)
Age, years	47.4 (14.2)	47.8 (15.5)	51.1 (15.2)
Age at first self-reported stone event	37.1 (14.1)	36.2 (15.0)	29.3 (9.0)
Age at first stone composition analysis	45.6 (14.3)	46.6 (16.7)	48.3 (17.7)
Body mass index, kg/m^2	27.0 (4.8)	28.1 (5.4)	24.9 (2.7)
eGFR creatinine Equation CKD-EPI 2009, mL/min per 1.73 m^2 BSA	95.4 (18.7)	93.6 (25.0)	80.2 (25.4)
Hypertension medication usage	232 (27.4%)	34 (28.8%)	2 (25.0%)
Diabetes	94 (11.1%)	20 (16.9%)	0 (0.0%)
Stone recurrence (≥ 2 stone events)	689 (81.3%)	96 (81.4%)	8 (100.0%)
Medications affecting plasma 25(OH) Vitamin D3	55 (6.5%)	15 (12.7%)	1 (12.5%)
Loop diuretics	11 (1.3%)	3 (2.5%)	0 (0.0%)
Thiazide diuretics	115 (13.6%)	11 (9.3%)	1 (12.5%)
DEXA parameters			
Femoral neck BMD, g/cm^2	0.84 (0.76, 0.93)	0.79 (0.72, 0.88)	0.79 (0.65, 0.84)
Femoral neck T-score, SD	-0.50 (-1.20, 0.10)	-0.80 (-1.48, -0.20)	-1.05 (-2.00, -0.70)
Lumbar spine BMD, g/cm^2	1.02 (0.94, 1.11)	0.98 (0.85, 1.08)	1.01 (0.97, 1.06)
Lumbar spine T-score, SD	-0.60 (-1.28, 0.30)	-0.70 (-2.00, 0.18)	-0.70 (-0.98, -0.28)
Blood parameters			
Total plasma calcium, mmol/L	2.34 (2.28, 2.39)	2.32 (2.26, 2.39)	2.43 (2.34, 2.53)
Corrected plasma calcium, mmol/L	2.36 (2.30, 2.42)	2.36 (2.29, 2.43)	2.46 (2.34, 2.54)

Ionized calcium, mmol/L	1.20 (1.17, 1.22)	1.20 (1.18, 1.23)	1.24 (1.21, 1.26)
Phosphate, mmol/L	1.00 (0.88, 1.11)	0.98 (0.87, 1.10)	0.96 (0.91, 1.01)
25(OH) Vitamin D_3, ng/mL	22.00 (16.00, 30.00)	12.00 (8.30, 18.00)	12.50 (9.00, 31.30)
1,25$(OH)_2$ Vitamin D_3, pmol/L	100.00 (76.00, 127.00)	90.00 (67.00, 121.00)	81.50 (72.50, 105.00)
Total 24,25$(OH)_2$ Vitamin D ng/mL	1.65 (0.96, 2.44)	0.37 (0.24, 0.57)	0.12 (0.07, 0.14)
Intact PTH, ng/L	38.60 (30.40, 49.00)	44.00 (32.00, 59.50)	42.00 (21.40, 45.00)
cFGF23, RU/mL	69.30 (54.20, 94.80)	71.10 (55.20, 111.50)	94.80 (65.70, 236.20)
Urine parameters			
Urinary calcium, mmol/24h	5.20 (3.43, 7.48)	4.51 (2.66, 7.15)	9.52 (6.07, 10.30)
Fractional excretion of calcium, %	2.72 (1.84, 3.56)	2.66 (1.55, 3.55)	3.59 (2.95, 5.24)
RSS for calcium oxalate	4.00 (2.00, 7.50)	5.00 (2.70, 8.60)	5.70 (2.60, 7.90)
RSS for brushite	0.70 (0.30, 2.00)	1.00 (0.30, 3.10)	1.30 (0.40, 4.70)
Relative kidney stone composition ≥ 50%			
Total calcium oxalate	525 (79.4%)	65 (77.4%)	5 (83.3%)
Calcium oxalate monohydrate	354 (54.4%)	44 (53.7%)	1 (16.7%)
Calcium oxalate dihydrate	112 (17.2%)	16 (19.5%)	4 (66.7%)
Total calcium phosphate	76 (11.5%)	14 (16.7%)	1 (16.7%)
Apatite	53 (8.0%)	9 (10.7%)	1 (16.7%)
Brushite	12 (1.8%)	3 (3.6%)	0 (0.0%)
Uric acid	57 (8.6%)	3 (3.6%)	0 (0.0%)
Cystine	8 (1.2%)	1 (1.2%)	0 (0.0%)

Table 3 | Univariable and multivariable linear regression analysis. Association between Vitamin D metabolite diagnostic ratio with clinical traits as outcome variables, adjusted for age, sex, BMI, eGFR and 25(OH) Vitamin D_3. BMI, body mass index; eGFR, estimated glomerular filtration rate; PTH, parathyroid hormone; BMD, bone mineral density; RSS, relative supersaturation; *, natural logarithm transformed; **, square root transformed. Number of observations (N obs), beta coefficients (β), 95% confidence intervals (95% CI) and *p*-values are indicated for each comparison.

	Univariable Model				Multivariable Model			
Outcome variable	**N**	**β**	**95% CI**	***p*-value**	**N**	**β**	**95% CI**	***p*-value**
Blood parameters								
Total plasma calcium, mmol/L	960	0.009	0.002, 0.016	0.031	959	0.009	0.002, 0.016	0.016
Ionized calcium, mmol/L	601	0.005	0.002, 0.009	0.002	599	0.005	0.002, 0.008	0.004
Phosphate, mmol/L	962	-0.003	-0.014, 0.008	0.570	961	-0.004	-0.015, 0.007	0.460
**1,25$(OH)_2$ Vitamin D_3, pmol/L	956	-0.052	-0.174, 0.07	0.400	953	0.029	-0.082, 0.139	0.610
*Intact PTH, ng/L	942	0.019	-0.007, 0.045	0.150	939	0.008	-0.018, 0.033	0.560
**cFGF23, RU,mL	356	0.065	0.023, 0.108	0.002	352	0.045	0.004, 0.086	0.030
Urinary parameters								
*Urinary calcium, mmol/24h	947	0.022	-0.023, 0.067	0.330	944	0.054	0.010, 0.097	0.015
**Fractional excretion of calcium, %	931	0.041	0.013, 0.069	0.004	931	0.046	0.018, 0.074	0.001
*RSS for calcium oxalate	801	0.042	-0.046, 0.129	0.350	807	0.068	-0.017, 0.154	0.118
*RSS for brushite	812	0.077	-0.062, 0.217	0.277	796	0.128	-0.005, 0.261	0.059
DEXA parameters								
**Lumbar BMD, g/cm^2	358	-0.002	-0.007, 0.003	0.470	352	-0.001	-0.006, 0.004	0.650
**Femoral BMD, g/cm^2	359	-0.008	-0.013, -0.002	0.008	353	-0.005	-0.010, -0.001	0.036

Table 4 | Univariable and multivariable logistic regression analysis. Univariable and multivariable association between Vitamin D metabolite diagnostic ratio with relative kidney stone composition analysis ≥ 50%. Multivariable model adjusted for age, sex, BMI, eGFR and 25(OH) vitamin D_3. BMI, body mass index; eGFR, estimated glomerular filtration rate. Number of observations (N obs), Odds ratios (OR), 95% confidence intervals (95% CI) and *p*-values are indicated for each comparison.

	Univariable Model				Multivariable Model			
Relative kidney stone composition ≥ 50%	**N obs**	**OR**	**95% CI**	***p*-value**	**N obs**	**OR**	**95% CI**	***p*-value**
Total calcium oxalate	751	0.912	0.737, 1.142	0.370	745	0.961	0.775, 1.237	0.720
Calcium oxalate monohydrate	739	0.656	0.469, 0.873	0.009	733	0.558	0.377, 0.785	0.002
Calcium oxalate dihydrate	739	1.406	1.103, 1.887	0.015	733	1.636	1.217, 2.353	0.004
Total calcium phosphate	751	1.170	0.924, 1.476	0.150	745	1.152	0.906, 1.493	0.220
Apatite	751	1.158	0.879, 1.463	0.200	745	1.104	0.845, 1.454	0.420
Brushite	751	1.128	0.546, 1.505	0.550	745	1.235	0.479, 1.836	0.500

Table 5 | Confirmatory analysis for idiopathic calcium stone formers – linear regression analysis. Multivariable association between Vitamin D metabolite diagnostic ratio with clinical traits as outcome variables, adjusted for age, sex, BMI, eGFR, 25(OH) vitamin D_3, urinary sodium excretion, month of VMDR measurement, loop and thiazide diuretics, and medications that interfere with plasma 25(OH) Vitamin D3 concentration. Non-calcium stone formers, patients without available stone analysis and with secondary causes for kidney stones were excluded (primary hyperparathyroidism, sarcoidosis, complete dRTA, primary or enteric hyperoxaluria). BMI, body mass index; eGFR, estimated glomerular filtration rate; PTH, parathyroid hormone; BMD, bone mineral density; dRTA, distal renal tubular acidosis; *, natural logarithm transformed; **, square root transformed. Number of observations (N obs), beta coefficients (β), 95% confidence intervals (95% CI) and p-values are indicated for each comparison.

		Vitamin D Metabolite Diagnostic Ratio		
Outcome variables	**N obs**	**β**	**95% CI**	***p*-value**
Blood parameters				
Total plasma calcium, mmol/L	606	0.014	0.003, 0.025	0.011
Ionized calcium, mmol/L	379	0.009	0.001, 0.016	0.025
Phosphate, mmol/L	607	-0.007	-0.024, 0.009	0.398
**1,25$(OH)_2$ Vitamin D_3, pmol/L	605	0.060	-0.111, 0.23	0.491
*Intact PTH, ng/L	593	0.002	-0.039, 0.042	0.938
Urinary parameters				
*Urinary calcium, mmol/24h	607	0.077	0.018, 0.136	0.011
**Fractional excretion of calcium, %	601	0.051	0.01, 0.093	0.016
*RSS for calcium oxalate	504	0.051	-0.045, 0.148	0.298
*RSS for brushite	501	0.071	-0.076, 0.217	0.345
DEXA parameters				
**Lumbar BMD, g/cm^2	232	-0.007	-0.018, 0.004	0.189
**Femoral BMD, g/cm^2	233	-0.018	-0.030, -0.007	0.002

Table 6 | Confirmatory analysis for idiopathic calcium stone formers – logistic regression analysis. Multivariable logistic regression between Vitamin D metabolite diagnostic ratio with relative kidney stone composition analysis ≥ 50%. Multivariable model adjusted for age, sex, BMI, eGFR , 25(OH) vitamin D_3, urinary sodium excretion, loop and thiazide diuretics, and medications that interfere with plasma 25(OH) Vitamin D3 concentration. Non-calcium stone formers, patients without available stone analysis and with secondary causes for kidney stones were excluded (primary hyperparathyroidism, sarcoidosis, complete dRTA, primary or enteric hyperoxaluria). BMI, body mass index; eGFR, estimated glomerular filtration rate; dRTA, distal renal tubular acidosis. Number of observations (N obs), Odds ratios (OR), 95% confidence intervals (95% CI) and *p*-values are indicated for each comparison.

		Vitamin D Metabolite Diagnostic Ratio		
Outcome variables	**N obs**	**OR**	**95% CI**	***p*-value**
Total calcium oxalate	595	0.844	0.668, 1.067	0.157
Calcium oxalate monohydrate	588	0.404	0.258, 0.632	<0.001
Calcium oxalate dihydrate	588	1.62	1.147, 2.289	0.006

Figure Legends

Figure 1. Associations between 25(OH) Vitamin D_3, total 24,25$(OH)_2$ Vitamin D and VMDR. Panel (A) shows a scatterplot of the association between 25(OH) Vitamin D_3 and total 24,25$(OH)_2$ Vitamin D levels, including linear and natural cubic spline regression lines with green shadowed areas representing the 95% confidence bands. The association appears to be approximately linear at 25(OH) Vitamin D_3 concentration between 0 to 50 ng/mL, and total 24,25$(OH)_2$ Vitamin D between 0 to 4 ng/mL. Panel (B) shows the association between 25(OH) Vitamin D_3 and VMDR restricted to values ≤ 150 and a natural cubic spline regression line. Panel (C) shows a scatterplot of the association between total 24,25$(OH)_2$ Vitamin D and VMDR restricted to values ≤ 150 and a natural cubic spline regression line.