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Evaluating *VDR* Gene Expression and Vitamin D3 Levels in Calcium Oxalate Kidney Stone Patients: A Comparative Study with Healthy Controls

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Abstract: Kidney stones, also known as urolithiasis, is a urological condition with a high prevalence and recurrence rate, significantly impacting both individual health and the healthcare system. The progress in preventing their recurrence remains limited. Calcium oxalate (CaOx) stones are the most common type of kidney stone, accounting for over 70% of all cases. Vitamin D receptor (*VDR*) and vitamin D (VD) are associated with kidney stones in a multidimensional complex relation involving numerous physiological and metabolic pathways. In this study aims to evaluate and compare *VDR* gene expression and VD3 serum levels in kidney stone patients with healthy controls and investigate the relationships that may interfere with kidney stone formation and recurrence. The central research hypothesis is that an alteration in *VDR* and VD values in patients with kidney stones affects calcium homeostasis, which plays a role in kidney stone formation. To accomplish this objective, the RT-qPCR method was employed to assess *VDR* gene expression and serum levels of vitamin D (VD) and calcium in patients with kidney stones. These measurements were then compared with those of healthy individuals. The key findings of the research revealed that VD deficiency was detected in 56% of patients and 7.5% in controls groups, significant differences in vitamin D levels, which were (20.66±7.07) and (33.90±10.40), and *VDR* gene expression, which were (37.61±32.19) and (10.39±5.07) between patients and healthy controls groups, respectively; suggesting a potential link with Kidney Stone formation, and a good target for treatment developing strategies aiming to maintain healthy calcium metabolism and reduce kidney stone formation risk.

Keywords: CaOx Kidney Stone, Vitamin D, Vitamin D Receptor, Calcium, Gene Expression.

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Introduction

Kidney stones (nephrolithiasis) is a multifactorial disease; it is a common and often excruciating medical condition with a rising prevalence that affects approximately 13% of men and 7% of women worldwide (1-3). Kidney stones are solid crystalline formations that develop within the urinary tract, primarily in the kidneys. It is often a recurrent urological condition with a recurrence rate ranging from 6.1% to 66.9% that significantly burdens individual health and the healthcare system (4-6). Despite the significant improvement in the surgical treatment of renal kidney stones, the progress in preventing renal calculi recurrence is limited (7). High recurrence rate of kidney stones lead to recurrent urinary tract infection, urinary tract obstruction, increased risks of renal function reduction, chronic renal injury, and even renal failure, which have a severe impact on the patients' health (2,8). Calcium-containing stones are the most

common type; more than 70% of all kidney stones are Calcium oxalate (CaOx) (9,10). Multiple factors influence kidney stones development and formation, such as dietary habits, metabolic imbalances, genetic predisposition, and underlying medical conditions (11,12).

Vitamin D3 (VD3) (Cholecalciferol) is a fat-soluble vitamin that plays a crucial role in various physiological processes within the human body; it has been shown to influence calcium and phosphorus metabolism, bone health, and regulating immune function (13-16). VD3 affects kidney stone formation indirectly by increasing the efficiency of intestinal calcium absorption and regulating calcium renal excretion, also it is pivotal in oxalate metabolism (17,18).

VD3 deficiency is detected when the serum concentration levels of VD3 is less than 20 ng/mL (19). VD3 deficiency may elevate the risk of kidney stone formation by inducing secondary hyperparathyroidism as a compensatory physiological response by the body to maintain calcium blood levels homeostasis. To achieve this, PTH secretion is increased, promoting calcium release from bone into the bloodstream and inhibiting renal reabsorption, leading to increased urinary calcium excretion and hypercalciuria (18,20). Furthermore, it may induce oxidative stress and overexpression of inflammatory mediators in renal tissue (21).

Vitamin D receptor (*VDR*) (encoded by the *VDR* gene, located on chromosome 12) is a nuclear receptor and transcription factor found in almost all cells (22,23). Playing an essential role in the regulation of various physiological processes, VDR is the primary mediator of vitamin D's biological effects (24). Acting as a ligand-activated transcription factor, VDR binds to vitamin D and establishes a heterodimer with the retinoid x receptor (RXR), which ultimately regulates the expression of target genes involved in a diverse array of cellular processes (25,26).

Materials and methods

Study design and samples collection

This study was designed as a case-control study. It included two groups of samples: 50 patients diagnosed and confirmed with (CaOX) kidney stones and 40 healthy controls (Only individuals with no history of kidney stones, a clean renal ultrasound, a clean urine analysis, and evaluation by a urologist to confirm the absence of kidney stones and any urological issues were included as controls.). All the samples did not have diabetes. Under sterile conditions, whole blood samples (5 mL) were collected in EDTA tubes. Kidney stone samples were primarily collected from patients undergoing urological procedures at the hospital. Most stones were obtained during surgical interventions such as Percutaneous Nephrolithotomy (PCNL) or Ureteroscopy (URS), while additional samples were collected from patients who underwent Extracorporeal Shock Wave Lithotripsy (ESWL). The samples were collected from Al Yarmouk Teaching Hospital and Ghazi Al-Hariri Hospital for Surgical Specialties in Baghdad from October 2023 to September 2024. The study was approved by the Iraqi Ministry of Health's ethics committee, and participants signed the ethical approval.

Biochemical analysis and stone analysis

Serum calcium, urea, and creatinine levels (mg/dL) were measured using an automated biochemical analyzer (AU5800, Beckman Coulter Inc, Japan), Vitamin D3 serum levels (ng/mL) were

measured using a fully automated system (Access 2, Beckman Coulter Inc, Germany), and the assays were performed according to the manufacturer's instructions. To confirm that all participants did not have diabetes, glycated hemoglobin (HbA1c) levels were measured using (BR2200220, Bio-Rad D-10 Hemoglobin Testing System, Germany) less than 6.5% was used as a cut-off value to confirm non-diabetic status.

Kidney stone samples were analyzed using (Stone Analysis Set, Biolabo, France) to determine the main components of the urinary stones and their type; the analysis was performed according to the manufacturer's instructions.

RNA extraction

For extracting mRNA from whole blood, the QIAamp RNA Blood Mini Kit (QIAGEN, Germany) was used according to the manufacturer's protocol. To determine the purity and concentration of mRNA samples, we evaluated the A260/A280 ratio using the NanoDrop™ OneC Microvolume UV-Vis Spectrophotometer (Thermo Scientific), with all samples showing approximately 2.0 of purity.

Quantitative real-time PCR (qRT-PCR)

VDR gene expression was quantified using a one-step RT-qPCR assay on the QuantStudio™ 5 Real-Time PCR System (Thermo Fisher Scientific, USA) with Absolute Quantification.

The reactions were performed using the TaqPath DuraPlex 1-Step RT-qPCR Master Mix (Thermo Fisher Scientific, USA) according to the manufacturer's protocol. Universal primers for cDNA synthesis and specific mRNA *VDR* gene primers (Forward 5′-TGAAGGCTGCAAAGGTTTCT-3′ and Reverse 5′-TAGCTTGGGCCTCAGACTGT-3′) (27) were added. A standard curve was generated using serial dilutions of known *VDR* gene concentrations. The absolute number of *VDR* copies was determined by automatically comparing the Ct values of the samples with the standard curve.

Data analysis

Ct values were automatically converted into absolute copy numbers by the QuantStudio™ 5 Real-Time PCR System using standard curve interpolation.

Statistical analysis, mapping, and graphing were conducted using SPSS (IBM Corp., Armonk, NY, USA) and GraphPad Prism 10.3 (GraphPad Software, Boston, Massachusetts, USA). Data distribution normality was evaluated using the Kolmogorov-Smirnov test. Data that followed a normal distribution were analyzed using parametric tests (Mean, Independent t-tests). Nonparametric tests (Median, Mann-Whitney U tests) were used to analyze non-normally distributed data to compare differences between patients and controls.

Result and discussion

Study population characteristics and biochemical analysis

The study included 90 participants divided into two groups: 50 kidney stone patients and 40 healthy controls. All patients' stones were confirmed to be (CaOX) kidney stones type. The mean age of the patient group was 35.78 ± 11.28 years, while the control group had a mean age of 38.20 ± 8.95 years ($p = 0.173$). The male-to-female ratio was 2:3 for both the patients and the controls. The mean HbA1c levels were 5.35 ± 0.737 for the patient group and 5.26 ± 0.66 for the control group ($p = 0.520$).

Biochemical analysis showed that serum calcium, urea, and creatinine levels were higher in patients than in

controls. However, statistical analysis showed non-significant differences between the two groups, while VD3 serum levels were lower in patients (20.66±7.07) than in controls (33.90±10.40) and had a significant difference (p= 0.0001) between the two groups. It was noted that VD3 deficiency was more prevalence in patients 56% than in controls 7.5%. All are detailed in (Table 1).

Table (1): General characteristics and Biochemical analysis of the studied groups

Characteristic	Patients (n=50)	Controls (n=40)	*p-value Sig. (p<0.05)*
Age (years)	35.78±11.28	38.20±8.95	*p*=0.173
Age Group			
19-28	15 (30%)	6 (15%)	-
29-38	18 (36%)	14 (35%)	-
39-48	10 (20%)	15 (37.5%)	-
49-63	7 (14%)	5 (12.5%)	-
Sex (M/F)	2:3	2:3	-
Male	20 (40%)	24 (60%)	-
Female	30 (60%)	16 (40%)	-
Stone Type CaOX	50(100%)	-	-
HbA1c	5.35 ± 0.737	5.26 ± 0.66	*p* =0.520
Calcium	9.44 ± 0.530	9.33 ± 0.264	*p*=0.202
Urea	37.96 (30.47-49.70)	42.25 (40.15-44.35)	*p*=0.366
Creatinine	0.99(0.82-1.32)	0.94(0.91-0.98)	*p*=0.348
VD3 Levels	20.66±7.07	33.90±10.40	*P* < 0.0001
Deficient VD_3	28(56%)	3(7.5%)	*p* < 0.0001
Insufficient VD_3	16(32%)	15(37.5%)	*p* = 0.5874
Sufficient VD_3	6(12%)	22(55%)	*p* < 0.0001

VDR gene expression results

The absolute number of *VDR* expression copies ranged from 4.900 to 134.8 copies/µL in (CaOX) kidney stone patients, compared to healthy controls, which ranged from 3.500 to 23.70 copies/µL. The median absolute expression of *VDR* in patients was 26.20[11.98–53.03] and 9.400[7.625–11.68] in controls (Figure 2). A Mann-Whitney U test confirmed that *VDR* expression was significantly higher in patients than in controls (p <0.0001) revealing that *VDR* expression was upregulated in the patients compared to the controls.

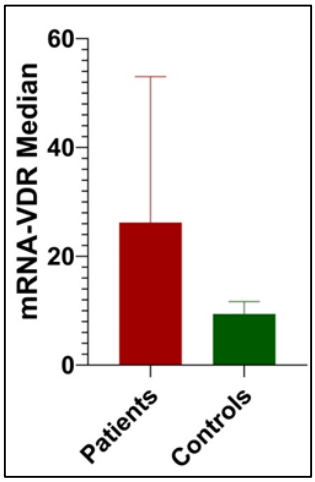

Figure (1): Median of absolute *VDR* mRNA expression

ROC Curve analysis of *VDR* expression and VD3 levels

A Receiver Operating Characteristic (ROC) curve was used to evaluate the diagnostic potential of *VDR* expression and VD3 Levels. The *VDR* expression curve showed an AUC of 0.858 (P<0.0001), and the VD3 levels curve showed an AUC of 0.857 (P<0.0001), indicating excellent discrimination in both variables between calcium oxalate kidney stone patients and healthy controls (Figure 3).

These findings suggest that *VDR* expression and VD3 Levels could be a potential biomarker for distinguishing between patient and control groups.

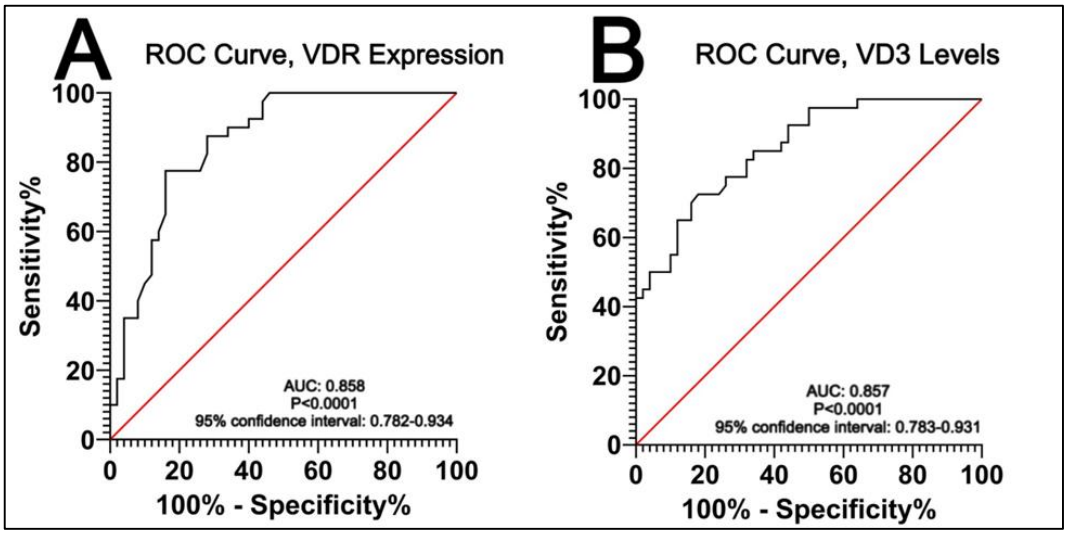

Figure (2): Receiver Operating Characteristic (ROC) curve between the patient group and control group A. For *VDR* expression B. For VD3 levels

VDR and VD play a pivotal role in calcium and oxalate metabolism, and regulating calcium deposition in renal tissues through modulation of renal calcification inhibitory factors activity (28). When imbalanced, it may result in the abnormal deposition of calcium salts in the kidneys, which can induce stone formation. Vitamin D affects the renal acid-base regulatory mechanism, modulating the solubility of calcium and oxalate by altering the pH of the urine. Changes in urinary pH directly impact stone formation (29).

The current study results showed upregulation in *VDR* expression and significant difference between CaOx kidney stone patients and controls groups (p <0.0001) which consistent with previous studies on rats models of kidney stone that showed *VDR* upregulated expression (27,30), the VD3 levels reduced in patients compered to controls (P < 0.0001), (56%) of the patients group were diagnosed with VD3 deficiency aligning with other studies (31-33) ,suggesting a potential link with Kidney Stone formation.

However, the present study results showed a slight non-significant difference in calcium serum levels between patients and controls groups, as the mean calcium level for both groups was found to be within the normal range, consistent with a previous study (34,35). Although the balance of calcium blood levels appears to be stable and unaffected by changes in other factors' values, maintaining this balance is the result of multiple regulatory factors and mechanisms. These include VD, *VDR*, PTH function, renal calcium reabsorption, and urine excretion. These mechanisms strive to maintain this balance, as calcium is one

of the most important minerals in the body; it has an essential role in regulating muscles and heart functioning, transmitting nervous system messages, and playing many other vital roles. Kidney stone formation may be a consequence of maintaining calcium balance when regulatory factors are disrupted for various reasons.

ROC curve analysis revealed an AUC of 0.858 for *VDR* expression and 0.857 for VD3 levels, indicating an excellent discrimination power of *VDR* expression and VD3 levels as a potential biomarker, and a good target for treatment developing strategies aiming to maintain healthy calcium metabolism and reduce kidney stone formation risk.

Conclusion

Upregulation of *VDR* expression and lower levels of VD3 were observed in CaOx kidney stone patients, suggesting an effect on calcium homeostasis and influencing kidney stone pathology. These variables can be used as biomarkers and in developing target therapy strategies.

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Approval

The local ethics council at the University of Baghdad approved this study under the official letter No. 6343 on 29/10/2023.

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