Hormonal and molecular characterization of calcium oxalate stone formers predicting occurrence and recurrence

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Research Article

Keywords:

Posted Date: October 13th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-2147324/v1

Additional Declarations: No competing interests reported.

Version of Record: A version of this preprint was published at Urolithiasis on April 24th, 2023. See the published version at https://doi.org/10.1007/s00240-023-01440-8.

Abstract

Abstract Purpose: To investigate the role of sex hormones, androgen receptors (ARs) and mi-RNA/CSF-1 in occurrence and recurrence of calcium oxalate (CaOx) renal urolithiasis. Materials and Methods: In this prospective study, 74 patients with CaOx stones; stone formers group (SFG) and 40 healthy subjects; control group were compared. SFG includes both denovo and recurrent cases. Steroid sex hormone plasma assay including testosterone, free testosterone, dihydrotestosterone, estradiol, and sex hormone binding globulin were analyzed. ARs, mi-RNA 185-5p and CSF-1 expression were compared between groups. Results: SFG showed significant higher ARs and mi-RNA 185-5p expression (3.7 ± 1.3, 1.8 ± 0.4, respectively) than control group (1 ± 0.08 and 1± 0.07, respectively) ($p < 0.05$). However, CSF-1 expression was significantly lower in stone formers than control group. (0.4 ± 0.19 vs 1 ± 0.1, respectively) ($p < 0.05$). No differences were detected between denovo and recurrent SFG regarding sex hormones, AR, mi-RNA or CSF-1 expression. Conclusion: Our data suggest the important role of AR. mi-RNA and CSF-1 signaling in human nephrolithiasis pathogenesis.

Introduction

Urolithiasis represents one of the most common urologic health care problems worldwide. Estimates of calcium oxalate (CaOx) / calcium phosphate (CaPh) stones occurrence are as high as 80% of all renal stones. The underlying pathophysiology of the calcium based renal stones is complex and multifactorial with most of these stones occur in healthy individuals because of metabolic derangements which involve an imbalance between stone formers and stone inhibitors. The most common of these derangements are idiopathic hypercalciuria, hyperoxaluria and hypocitraturia.[1].

CaOx stones formation can be affected by infiltrating macrophages which include pro- inflammatory macrophages or M1 promoting crystals deposition and anti-inflammatory or M2 macrophages phagocytosing ca-oxalate stone formation directly [2].

Regarding the age and sex prevalence of renal stones; it is more common in males compared to females by about 3:1. This prevalence gap is mainly present in reproductive age between 15–49 years and lowest in postmenopausal females with low estrogen which propose that estrogen can serve as a protective hormone against urolithiasis [3] [4].

Also, androgen deprivation therapy (ADT) use in patients with Prostate cancer results in approximately one-third lower risk of subsequent renal calculi [5] which may suggest a promoting effect of male androgens on renal stones formation. This effect has been established experimentally in rats in some trials [6].

Testosterone appears to promote stone formation by suppressing osteopontin expression in the kidneys and increasing urinary oxalate excretion, this action has been found that is reversed with estrogen [7]. Another possible mechanism for promoting role of testosterone on calcium oxalate stone formation may

be due to increase urinary uric acid excretion [3] and increase hepatic synthesis of glycolic acid oxidase (GAO), an enzyme essentially involved in primary hyperoxaluria [8]

This action of sex hormones could be done either through alternation of serum hormone levels or expression of their receptors. It was found that androgen receptors (AR) stimulation increases miRNA-185-5p which inhibit macrophages colony stimulating factor 1 (CSF-1) result in inhibition of M2 mediated CaOx crystals phagocytosis [9–11]. However, this action was demonstrated only using human cell lines via invitro studies and rat models. Furthermore, higher testosterone levels were reported in renal stones patients. Considering the role of estrogen, data is sparse due to lack of assessment of the role of estrogen or absence of depictable inhibitory role of estrogen on stones formation. [9–11]. Also, the relation between sex hormones and calcium oxalates stones recurrence was not assessed before in any trial.

Until now, there is a shortage of data regarding the association between sex hormones and the prevalence of urolithiasis and the utility of this association in clinical practice. This study was conducted looking for an association between sex hormones and calcium oxalate stone formation. Moreover, the relation between sex hormones and recurrence of calcium oxalate stones was assessed.

Patients And Methods

- Study design and groups

This is a prospective controlled study that was performed between June 2020 and June 2021 after approval of the local ethical committee.

Patients who were diagnosed with calcium oxalate (Ca.ox) stones on further stone analysis either as pure Ca Ox stones or mixed stones were allocated as stone formers group **(SFG)** and compared against healthy subjects who were selected from same age range with no history or radiological evidence of urinary stones and considered as **"control"** group.

Stone formers group was subdivided into: **"denovo"** group included patients who presented with renal stones for the first time, and they were compared against **"recurrent"** group who presented with previous episodes of renal stones that necessitated active intervention.

- Inclusion and exclusion criteria

Inclusion criteria included male patients between 18–60 years who were diagnosed with renal stones and were admitted to our center for active stone intervention. Eligible subjects were asked to sign an informed consent.

Exclusion criteria included patients with known hyperthyroidism, hyperparathyroidism, recurrent urinary tract infections, renal and liver diseases, intestinal malabsorption, or metabolic syndromes were excluded from the study. Also, patients with known structural or functional abnormality of the urinary tract like

vesico-ureteric reflux, neuropathic bladder or pelvi-uretral junction obstruction were also excluded from the study.

- Intervention

All patients who were diagnosed with renal stones were managed with standard percutaneous nephrolithotomy (PNL) or retrograde intrarenal surgery (RIRS) depending on stone volume, location, and surgeons' preference. Retrieved stones were submitted for stone analysis and calcium oxalate (Ca.ox) stone formers were included.

- Study work-up

All clinical data including history, clinical examination and routine laboratory investigation were collected. The sonographic and computerized tomographic evaluations of the kidneys and urinary tract systems and the diagnosis of renal stone were performed by the attending expert radiologists.

Sample collection for laboratory and molecular analysis was done as following:

24-hour urine samples collection for determination the levels of calcium, uric acid, citrate and oxalate.

Stone samples for analysis using FT-IR (Fourier Transform- Infrared) spectroscopy looking for type of stone.

Blood samples were taken for evaluation the level of sex hormones and gene expression.

A. Hormone analysis

For hormone analysis, all samples of blood were collected, and each sample was centrifuged at 3000 g for 15 min and the separated plasma then fractionated and stored at -20C until hormone assay. Hormones in the plasma samples including testosterone (T), free testosterone (FT), dihydrotestosterone (DHT), estradiol (E2), and sex hormone binding globulin (SHBG) were analyzed by ELISA.

B. Gene expression of AR and CSF- 1 using Real-time PCR

AR mRNA level were assessed by quantitative RT-qPCR method. Total RNA (0.5 μg), was isolated from the blood samples, reverse transcribed. Gene expression for Primers for the AR and CSF-1 and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were examined by real-time qPCR, using AR primers (Applied Biosystems, Foster City, CA, USA). Gene expressions all data were normalized to GAPDH; expression of one tumor was assumed to be 1 and used as reference. The 2 − ΔΔCT method was used to calculate relative amounts of target genes.

C. Gene expression of miRNA-185-5p

miRNA technology ingeniously integrates a universal tailing and reverse transcription reaction specific for mature miRNA combined with state-of-the-art primer-assay design technology to enable the accurate

expression level measurement of miR-2909.

miRNA was extracted from plasma samples using an miRneasy Mini Kit. cDNA was synthesized from 1 µg of total miRNA. Amplification and detection was performed using real time PCR (step one plus).

AR, CSF- 1 and miRNA-185-5p

- Study outcomes

The primary outcome was to assess the association between sex hormones and occurrence of calcium oxalate renal stones.

While the secondary outcome was to assess the association between these hormones and recurrence of calcium oxalate stones over time.

• Statistical Analysis

Continuous variables were presented as mean ± SD for normally distributed and as median and range for non-normally distributed variables. Ordinal and nominal variables were presented as frequency and percentage. Comparison between study and control groups was performed by Student's t-test for normally distributed continuous variables and by Mann-Whitney test for asymmetric continuous variables. Chi square and Fisher exact tests was used for comparison between categorical variables. Multivariable analysis by logistic regression was done as appropriate. A P value < 0.05 was considered significant. A software SPSS was used for storage and analysis of data.

Results

Demographic and Perioperative Data

Out of 91 male patient who were diagnosed with renal stones and underwent endoscopic treatment with PNL or RIRS between June 2020 and June 2021, 74 patients were diagnosed as Ca Ox stones and included in the study. Then, they were compared against 40 healthy subjects.

After that, the 2 subgroups of SFG were compared against each other: 40 patients of denovo group and 34 patients of recurrent group as shown in study`s flow chart (Fig. 1).

The mean ± SD age and BMI in SFG and control groups were (52 ± 14 and 54 ± 10, P 0.4) and (30.6 ± 7.3 and 29 ± 5.3, P 0.09) respectively. Also, patients demographics and stone characteristics of denovo and recurrent stone formers group were comparable with no significant difference as shown in Table 1.

The primary outcome

As shown in table 2, The mean ± SD levels of RQ-AR, mi-RNA-185 and serum estradiol were significantly higher in SFG 3.7 ± 1.3, 1.8 ± 0.4 and 69.5 (20–300), respectively compared to control group 1 ± 0.08, 1 ± 0.07 and 34 (20–55), respectively with $p < 0.001$.

However, RQ-csf-1 was significantly lower in SFG than control group (0.4 ± 0.19 vs 1 ± 0.1, respectively) with $p < 0.001$. While serum androgens and SHBG levels did not show significant difference between 2 groups.

The secondary outcome

Table 2 summarizes data of SFG with comparative analysis of its 2 subgroups. Both denovo and recurrent stone formers group were comparable regarding serum androgens, estradiol levels and molecular gene expression of RQ-AR, RQ-csf-1 and Mirna 185 with $p > 0.05$.

Table 3 summarizes 24-hours urinary metabolites among the 2 subgroups of stone formers with only urinary citrate level that showed significant higher median concentration in denovo stone formers (< 0.001).

Discussion

Kidney stones prevalence had markedly increased over the past two decades. According to the latest report from the National Health and Nutrition Examination Survey (NHANES 2007–2010), the prevalence of kidney stones among American adults was 8.8%: 10.6% among men and 7.1% among women [10].

Renal stones has major economic and medical burdens due to cost of treatment, sick leaves from work, risk of renal impairment and renovascular hypertension [12]. Another major problem of stone diseases is the risk of recurrence, 50% of patient with kidney stones have chance of development another episode of stone disease over 7 years [13].

CaOx stones are formed through a complex and multifactorial process including underlying genetic and metabolic abnormalities, life style pattern, obesity and hot climatic environment [1]. Recently, a strong association was found between sex hormones and kidney stones. Testosterone and dihydrotestosterone hormones were found to be a potential promoting factor in occurrence of CaOx urolithiasis, while estrogen hormone was found to be a protective against urolithiasis [11] [3].

This role was supported by age and sex prevalence of urolithiasis. It was found that kidney stones are more common 3 times in males than females and this gap is significantly decreased in postmenopausal females [14]. Also, hyperoxaluria was found to be significantly lower in castrated male rates compared to normal ones [15]. Polycystic ovary syndrome which characterized by clinical and laboratory evidence of hyperandrogenism is a known risk factor in kidney stones formation [16]. In some animal studies, administration of testosterone increases urinary oxalate excretion and enhances the formation of calcium oxalate stones [17].

This promoting effect of testosterone in urolithiasis pathogenesis is mediated through different mechanisms including increase hepatic synthesis of glycolic acid oxidase (GAO) which results in hyperoxaluria [8]. Also, testosterone was found that it inhibits renal osteopontin expression and subsequent increase renal excretion of oxalate [7]. In serum, 95% of testosterone binds sex hormone

binding globulin and only 1–2% is free testosterone [18]. Free testosterone diffuses into target cells, where it binds to AR, which has the pivotal role in androgen signaling.

ARs were found to be expressed in nuclei of normal distal renal tubular epithelial cells. Moreover, they were found to be up regulated in tubular epithelial cells of patients with hyperoxaluria which suggests the important role of androgen/AR axis enhancement in kidney stones formation [6].

The molecular mechanisms of action of ARs in pathogenesis of hyperoxaluria and CaOx stones formation were demonstrated in 2 animal trials on mice [9, 19].

First, on physiological basis, there are Types of intrarenal macrophages: M1 macrophages which induce kidney epithelial injury and inflammation and not phagocytose CaOx crystals and M 2 macrophages which promote phagocytosis of CaOx crystals.

In this trial, they found that CSF- 1 is mainly secreted from renal tubular epithelium and play an important role in M2 macrophages proliferation and in extension suppression of hyperoxaluria.

However, they found that AR increase miRNA expression which inhibit CSF-1 resulting in M2 macrophages inhibition and promoting hyperoxaluria.

In the second trial [19], they found that AR could directly up-regulate hepatic glycolate oxidase and kidney epithelial NADPH oxidase subunit p22-PHOX at the transcriptional level. This up-regulation might then increase oxalate biosynthesis and oxidative stress that resulted in induction of kidney tubular injury. Targeting AR with the AR degradation enhancer dimethyl curcumin (ASC-J9) led to suppression of CaOx crystal formation. However, these results were demonstrated only in animal studies.

Only one trial assessed the levels of ARs in human patients with CaOx stones [6]. This study included 68 participants, 37 male patients with CaOx/CaPh stones and 31 healthy controls. ARs were detected by immunohistochemistry in nuclei of normal distal renal tubular epithelial cells. Moreover, they were found to be up regulated in tubular epithelial cells of patients with hyperoxaluria which suggests the important role of androgen/AR axis enhancement in kidney stones formation. However, this study has some limitations of being small sample size and the detailed mechanism of ARs could not yet be fully elucidated.

From clinical point of view, knowing that the risk of recurrence of stone formation is about 50% [13], there was a need for a trial looking after molecular and genetic differences and if they have a role in stone recurrence. This might have clinical impact on attempts to reduce stone recurrence.

To the best of our knowledge, this prospective trial is the first one to compare the difference in sex hormones between denovo and recurrent stone formers and, the first one to study the relation between AR and genetic expression of miRNA and CSF-1 in stone former patients. Moreover, it includes a sample size larger than any other previous study.

In the current study, AR and miRNA were significantly higher and CSF-1 was significantly lower in SFG compared to control group. These results go in parallel with the previous result of [9] animal studies. However, no difference between both groups was observed regarding blood androgen level. Interestingly plasma estradiol level was higher among SFG in the current study, yet this could be explained by the higher rate of conversion of testosterone to estradiol in the testosterone metabolic pathway. However, this higher level did not seem strong enough to exert stone inhibiting effect in males. [10]

Nevertheless, denovo and recurrent stone formers were not different regarding all studied parameters. Further studies are warranted to look for the clinical utility of those findings.

In conclusion, CaOx renal stones was found to positively correlated with genetic expression of AR and miRNA and it inversely related with CSF-1. However, renal CaOx stones` recurrence was not associated with changes in sex hormone levels nor its related molecular pathway.

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Tables

Table 1: Baseline demographic characteristics of denovo and recurrent stone formers groups.

Variable:		Denovo group *N:40*	Recurrent group *N:34*	P value
Patients' characteristics				
Age (years)	*Mean± SD*	51 ± 10	53 ± 8	0.3*
BMI (kg/m2)	*Mean± SD*	30.1 ± 5.3	28 ± 8.3	0.07*
DM:	*NO (%)*	4 (10%)	7 (20.6)	0.3#
Urinary tract characteristics				
Urine PH	*Mean* ±SD	5.7 ± 0.7	5.4 ± 0.7	0.02*
S Cr	*Median (range)*	0.9 (0.6-4)	1 (0.4-3)	0.1**
Stone characteristics				
Stone density	*Mean* ±SD	1015 ± 306	1021 ± 266	0.9*
Stone diameter	*Median (range)*	13 (4-30)	14 (1-40)	0.7**
Stone length	*Median (range)*	16 (2-40)	15 (1.6-42)	0.7**
Stone side	*NO (%)*			
RT		15 (37.5)	11 (32.4)	
LT		21 (52.5)	17 (50)	0.6#
Bilateral		4 (10)	6 (17.6)	
*Independent sample t test, ** Man-Whitney U test, # Chi square test				
S Cr : serum creatinine, **BMI**: body mass index				

Table (2): Comparison of hormonal and molecular characteristics of SFG (with its 2 subgroups) and control group

Variable	Stone formers group *N: 74*			Control group *N:40*	P1	P2
	Denovo *N:40*	Recurrent *N:34*	Total *N:74*			
Testosterone (2.62-16 ng/ml). *Median (range)*	7 (1.5 -17.5)	4.5 (1-20)	5.3 (13.3-20)	7 (1.4-16)	0.8**	0.6**
Free Testosterone (10-40pg/ml) *Mean* ± SD	24 ± 11	23.5 ± 11	24 ± 11	24.7 ± 7	0.7*	0.8*
DHT (52-90 ng/dl): *Mean* ± SD	40 ± 15	41 ± 15	40.6 ± 15	37.5 ± 10	0.2*	0.7*
SHBG (10-71 nmol/l) *Mean* ± SD	37 ± 9	39 ± 9	37.8 ± 9	37.7 ± 9	0.9*	0.5*
Estradiol (up to 56 pg/ml) *Median (range)*	65 (20-226)	73 (20-300)	69.5 (20-300)	34 (20-55)	<0.001**	0.8**
RQ-AR *Mean* ±SD	3.8 ± 1.3	3.5 ± 1.4	3.7 ± 1.3	1± 8	<0.001*	0.5*
RQ-csf-1 *Mean* ±SD	0.5 ± 0.19	0.4 ± 0.19	0.4 ± 0.19	1 ± 0.1	<0.001*	0.6*
Mirna 185 *Mean* ±SD	1.8 ± 0.3	1.9 ± 0.4	1.8 ± 0.4	1± 0.07	<0.001*	0.4*

*Independent sample t test, ** Man-Whitney U test, **SHBG;** sex hormone binding globulin**,** **DHT;** dihydrotestosterone**.**

P1: Total stone formers group Vs control group

P2: Denovo Vs control group

Table 3: comparison of metabolic work up between denovo and recurrent stone formers group:

Variable:	Denovo group *N: 40*	Recurrent group *N: 34*	P value
24 hrs urinary Ca *Median (range)*	327 (125-3511)	410 (123-710)	0.3**
24 hrs urinary uric acid *Median (range)*	240 (122-921)	254 (201-698)	0.2**
24 hrs urinary Oxalate *Median (range)*	55 (22-80)	47 (41-84)	0.2**
24 hrs urinary Citrate *Median (range)*	354 (30-651)	526 (236-652)	<0.001**
Hypercalciuria *NO (%)*	37 (92.5)	32 (94.1)	0.7#
Hyperuricosuria *NO (%)*	2 (5)	0	0.2#
Hyperoxaluria *NO (%)*	31 (77.5)	22 (64.7)	0.2#
Hypocitraturia *NO (%)*	14 (35)	5 (15.2)	0.05#
** Man-Whitney U test, # Chi square test **Reference range**; hypercalciuria; >200 mg/day, hyperuricosuria; >750 mg/day, hyperoxaluria; >45 mg/day and hypocitraturia; <320 mg/day			

Figures

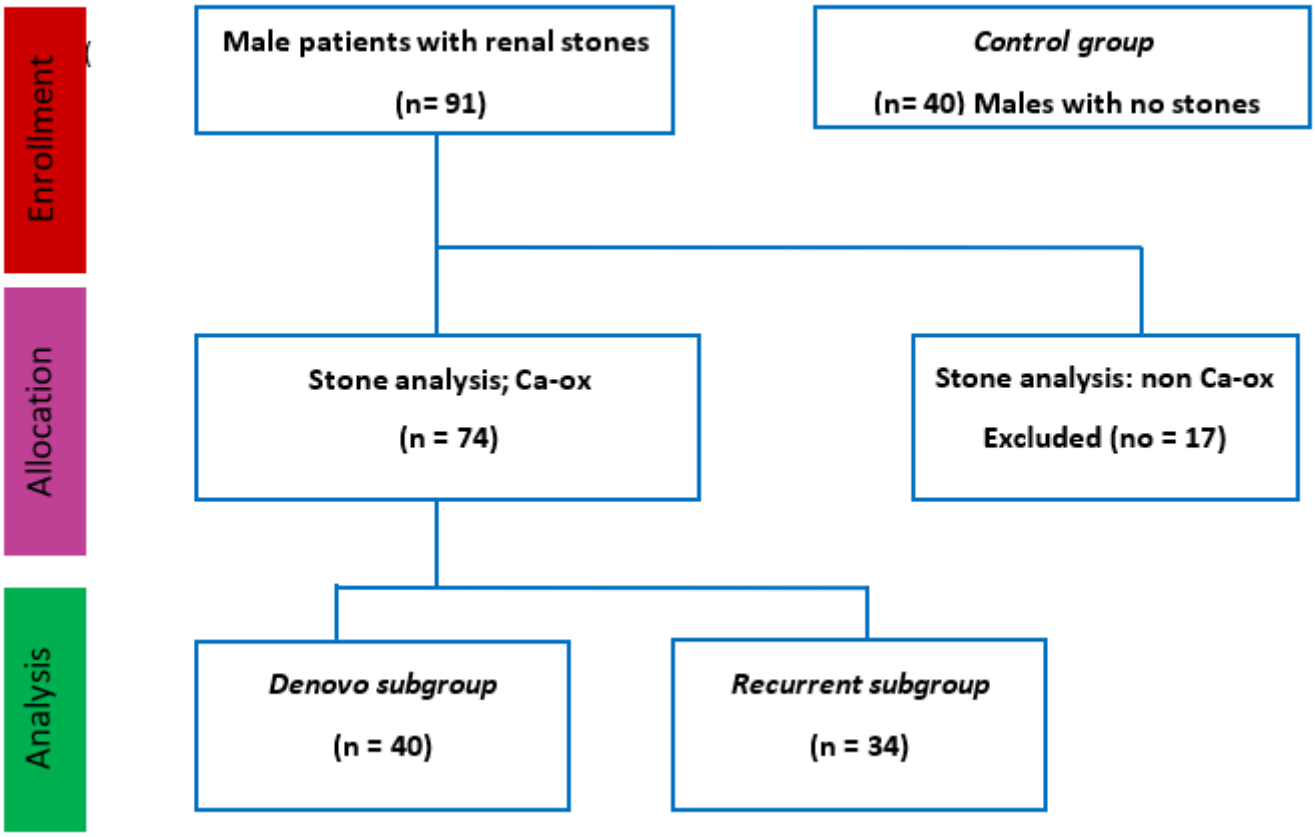

Figure 1

Legend not included with this version.