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ORIGINAL PAPER

The relationship between calcium oxalate lithiasis and chronic proinflammatory intestinal dysbiosis pattern: a prospective study

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Abstract
The objective is to establish whether a pattern of intestinal dysbiosis exists in calcium oxalate (CaOx) lithiasis and, if so, to identify its characteristics and explore whether there are differences in the pattern between CaOx dihydrate (COD) and monohydrate (COM) lithiasis. With this aim 24 patients diagnosed with CaOx lithiasis by means of optical microscopy and spectrometry were prospectively recruited. Faecal analysis was carried out by means of RT-PCR 16S rRNA assay and agar plate culture according to the methodology proposed by the *Institute of Microecology* (*Herborn*, *Germany*). The total number of bacteria was depleted due to COD lithiasis ($p=0.036$). The mean values of immunoregulating microbiota were normal, but the percentage of normal values was lower in the COD group (30%) than in the COM group (69.2%) ($p=0.062$). The total mean values of protective microbiota were normal in both groups. There was a large decrease in the mean values of the muconutritive microbiota *Akkermansia muciniphila* and *Faecalibacterium prausnitzii*, the most intense decline being observed in the COD group ($p=0.019$). Levels of proteolytic microbiota were elevated in both groups, without differences between them. We conclude that patients with CaOx lithiasis have a chronic pro-inflammatory intestinal dysbiosis pattern characterised by a reduction in the total number of bacteria, a reduction in immunoregulating microbiota and a large reduction in muconutritive microbiota that is significantly more intense in COD lithiasis than in COM lithiasis.

Keywords Calcium oxalate lithiasis · Calcium oxalate monohydrate lithiasis · Calcium oxalate dihydrate lithiasis · Microbiota · Intestinal dysbiosis

Introduction

Urinary lithiasis has a prevalence of 5–10% and affects many millions of people throughout the world [1]. The most common type of urinary lithiasis is calcium oxalate (CaOx), which can be monohydrate (COM), more closely linked to problems of hyperoxaluria, or dihydrate (COD), more closely linked to problems of hypercalciuria [2].

The first studies on the relationship between urinary lithiasis and intestinal microbiota were carried out in relation to COM lithiasis caused by primary hyperoxaluria [3–9]. Although primary hyperoxaluria is a very serious disease, it accounts for only a small percentage of the cases of COM lithiasis. Various studies demonstrated the absence of *Oxalobacter formigenes*, a gram-negative, obligate anaerobic bacteria with a complete metabolic dependence on oxalate [3–7], in the intestinal microbiota of patients affected by hyperoxaluria. Subsequently, further studies showed a decrease in *O. formigenes* not only in the infrequent cases of primary hyperoxaluria but also in other cases of calcium oxalate lithiasis [8, 9]. Some studies indicated that *O. formigenes* could have a protective role in calcium oxalate stone disease [10–12]. On the other hand, low levels of *O. formigenes* have been found in diseases and contexts traditionally related to CaOx lithiasis, such as Crohn's disease, inflammatory bowel disease and jejunoileal bypass surgery [12–14]. In addition, it has been demonstrated that bacteria other than *O. formigenes* have the ability to catabolise the oxalate as some *Lactobacillus* sp. (*L. paracaseii, L. gasseri, L. acidophilus*), *Bifidobacterium* sp. (*B. animalis*) and *Bacillus* sp. [4, 15–20].

Another interesting study [21] found that while, compared with a control group, people who suffer from urinary lithiasis

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have significantly higher levels of *Bacteroides* and lower levels of *Prevotella*, there is an inversely proportional correlation between *Eubacterium* levels and oxalate levels and also between levels of *Escherichia* and citrate, which is an inhibitor of stone formation.

All this has led to the suggestion that abuse of antibiotic treatment, which alters intestinal and urinary microbiota, is one of the factors promoting urinary lithiasis [9, 11, 22–24] and that re-establishment of intestinal microbiota with probiotics could represent a preventative treatment [25–31]. Nevertheless, these studies' findings were contradictory. There have even been investigations into possible treatments with oxalate-degrading micro-organisms or enzymes [32].

Nevertheless, most of these studies have focussed on the analysis of a limited number of intestinal bacteria: *O. formigenes*, other oxalate degraders or bacteria classified as protective microbiota. Only a few studies have performed a global analysis of all intestinal microbiota. Among these, one study carried out in dogs found different microbiota in healthy animals and those with CaOx lithiasis formation [33]. Two other studies found significant depletion of *Faecalibacterium, Enterobacter* and *Dorea* [34] and a characteristic microbiome pattern in lithiasis-forming patients [35]. A further study analysed the microbiome of calcium lithiasis and compared it with that of the surrounding urine [36]. Finally, an interesting recent study evidences the relation between urinary lithiasis and the changes caused in the microbiome by the use of antibiotics, specifically a decrease in *Lactobacillus* and subsequent recolonization by *Enterobacteriaceae*, which are, respectively, protective against and promoters of the formation of urinary lithiasis [37].

A technique for analysing intestinal microbiota exists, namely the Institute of Microecology's (*Herborn, Germany*) KyberkompaktPRO, which offers information not only on protective microbiota but also on proteolysis, fungi, immunoregulators and muconutritives. This method of analysis, performed on stool samples, is used in many countries to study microbiota disorders related to digestive and non-digestive pathologies [38–41]; to date, however, no studies have used the method to investigate the relation of such disorders to urological conditions. With this in mind, in the present study this methodology was employed in a series of patients with CaOx lithiasis (COD/COM) in order to establish whether a pattern of intestinal dysbiosis exists in this condition, and, if so, to identify its characteristics and explore whether there are differences in the pattern between COD and COM lithiasis.

Patients and methods

The study population comprised 24 patients who met the following criteria: adult age, recent expulsion of a lithiasis in the last 3 months and an analysable sample of pure CaOx stone. Exclusion criteria were: urinary infections, neoplasia, active inflammatory bowel disease, use of antibiotics in the last month and a history of bowel resection or intestinal bypass surgery.

The analysis of kidney stones was carried out using optical microscopy and spectronomy, as explained previously [42]. Stones were classified as belonging to either the COD or the COM group when at least 60% of the CaOx was in one of these forms.

Faecal analysis was carried out by means of RT-PCR 16S rRNA assay and agar plaque culture, according to the technique proposed by the *Institute of Microecology* [38, 39, 41]. The patient collected a single sample in a standardised container for biological sample collection. The container was transported inside a hermetic bag approved for the transport of biological material, which was part of the kit provided to the patient by the laboratory. The sample was sent by courier and arrived at the laboratory within 24 h; the laboratory started to process the sample on the same morning. The sample was divided into parts that were prepared in accordance with the parameters requested; cultures were started immediately, as was automated extraction of bacterial DNA. The latter operation was performed by means of QUIcube (Qiagen) and the QIAamp DNA stool mini kit (also from Qiagen), with a specific program for extraction of DNA from pathogens. This extracted DNA was stored in a refrigerator, where it is kept until the start of the set of RT-PCRs for each bacterium.

For culturing, 10 µl was collected with an inoculation loop and transferred to 2250 µl of NaCl; a serial dilution was then made and 50 µl was inoculated in Endo (in order to determine *E. coli* and *E. coli biovare*), Orientation (enterococci, KESC group bacteria and *Pseudomonas*), Rogosa (lactobacilli and H_2O_2) and SPM (*Clostridia*). A further 10 µl was taken with an inoculating loop and transferred from the original sample to 2500 µl of trypsin. The latter was incubated for 15 min at room temperature under stirring. Four hundred microliters was passed into 1600 µl of PBS. One hundred microliters was inoculated in Sabouraud containing gentamicin and chloramphenicol to determine and quantify fungi and yeasts. The number of colonies was determined de visu. Each colony was equivalent to 2×10^4 CFU/g (colony forming units per gram) due to the correction factors that must be applied for Endo, Orientation, SPM and Rogosa. In the case of Sabouraud a correction factor was applied such that each colony equalled 1×10^3 CFU/g. Rogosa, which contains tetramycin, is used for the growth of *Lactobacillus* spp. and peroxides.

The procedure for strict anaerobes was to homogenise the sample, collect a small amount of faeces (approx. 0.25 g) and dilute in 2.25 ml of NaCl; stirring was performed to achieve a homogeneous suspension. Subsequently, a serial dilution was performed with 100 µl of the homogeneous

suspension and 900 µl of NaCl until a 10^3 dilution was achieved. Fifty microliters of the 10^3 dilution was inoculated in SPM medium and allowed to incubate in a 37 °C oven for 48 h within an incubator with envelopes for anaerobes.

The fact that multiple relationships and interactions are established among bacteria makes interpretation of the results very difficult. With this in mind, bacteria were categorised by functional groups to facilitate clinical interpretation of the results.

Statistical analysis was performed using the chi-square test for categorical variables and Student's *t* test for continuous variables. Subdivision into two groups was performed, according to whether the major component was COM or COD. Differences between the groups were analysed. Statistical significance was set at $\alpha = 0.05$, and analysis were performed using SPSS version 17.0.

Results

The analysis was not carried out in one of the 24 participants in the study, so the adherence rate was 96%. The average age of the participants was 54 years; 78% were men and 22% women. Regarding the composition of the stones, 13 (56.5%) were COM and 10 (43.5%) were COD. Of the 13 COM stones, 12 were pure while in one the composition was 60% COM. Of the ten COD stones, five were pure, while in three, one and one the composition was 60%, 70% and 80% COD, respectively. The average pH of the faeces was 6.21 (95% CI 5.97–6.46). Concerning the consistency of the faeces, 47.5% were slightly pasty, 42% pasty and 10.5% hard.

Overall the mean total number of bacteria was 0.79×10^{11}, with depletion in patients with COD stones (0.49×10^{11}) but normal levels in those with COM stones (1.015×10^{11}). This difference was statistically significant ($p = 0.036$).

The overall mean values of immunoregulating microbiota, as well as the two main bacteria which compose it, i.e. *E. coli* and *Enterococcus,* were normal, without statistically significant differences between the COM and COD groups (Table 1). Nevertheless, when we analysed the percentage of patients who had normal levels (Table 2), we found that only approximately half of all patients (52.2%) had normal *E. coli* levels, with an almost significant difference ($p = 0.062$) between those with COD stones (30%) and those with COM stones (69.2%).

With regard to protective microbiota, both the total mean levels and the levels of the constituent species, i.e., *Bifidobacterium, Bacteroides, Lactobacillus* and H_2O_2-producing *Lactobacillus*, were normal in the entire series as well as in the COM and COD groups (Table 3). However, compared with the COM group, a lower percentage of patients in the COD group were found to have normal levels of total microbiota, *Bifidobacterium* and *Bacteroides*, whereas a higher percentage had normal *Lactobacillus and* H_2O_2-producing *Lactobacillus* levels (Table 4).

Table 1 Mean levels of immunoregulating microbiota in the overall patient population and the COM and COD groups (SD = standard deviation/*R* = range)

Total	COM	COD	*p*
Escherichia coli			
8.32×10^6	9.07×106	7.35×106	0.819
SD 17.2	SD 18.56	SD 16.2	
R 0.01–70.0	*R* 0.01–70	*R* 0.04–50.0	
Enterococcus			
3.11×10^6	4.43×106	1.39×106	0.295
SD 7.44	SD 9.49	SD 3.08	
R 0.01–30.0	*R* 0.01–30	*R* 0.01–10	

Table 2 Percentage of patients with normal levels of immunoregulating microbiota in the overall patient population and the COM and COD groups

	Microbiota total	*E. coli*	*Enterococcus*
COM	7.7	69.2	23.1
COD	10	30	30
Total	8.7	52.2	26.1
p	0.347	0.062	0.708

Analysis of muconutritive microbiota showed a reduction in mean values both overall and with respect to the two bacteria *Faecalibacterium prausnitzii* and *Akkermansia muciniphila* (Table 5). This decline was observed in both the COM and the COD group, the difference between them being most marked for *F. prausnitzii* ($p = 0.019$). The marked depletion in muconutritive microbiota was also seen when the percentages of patients with normal levels were analysed (Table 6), the reduction being even more dramatic in the COD group, where not even one instance of a normal level was found.

Mean overall levels of proteolytic microbiota species such as *E. coli biovare, Pseudomona* and *Clostridium* were higher in the COM group (Table 7). Nevertheless, analysis of the percentage of normal levels in each group revealed no statistically significant differences (Table 8).

Finally, the mean yeast level was significantly higher in patients with COD stones (4.5×10^3) than in those with COM stones (1×10^3) ($p = 0.034$).

Table 3 Mean levels of protective microbiota in the overall patient population and the COM and COD groups (SD = standard deviation/*R* = range)

Total	COM	COD	*p*
Bifidobacterium			
4.4 × 108	5.3 × 108	3.24 × 108	0.546
SD 7.87	SD 9.15	SD 6.07	
R 0.30–30.0	*R* 29.7	*R* 19.7	
Bacteroides			
2.03 × 109	2.61 × 109	1.27 × 109	0.053
SD 1.66	SD 1.66	SD 1.40	
R 0.03–6.0	*R* 5.0	*R* 3.97	
Lactobacillus			
22.31 × 105	29.6 × 105	12.85 × 105	0.186
SD 31.84	SD 38.07	SD 19.23	
R 0.1–90.0	*R* 89.9	*R* 59.9	
H_2O_2-*Lactobacillus*			
7.62 × 105	11.23 × 105	2.93 × 105	0.242
SD 18.40	SD 24.09	SD 3.28	
R 0.1–70.0	*R* 69.9	*R* 9.9	

Table 5 Mean levels of muconutritive microbiota in the overall patient population and the COM and COD groups (SD = standard deviation/*R* = range)

Total	COM	COD	*p*
Faecalibacterium prausnitzii			
0.35×10^9	0.53×10^9	0.11×10^9	0.019
SD 0.46	SD 0.54	SD 0.11	
R 0.0–2.0	*R* 1.99	*R* 0.30	
Akkermansia muciniphila			
0.103×10^8	0.16×10^8	0.025×10^8	0.443
SD 0.41	SD 0.55	SD 0.063	
R 0.0–2.0	*R* 1.99	*R* 0.19	

Table 6 Percentage of patients with normal levels of muconutritive microbiota in the overall patient population and the COM and COD groups

	Microbiota total	*F. prausnitzii*	*A. muciniphila*
COM	0	23.1	7.7
COD	0	0	0
Total	0	13	4.3
p	0.244	0.103	0.37

Discussion

The objective of this study was to establish whether an intestinal dysbiosis pattern exists in patients with CaOx lithiasis, and to identify any differences between patients with COD lithiasis and those with COM lithiasis.

In an intestinal microbiota study, the pH of the faeces is the first parameter to be analysed. Reduction in pH can suggest, among other things, a metabolic block in carbohydrate absorption or the presence of ammonium ions in the form of ammonium salts, which are acids that the intestinal epithelium has difficulty absorbing and are therefore excreted in the faeces, acidifying them. On the other hand, elevated faecal pH suggests an intestinal dysbiosis due to excess proteolytic microbiota, which causes an elevated production of ammonia that in turn leads to inflammation, an over-burdened liver and possible changes in pH [53–56]. The study patients showed a faecal pH range (95% CI 5.97–6.46) similar to that observed in the normal population (5.8–6.4).

When we next analysed the total number of bacteria in the faeces, we observed a depletion due to COD ($p = 0.036$). The reduction in the total bacterial load indicates that a pro-dysbiotic pattern is present in patients with COD lithiasis.

Beyond its role in immunomodulation, the immunomodulating microbiota is relevant in other processes, such as the conversion of vitamin K_1 to K_2. *E. coli* has a first class immunomodulator role. In the COD group, only 30% of patients had normal *E. coli* levels, compared with 69.2% in the COM group ($p = 0.062$ Table 2), which corresponds to the dysbiotic pattern already mentioned with respect to depletion of the total number of bacteria. *Enterococcus* has a relevant role in mucous immunity by promoting the synthesis of IgA secretors. In most studies, the alteration in *Enterococcus* has tended to be more pronounced than that

Table 4 Percentage of patients with normal levels of protective microbiota in the overall patient population and the COM and COD groups

	Microbiota total	*Bifidobacterium*	*Bacteroides*	*Lactobacillus*	H_2O_2-*Lactobacillus*
COM	30.8	53.8	100	61.5	38.5
COD	20	40	40	80	70
Total	26.1	47.8	73.9	69.6	52.2
p	0.021	0.51	0.001	0.34	0.133

Table 7 Mean levels of proteolytic microbiota in the overall patient population and the COM and COD groups (SD = standard deviation/*R* = range)

Total	COM	COD	*p*
E. coli biovare			
85×10^4	60.6×10^4	116.7×10^4	0.502
SD 193.02	SD 164.42	SD 230.31	
R 1.0–600.0	*R* 1.0–600.0	*R* 1.0–600.0	
Pseudomona			
12.6×10^4	5.53×10^4	21.8×10^4	0.377
SD 42.6	SD 16.36	SD 62.67	
R 1.0–200.0	*R* 59.0	*R* 199.0	
Other proteolytic bacteria			
138.05×10^4	170×10^4	83.2×10^4	0.707
SD 465.08	SD 576.33	SD 185.48	
R 1.0–2000.0	*R* 1999.0	*R* 499	
Clostridium			
2.961×10^5	0.623×105	6×105	0.304
SD 10.36	SD 1.41	SD 15.54	
R 0.1–50.0	*R* 4.9	*R* 49.9	

in *E. coli*. In this study, normal levels were found in only 23–30.5% of patients (Table 2). A decline in *Enterococcus* levels implies a dysbiotic pattern, which makes infection more likely.

As regards protective microbiota, *Bifidobacterium* is the major coloniser of the large intestine and is considered a probiotic marker. When its level is altered, butyrate levels diminish, causing an increase in inflammation and affecting the trophism of the epithelium. In this study, the values were normal. *Bacteroides* is the quantitatively most abundant bacteria species, and, as with *E. coli,* its levels are not normally affected. When they are depleted, a chronic dysbiotic pattern is indicated. The promotion of acetate production then leads to a reduction in the energetic yield as well as in the availability of substrates to form other short-chain fatty acids. In this study, the mean *Bacteroides* level was normal (Table 3), but only 40% of COD patients had normal values compared with 100% of COM patients ($p = 0.001$, Table 4). A further group of protective microbiota is the *Lactobacillus* species, including H_2O_2-producing *Lactobacillus* species, whose overall functional efficiency is antibacterial and antifungal. They are found in the small intestine and contribute to digestion and also to the conversion of primary bile acids into secondary bile acids. In our series, mean levels were normal (Table 3), but the percentage of patients with normal levels was higher in the COD group than in the COM group (Table 4).

Muconutritive microbiota is the first to be affected when there is a dysbiotic pattern, as was the case in our series. A large reduction in *F. prausnitzii* and *A. muciniphila* was observed in both groups (Table 5), though it was more intense in the COD group, the difference with respect to *F. prausnitzii* reaching significance ($p = 0.019$). *F. prausnitzii* is quantitatively the second most important species in the intestine and is also the greatest producer of butyrate and the largest consumer of acetate. That is why its depletion causes a shortage of butyrate, which in turn results in an inflammatory pattern. In addition, as butyrate contributes to the throphism of the epithelium, its depletion contributes to functional instability of the epithelium in the gastrointestinal tract and elsewhere, including the urothelium. *A. muciniphila* activates the calciform cells so that they segregate the glycoproteins that form the mucus layer, which performs a protective function with respect to the mucous membrane, contributing to its homeostasis. An epithelium with a poor protective layer will favour a pro-inflammatory dysbiotic pattern due to its depleted barrier. A depletion in the number of *A. muciniphila* has also been found in disorders related to metabolic syndrome. In this study, a large reduction in *A. muciniphila* values was observed, both overall and, especially, in the COD group (Tables 5, 6).

In normal intestinal microbiota, proteolytic bacteria are found. However, when there is a large increase in proteolytic bacteria, proteolytic pathways are activated; this increases the ammonia burden, creating biogenic amines, indoles and cresols, with consequent renal and hepatic overload. Of all the proteolytic bacteria, *Clostridium* is the most harmful because it is the most inflammatory. In the present study, proteolytic microbiota was greatly increased, especially in the COD group; this held true for all bacteria, but the increase in *Clostridium* was only slight (Tables 7, 8).

Finally, a higher level of yeast was found in the COD group compared with the COM group ($p = 0.034$), which coincides with the presence of a more pronounced dysbiotic pattern in that group.

The results of this study support two interesting hypotheses that could constitute the grounds for future lines of

Table 8 Percentage of patients with normal levels of proteolytic microbiota in the overall patient population and the COM and COD groups

	Microbiota total	*E. coli biovare*	*Pseudomona*	Others	*Clostridium*
COM	53.8	61.5	80	66.7	92.3
COD	40	77	92.3	42.9	80
Total	47.8	65.2	87	57.9	87
p	0.642	0.673	0.385	0.311	0.385

investigation: that an intestinal dysbiosis pattern exists in patients with CaOx lithiasis and that the pattern is more evident in patients with COD lithiasis than in those with COM lithiasis. A statistically significant depletion in the total number of bacteria is present in patients with COD stones. Among the different groups of microbiota, those most affected, overall and specifically in the COD group, are the muconutritive and the proteolytic, with reduced levels of the former and increased levels of the latter. Among all the previously explained consequences, we would highlight two in relation to lithiasis: the inflammatory state and alteration in the throphism of the epithelium. *F. prausnitzii,* together with *Bifidobacterium*, which is more reduced in patients with COD lithiasis, is the main producer of butyrate. The inflammatory state is due, on the one hand, to the dearth of butyrate, which has a high anti-inflammatory activity, and, on the other, to a direct effect of the ammonia ion and other metabolites that produce proteolytic microbiota. In addition, the butyrate systematically contributes to trophic levels of the epithelium, so a scarcity provokes problems in epithelium throughout the body, including urothelium. The fact that the COD group included a significantly lower percentage of patients with normal *Bacteroides* levels suggests that this is a chronic dysbiotic pattern.

To sum up, in patients with CaOx lithiasis, and in particular those with COD, a chronic pro-inflammatory intestinal dysbiosis pattern was found, with alterations in the trophic level of the epithelium. The aetiology of lithiasis is multifactorial and in the case of COD lithiasis the main accepted causative factor is a metabolic calcium disorder, specifically hypercalciuria. The link between other disorders, such as arteriosclerosis, and inflammation and calcium has long been known, as vitamin K_2 treatment, which removes calcium from the vascular endothelium, not only prevents but can reverse the formation of atheromatous plaques. All of this suggests that in different diseases related to calcium, there could be a relation to inflammation. The present study relates a pathology associated with alterations in the metabolism of calcium, i.e., CaOx lithiasis, to an intestinal dysbiosis characterised by a high inflammatory pattern. This study should stimulate further research based on this premise, with the aim of opening the door to improved management of CaOx lithiasis and taking into account the relationship with other variable factors not contemplated in this preliminary study such as: stone events, stone type and the metabolic study. Recommendations might include diets to diminish the proteolytic microbiota, prebiotics to increase muconutritive microbiota bacteria, probiotics to re-establish intestinal microbiota balance and immunomodulatory therapy with bacterial lysates, as is done with other pathologies such as atopic dermatitis, recurrent sinusitis, recurrent bronchitis and irritable bowel syndrome [43–52].

Compliance with ethical standards

Conflict of interest There is no conflict of interest.

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