

# **Electron Microscopy Investigation of Urine Stones suggests how to prevent Post-operation Septic Complications in Nephrolithiasis**

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## **Abstract**

This research was conducted on renal concretions from patients suffering from nephrolithiasis, a pathology caused by the presence of kidney stones. This disease provokes inflammation of the interested area, pain and functional alterations of the organ. Microorganisms play a leading role in stones formation being at the base of septic complications. Urease producing microorganisms (*Escherichia coli* among them) are typical microorganisms participating in stones formation. Scanning Electron Microscope (SEM) and Focused Ion Beam/Scanning Electron Microscope (FIB/SEM) techniques show that microorganisms remain in renal *calculi* for a long time and produce biofilm, a mucoid matrix with several physicochemical microenvironments in which bacteria act as a community. Bacteria, organized in microcolonies within the biofilm, cause infectious processes and provoke commissures formations, which fix stones to kidneys. Electron microscopy observation of stones from patients suffering from nephrolithiasis who underwent surgery or lithotripsy shows the presence of collagen fibers on the concrements. This suggests that it is required a proper surgical methodology for *calculi* removal, in order to prevent the infection from spreading and to avoid a relapse of nephrolithiasis.

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**Keywords:** nephrolith, renal *calculi*, kidney, nephrolithiasis, lithotripsy, Scanning Electron Microscope (SEM), Focused Ion Beam/Scanning Electron Microscope (FIB/SEM), Scanning Electron Microscope with X-ray Microanalysis (SEM/EDS)

## 1 Introduction

Nephrolithiasis is a widely spread pathology caused by the presence of renal *calculi* in the urinary system. This disease, provoked by genetic factors and secondary metabolic disorders [1-3], gives inflammation of the interested area, pain and functional alterations of the organ.

Microorganisms can be at the base of septic complications playing a leading role in stones formation [4-9]. Microbes penetrate in the renal pelvis, the proximal part of the ureter in the kidney, as a result of the kidney filtration so that while the urine continuously flows, bacteria remain in the urinary duct provoking urinary duct infections [9, 10]. Urine is a water solution of proteins, sugar, mineral salts, urea, creatinin, urobilin and ammonia; it meets the trophic requirements of various microorganisms whereas the renal pelvis represents an attractive niche for bacteria capable to use this resource [7, 11]. The only obstacle to the development of this niche is the continuous flow of urine; microbes can overcome this impediment anchoring themselves at already present concrements, thus avoiding to be removed from the surface and getting access to nutrients in urine and to organic and inorganic substances in stones.

The primary cause of urinary stones appears to be the urinary supersaturation of stone-forming salts [2, 12]. Urine alkalinity is provoked by urease, an hydrolase produced by several bacteria, such as *Proteus*, *Klebsiella*, *Pseudomonas* and *Staphylococcus* species and 1% of *E. coli* [9, 13, 14], necessary to split urea to ammonia and CO<sub>2</sub>. As a result, ammonia ions can form and at the same time alkaline urine develops (pH > 7.5), both being preconditions for the formation of struvite and carbonate apatite crystals [9, 13, 15]. As a consequence of the deposit of these crystals, infection stones form and they are approximately 15% of the urinary stones diseases [13].

A good review of the primary pathophysiology of nephrolithiasis can be found in Moe *et al.* [16].

Microorganisms of the renal pelvis act like a microbioma and as a trigger for stone formation [5-7], moreover they are able to use stones for biofilm formation in order to survive to adverse environment. Biofilm is an aggregate of microorganisms in which cells that are frequently embedded within a self-produced matrix of extracellular polymeric substance (EPS) adhere to each other and/or to a surface [17]. Biofilm formation is a process characterized by different steps: the initial attachment of bacterial cells, the formation of microcolonies, the maturation of the biofilm and the detachment of planktonic cells from the biofilm that can start a new cycle of biofilm formation elsewhere [18-20]. Bacterial population, organized in the biofilm in several physiochemical microenvironments, is protected from adverse conditions for their survival and from antibiotics [9, 18-20], disinfectants, phagocytosis [21] and other components of the innate and adaptive immune and inflammatory defense system of the host, thus becoming the locus of chronic infectious processes in kidneys.

Under these conditions the localization of the bacterial locus in kidneys by the immune system is limited since there is no possibility of direct interaction of cellular and humoral immunity with the infectious agents [22]. Moreover, in the renal pelvis there are no cells

producing mucus as antibacterial protection [23, 24].

Kidney stones provoke partial or total ureteral obstruction, causing the urine stagnation and the augmentation of the size and the number of the already present *calculi* [25].

The presence of biofilm on the stones' surface [19] is accompanied by the action of the connective tissue [26] with the appearance of collagen fibers fixing the stones to the internal surface of renal pelvis, so the concrements cannot be removed by the urine flow.

The aim of this work is to show that electron microscopy highlights aspects of the dynamics between bacteria and nephroliths, focusing on the correct surgical methodology for *calculi* removal, in order to prevent the infection from spreading and to avoid a relapse of nephrolithiasis.

## 2 Materials and Methods

Twelve samples of renal stones (coral shaped and single) were surgically taken from the renal pelvis of patients suffering from nephrolithiasis.

The analysis of mineral structure of urinary stones was carried out with a Fourier Transform InfraRed spectroscopy (FT-IR) technique (Thermo Scientific Nicolet, USA).

Samples were prepared by fixation of stones with the Ito-Karnovsky method [27] and prepared for the Transmission Electron Microscope (TEM) analysis [28]. Ultrathin sections were stained according to the Reynolds method [29] and analyzed with JEOL 100B (Japan).

For the Scanning Electron Microscope (SEM) analysis samples were coated with a 5-nm-thick gold or carbon layer in a SPI-Module Sputter/Carbon Coater System (SPI Inc., USA). The conductive coating is important because it prevents the charging of the specimen surface during the observation with the SEM, especially when the microscope is operated at high vacuum; moreover the coating is needed for the micro elemental analysis as well. Even though the coating is considered a main passage in sample preparation for the electron microscopy analysis, it is reported that imaging of samples with no preparation (only air drying) is possible [30, 31].

Samples were analyzed with a dual beam Focused Ion Beam/Scanning Electron Microscope (FIB/SEM) Quanta 200 3D (FEI Company, USA) in a regime of high vacuum. The microscope was used to obtain SEM images at both 5 kV and 10 kV acceleration beam with electrons generated from a tungsten source. It has been proved that these are the proper voltages to observe biological specimens [32, 33]. Getting good images using lower voltages ( $\leq 1\text{kV}$ ) [34] is also possible, in fact the low beam penetration provides better superficial details and the resulting electron beam penetration is similar to that of a ionic beam obtained on a FIB/SEM. The focused ion beam operated at low beam currents is used for imaging, at high beam currents is used for site specific *in situ* milling [35]. The stones' surfaces were investigated with the Scanning Electron Microscope with Energy Dispersive X-ray Microanalysis (SEM/EDS) technique, which requires an electron beam acceleration higher than 10 kV. The micro elemental analysis of stones was conducted with a Genesis XM 2 (EDAX, USA). In order to identify the microorganisms present on stones, scraps of the shattered stones were put in a nutritious broth (Luria-Bertrani) and incubated for 24-48 hours at 37°C. The specific identification of bacteria was carried out with the Apex system. A bacteriological research of stones revealed the presence of *Escherichia coli*, *Enterococcus faecalis*, *Enterococcus faecium*, *Acinetobacter* species, *Klebsiella pneumoniae* and *Proteus mirabilis*.

### 3 Results

The analysis of stones' mineral structure revealed phosphates, struvites, uric acid, sodium urate, ammonium urate, carbonate apatite and calcium oxalate.

The microscopic analysis showed several concretions' surfaces: almost smooth, hilly, with inclusions of crystals with keen edges, with roundish porous structure, united in conglomerates.

Microorganisms anchor themselves to renal *calculi* [25] and start the biofilm formation [18-20]. The presence of biofilm on renal stones promote chronic infectious processes [18-20] in kidneys and anchors the concretions at the walls of the renal pelvis.

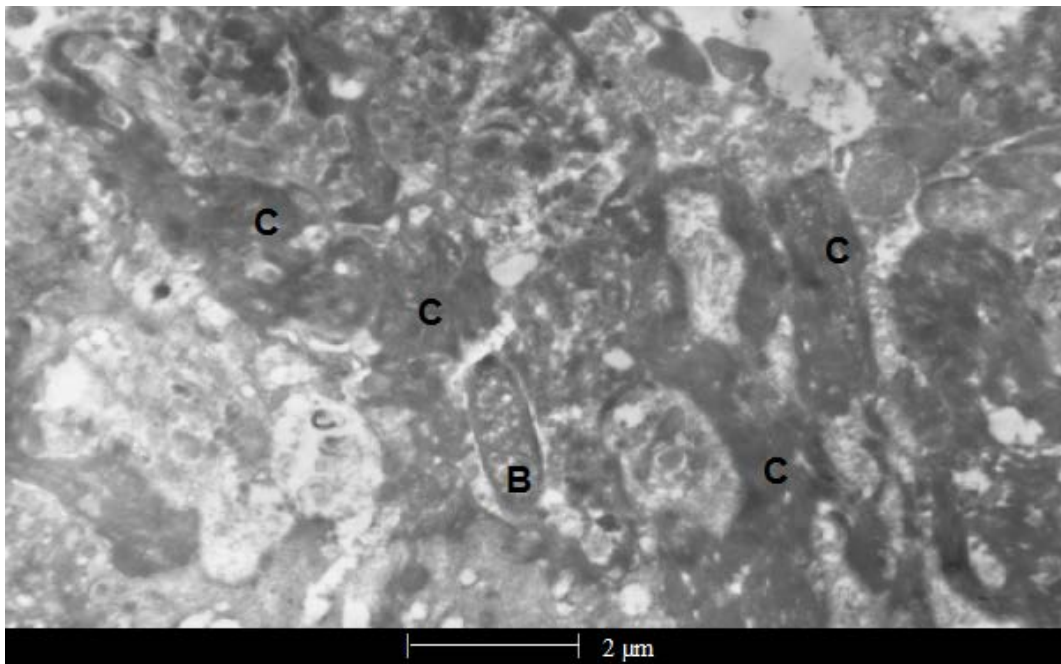


Figure 1: Image of the surface of a kidney stone obtained with TEM JEOL 100B (Japan). The biofilm covering the nephrolith is composed by different bacterial populations (B): Gram positive and Gram negative bacterial and vegetative forms. Bacteria were localized between collagen fibers (C) and cell debris.

It was observed that bacteria produce a biofilm that traps several cell types, such as fibrocytes (Figure 2), leucocytes (Figures 2-3) and erythrocytes (Figure 4). In both Figure 2 and Figure 4 it is possible to observe collagen fibers fixing the stones to the internal surface of the renal pelvis so that the concretions cannot be easily removed by the urine flow. The connective tissue proliferation is an universal reaction [26] of a macroorganism in order to isolate the abiotic or infectious agents.

SEM images show the biofilm perfectly preserved thanks to the use of the proper fixation technique. It is also notable that despite the presence of the biofilm, which typically causes a blurring effect, it was possible to obtain clear images of the cells and the fibers embedded in it. In particular, in these images obtained from *ex vivo* specimens, the erythrocytes are clearly visible. The comparison with images of samples from forensic investigations suggests that the preparation method adopted in this work shall be used in

haemotaphonomy as well [36, 37].

Figures 3, 4, 5a, 5b show that the connective tissue, represented by the collagen fibers, fastens the fibrocytes, erythrocytes and leucocytes forming a sort of tridimensional web that keeps the cells sunk in the biofilm. The connective tissue firmly anchors the cells in the urinary duct forming a complex and stable structure embedded in the biofilm.

In Figure 6 it is notable the presence of erythrocytes (E) in *hiatus* (aperture forming as a consequence of a trauma). The presence of blood cells can be explained as a result of a mechanical trauma of the wall of the renal pelvis with damage of vessels and bleeding.

The micro elemental analysis (Figure 7) of stones shows the relation between distinct stone surfaces' structures (different roughness) and their composition (presence of specific chemical elements), proving that several structures can either promote or inhibit biofilm formation. Besides carbon and oxygen on smooth surfaces it was found calcium only, on surfaces with keen edges were revealed both calcium and phosphorus, whereas on spherical porous structures nitrogen, magnesium, sulfur, calcium and phosphorous were detected.

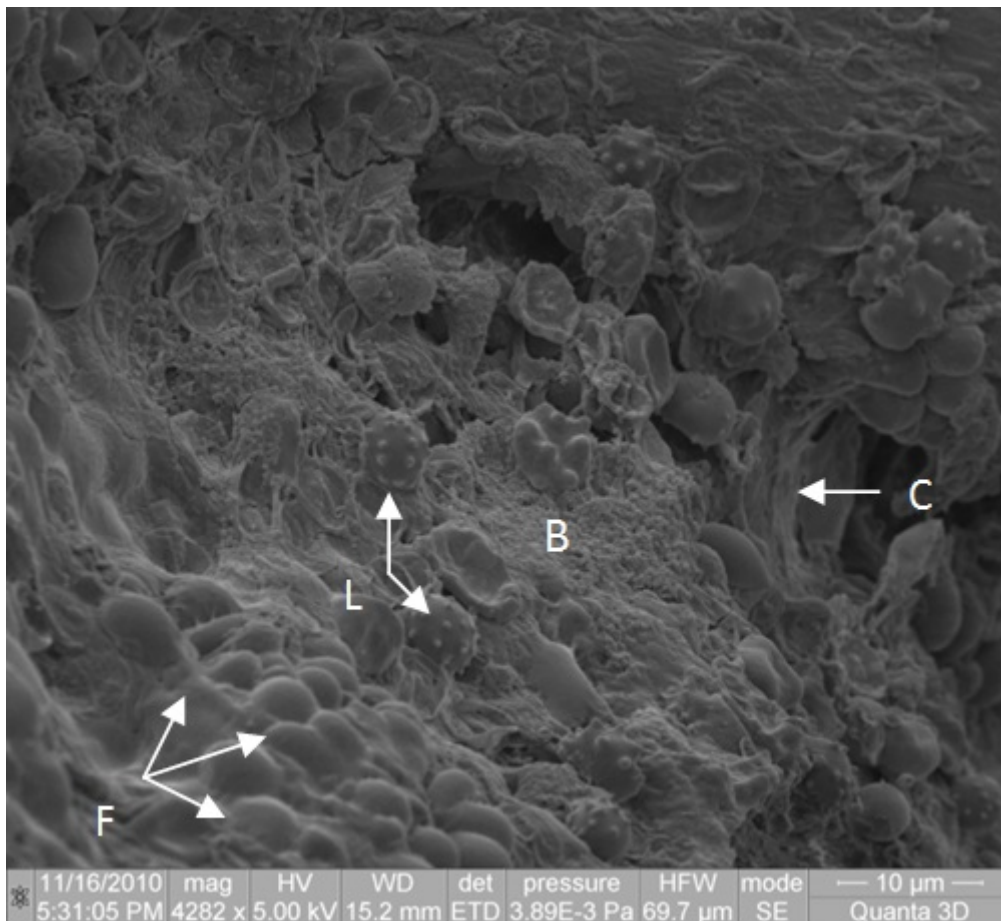


Figure 2: SEM image of the surface of a kidney stone obtained with a FIB/SEM by secondary electrons ETD at 5 kV electron beam acceleration in high vacuum. It is possible to observe fibroblasts (F), leucocytes (L) and collagen fibers (C) embedded in the extracellular matrix of the biofilm (B).

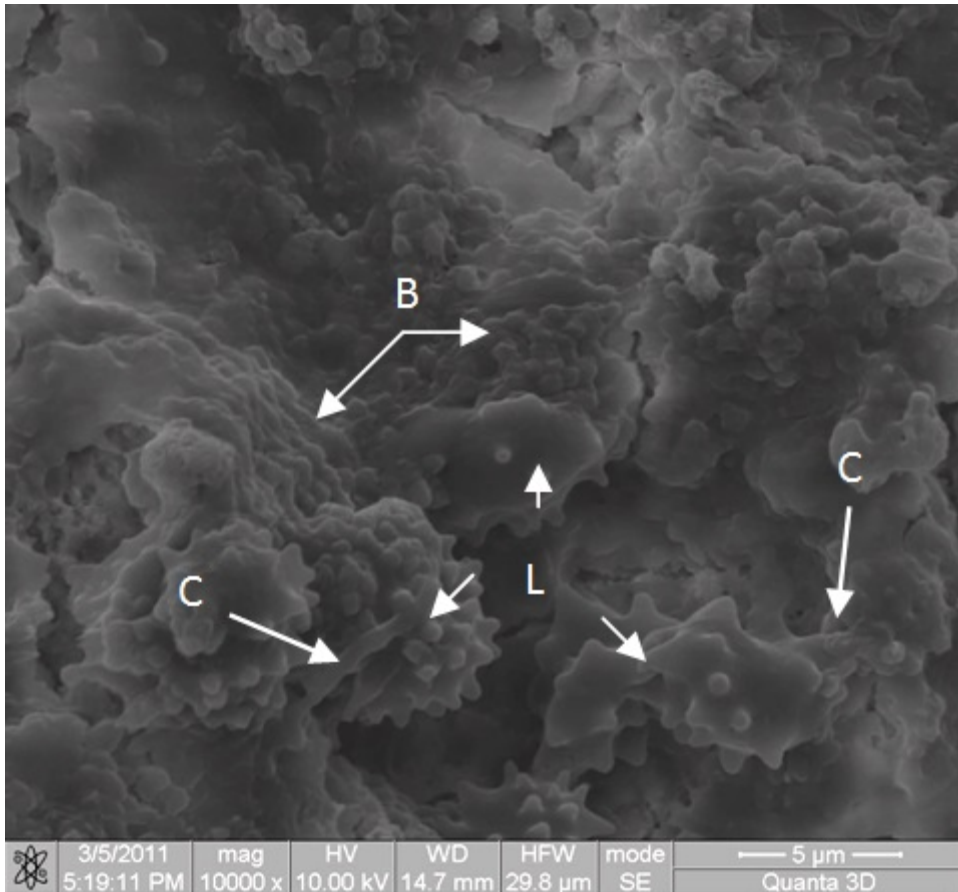


Figure 3: SEM image of the surface of a kidney stone obtained with a FIB/SEM by secondary electrons ETD at 10 kV electron beam acceleration in high vacuum. This image gives further details about the linkage of the elements embedded within the biofilm on kidney stones. The neoformed connective tissue, collagen fibers (C), traps the leucocytes (L) and the biofilm (B) in order to isolate the infectious agents.

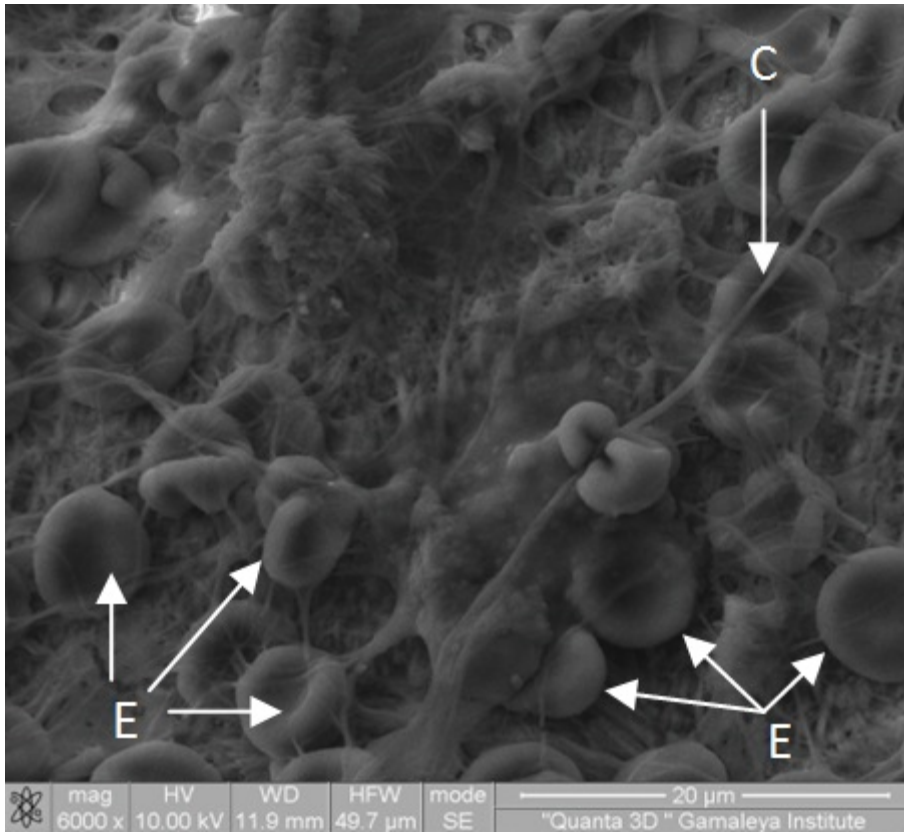


Figure 4: SEM image of the surface of a kidney stone obtained with a FIB/SEM by secondary electrons ETD at 10 kV electron beam acceleration in high vacuum. It is clearly visible the presence of erythrocytes (E) and of collagen fibers (C) on the kidney stones.

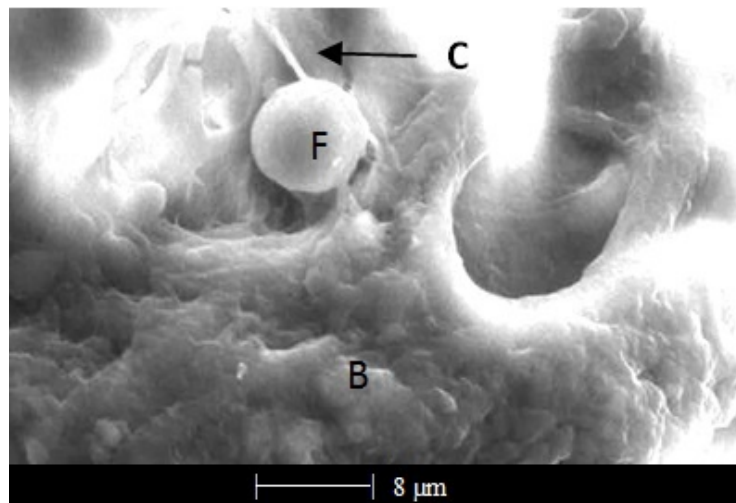


Figure 5a: SEM image shows a fibrocyte (F) tightly linked through collagen fibers (C) at the biofilm (B). The connective tissue, represented by the collagen fibers, fastens the fibrocyte keeping the cell sunk in the biofilm.

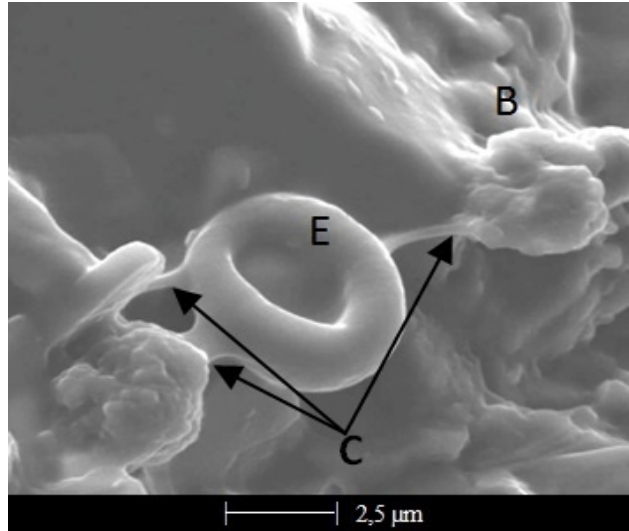


Figure 5b: SEM image shows an erythrocyte (E) which is stuck in the biofilm (B) through collagen fibers (C). The connective tissue firmly anchors the red blood cell forming a complex and stable structure embedded in the biofilm.

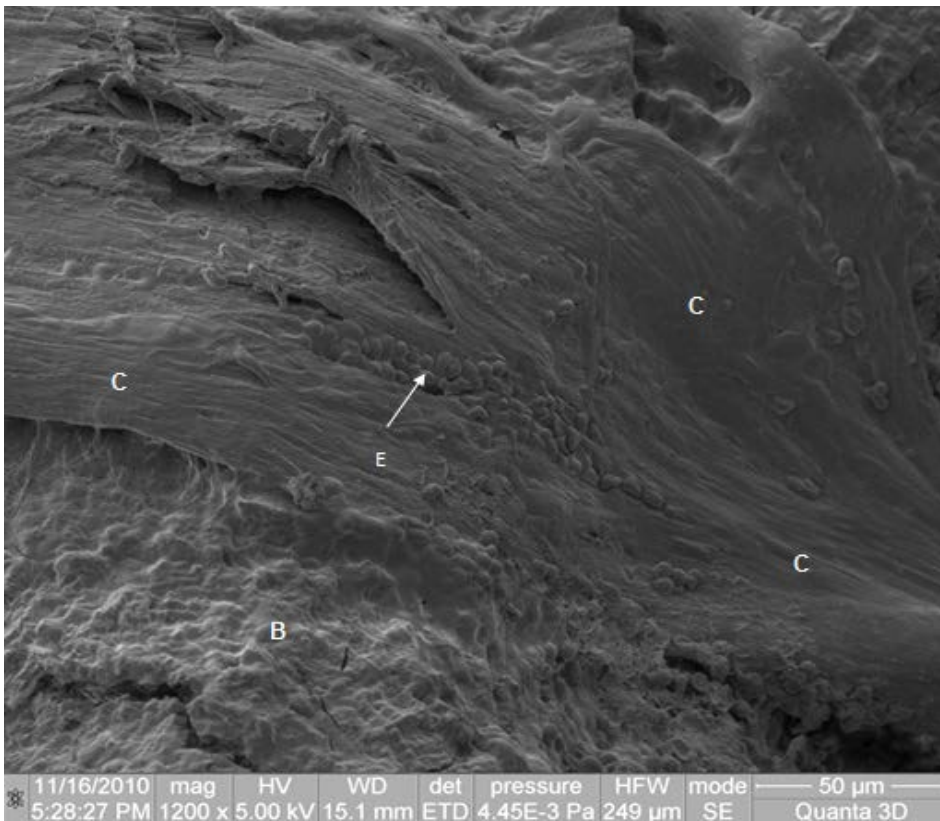


Figure 6: SEM image of the surface of a kidney stone obtained with a FIB/SEM by secondary electrons ETD at 5 kV electron beam acceleration in high vacuum. It is notable the presence of erythrocytes (E) in *hiatus* (capillary vessel forming *de novo*, embedded in the biofilm (B) and in the collagen fibers (C) on the kidney stone's surface).



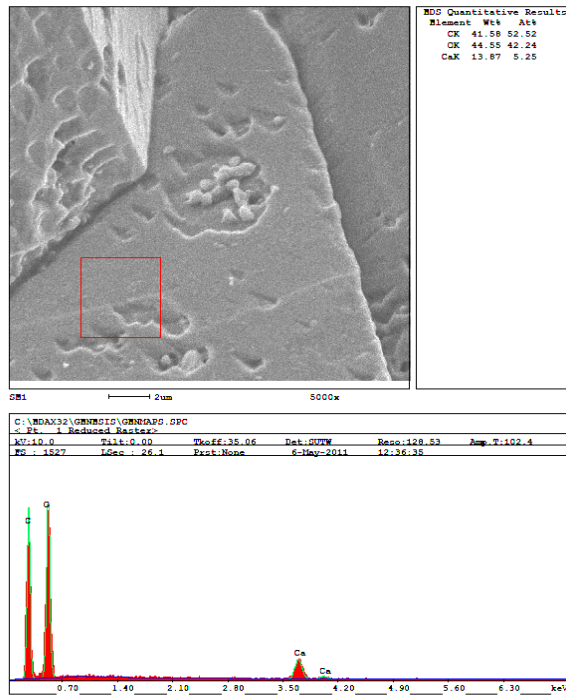


Figure 7a.

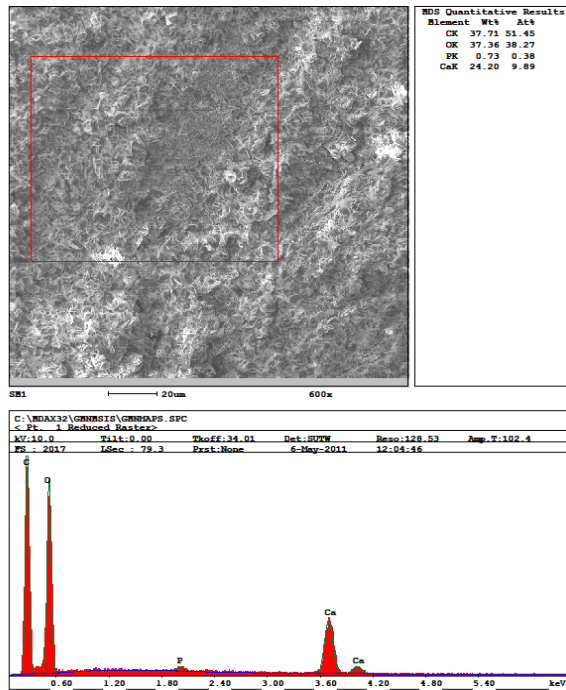


Figure 7b.

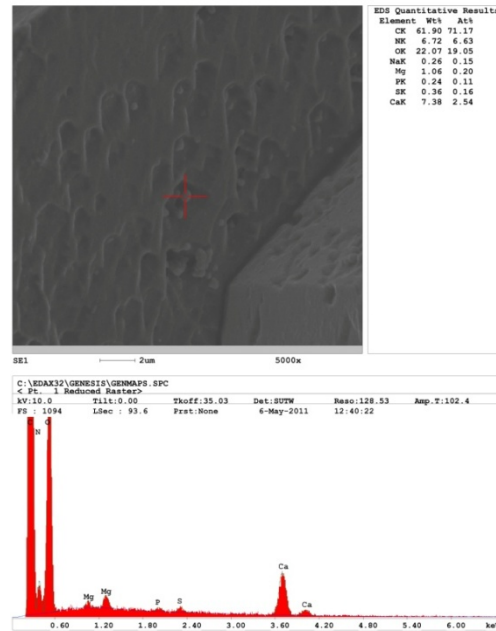


Figure 7c.

Figure 7a, 7b, 7c: Renal concretions were analyzed using Energy Dispersive X-ray microanalysis in association with SEM.

The different roughness of the nephroliths' surfaces seems to be strictly correlated to the presence of distinct chemical elements [26]. Besides carbon and oxygen several elements were detected according to the surface' structure.

Figure 7a shows that on a smooth surface only calcium was found.

In Figure 7b it has been analyzed a surface with keen edges and both calcium and phosphorus were revealed.

Figure 7c represents the chemical analysis of a spherical porous structures in which nitrogen, magnesium, sulfur, calcium and phosphorous were detected.

Comparable results can be found in Khan *et al.* [26].

#### 4 Discussion and Conclusion

Bacteria and other cell types such as nanobacteria can initiate pathologic calcifications acting as crystallization centers for kidney stones formation [5-7]. Although many authors feel doubtful about the actual existence of nanobacteria [8, 9, 13], thanks to biochemical analysis and electron microscopy investigations nanobacteria have been detected in kidney concretions [5-7]. Nanobacteria are not urease producing organisms, their diameter goes from 50 nm to 200 nm, they are poorly disruptable, highly resistant to heat [5-7] and can infect phagocytosing cells [5]. Nanobacteria are transported from blood into urine as living organisms [5-7] and kidneys represent a preferable niche for these bacteria to adhere and grow, resulting in the biocrystallization [7]. The formation of carbonate apatite crystals can occur both intra- and peri-bacterially [13].

Besides nanobacteria also a particular deposit composed of calcium phosphate apatite and carbonated calcium phosphate, termed Randall's plaque, can serve as a nucleus for *calculi*

formation [38].

Microorganisms producing crystals of struvite and carbapatite are eventually embedded in a biofilm. Biofilm in the renal pelvis protects microbes from adverse factors becoming the locus of chronic infectious processes [5-7, 19, 20].

The presence of a biofilm on a stone surface induce the reaction of the connective tissue with the migration of fibroblasts and the formation of an intercellular web of collagen fibers that mechanically isolate the *calculus* (Figures 2, 3, 4, 5a, 5b). In Figure 8 it is possible to see that as a result of this braiding, collagen fibers fix the stone to the internal surface of the renal pelvis, therefore even small stones cannot be removed by the urine passage and can enlarge their size [25, 26, 39]. The connective tissue is characterized by the presence of fibroblasts and collagen fibers; it is richly vascularized, then it is possible to find erythrocytes, leukocytes and neutrophils.

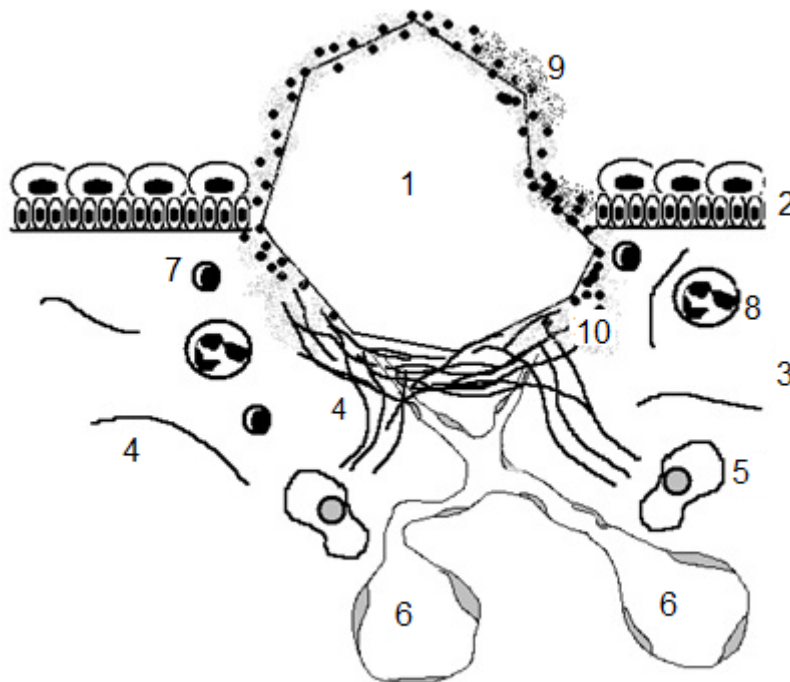


Figure 8: Pre-operation situation. Kidney stone in the renal pelvis and infection.

- 1) Renal stone / nephrolith
- 2) Transitional epithelium
- 3) Fibrous connecting tissue
- 4) Collagen fibers
- 5) Fibroblasts
- 6) Capillary vessels
- 7) Leucocytes
- 8) Neutrophils
- 9) Biofilm and bacteria (black points)
- 10) Inflammation *locus* and fibrous connecting tissue formation

Chemical elements play a key role in the microorganisms trophic requirements. Bacteria get nutrients from urine and from minerals derived from kidney *calculi*, indeed their presence is related to the stone's chemical composition. Microbes are located in sites where phosphorus, nitrogen, sulfur and magnesium are present, but they are not found in sites where only calcium is detected (Figures 7a, 7b, 7c). Calcification is a protective reaction: a calcium incrustation prevents an exit of vegetative forms of bacteria from the infectious *locus*, thereby limiting their harmful effects on an organism.

Studies revealing information about the linkage of biofilm and collagen fibers on kidney stones in the renal pelvis are important to understand the pathogenicity of nephrolithiasis [1-3]. Planktonic cells can detach from the biofilm and migrate through vessels into the bloodstream being the reason of pyelonephritis [9, 10, 13-15]. The clinical manifestations of this infection are hyperthermia, moderate pains in kidneys, increase of leukocytes in blood and in urine, a massive bacteriuria [9, 16].

The therapy for patients suffering from nephrolithiasis can be lithotripsy or the surgical intervention [9, 13, 16]. Lithotripsy is a medical procedure that uses high-energy shock waves, laser or high intensity focused ultrasound (HIFU) [9, 25, 40] to break up stones in kidneys or ureter into large fragments (~ 1 mm) [9, 40]; some authors propose the probable use of histotripsy, a new technique based on pulsed focused ultrasound used to control cavitation activity for a comminution of renal concretions in fine debris, in synergistic interplay with lithotripsy [25]. To intervene with lithotripsy or histotripsy on *calculi* embedded in the biofilm has numerous contraindications. Since these treatments blast the stone wrecking it into several fragments [25], it is implied that a part of the small scraps of stone covered with biofilm would be spread to other *loci* in the kidney diffusing the infection, and the remaining part of fragments stays trapped in the intertwined web of collagen fibers, promoting additional stone growth [25] and causing steinstrasse [39, 41].

In the surgical intervention if the surgeon manages to eradicate the whole *calculus* and the biofilm, the patient should not have a relapse of nephrolithiasis. In some cases the stone within the biofilm is not completely removed and a few fragments could remain in the kidney [25, 41]. In this situation as well two main problems appear: relapse of nephrolithiasis and infection spread. The *calculus* removal can be incomplete and little splinters of stone embedded in the biofilm could remain in the renal pelvis (Figure 9). This could lead to a relapse of nephrolithiasis (relapse occurs in one fifth of the treated patients within 5 years [41]) because the small stones could newly attract other concretions and bacteria, and thus enlarge their size [6, 25].

It has already been said that the connective tissue is richly vascularized. The energy deposited on the tissue by shock waves, laser or ultrasounds lithotripsy as well as the surgical intervention for the removal of a kidney stone inevitably provokes a local trauma, breaking the capillary vessels and causing *hiati*, i.e. apertures, through which planktonic bacteria could enter in the bloodstream and spread the infection to other body districts.

Since connective filaments are not visible with the naked eye and electron microscopy shows how many pathogenicity factors are present, at least optical microscopy equipment (with a magnification not lower than 100x) is strongly recommended for this intervention in order to guarantee that the fibers are properly cut.

In conclusion electron microscopy is a necessary tool to identify and study several renal structures and their behaviour as a consequence of the *calculi* removal, so that it is possible to supply important information for the surgical ways of intervention in order to prevent a relapse.

Nanobacteria (20-200 nm) and Randall's plaques play a key role in the stones formation

acting as *nuclei*. The formation of Randall's plaques involves components of extracellular matrix including collagen fibers and small membrane bound vesicles [26]. The production of membrane bound vesicles can be induced also by the biodestruction of plastic materials (polyurethane) carried out by bacteria (*S. aureus*) [42, 43]. A consequence of this biodestructive process [44] is the dissemination of membrane bound vesicles in different body districts operated by bacteria.

Due to the presence of elements such as nanobacteria and membrane bound vesicles it is implied that electron microscopy is irreplaceable in this kind of investigation to examine and determine the presence of biocrystallization centers and their dynamics starting from the nucleation of the crystals.

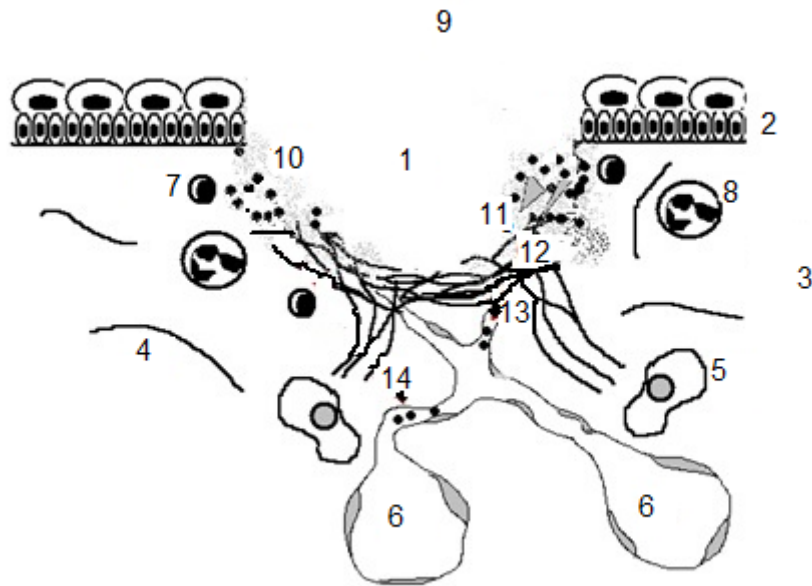


Figure 9: Post-operation condition. Reasons of relapse of nephrolithiasis and septic complications.

- 1) Space left by the *calculus* removal
- 2) Transitional epithelium
- 3) Fibrous connecting tissue
- 4) Collagen fibers
- 5) Fibroblasts
- 6) Capillary vessels
- 7) Leucocytes
- 8) Neutrophils
- 9) Site for the formation of new stones with biofilm
- 10) Fragments of biofilm
- 11) Scraps of stones
- 12) Rest of collagen fibers
- 13) Bacterial migration in central blood circulation
- 14) Bacterial migration through intercellular space into vessels

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