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Review

What Causes Calcium Oxalate Kidney Stones to Form? An Update on Recent Advances

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Abstract: Kidney stone disease affects 12% of the global population with a prevalence that continues to increase. It is recurrent in up to 50% of patients within 5 years and is associated with major health concerns including coronary artery disease and chronic kidney disease. Thus, kidney stones pose a substantial health and economic burden. However, despite kidney stone disease being one of the oldest known and most common diseases worldwide, our understanding of the mechanisms underlying stone formation is lacking. Moreover, recent data have raised questions about the efficacy of currently used therapeutic options for calcium oxalate stones, which account for 75% of all kidney stones. Development of new therapeutics for the successful prevention and management of this disease will require improved understanding of the causes of kidney stones. Recent advancements have shed light on the nuanced contribution of diet, environment and genetics as well as the more fundamental roles of calcium oxalate crystallization, Randall's plaque formation, inflammation and even a possible contribution of the recently discovered urinary microbiome. This review provides a comprehensive overview of our current understanding of kidney stone pathogenesis and identifies new frontiers and remaining gaps in our knowledge of this disease.

Keywords: kidney stones; nephrolithiasis; pathogenesis; Randall's plaques; supersaturation; crystal; biomineralization

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1. Introduction

Nephrolithiasis, or kidney stone disease, affects over 35 million North Americans annually. This disease can cause urinary tract infection, kidney damage, and, in severe cases, sepsis. Calcium oxalate (CaOx) is the most common component of stones, accounting for about 80% of all cases [1,2]. Several factors including metabolic syndrome, diet and vitamins, hydration, and sex hormones have been postulated to be involved in the formation of CaOx stones [3–7]; however, the underlying mechanism of stone pathogenesis remains incompletely understood, impeding the development of successful therapeutic approaches and preventive strategies [8]. Thus, the rate of incidence and recurrence of the disease is high [1,2]. To develop treatments that are effective and validated, it is crucial to have a more comprehensive understanding of the pathogenesis of the disease and the underlying mechanisms that contribute to stone formation. Over the past few decades,

substantial research has been conducted through clinical, translational, and scientific studies to investigate various aspects of stone formation. These investigations have covered a broad range of areas including the physicochemical properties of CaOx stones, factors contributing to their development, sites of their attachment inside the renal system, causes of stone retention, and the role of immune responses. These studies suggest that CaOx stone formation is a complex biochemical crystallization process that involves multiple thermodynamic events, followed by a cascade of immunological responses. In this paper, we will review current understanding of various aspects of the stone formation mechanism and discuss open questions as well as potential research opportunities in this field. The following sections and subsections will be addressed in this paper:

2. Discussion

2.1. Epidemiology

Kidney stone disease is a prevalent urological condition that affects more than 35 million North Americans annually, which is about 10% of the population. The formation of kidney stones is also associated with other renal and vascular disorders such as hypertension and chronic and end-stage kidney diseases. The recurrence rate is high, and around half of patients will experience another stone episode within 5 years of their initial treatment, while 74% of stone-formers will have another episode within 20 years of their first treatment [1,2,9].

The incidence and prevalence of kidney stones are increasing worldwide, and people of all ages and genders, including women and children, are affected more than ever before. Since the 1990s, the number of individuals affected by kidney stone disease has significantly increased from 1 in 20 to 1 in 11 [2]. The financial impact of kidney stones on the medical system is also substantial, with an estimated annual cost of over $5 billion in direct and indirect expenses in the United States alone [2,10].

2.2. Kidney Stones and the Renal System

Kidney stones are an aggregation of individual crystals that form from urine, in which specific minerals such as calcium may become supersaturated; stones are named according to their main crystalline components. Approximately 80% of stones are predominantly composed of calcium oxalate (CaOx), in pure composition or mixed with calcium phosphate

(CaP) at varying percentages. After formation within the renal tubules, dissolved phases of CaOx crystals are thought to deposit in the kidney's calyces and pelvis (Figure 1). In a cascade of events, the deposited crystals retained inside the urinary tract grow, agglomerate, and transform into kidney stones [11]. But how does urine become supersaturated with stone-forming minerals?

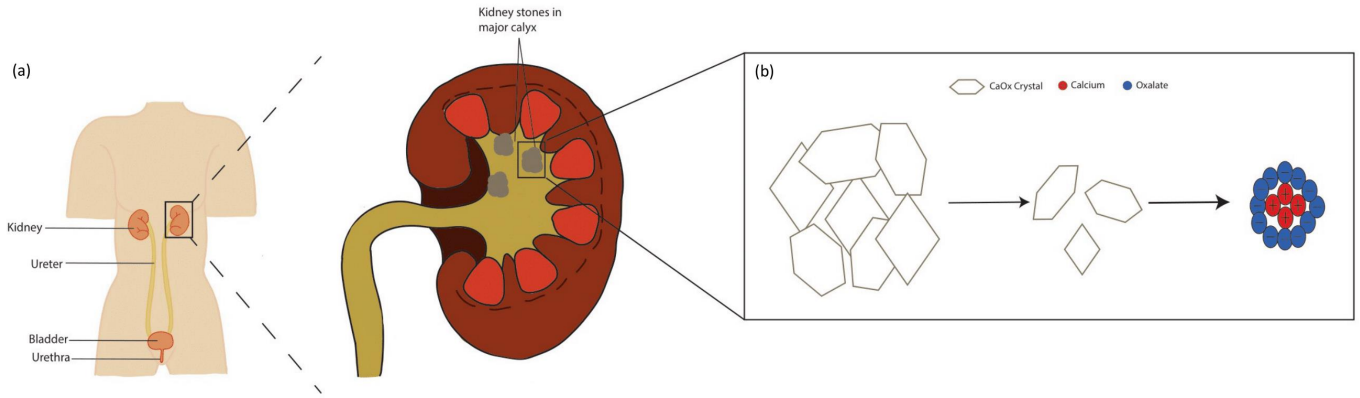


Figure 1. (**a**) Locations of kidney stones inside the renal system. (**b**) Aggregation of CaOx crystals inside the urinary tract.

Within the kidney, the functional filtering unit is called the nephron, which is composed of two main parts: the glomerulus and the long renal tubule (Figure 2). The (1) glomerulus consists of a network of branching glomerular capillaries, which are highly water-permeable barriers that restrict the passage of large plasma proteins. The (2) renal tubule is mainly responsible for reabsorption and secretion and is divided into three main segments: the proximal tubule, the loop of Henle, and the distal tubule, which connects to the collecting duct. The collecting ducts progressively merge to form larger ducts that lead to the renal calyces and pelvis through the tips of the renal papillae (Figure 2).

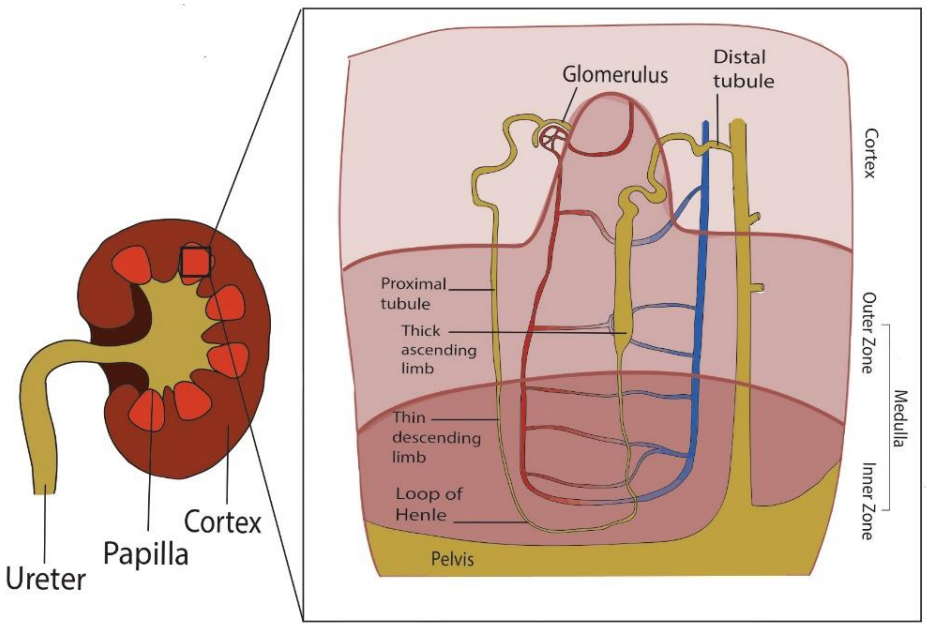


Figure 2. Tubular segments of the nephron.

As the glomerular filtrate passes through the renal tubule, its volume and content are altered by several processes, including reabsorption and secretion. These processes are mainly localized to different parts of the tubules. Solute reabsorption occurs mainly in

the proximal tubules. Further urine concentration occurs in the loop of Henle, which is a specialized segment of the nephron that creates a concentration gradient in the surrounding tissue. This gradient allows for the reabsorption of water and electrolytes, resulting in a more concentrated urine.

The distal tubule and collecting ducts are the final site for adjustment of urine composition, secretion, and acid-base balance. These structures regulate the amount of sodium, potassium, hydrogen, and other ions in the urine, which can affect its acidity and overall composition. During this process, urine may become extensively supersaturated with minerals, resulting in crystal nucleation within the tubular lumens or interstitium [3,12]. After passing through the nephron, the final urine exits the kidney through the pelvis and into the ureter, which carries it to the bladder for storage and eventual elimination.

During the urine concentrating process, CaOx crystals are continuously formed and are typically washed off through the urine stream. However, individuals who are prone to kidney stone formation have an increased risk of developing urinary crystals that can reside in the renal system and ultimately cause blockages. These stones can result in dilation and stretching of the ureter and renal pelvis and, in trying to pass through the urinary tract, can cause severe pain and possible kidney damage [13].

Types of Kidneys Stones

Approximately 80% of kidney stones are predominantly composed of calcium oxalate and/or calcium phosphate with a distribution of 50% pure calcium oxalate, 5% calcium phosphate, and 45% a mixture of both. Other types of stones composed of struvite (magnesium ammonium phosphate), uric acid, and cystine are less common, each accounting for 9%, 10%, and 1% of stones, respectively [3,7]. In this paper, we exclusively focus on calcium oxalate stones.

Calcium oxalate can crystallize in three hydrate forms and different morphologies. These include (i) calcium oxalate monohydrate (COM) with monoclinic prismatic, hexagonal, or dendrite shapes; (ii) calcium oxalate dihydrate (COD) with tetragonal bipyramidal (weddellite) morphologies; and (iii) calcium oxalate trihydrate with triclinic or needle-shape structures. During crystal formation, several urinary factors such as the urine pH, composition, and modulators determine the types, morphologies, and sizes of single CaOx crystals. Additionally, different types of CaOx crystals can be transformed into each other. For instance, when acid-rich proteins are present in the urine, COM can exhibit evidence of reprecipitation of COD [12–14].

3. Physicochemical Properties

The physicochemical analysis of kidney stones involves a thorough examination of their chemical, mineral, and crystalline properties, as well as their structure and morphology. Physical methods such as X-ray diffraction (XRD), Fourier transform infrared spectroscopy (FT-IR), X-ray fluorescence (XRF), time-of-flight secondary ion mass spectroscopy (TOF-SIMS), and laser-induced breakdown spectroscopy (LIBS) have been commonly employed for stone chemical and crystalline analysis. These methods provide a precise identification of the stone's chemical composition and a quantitative determination of the proportions of various elements. Additionally, these methods are capable of accurately determining the distinct crystalline phases and the relative proportions of each phase [13].

In addition to chemical analysis, morphological examination is also crucial in describing the size, color, and roughness of the stone's surface. This examination provides information about the size of the individual crystals that make up the stone [15], the organization of the stone's inner structure, and the location and properties of the core [16]. This information is important because stones with similar chemical or crystalline compositions

may appear differently in terms of their morphology [16–18]. The morphology of urinary stones is influenced by various factors, including the size and form (habit) of individual crystals, the kinetics of crystal growth and aggregation, and the location of stone nucleation within the urinary tract [18]. In this section, we will review the composition and structure of CaOx kidney stones, which have been derived and examined from stone-formers.

3.1. Chemical and Composition Phase Analysis of CaOx Kidney Stones

CaOx stones are classified based on their predominant mineral and crystalline phases, including calcium oxalate monohydrate (Ca (C_2O_4). H_2O) (COM), calcium oxalate dihydrate (Ca (C_2O_4). $_2H_2O$) (COD), or a mixture of both. Calcium oxalate trihydrate is rarely found in Ca-based stones. The stones are formed by the aggregation of single crystals that range in size from 50 to 3000 nm and are surrounded by an organic matrix of urinary macromolecules which make up a portion of the stone, around 10% [7,18]. These macromolecules, including glycosaminoglycans, lipids, carbohydrates, and proteins that exist at low concentrations in the urine, have been shown to play a significant role in stone formation, from the early stages of crystal nucleation and continuing through crystal growth and aggregation, ultimately leading to the development of stones. Micro-elemental analysis has also shown that the stone matrix contains high concentrations of heavy metals, including lead (Pb), strontium (Sr), zinc (Zn), sulfur (S), chromium (Cr), and magnesium (Mg) [16,17]. The role of these minerals in kidney stone formation remains to be fully elucidated.

3.2. Morphology and Microstructure Analysis of CaOx Kidney Stones

CaOx stones can vary in size ranging from a few micrometers to a few millimeters, and their surface can be smooth, rough, or spiky. Depending on their composition and the formation kinetics, stones can be brown, yellow, or gray in color [7,18]. Scanning electron microscope images of polished stone sections reveal that CaOx kidney stones are highly heterogeneous and the inner structure is composed of layers of amorphous and crystalline materials with a core nidus [19].

Additionally, microscopic analysis of thin sections of CaOx kidney stones by Tanaka and colleagues revealed that the mineral phases of stones are mainly composed of three distinct textures: (i) The first is an irregular texture made up of euhedral COD crystals, which are predominantly observed on the periphery of some stones (Figure 3a). (ii) The second texture is the mosaic texture, consisting of irregularly oriented COM crystals (Figure 3d). (iii) Finally, the third texture is the concentrically laminated COM crystals (Figure 3g). The mosaic and laminated textures are the most frequently observed textures in CaOx stones [20].

The application of super-resolution auto-fluorescence microscopy on thin sections of CaOx stones revealed that laminated regions comprise a high frequency of alternating layers consisting of mineral and organic matter. The thickness of mineral layers in the laminated areas is estimated to be between 10 and 100 nm [21]. Several groups have proposed that the presence of these layers of agglomerated single crystals within the inner structure of CaOx stones suggests that the stones are formed as a result of a series of recurring events that involve mineral precipitation, crystal nucleation, growth, and aggregation [21]. In the case of slow crystallization, single crystals are agglomerated in a well-organized and compact centric layer with a radiating pattern starting from the nucleus. Conversely, rapid crystallization leads to the formation of poorly organized concentric layers with a loose nucleus [22]. Periodically, the formed layers of crystals may be coated by urinary macromolecules fixed on the surface of crystals, which are further covered by new agglomerated crystals, and this process continues [23]. This dark color of the stones is attributed to the urinary pigments and macromolecules that are incorporated into the stone

matrix during the lithogenic process [22]. However, the regulating mechanism controlling the deposition of minerals and organic matter is not entirely clear. Furthermore, over time, some minerals may dissolve, and some embedded biomolecules may degrade and retract from the matrix, leading to the formation of cavities, cracks, and porosity in the stone matrix [18,24].

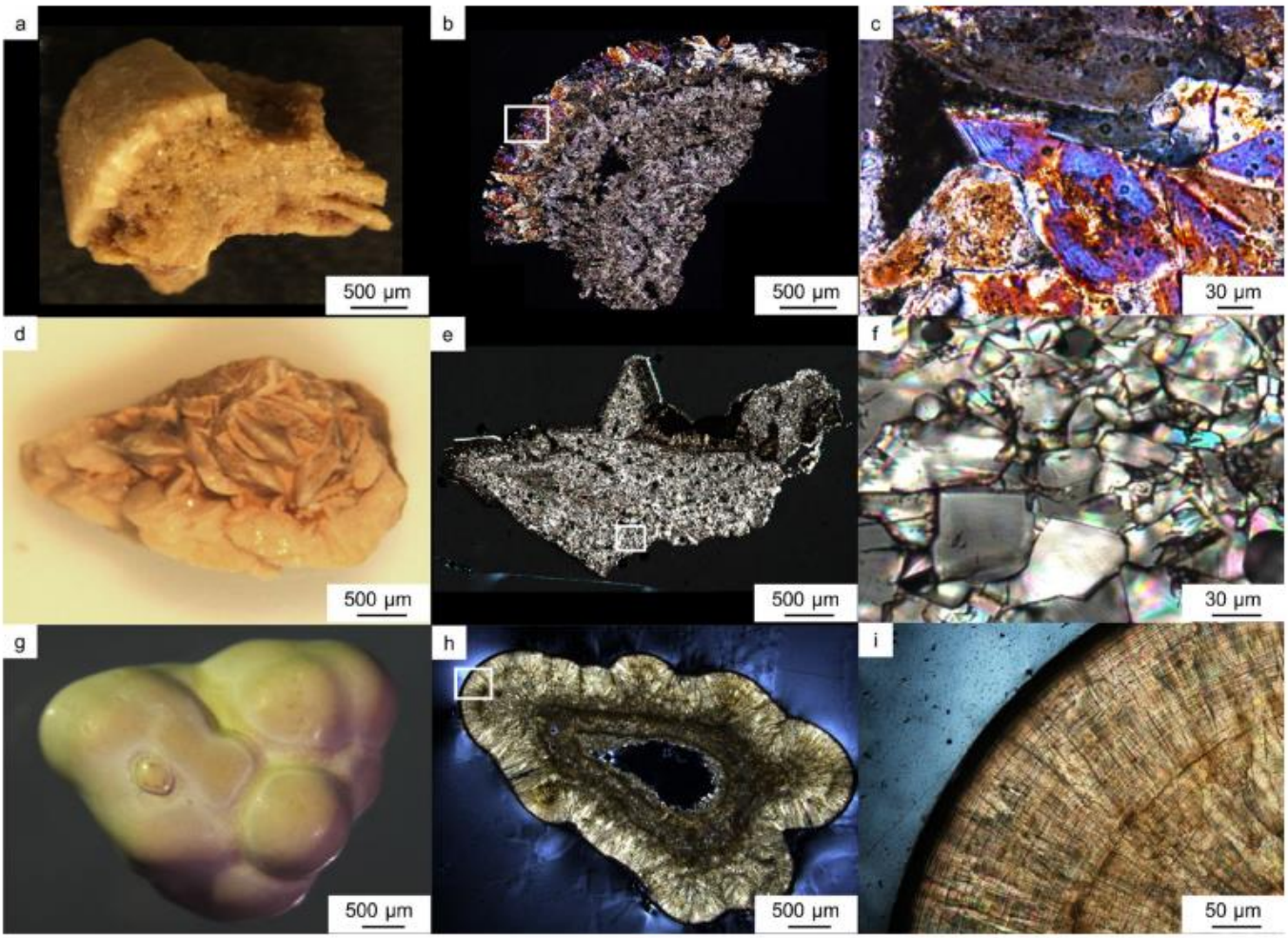
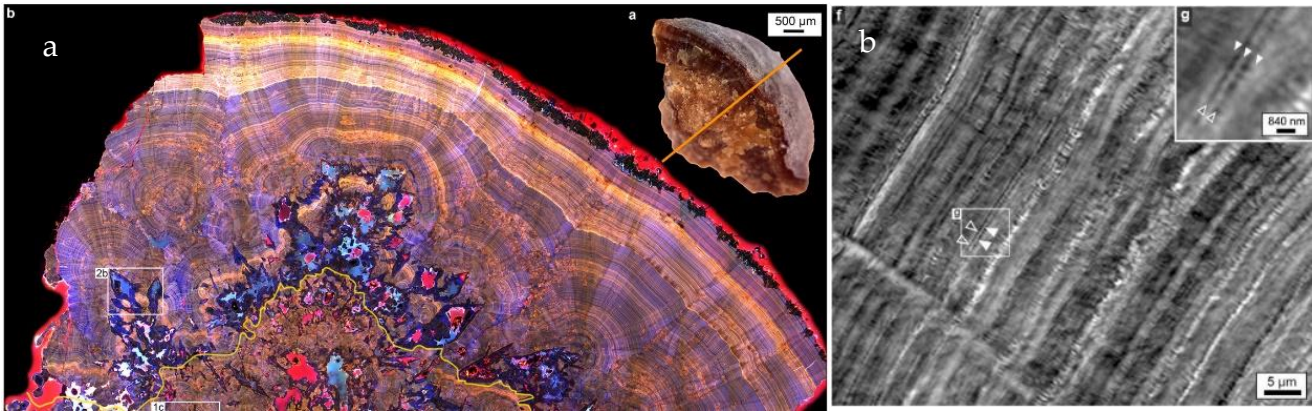


Figure 3. Main structural domains observed in CaOx kidney stones [20]. (**a**) Sample 1, (**b**) microscopic image of a thin section from sample 1, (**c**) zoomed on white box area in (**b**), (**d**) sample 2, (**e**) microscopic image of a thin section from sample 2, (**f**) zoomed on white box area in (**e**), (**g**) sample 3, (**h**) microscopic image of a thin section from sample 3, (**i**) zoomed on white box area in (**h**). Figure obtained with permission from Scientific Reports.

3.3. Biomineralized CaOx Stones and the Role of the Microbiome

The hybrid composition of CaOx stones, consisting of alternating layers of organic matter and crystalline-rich material (Figure 4), suggests that the stone formation occurs through periodic events of mineral–organic precipitation, similar to the process of biomineralization-a process in which living microorganisms actively induce and regulate crystallization. Moreover, bacteria and fungi have been detected in samples of stones collected from patients who did not have active urinary infection. In a recent study, next-generation sequencing was utilized to examine 18 CaOx stones; the results showed that 7 stones contained bacterial 16S rRNA and human host nonribosomal DNA fragments, while fungal amplicon sequences were detected in 9 stones [19]. This identification of living microorganisms within the matrix of CaOx stones provides further evidence that the formation of kidney stones may occur through pathological biomineralization [25]. Pathological biomineraliza-

tion of CaOx kidney stones arises from various microbial activities that affect the urinary supersaturation state, catalyze mineral precipitation, facilitate nucleation, and promote crystal growth and aggregation [5,21,25]. Several groups have suggested the existence of a kidney stone microbiome which may contribute to nephrolithiasis, while others have implicated the gut and urinary microbiomes [25,26].



(**a**) (**b**)

Figure 4. (**a**) Tiled confocal auto-fluorescence (CAF) image of layered crystalline structure of CaOx kidney stone. (**b**) Black and white circular polarization phase contrast (CPOLPC) image of the alternating light layers of organic and dark layers of mineral matter [21]. Figure reproduced with permission from Scientific Reports.

Biomineral crystals differ distinctly from their inorganically formed equivalents in shape, size, crystallinity, and isotopic and trace element compositions [27]. In the organic matrix of kidney stones, over 1000 proteins have been identified, which have been suggested to influence stone formation [28]. These proteins generally have anionic functionalities and may serve as adhesives to promote stone growth, aggregation, and attachment to renal epithelial cells [29].

4. Proposed Models of CaOx Stone Formation

There are currently three main pathological mechanisms that are hypothesized to be involved in the formation of CaOx kidney stones [7,21,30–34]. The first mechanism is known as the "free-particle model" and involves the deposition, growth, and aggregation of dissolved mineral phases of CaOx within renal tubules due to urinary supersaturation [30]. This model suggests that the formation of stones occurs entirely outside of cells in renal tubules. The second mechanism, referred to as the "fixed-particle" model, proposes that the initial step in stone formation is the attachment of crystals to the renal epithelium, creating the first sites of nucleation. Subsequently, the attached crystal nuclei further grow and aggregate to form larger crystals. Evidence from cell and animal studies supports the role of specific proteins in mediating crystal attachment to cells [7,35]. The third mechanism involves the formation of a Randall's plaque. This model hypothesizes that the formation of CaOx stones begins with the deposition of calcium phosphate (CaP) in the interstitial tissue of the renal papilla, which is known as a Randall's plaque. Initially, the Randall's plaque is buried in the renal papilla, but eventually, it grows and loses its covering of epithelial cells. When it becomes exposed to the urine in the pelvis, it serves as a nidus for CaOx stone formation [36]. These three mechanisms are illustrated in Figure 5.

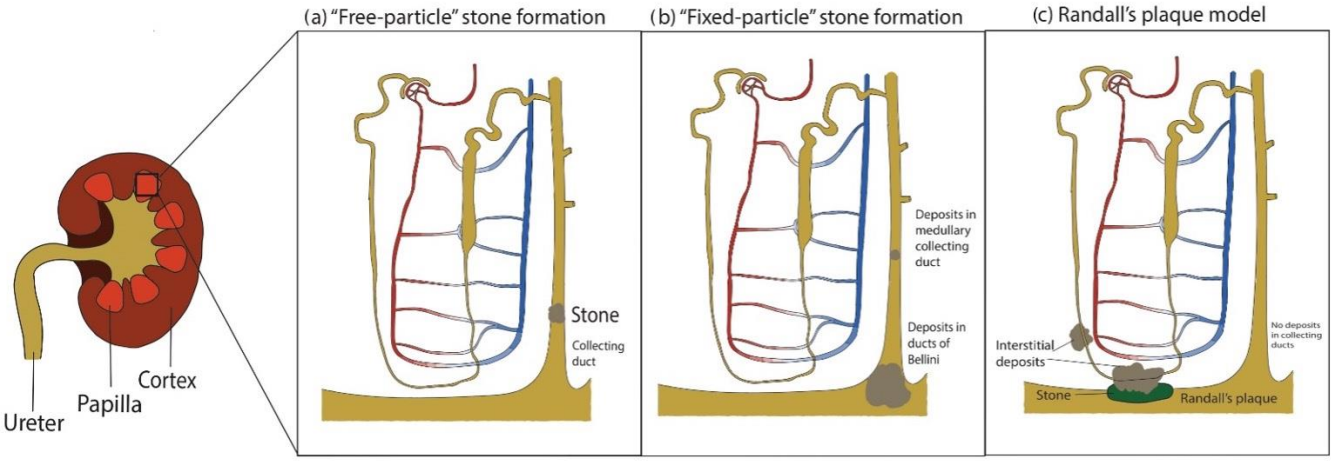


Figure 5. Three main proposed mechanisms of CaOx kidney stone formation (**a**) free-particle, (**b**) fixed-particle, and (**c**) Randall's plaque models.

Regardless of the model, the basic underlying process involves the formation of CaOx crystals which is currently best described by prevailing theories of crystallization, including a series of events of supersaturation, nucleation, growth, and aggregation. Additionally, this process triggers inflammation and stimulates immune responses in the body, ultimately leading to the development of CaOx kidney stones.

4.1. Supersaturated Urine

From a thermodynamic perspective, the formation of CaOx crystals is primarily driven by the urinary supersaturation with respect to calcium (Ca^{2+}) and oxalate ($C_2O_4^{2-}$) ions, resulting in precipitation of CaOx. This precipitation occurs when the concentration of the dissolved phase of the salt, C, exceeds its solubility, C^*, in urine at body temperature. This ratio is known as urine supersaturation, S, and is calculated using Equation (1) [4]. However, it is important to note that the state of supersaturation in urine is not a fixed thermodynamic value and is influenced by various factors such as the ionic strength, concentration of urinary biomolecules that promote or inhibit CaOx crystal formation, solute concentration, and pH [37].

$$S = \frac{C}{C^*} \quad (1)$$

When the concentration of calcium and oxalate ions in the urine exceeds their solubility limit (i.e., $S > 1$), the urine becomes supersaturated, which can lead to spontaneous precipitation of minerals and the formation of crystal nuclei. Furthermore, supersaturation can result in the growth and enlargement of existing crystals, potentially leading to the formation of CaOx kidney stones [4]. Previous studies have confirmed the supersaturation theory as a governing factor for CaOx crystallization, as they have shown that a higher urinary excretion of calcium and oxalate is associated with an increased risk of developing CaOx kidney stones. Thus, there is a direct relationship between the levels of these ions in urine and the likelihood of developing kidney stones [38].

4.2. Homogenous and Heterogeneous Nucleation

The nucleation process plays a crucial role in determining the properties of the resulting crystalline phase and the overall mechanism of stone formation. Nucleation can take place in two ways, either homogenously in a solution or heterogeneously on a foreign substrate in the solution. Classical nucleation theory is often used to describe the process of crystal nucleation in solution. Nucleation, or the initial formation of a microscopic solid particle from the liquid phase (or from the solution phase, which is more appropriate for

stone formation), is regarded as the first step in the formation of a new macroscopic solid phase. The kinetics that governs the formation of the bulk crystal is often dominated by the timescale required for nucleation, which can vary broadly. Nucleation can occur in two ways. Homogeneous nucleation occurs spontaneously from the solution. Heterogeneous nucleation occurs on a surface or an impurity. In homogeneous nucleation, the Gibbs free energy change as a function of the radius r of the nucleus is formulated as a sum of two terms, a negative 'volume term' proportional to the volume of the nucleus, and a positive surface 'term' proportional to the surface area of the nucleus multiplied by the surface tension between the nucleus and its solution environment. The surface term dominates at small radii, and the volume term dominates at large radii, resulting in a Gibbs free energy change that is positive for small r and negative for large r. In fact, for a specific intermediate value of r_c, the free energy change is maximized, and the probability of nucleus formation is minimized. Addition of new solute molecules to nuclei larger than this radius r decreases the free energy so the probability of formation is increased. In other words, above this radius r_c, formation of nuclei becomes progressively more probable. This forms the conceptual basis of a minimal critical nucleus size for crystal growth.

In summary, nucleation requires overcoming a free energy barrier. The critical size for the formation of a stable nucleus is a function of a number of parameters, including the concentration of the solution. A higher concentration of solutes makes it easier for nuclei to form. Homogeneous nucleation is usually slow and requires a high degree of supersaturation [4,39,40].

Heterogeneous nucleation can occur in the urinary system when an existing substrate is present. Renal epithelial cells, urinary macromolecules (such as proteins and lipids), or crystals of other minerals can all serve as templates for new crystals in urine. Injured cells in the renal system can also promote the crystallization of CaOx. These injured cells produce debris and lipid vesicles that may serve as templates for CaOx crystallization. The association between cell injury and CaOx kidney stone formation is further discussed in Sections 4 and 5. The use of a template lowers the interfacial energy and, therefore, the total free energy of nucleation. The interfacial energy between the formed clusters of ions and a solid substrate is lower than that of the cluster in contact with the solution, allowing the formation of smaller and more stable nuclei. This nucleation mechanism requires a lower level of urinary supersaturation and is, therefore, the more likely mechanism through which crystal nucleation initiates in the renal system [13,39,40].

Recent evidence suggests that CaOx nucleation may proceed through a non-classical pathway known as the prenucleation pathway, which is an alternative to classical nucleation theory. This theory proposes that under confinement, CaOx biomineralization begins with the precipitation of the amorphous phase of CaOx minerals from the aqueous solution. Studies employing techniques that enable the characterization of nanoscale processes have demonstrated that calcium (Ca^{2+}) and oxalate ($C_2O_4^{2-}$) ions bond into stable multi-ion complexes, known as pre-nucleation clusters, which exhibit no phase boundary with the surrounding solution and evolve to produce the amorphous phase of CaOx. Depending on the bonding strength of ions in pre-nucleation clusters, the amorphous solid can give rise to the formation of different crystalline phases of CaOx [32,33,41]. This novel perspective on nucleation suggests that with prenucleation, the process of crystallization is more likely to occur. Addressing prenucleation also provides new strategies to control crystallization. For instance, in one study, poly(acrylic) acid (PAA) was introduced into the crystallization system to interrupt CaOx crystal formation by stabilizing pre-nucleation clusters at a very early stage of nucleation [32]. In another study, the modulatory role of citrate was demonstrated in CaOx crystal nucleation at early stages by stabilizing the pre-nucleation clusters, thereby inhibiting crystal formation [33].

Regardless of the underlying mechanism, one of the notable characteristics of CaOx stone formation in the urinary system is the nucleation process taking place in a confined environment of the urinary tract. The confined space of the urinary tract can affect the nucleation rate, crystal size, morphology, and orientation. Therefore, it is crucial to use an appropriate model to mimic the urinary system in studies focused on understanding the disease pathogenesis of CaOx formation. Additionally, another critical issue in this research field is determining the site of initial crystal nucleation in the urinary system. Understanding whether crystallization begins in the interstitium, the tubular lumen, or the renal calyx would significantly advance our knowledge of CaOx stone pathogenesis. According to some studies, CaOx crystallization may start in the collecting ducts of nephrons. These studies have reported a range of sizes for a single crystal in the stone, from 50 to 3000 nm in one study to 0.5–1.5 μm in another [18,42]. Considering the size of the constituent crystals and the time taken for urine to travel through a nephron to the collecting duct, these studies suggest that crystal nucleation may occur preferentially in the collecting ducts [15].

4.3. Crystal Growth and Aggregation

Crystal nucleation is often followed by crystal growth and aggregation. Once the crystal nuclei reach their critical size and become stable, the total free energy decreases through crystal growth and the addition of new ions to the existing nuclei. Epitaxial crystal growth, which involves layer-by-layer advancement, is a dynamic process governed by the transport of ions to the growing crystals. While ions in a bulk solution are mainly transported through advection and convection, these mechanisms are not dominant in the confined environment of a micro-sized channel of the urinary tract. Instead, ion diffusion plays a significant role in crystal growth. Moreover, crystals may also grow and aggregate by the assembly of ion clusters or crystalline nanoparticles onto the existing crystals [13,29,33,43].

Once the crystals settle inside the renal system, they are exposed to the urine stream, which further promotes their growth and aggregation. Crystal aggregation refers to the process of individual crystals sticking together to form larger particles [6]. It has been observed that the presence of urinary promoters can enhance this process. Urinary promoters are macromolecules that can attach to crystal surfaces, providing more favorable binding sites for other crystals to attach. Essentially, promoters act as adhesive glue between individual crystals. Conversely, certain urinary macromolecules act as inhibitors by coating crystal surfaces and preventing their adhesion to renal epithelial cells. In the following section (Section 4.4), we will review the most common urinary promoters and inhibitors.

However, it should be noted that the residence time of crystals inside the kidney is relatively short (around 5 to 10 min), which does not allow them to grow enough to become trapped in the urinary system. This highlights the complexity of stone formation and emphasizes the crucial role of cell–crystal interaction and immunity in retaining crystals within the urinary tubular system. Understanding the mechanisms underlying these interactions is key and will be addressed in Sections 4 and 5.

4.4. Promoters and Inhibitors of CaOx Crystallization

The matrix of a kidney stone is composed of a mineral phase embedded in an organic matrix. The organic matrix comprises only 2–5% of the total weight and is made up of various urinary biomolecules, including lipids (7–12%), glycosaminoglycans (20%), carbohydrates (8%), and proteins (64%) [44]. These macromolecules are thought to play a crucial role in the formation of kidney stones at different stages of crystallization, promoting or inhibiting nucleation, growth, aggregation, and adhesion of crystals to renal epithelial cells. For instance, acidic phospholipids with negatively charged headgroups

from injured cell membranes facilitate heterogenous nucleation by catalyzing CaOx precipitation from a metastable solution. Some studies also demonstrate the dual roles of urinary glycosaminoglycans as both nucleation-promoters and growth-inhibitors of CaOx crystals [45].

Proteins are the most abundant macromolecules found in the matrix of CaOx stones. Recently, Yang et al. [46] conducted a comprehensive literature study and identified 1409 proteins present in the stone matrix. Some of these proteins are normally present in urine at low concentrations, while others are thought to be introduced into the matrix due to cellular injury. Yang et al. also identified the top 20 most frequently observed proteins in the stone matrix: uromodulin, albumin, osteopontin (OPN), lactotransferrin, vitamin K-dependent protein Z, prothrombin, hemoglobin subunit beta, myeloperoxidase, mannan-binding lectin serine protease 2, lysozyme C, complement C3, serum amyloid P-component, cathepsin G, vitronectin, apolipoprotein A-1, eosinophil cationic protein, fibrinogen alpha chain, S100A8, S100A9, and apolipoprotein D [46]. These proteins mainly participate in immune and inflammatory responses, indicating the role of inflammation in the development of CaOx kidney stones. In agreement with this study, Narula et al. also showed that 12 proteins of the organic matrix are involved in apoptosis, calcium binding, as well as inflammatory and stress response pathways [44].

Proteins play a crucial role as principal modifiers in biomineralization. They bind to specific crystallographic surfaces and regulate crystal growth, aggregation, and attachment to renal cells by promoting or inhibiting further precipitation of minerals [29]. Cationic and anionic proteins with net positive and negative charges, respectively, have been identified in the organic phase of CaOx stones [29]. Anionic proteins with carboxylate, poly (acrylic acid), poly (aspartic acid), and poly (glutamic acid) side chains adhere to specific crystallographic surfaces and hinder the attachment of ions from the solution to crystals, thus inhibiting crystal growth [29,47,48]. On the other hand, cationic proteins with specific subdomain characteristics can promote crystallization by increasing the size or number of nuclei, the rate or degree of intercrystallite aggregation, and enhancing the epitaxial growth of crystals at interfaces. Lysozymes and lactoferrin are two well-studied examples of cationic proteins that promote the kinetics of CaOx crystal growth by increasing the strength of the protein–crystal interaction, due to their rich basic side chains and specific spatial amino acid sequences [29]. Tamm–Horsfall protein (THP), OPN, and Inter-α-Inhibitor (IαI) are also anionic protein inhibitors that are involved in hindering crystal growth, depending on their anatomical source, specific molecular structure, concentrations in the solution, and the crystallographic surfaces and phases of CaOx crystals [48,49].

As discussed, proteins play a significant modulatory role in biomineralization, and this role is not only influenced by their physical properties and total charge, but also by specific domains within their molecular structure. For instance, calcium-binding proteins have been found to be critical in various stages of crystallization. These proteins possess local affinities for positively charged Ca^{2+} ions on crystalline surfaces, allowing them to interact, adhere, and incorporate into specific surfaces of CaOx crystals. This incorporation inhibits mineral attachment and slows down the epitaxial growth rate of crystals along these surfaces [26]. Some calcium-binding proteins that have been found to inhibit CaOx crystallization due to their Ca-binding domains, along with their physical properties and charges, include OPN, renal prothrombin fragment I, calgranulin, and nephrocalin [49].

On the other hand, protein aggregation can also have an impact on CaOx crystallization. Research has shown that the stone matrix contains a high concentration of strongly anionic proteins, such as OPN, and strongly cationic proteins, such as histones [50]. At low concentrations, these proteins interact with each other to form an aggregate composed of polyanion/polycation proteins. Once this aggregate is formed, other urinary proteins with

lower solubility in water can also interact with it, leading to their incorporation. Ultimately, this final aggregate has been shown to facilitate the formation of CaOx stones [51].

In addition to lipids and proteins, other well-known urinary biomolecules with CaOx-growth-inhibitory functions include citrate, magnesium, phytate, pyrophosphate, diphosphonates, and chondroitin [6]. At present, one of the most active areas of research is focused on the identification of promoters and inhibitors of CaOx crystals and the elucidation of the underlying molecular mechanisms that govern their regulatory performance. This area of investigation holds significant potential for advancing our understanding of the complex biological processes that regulate biomineralization with respect to CaOx stone disease.

4.5. Crystal–Cell Adhesion and Interaction

The formation of a CaOx crystal nucleus occurs from supersaturated urine in renal tubules and capillaries. Once formed, the crystal can reside in the renal system through various mechanisms. CaOx crystals can grow and aggregate with other crystals, eventually forming a large mass that gets trapped inside the kidney (known as the free-particle model) due to enlarged aggregate size and disturbed urinary flow. The formed crystals and aggregates also can stick to the brush border of the cells [36]. Moreover, CaOx crystals can adhere to the renal epithelial cells (the fixed-particle model) through surface receptors or binding molecules which have high affinity for crystals [52]. Adhered crystals are exposed to supersaturated urine and will continue to grow. Additionally, CaOx crystals can be internalized inside the interstitium, developing Randall's plaques (Section 4.5.1) [53]. The process of CaOx crystal adhesion to or internalization within renal epithelial cells is referred to as crystal–cell interactions and is an early and critical step in kidney stone formation [53,54]. Adhered crystals can interact with renal cells, altering cell function and the extracellular environment and triggering a range of cellular responses. This leads to oxidative stress, the generation of reactive oxygen species (ROS), lipid peroxidation, and local inflammation, ultimately resulting in cell injury. In addition to microcrystals of CaOx, the abnormal alternation in the levels of urinary oxalate, calcium, citrate, and phosphate may cause cellular injury and initiate the development of CaOx stones [55].

In fact, cellular injury is a major contributor to stone development [56,57]. After the injury occurs, the basement membrane is exposed to the urinary flow, allowing microcrystals to internalize or reside on the surface and continue to grow and aggregate. Moreover, the detached and dead epithelial cells can disintegrate into membranous vesicles, which are released into the urine and can serve as a favorable substrate for nucleation, leading to further crystallization [58]. Fragments of dead cells and degraded membranes can also slow the urinary flow through the renal tubules, resulting in a longer resistance time of crystals inside the urinary tract, facilitating crystal growth and aggregation [59].

Alternatively, cell injury can also trigger inflammation and alter gene expression, resulting in the production of proteins, cytokines, and chemokines that promote CaOx formation and adhesion to renal epithelial cells. For instance, it has been observed that high levels of ROS can activate p38 mitogen-activated protein kinase (MAPK) and cJun N-terminal kinase (JNK), leading to the production of transcription factors such as nuclear factor NF-κB and activator protein-1 (AP-1) [60]. These transcription factors can induce expression of stone modulator proteins including OPN, bikunin, heparan sulfate proteoglycan, monocyte chemoattractant protein-1, and prostaglandin E_2 [55]. These urinary proteins can promote crystal growth and aggregation. Furthermore, these proteins can mediate crystal adhesion to renal cells. Research on stone-forming rats has shown that isolated CaOx crystals are coated with OPN. This OPN coating promotes the binding of CaOx crystals to multinucleated inflammatory cells in the surrounding interstitium, resulting in the crystals being submerged within the interstitium [53].

In addition, the significant elevation of IL-6 and monocyte chemoattractant protein-1 (MCP-1) identified in the urine and renal tissue of stone-formers supports the role of inflammation in the development of CaOx crystals [61,62]. The secretion of these chemokines and cytokines plays a crucial role in attracting, recruiting, and activating macrophages and monocytes at the sites of inflammation. Monocytes / macrophages are thought to play a crucial role in crystal clearance [61]. However, if they are unable to properly clear crystals, their prolonged exposure to the crystals may induce their polarization toward an anti-inflammatory phenotype, resulting in increased inflammation, oxidative stress, and tissue injury [62,63]. Moreover, secreted proteins can also play a role in regulating further inflammatory responses and cell signaling pathways, which can ultimately contribute to the formation of kidney stones. For instance, the presence of CaOx crystals was found to be associated with higher levels of FKBP4 expression, which can subsequently inhibit TRPV5 activity and reduce Ca^{2+} intake. This can lead to hypercalciuria and, consequently, an increased chance of developing kidney stones [64].

4.5.1. Development of Randall's Plaques

A significant proportion of kidney stones develop attached to the surfaces of kidney papillae, specifically at sites where pre-formed calcium phosphate (CaP) subepithelial plaques exist [36]. These plaques are commonly known as Randall's plaques (Figure 6) and are found in both stone-formers and non-stone-formers. However, not all plaques necessarily lead to the formation of kidney stones (Figure 6) [65].

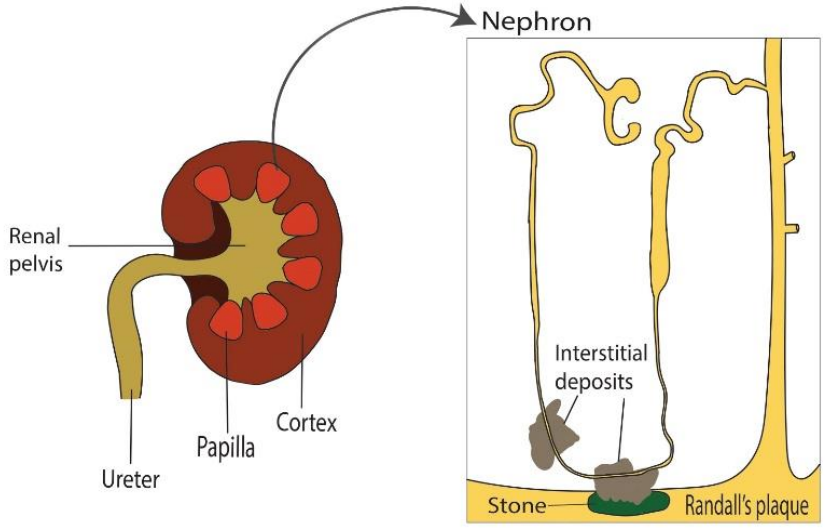


Figure 6. Development of CaOx stones attached to Randall's plaque on kidney papillae.

In recent years, there has been increasing evidence of kidney stones associated with Randall's plaques, especially among children and young women [36]. For CaOx kidney stones that are found on papillary surfaces, the role of Randall's plaques in stone formation is postulated to be crucial. According to this mechanism, lithiasis is thought to occur independently of urinary supersaturation, with the renal papilla tip serving as the initiating site of stone formation. It is further hypothesized that this process begins with the formation and deposition of calcium phosphate (CaP) crystals into the renal interstitium [7,36]. This is followed by the growth of the formed CaP-based plaque, which can damage the epithelial cells and become exposed to pelvic urine. The exposure to urine allows for the deposition and development of CaOx on the surface of the plaque. This interplay between CaP and CaOx crystals is an important factor in the formation of kidney stones associated with Randall's plaques.

However, the exact mechanisms involved in the formation and growth of Randall's plaque remain unclear. This is due to several factors, including limitations in data related to supersaturation and early-stage CaP formation in the kidney interstitium, lack of experimental approaches to study this phenomenon, and the absence of an appropriate animal model to stimulate CaOx stone formation on Randall's plaques [36,65]. However, ongoing research continues to shed light on this complex phenomenon.

The composition of the matrices of Randall's plaques includes calcium phosphate embedded with calcified collagen fibers, membrane-bound vehicles, some products of cellular degradation, and unidentified fibrillary materials [30]. This unique composition has led to speculation about the potential role of inflammation in causing the formation of Randall's plaques.

According to the current working hypothesis, Randall's plaques originate in the basement membranes of the ascending thin limb of the loop of Henle as small, round nanoparticles with concentric layers of mineral and organic matter [66]. This process begins with the formation of CaP crystals in the tubular lumen, which are then endocytosed in by renal epithelial cells on the luminal side and exocytosed on the basolateral side, leading to the deposition of calcium phosphate on the basement membrane. In addition to this pathway, an alternative route for calcium phosphate deposit formation involves the calcification of membrane-bound vesicles and collagen that are released from renal epithelial cells as a result of inflammation. Reactive oxygen species (ROS)-induced inflammation triggers the transformation of renal epithelial cells into a mesenchymal (EMT) or osteoblast (EOT) phenotype, which results in the secretion of membrane-bound vesicles and collagen, respectively, which subsequently undergo calcification [56]. Figure 7 depicts a model of this hypothesis. This calcification mechanism shares many features with bone development. The involvement of bone-related proteins including osteopontin, osteocalcin, and bone sialoprotein 2, as well as cellular debris and matrix vesicles in kidney papillary tissues, stone matrix, and the urine of stone-formers further supports this hypothesis [67,68].

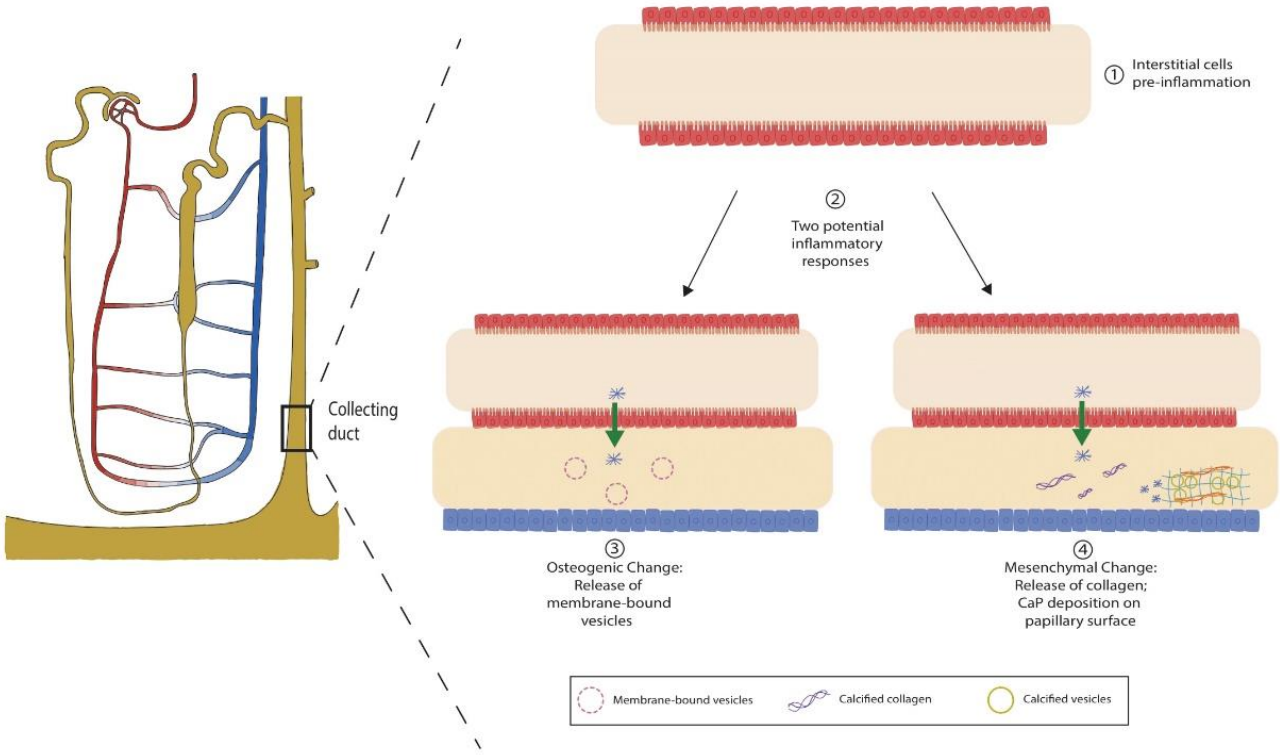


Figure 7. Proposed models for the deposition of CaP around the basement membrane of the ascending thin limb of the loop of Henle.

Additionally, vascular calcification may be involved in the early stages of Randall's plaque formation. The coexistence of arterial calcification and kidney stones, along with the presence of osteogenesis markers in the matrix of kidney stones, support this hypothesis. The vascular bed at the tip of the renal papilla is thought to be prone to injury due to the hypoxic and hyperosmolar environment around the vasa recta and capillaries, as well as turbulent blood flow at the papillary tip. As with vascular injury, calcification may occur during the repair process, which eventually spreads to the interstitium [68].

Over time, the deposited CaP plaques gradually spread through the interstitium towards the urothelium, and during this transition, crystals continue to grow and push through the basement membrane, physically injuring cells in the process [36]. Furthermore, cytokines and chemokines released at the site of cell injury attract and accumulate infiltrating inflammatory cells, such as monocytes, macrophages, and polymorphonuclear leukocytes, into the adjacent interstitium. The recruited inflammatory cells attack the crystals adhered to the interstitium, releasing their contents around the crystals. These contents include an arsenal of mediators, proteolytic enzymes, and other biomolecules that cause significant damage to the local tissue and create a pathway for interstitial crystals to further penetrate into the tissue. These plaques continue to grow and penetrate deeper into the interstitium, eventually moving towards the urothelium, which is exposed to the lumen of the urinary drainage system. Once there, they come into contact with various biomolecules found in urine, such as OPN and Tamm–Horsfall protein, as well as other CaOx crystals that form due to supersaturation. This interaction causes the CaOx crystals to grow and aggregate, ultimately leading to the formation of CaOx stones within the lumen [7,36].

The cause of early-stage deposition of CaP around the segments of the loop of Henle remains unclear, though some studies suggest that imbalances such as hypercalciuria and hyperoxaluria may be risk factors for plaque formation. Coe et al. hypothesize that decreased calcium reabsorption at the proximal tubules leads to increased calcium concentrations in the loop of Henle, thereby promoting Ca reabsorption into the interstitium and initiating the deposition of CaP [64]. However, subsequent animal studies indicate that hypercalciuria and hyperoxaluria cause the formation of intratubular but not interstitial deposits without deficiencies in crystal inhibitors and specific genes, including mutations in the Na^+-dependent phosphate transport protein 2A (NPT2), ATP binding cassette subfamily C member 6 (ABCC6), and ectonucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1) genes [36]. Research is ongoing to better understand the mechanistic underpinnings of this deposition.

4.6. The Role of Vitamins

Recent publications have provided a comprehensive review of the potential contributions of various vitamins as well as the gut microbiome in the process of nephrolithiasis [25,65].

5. Conclusions

This review covers several important aspects of CaOx kidney stones, including their epidemiology, physicochemical properties, and proposed model of stone formation. The review underscores the complexity of CaOx stone formation and the continued need for a thorough understanding of the multifactorial processes that influence pathological biomineralization. Factors such as urinary supersaturation state, catalysis mineral precipitation, facilitation of nucleation, and promoters of crystal growth and aggregation all play important roles. By better understanding the disease pathogenesis and identifying the underlying mechanisms, we can potentially develop more effective therapeutic strategies for preventing and treating this condition.

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