Nephrocalcinosis: new insights into mechanisms and consequences

Benjamin A. Vervaet, Anja Verhulst, Patrick C. D’Haese and Marc E. De Broe

Department of Medicine, Laboratory of Pathophysiology, University of Antwerp, Antwerp, Belgium

Correspondence and offprint requests to: Patrick C. D’Haese; E-mail: Patrick.Dhaese@ua.ac.be

The most common form of renal stone disease, calcium nephrolithiasis, is defined as the presentation of a macroscopic concrement of inorganic (calcium phosphate and/or calcium carbonate) and organic material in the renal calyces and/or pelvis, either adhered to the papillae or pelvic urothelium or not. In search of the mechanism underlying calcium nephrolithiasis, in vitro and in vivo studies and observations in human biopsies have shown the presence of two distinct types of renal microscopical crystal deposition processes; one taking place within the tubular lumen (intratubular nephrocalcinosis), and the other in the interstitium (interstitial nephrocalcinosis). Recent observations, however, strongly suggest that nephrocalcinosis and calcium nephrolithiasis are to be considered two independent pathologies and that nephrocalcinosis may cause calcium nephrolithiasis only in particular conditions. In this review, we discuss our current understanding of the mechanisms involved in both types of nephrocalcinosis (intratubular and interstitial), their possible consequences and their relation to calcium nephrolithiasis.

Intratubular nephrocalcinosis

Mechanisms of intratubular nephrocalcinosis

Two key processes determine the development of intratubular nephrocalcinosis, crystal formation and crystal retention. Crystals (mainly calcium oxalate, CaOx, and calcium phosphate, CaP) are thought to frequently form in the tubular fluid as a result of supersaturation mainly in the distal nephron and can be considered a renal mechanism to excrete an increased amount of waste per unit of volume [1–4]. To ensure safe tubular crystal passage, the healthy kidney on the one hand presents a non-crystal binding epithelium [5–9] and on the other hand is able to keep crystal nucleation, growth and aggregation under control via urinary micro- and macromolecular constituents such as citrate, magnesium and proteins [10–13]. In addition, at the physiological level, a high intratubular calcium concentra-

1. In vitro renal cell-lines as well as primary human epithelial cell cultures only bind crystals firmly in their subconfluent phase, i.e. when cells are proliferating and migrating, and lose this capacity once they become confluent and fully polarized [6,8,17,24,27]. It has to be noted that this is particularly true for epithelial cells of distal origin, thought to be frequently exposed to intraluminal crystals [28,29].

2. In rats treated with ethylene glycol (EG, a model of hyperoxaluria/crystalluria) already after 1 day of treatment, the numerous crystals present in the tubular fluid were not adhering to the normal differentiated intact epithelium. After 4 days of treatment, however, crystals...
were found adhering to dedifferentiated/regenerating cells [5]. Interestingly, during washout, shortly after arrest of a 4-day EG-administration period, the number of regenerating tubular cells markedly increased, as did crystal retention, while crystalluria decreased to control values (Vervaet et al., unpublished results). Additionally, in another study mild EG administration did not result in nephrocalcinosis until a nephrotoxic agent was injected intraperitoneally [30].

3. In a transplant protocol biopsy study of our group, all patients showed tubular luminal expression of osteopontin and hyaluronan at first biopsy 12 weeks post-transplantation, while only 20% of these patients presented nephrocalcinosis. At second biopsy, 12 weeks later, osteopontin and hyaluronan expression was still present in all patients; however, it was associated with 100% nephrocalcinosis, evidencing luminal expression of these molecules to precede crystal adhesion [16]. This early and maintained expression of particular molecules may be related to recovery from ischaemia–reperfusion and subsequent sustained renal stress by exposure to potential nephrotoxic immunosuppressive drugs. In the same study, it was found that preterm infants, who are well known to be born with an immature epithelium, also presented luminal osteopontin and hyaluronan; however, nephrocalcinosis did not develop until several days of life [16]. This delay most likely is due to the time it takes for diet and/or medication protocols to induce crystalluria and subsequent crystal adhesion [16,31,32].

In contrast, in some histological studies in rat and man, intratubular crystals were observed adjacent to epithelial cells that appeared morphologically normal [33,34]. These observations may either suggest crystal adhesion to normal differentiated cells or alternatively may represent a ‘snapshot’ of transient (non-adhesive) crystal–cell interactions. Although it is not known whether these epithelia actually present a normal apical membrane in terms of protein and phospholipid composition, the latter possibility seems more likely. Indeed, animal models of mild hyperoxaluria and crystalluria (such as EG or minipump-infused oxalate) do not immediately develop renal crystal retention by adhesion or obstruction, but apparently require several days to weeks of exposure [5,35]. This ‘incubation’ period, during which nephrotoxic EG metabolites and/or transient toxic or mechanical crystal–cell interactions may affect the tubular epithelium, is in line with the need of a shift in the epithelial phenotype prior to crystal adhesion [29,36,37].

In the case of severe injury, crystals may adhere to apoptotic and/or necrotic cells (known to present altered membrane surfaces) and even to denuded basement membranes after cells have been lost from the epithelium [26,38,39]. Furthermore, besides crystal adhesion, nucleation of crystals onto the tubular epithelium has been suggested to be a potential mechanism underlying intratubular nephrocalcinosis [25]. In this process, crystallization starts at particular sites on the epithelial surface instead of starting freely in the tubular fluid. Remarkably, the composition of the cell surface appears also to be a critical determinant in modulating this process [25,40].
Altogether, whereas excessive crystal formation/aggregation may result in tubular obstruction and its deleterious consequences, crystal adhesion turns out to be a consequence of epithelial phenotypical changes, which can be induced by any renal insult/condition and, possibly, also by passage of crystals/oxalate.

**Consequences of intratubular nephrocalcinosis**

Intratubular nephrocalcinosis is as harmful to renal function as the number of tubules it functionally impairs. Whereas the mechanism of tubular impairment is straightforward for obstruction, it is harder to ascribe any direct deleterious effect to crystal adhesion. Since both processes differ in their nature, different ways of affecting renal function are to be expected. While obstruction presents itself rather acutely, adhesion most likely exerts chronic effects adding to the severity of an already underlying pathology or condition.

**Tubular obstruction** acutely impairs tubular function by mechanical blockage of tubular fluid flow, followed by tubular atrophy, interstitial inflammation and interstitial fibrosis, and hence chronic renal damage/insufficiency develops [41,42]. Histological evidence here to was found in pathologies with acute and/or excessive forms of crystal formation and subsequent intratubular retention such as acute phosphate nephropathy [43], primary hyperoxaluria, jejunooileal bypass-induced enteric hyperoxaluria [33,44] and several drug-induced crystal nephropathies (methotrexate, acyclovir) [45,46]. Since the histopathology in these different types of crystal deposition shows important parallels with classical (ureteral) obstructive nephropathy, the bulk of the associated tubulo-interstitial changes most likely results from obstruction itself rather than of a chemical (nephrotoxic) effect/contribution of the different types of retained crystals. With respect to nephrolithiasis, it has been observed that in patients with primary hyperparathyroidism and calcium phosphate stones, patients with brushite or cystine stones and patients with distal renal tubular acidosis, tubular obstruction presents itself as calcium phosphate (cystine in cystinuria) crystal plugging of the ducts of Bellini with crystals protruding out of the papillary slits/mouths into the pelvic lumen [47–50]. It is hypothesized that these crystal plugs, besides inducing fibrosis, tubular atrophy and even glomerular pathology [50], can form the nidus or platform for stone formation in these kidneys (see Figure 1).

In less severe/acute forms of nephrocalcinosis, as found in transplant patients and preterm infants, the effect of mere crystal adhesion might be more straightforward. Indeed, numerous *in vitro* studies, mimicking these non-obstructive crystal-cell interactions, investigate the epithelial reaction to CaP and CaOx crystal contact (either apical or basolateral) and report production of inflammatory mediators (MCP-1, PGE-2), reactive oxygen species (H2O2) and release of LDH, thereby marking crystal-induced injury [21,29,51–53]. However, despite these reactions *in vitro*, up to now a clinical detrimental effect of crystal–cell contact or adhesion is not unequivocally proven *in vivo*. In kidney transplant patients, Pinheiro *et al.* reported a 12-year allograft survival rate of 75% in the absence of nephrocalcinosis, whereas in the presence of nephrocalcinosis, allograft survival decreased to 48% [54]. Although these data suggest an association between nephrocalcinosis and an increased risk of allograft failure, it should be noted that half of the allografts survive despite the presence of nephrocalcinosis. Also, in several prospective and retrospective studies, in which preterm infants with nephrocalcinosis were compared with birth-weight- and postnatal (or gestational) age-matched controls without nephrocalcinosis, no clear evidence for an association between neonatal nephrocalcinosis and renal dysfunction in the long term was found [55–58].

Overall, it is likely that the individual renal outcome depends on numerous factors, such as the severity of the underlying disorder and the extent, rate and duration of crystal formation/adhesion on the one hand and the activity of renal crystal clearing mechanisms on the other (see further in the text) [34,59,60,61]. Possibly, adhered crystals may affect normal tubular redifferentiation/regeneration hampering restoration of a sufficient amount of functioning tubes and, in addition, may further enlarge by growth and aggregation with other crystals leading to obstructive tubulopathy.

**Interstitial nephrocalcinosis**

**Mechanisms of interstitial nephrocalcinosis**

The presence of crystals in the renal interstitium is defined as interstitial nephrocalcinosis. Two independent mechanisms may explain the appearance of these crystals in the interstitium: translocation of intratubular crystals and *de novo* interstitial crystal formation.

It has been hypothesized that translocation of crystals can be established via *transcytosis*, a process during which small intraluminal crystals are internalized within apical vesicles (either receptor mediated or not) and translocated transcellularly to the basolateral side where the crystals are released into the interstitial extracellular environment [62]. Although apical *endocytosis* of small crystals has been described [28,59,60], to the best of our knowledge, there is no evidence supporting the basolateral release of crystals into the interstitium. Instead, these crystals most likely disintegrate into lysosomes [60,61]. Recently, in an *in vivo* study we described an alternative mechanism of transepithelial crystal *translocation*, which also has been reported under the term ‘exotubulosis’ by De Bruijn and co-workers [34,63,64]. These studies demonstrated that intratubular adhered crystals can be overgrown by tubular epithelial cells adjacent to the crystal adhesion site. Within 2 weeks, these proliferating and migrating cells cover the crystals and differentiate into a new mature epithelium, with its basement membrane on top of the crystals and its apical side directed to the lumen, thereby restoring epithelial integrity of the affected tubule (see Figure 1).

Both *endocytosis* and *epithelial overgrowth* do not seem to be pathologic processes, and hence can be considered defence mechanisms against renal calcification since crystals disappear from the healthy kidney either in intracellular vesicles (lysosomes) or via the extracellular interstitium [34,60,61,65]. Currently, it is thought that crystals disintegrate/dissolve due to a local decrease in pH and
exposure to proteases [34,61]. Deficiency or saturation of these clearance mechanisms would reasonably result in tubular and/or interstitial crystal accumulation. It is currently unknown, however, what happens to the ionic constituents of the crystals after being dissolved. Are they immediately cleared from the kidney or do they accumulate by binding to interstitial macromolecular constituents (such as hyaluronan) and, at a later stage, initiate interstitial supersaturation and \textit{de novo} crystal formation during the turnover of these macromolecules?

Besides \textit{translocation}, crystals can also be formed \textit{de novo} in the interstitium. It is not known, however, whether this is merely due to a chemically driven supersaturation or whether cells are involved. Since up to now, the presence of osteoblast-like cells has not been reported in the parenchyma of calcified kidneys, no cell-mediated pathological bone-forming process, similar to that described for vascular calcification [66], can be suggested in the kidney at the moment. Regardless of the mechanism, \textit{de novo} crystal formation is thought to be the process by which the development of Randall's plaque is initiated (see Figure 1). In the clearest form of Randall's plaque formation, as found in biopsies of patients with idiopathic calcium oxalate nephrolithiasis (and some healthy controls), ultrastructural studies did not reveal intratubular or intracellular crystals. The smallest mineral deposits observed were scattered nano-sized, laminated apatite-protein particles in the basement membranes of the thin loops of Henle [33,67]. Although the specific mechanisms are still unclear, it is known that these particles may extend into the medullary interstitium and outgrow further towards the papillary surface [67]. There, as an aggregated mineral syncytium, they can either lie beneath the urothelium or be exposed to the urinary environment after the loss of urothelial integrity [33,67,68].

Various mineral types can be found in the interstitium, e.g. calcium phosphate, calcium oxalate and adenine [33,34]. Although translocation by epithelial overgrowth gives a plausible explanation for the appearance of these mineral deposits in the interstitium, \textit{de novo} formation of crystals other than calcium phosphate has to be considered. However, up to now, no 'non-calcium phosphate' interstitial crystal/particle outgrowth, histopathologically comparable to classical Randall's plaque formation, has been found [47,48,50]. Also, current methodology does not allow for the assessment of whether interstitial crystals originated in the tubular lumen or were formed directly in the interstitium. Therefore, it does not allow for the assessment of whether or not in acute phosphate nephropathy, for example, translocation is the main mechanism by which calcium phosphate occasionally appears in the interstitium [34,43]. Identifying specific urinary or interstitial proteins incorporated in the crystals might provide clues on their site of origin [69,70].

\textbf{Consequences of interstitial nephrocalcinosis}

Currently, no evidence has been provided showing that mere interstitial nephrocalcinosis, whether \textit{de novo} or acquired via translocation, impairs renal function. Randall's plaque formation is not associated with inflammation, does not damage epithelial cells and starts in basement membranes of morphologically normal epithelial cells of Henle's loop. Subsequently, when further interstitial crystal outgrowths lie in the vicinity of collecting ducts and ducts of Bellini, these epithelia show no morphological abnormalities [33]. Only when calcification completely surrounds the thin loops of Henle, is an association with epithelial injury found [33]. In addition, extensive calcification might present itself as a physical interstitial barrier impairing proper medullary/papillary function. Concentrating ability, however, seems not to be influenced since a correlation between low urinary volume (and high urinary calcium) and papillary plaque coverage was found [71]. Whether the overall morphological absence of epithelial injury and interstitial inflammation/fibrosis in the context of Randall's plaque formation is due to the crystal type (consistently being calcium phosphate) or to the site of origin is not known.

The main currently known clinically important feature of Randall's plaques is their proposed anchor function for stones found attached to the papilae of patients with idiopathic calcium oxalate nephrolithiasis [33,72]. Moreover, ultrastructural studies corroborate the hypothesis that Randall's plaques actually might serve as a platform for stone development by progressive alternation of successive protein matrix deposition and crystal nucleation (see Figure 1) [68]. This concept already was proposed by Alexander Randall in the late 1930s [73,74]. He observed calcium phosphate lesions (plaques) on the papillary surface and noticed that renal stones were intimately attached to them. In addition, the mineral composition of the plaque (being calcium phosphate) was different from that of the stones and the plaques occurred in higher incidence than clinical renal stones. Based on these observations, he concluded that renal stones were originating as a slow deposition/crystallization of urinary salts (calcium oxalate, calcium phosphate, uric acid) upon a lesion of the renal papilla. These observations have recently been confirmed and extended for patients with idiopathic calcium oxalate nephrolithiasis [33,68,72].

In contrast to Randall's plaques, initially appearing innocuous, it is known that interstitial CaOx crystals can be associated with inflammatory cells [34,75,76]. Whether this is due to a higher reactivity of CaOx is not known. Although it has been shown that fibroblasts can produce inflammatory mediators upon contact with oxalate ions and calcium oxalate crystals [53], it is of interest that interstitial crystals and associated inflammation are always associated with intraluminal crystal formation. The recruitment of inflammatory cells may therefore already be initiated by prior luminal crystal–cell interactions inducing the production of inflammatory mediators [21,29,51–53]. Altogether, it can be suggested that interstitial inflammation can only be found in disorders in which interstitial crystal deposition is accompanied with intraluminal crystal formation and/or handling.

\textbf{Concluding paragraph}

Nephrocalcinosis is a complex multifactorial histopathology presenting itself via different mechanisms, each having their own pathological course and mutually not exclusive. Recent findings have made it possible to gain some new insights into this complexity.
A sufficient number of experimental and clinical observations strongly suggest that crystals do not adhere to the normally differentiated tubular epithelium, but rather attach to dedifferentiated/regenerating epithelial cells [5,6,8,24,30]. We state that the shift of a normal tubular epithelial phenotype into a phenotype capable of binding crystals can be induced by many unrelated insults/conditions. Furthermore, if an epithelium binds crystals, kidneys can react by several crystal clearing mechanisms involving crystal degradation and dissolution, such as endocytosis and epithelial overgrowth (‘exotubulosis’) [34,60,61,63,64]. However, when clearance mechanisms are surpassed or compromised due to some underlying disease state or clinical condition, intratubular crystals may accumulate and add to the severity of that particular disorder. This might explain why half of the renal allografts with nephrocalcinosis deteriorate more rapidly [54]. Most likely, these grafts are already in a suboptimal state, favoring nephrocalcinosis, why half of the renal allografts with nephrocalcinosis deteriorate more rapidly [54]. Most likely, these grafts are already in a suboptimal state, favoring nephrocalcinosis, and hence accelerating chronic allograft deterioration. In this view, nephrocalcinosis may be considered a marker of a less potent allograft.

Whereas intratubular nephrocalcinosis seems to develop secondary to an underlying renal disorder/condition, the initiating event in interstitial nephrocalcinosis in the context of Randall’s plaque formation does not seem to be associated with any kind of prior tubulo-interstitial morphological aberration [33] or decreased renal function. However, determining the mechanism initiating Randall’s plaque formation is complicated by the lack of relevant experimental models. Importantly, a recent study showed that stone formation (one probable consequence of Randall’s plaques) is associated with a 60% increased risk of developing chronic kidney disease (Rule A et al. Abstract F-FC202 at the 41st Annual Meeting of the American Society of Nephrology, 2008). Although this study does not show causation, or does not include the type of stones or their possible initiating mechanisms, it demonstrates the importance for continuous efforts to unravel the exact mechanism of nephrolithiasis (and by extension that of nephrocalcinosis), which, in the long run, would allow for the establishment of preventive strategies.

Conflict of interest statement. None declared.

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