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Understanding the Pathophysiology of Nephrocalcinosis

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Abstract

Many *in vitro* and *in vivo* studies on the mechanisms underlying calcium nephrolithiasis have provided evidence of a frequently associated condition, i.e., a microscopic renal crystal deposition that can occur within the tubular lumen (intratubular nephrocalcinosis) or in the interstitium (interstitial nephrocalcinosis). Medullary nephrocalcinosis is the typical pattern seen in 98% of cases of human nephrocalcinosis, with calcification clustering around each renal pyramid. It is common in patients with metabolic conditions that predispose them to renal calcium stones. Cortical nephrocalcinosis is rare and usually results from severe destructive disease of the cortex. It has been described in chronic glomerulonephritis, but often in association with another factor, such as an increased calcium ingestion, acute cortical necrosis, chronic pyelonephritis or trauma. The most accredited hypothesis to explain the onset of interstitial nephrocalcinosis is purely physicochemical, relating to spontaneous Ca_2PO_4 crystallization in the interstitium due to oversaturation of Ca_2PO_4 salts in this milieu. The theory that nephrocalcinosis is a process driven by osteogenic cells was first proposed by our group. We review nephrocalcinosis in terms of its definition, genetic associations, and putative mechanisms, pointing out how much evidence in the literature suggests that it may have some features in common with, and pathogenic links to vascular calcification.

Keywords: nephrocalcinosis, genetics, Randall's plaque, calcium crystals, vascular calcification, osteogenic transdifferentiation

1. Introduction

Nephrolithiasis is a common disease, typically occurring between 30 and 60 years of age. It is the most often-diagnosed chronic condition involving the kidney, after hypertension. The symptoms and consequences are not life threatening for the majority of patients, but stones in the urinary tract are a major cause of morbidity, hospitalization, and days lost from work [1]. The incidence of nephrolithiasis is increasing. In Italy, for example, the number of patients given hospital treatment for this condition rose between 1988 and 1993 from 60,000 to 80,000 a year. About 12,000 patients a year required surgical treatment or urological maneuvers, and the number of extracorporeal shock wave lithotripsy sessions administered amounted to approximately 50,000 a year [2].

The metabolic characteristics of the urinary stones identified in patients with nephrolithiasis vary, but the most common (accounting for 75% of all cases) are calcium-containing stones. Calcium oxalate (CaOx) is the primary component of most stones [3], often combined with some calcium phosphate (CaP), which may form the stone's initial nidus. Crystal retention in the kidney is essential to stone formation and this occurs with several different patterns of deposition in the kidneys of stone formers, each pattern being associated with specific types of stone. Patients with idiopathic CaOx stones have white deposits on their papillae called "Randall's plaque" [4]. Biopsies of these areas reveal interstitial deposits of CaP in the form of biological apatite, which first develop in the basement membrane of the thin loops of Henle and which contain layers of protein matrix. These deposits may extend down to the tip of the papilla and, if the overlying urothelium is denuded, the exposed plaque can become an attachment site for stones [5]. Stones seem to start as deposits of amorphous CaP overlying the exposed plaque, interspersed with urinary proteins. With time, more layers of protein and mineral are deposited, and the mineral phase becomes predominantly CaOx.

By contrast, in patients whose stones consist mainly of CaP (apatite or brushite), these stones are not attached to plaque. Instead, many collecting ducts fill with crystal deposits that occupy the tubule lumen and may protrude from the openings in the ducts of Bellini. Generally speaking, most stone formers studied to date have had crystal deposits in the medullary collecting ducts, with the exception of those with idiopathic CaOx stones, who have no intratubular deposits, but abundant deposits of apatite in the papillary interstitium.

Calcium nephrolithiasis, the most common renal form of stone disease, is defined as the formation of macroscopic concretions of inorganic and organic material in the renal calyces and/or pelvis. Many *in vitro* and *in vivo* studies on the mechanisms underlying calcium nephrolithiasis have produced evidence of this condition frequently being associated with nephrocalcinosis, a condition involving microscopic renal crystal deposition.

2. Nephrocalcinosis

Strictly speaking, the term "nephrocalcinosis" refers to the generalized deposition of CaP or CaOX in the kidney, which can occur within the tubular lumen (*intratubular nephrocalcinosis*) or in the interstitium (*interstitial nephrocalcinosis*). Some authorities restrict the definition of

nephrocalcinosis to the deposition of CaP crystals in the interstitium. Randall's plaque could be an example of interstitial nephrocalcinosis.

It has been suggested in Refs. [6, 7] that nephrocalcinosis should be divided into three categories: *molecular nephrocalcinosis*, involving an increase in renal intracellular calcium without any crystal formation and essentially reflecting the renal dysfunction of hypercalcemia; *microscopic nephrocalcinosis*, in which CaP or CaOX crystals are visible on light microscopy, but not radiologically; and macroscopic nephrocalcinosis, when calcification is visible radiologically or on ultrasound scans. Nephrocalcinosis does not necessarily lead to renal stones, and renal stones may occur without any apparent macroscopic nephrocalcinosis, so these two conditions are distinct but closely related [6].

As for the sites involved, nephrocalcinosis can be divided into cortical and medullary nephrocalcinosis.

Cortical nephrocalcinosis is rare and usually results from severe destructive disease of the cortex. This condition has been described in chronic glomerulonephritis, though often in association with another factor, such as an increased calcium ingestion, acute cortical necrosis, chronic pyelonephritis, or trauma [8, 9], autosomal recessive polycystic kidney disease, primary and secondary oxalosis, chronic renal allograft rejection, or benign nodular cortical nephrocalcinosis [7]. Three different patterns of cortical nephrocalcinosis have been identified radiologically [10]. In the most common pattern, there is a thin peripheral band of calcification, often extending into the septal cortex. In a second type, there is a double line of calcification along the two sides of the necrotic zone in the cortex (what Lloyd Thomas et al. called "tram line" calcification in describing the pattern of nephrocalcinosis seen in obstetric cases of cortical necrosis [11]). The least common pattern consists of multiple punctate calcifications randomly distributed in the renal cortex.

Medullary nephrocalcinosis is the typical form seen in 98% of cases of human nephrocalcinosis. It forms clusters of calcification around each renal pyramid. It is common in patients with metabolic conditions (several of which are monogenic diseases) that predispose them to renal calcium stones. Knowing which genes are involved can help to shed light on the mechanisms behind nephrocalcinosis.

2.1. Genetics of Conditions predisposing to medullary nephrocalcinosis

Stone initiation and growth is prompted by the urine becoming supersaturated with a solute of calcium, oxalate, uric acid, and cystine, which leads the dissolved salts to condense into solids, thus forming the stone. But urinary CaOx supersaturation is a common finding in normal individuals too, who develop no stones. This is most likely due to the presence of crystallization inhibitors such as citrate or pyrophosphate in their urine. In addition to the concentration of solutes, urinary pH is a crucially important factor influencing crystal solubility. Supersaturation and crystallization in the urine also rely on the presence of macromolecules capable of binding and forming complexes with Ca and Ox. Mammalian urine contains numerous macromolecules that inhibit crystal formation, growth, and aggregation in the kidney. Levels of supersaturation and crystallization are kept under control by the proper functioning of a variety of cells lining the renal tubules [12, 13].

Conditions predisposing to medullary nephrocalcinosis may be either those that raise the urinary concentration of inductors of calcium crystal deposition or those that lower the concentration of the inhibitors of this process. The former category includes hypercalciuria, hyperoxaluria, the latter hypocitraturia and hypomagnesuria. Renal tubular acidosis (RTA), on the other hand, is responsible for changes in urinary pH, which has a fundamental role in favoring crystallization. In some cases, there may also be specific anatomical abnormalities that predispose to the onset of nephrocalcinosis, as in medullary sponge kidney (MSK).

Several genetic disorders have been found associated with conditions that predispose individuals to the development and progression of nephrocalcinosis. Most of them are tubular disorders associated with epithelial cellular and paracellular ion transport disruptions that result in the urinary excretion of higher levels of calcium, phosphate or oxalate and lower levels of citrate and magnesium. **Table 1** shows the genetic basis for the link between some inherited disorders and medullary nephrocalcinosis [7]. **Figure 1** shows the list of intrarenal transport defects that prompt a dysfunctional renal handling of the two most important divalent cations Ca^{2+} and Mg^{2+} [14]. As can be noted, not all cation-handling disorders are associated with nephrocalcinosis.

The kidney handles calcium, phosphate, and oxalate in the proximal tubules, in the thick ascending limb (TAL) of the loop of Henle, and in the distal convolute tubule (DCT), shown in **Figure 2** [6]. Knowing the site where the tubular exchanger and transporter proteins involved in regulating urinary calcium, phosphate, and oxalate work help us better understand the mechanisms underlying nephrocalcinosis.

Bearing in mind that 98% of interstitial crystal deposition occurs in the medulla around each pyramid, Sayer et al. proposed the model of nephrocalcinosis shown in **Figure 3**.

We focus our attention on the genetic defects that alter the kidney's homeostatic capacity.

2.2. Renal calcium handling

Of all the calcium filtered by the kidney, 98% is reabsorbed by the tubules, with the proximal tubule reabsorbing about 65%, the TAL of the loop of Henle accounting for approximately 20–25%, and 8–10% being reabsorbed in the distal tubule [15]. Hypercalciuria is an important, identifiable, and reversible nephrocalcinosis risk factor. It is a complex trait, caused by both environmental and genetic factors. It is not a disease per se, but represents the upper end of a continuum, rather like height, weight, and blood pressure, and—like these polygenic traits—urinary calcium excretion should be considered a graded risk factor [16]. **Table 2** shows a summary of the clinically and experimentally identified monogenic causes of hypercalciuria, pointing to the genetic causes of renal calcium leak.

It is worth remembering that the crystallization of calcium salts is a physiological event linked to biomineralization, i.e., the capacity of calcium, like other inorganic crystalline or non-crystalline minerals, to interact with and deposit around biomolecules, becoming an integral part of organic tissues to provide hardness and strength. Biomineralization is often arbitrarily distinguished as physiological or pathological. It would be more appropriate to say that pathological calcium crystallization is a physiological process occurring in the wrong place and at the wrong time [17]. Nephrocalcinosis might fit this definition.

Gene	Protein	Disorder	Mode of inheritance
ATP6V1B1	β 1 Subunit of H ⁺ - ATPase	Distal renal tubular acidosis (dRTA)	AR
ATPV0A4	α 4 Subunit of H ⁺ - ATPase	Distal renal tubular acidosis (dRTA) with neural deafness at birth or late onset	AR
SLC12A1	Sodium-potassium-chloride transporter (NKCC2)	Bartter syndrome type 1	AR
KCNJ1	Potassium channel (ROMK1)	Bartter syndrome type 2	AR
BSND	Barttin	Bartter syndrome type 4	AR
CLCNKB	Chloride channel ClC-Kb	Bartter syndrome type 3	AR
CASR	Calcium-sensing receptor (CasR)	Hypocalcemia with hypercalciuria	AD
CFTR	ATP-binding cassette transporter	Cystic fibrosis	
CLDN16	Claudin-16, tight junction	Familial hypomagnesemia with hypercalciuria and nephrocalcinosis	AR
CLDN19	Claudin-19, tight junction	Familial hypomagnesemia with hypercalciuria and nephrocalcinosis with ocular impairment	AR
SLC4A1	AE1	Distal renal tubular acidosis (dRTA) with neural deafness at birth or late inset	AD
SLC34A3	Sodium-dependent phosphate transporter protein 2C (NPT2C)	Hereditary hypophosphatemic rickets with hypercalciuria (HHRH)	AR
CLCN5	Chloride channel 5 (ClC-5)	Dent disease 1	XLR
OCRL	Phosphatidylinositol 4,5-bisphosphate 5-phosphatase (OCRL1)	Dent disease 2 or Lowe's syndrome	XLR
PHEX	Phosphate-regulating endopeptidase	Hypophosphatemic rickets	XLD
FAM20A	Pseudokinase FAM20A	MacGibbon-Lubinsky syndrome	AD
AGXT	Alanine glyoxylate aminotransferase	Primary hyperoxaluria (PH type I)	AR
GRHPR	Glyoxylate reductase	Primary hyperoxaluria (PH type 2)	AR
ELN, LIMK1	Elastin	Williams-Beuren syndrome (WBS)	AR

Gene	Protein	Disorder	Mode of inheritance
GDNF	Glial cell line-derived neurotrophic factor	Medullary sponge kidney (MSK)	AD
SLC7A9	B(0,+)-type amino acid Transporter 1 (BAT1)	Cystinuria	AD
ADCY10	Adenylate Cyclase 10, Soluble	Familial idiopathic hypercalciuria	AD
SCNN1G/B	Renal epithelium channel (β ENaC and α ENaC)	Liddle syndrome	AD
ATP7B	Copper-transporting ATP-ase	Wilson syndrome	AR

AD, autosomal dominant; AR, autosomal recessive; XLR, X-linked recessive; XLD, X-linked dominant.

Table 1. Inherited disorders associated to medullary nephrocalcinosis (NC).

2.3. Renal phosphate handling

The kidney's control over systemic phosphate homeostasis is crucial. About 80% of filtered phosphate is reabsorbed from the urine by transporters located in the proximal tubule and mostly in the juxtamedullary nephrons (**Figure 2**). At least three transporters are responsible for renal phosphate reabsorption, and they are precisely regulated by various cellular mechanisms and factors [18]. They are members of the Type II Na^+ -dependent phosphate cotransporter family encoded by the SLC34A1, SLC34A3, and SLC20A2 genes. Though it is not a

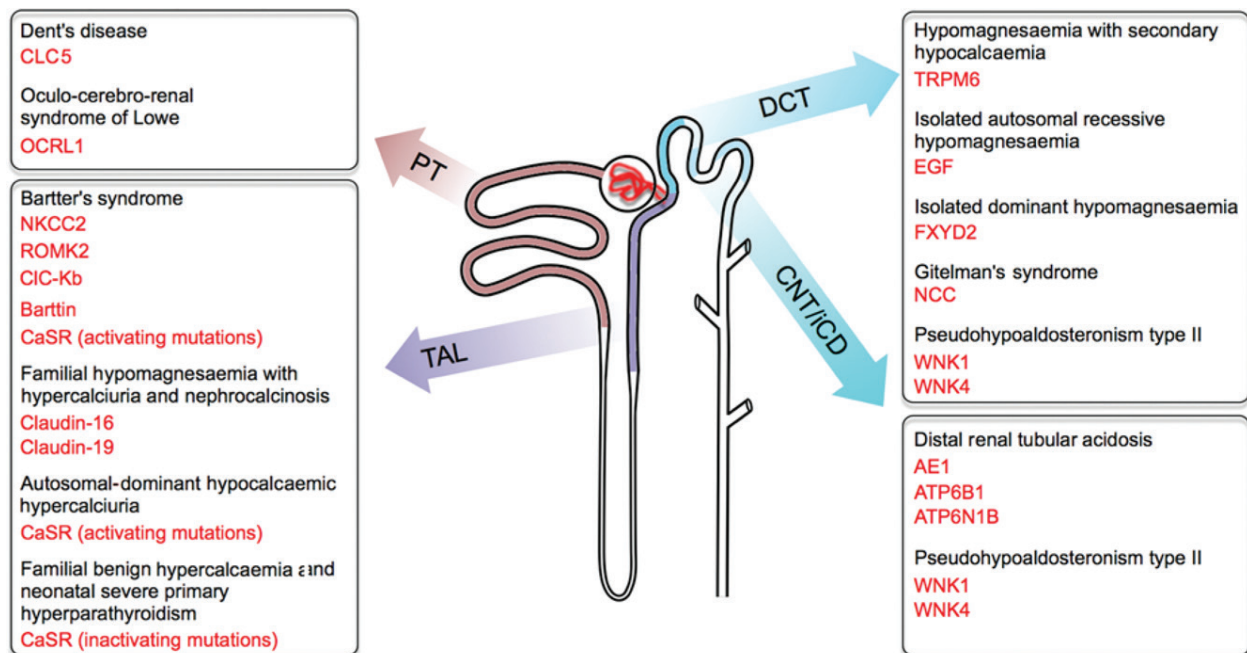


Figure 1. Schematic representation of the nephron listing the predominant origins of intrarenal transport defects causing dysfunctional renal handling of divalent cations. This figure was originally published in Ref. [14].

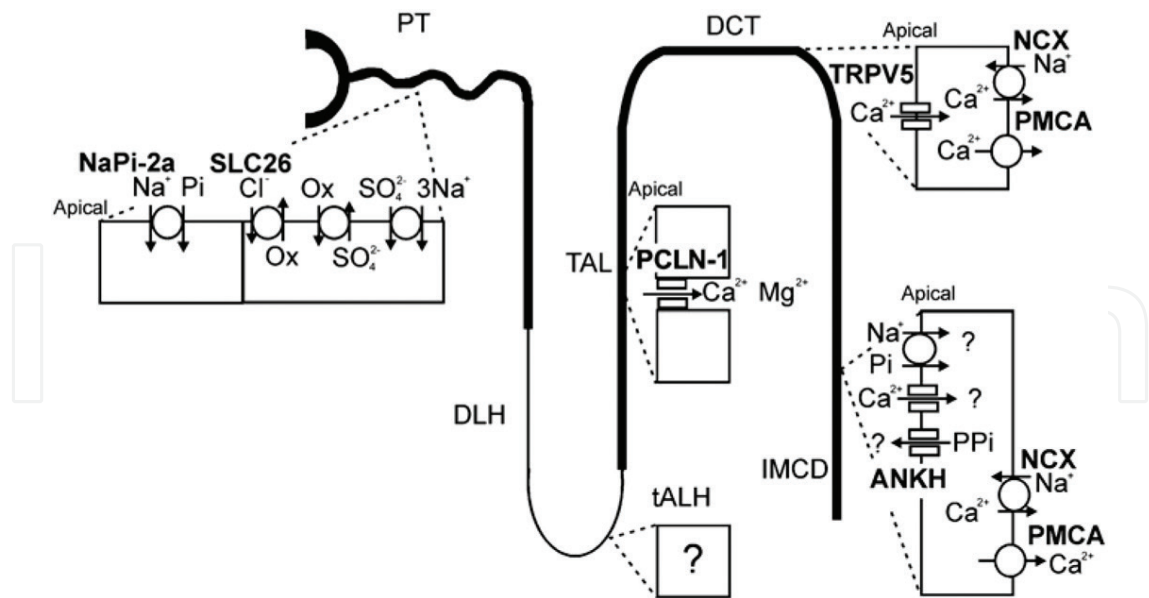


Figure 2. Tubular transport of calcium, phosphate, and oxalate. A nephron map of exchanger and transporter proteins involved in the regulation of urinary calcium, phosphate and oxalate is shown. In the proximal tubule (PT) apical Na/Pi cotransporters (NaPi-2a) mediate phosphate reabsorption, whereas several anion exchange proteins (including those of the SLC26 family) mediate transcellular oxalate (Ox) secretion and recycling. There is no evidence for calcium or phosphate transport in the thin descending limb (DLH) or the thin ascending limb (tALH) of the loop of Henle. The TAL of Henle allows paracellular calcium (and magnesium) reabsorption via paracellin channels (PCLN-1). The distal convoluted tubule (DCT) allows for regulated transcellular reabsorption of calcium via apical TRPV5 and the basolateral NCX, together with the basolateral PMCA. A calcium entry pathway exists within the IMCD, the molecular identity of which remains uncertain. It is likely that Na/Pi isoforms are also present in this nephron segment and their physiological function may be linked to the PPi transporter protein ANKH. Basolateral calcium exporters NCX and PMCA are also present in the collecting duct. This figure was originally published in Ref. [6].

transporter protein, the Na⁺/H⁺ exchanger regulatory factor (NHERF1) plays a crucial part in renal phosphate transport by binding to SLC34A1 in the proximal tubule. Alterations in the genes encoding these transporters result in phosphate wasting, and consequent hyperphosphaturia (**Table 3**). For the sake of completeness, **Table 3** also includes renal phosphate handling impairments due to extrarenal inherited defects.

2.4. Renal oxalate handling

Urinary oxalate is the most important risk factor for CaOx nephrocalcinosis/nephrolithiasis. Oxalate is filtered freely at the glomerulus [6]. Anion exchange proteins in the proximal tubule mediate oxalate excretion and recycling at the brush border membrane (**Figure 2**). These proteins belong to the SLC26 family, and they allow oxalate loss in exchange for chloride, then uptake oxalate in exchange for sulfate loss, energized by Na-sulfate transport in the proximal tubules [19]. The main causes of hyperoxaluria relate, however, to genetic defects that alter glyoxylate metabolism in the liver and erythrocytes, leading to endogenous oxalate overproduction. These hereditary autosomal recessive forms of hyperoxaluria are called primary hyperoxaluria type I, type II, and type III [20]. Defects in the genes responsible for oxalate reabsorption have recently been reported too. Recessive mutations in SLC26A1 gene were identified in two unrelated individuals with calcium oxalate kidney stones. Functional experiments have

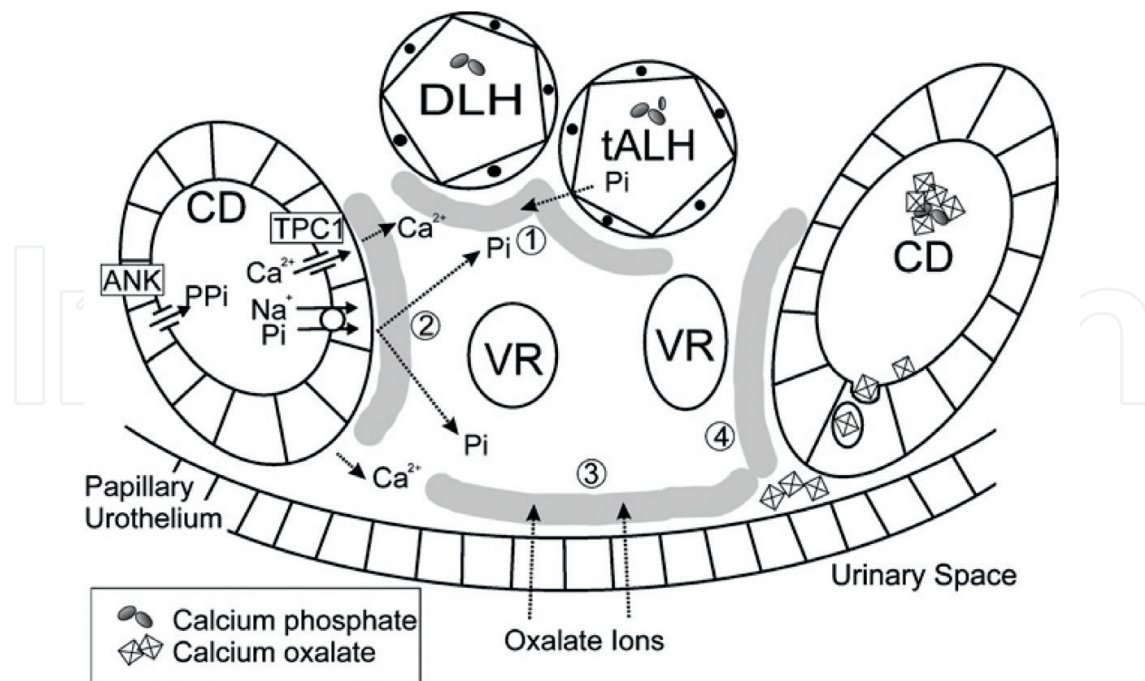


Figure 3. Renal papillary model of nephrocalcinosis. Collecting ducts (CD) together with descending loops of Henle (DLH) and ascending thin loops of Henle (tALH) and vasa recta (VR) are shown through a cross-section of a renal papilla. Possible initial sites of nephrocalcinosis are shown as shaded regions. Mechanisms leading to calcification may include: (1) Pi permeability at the papillary thin loops of Henle would allow interstitial loading of phosphate; (2) collecting duct absorption of calcium, possibly via apical calcium channels (e.g., TPC1) and basolateral calcium exit would allow delivery of calcium ions to the interstitium; Na/Pi cotransport at a collecting duct location would also provide additional phosphate ions, which may be derived from PPI delivery into the lumen via ANK; (3) concentration of oxalate from the urinary space into the papillary interstitium allows delivery of oxalate ions; (4) intraluminal crystal formation, both from the loop of Henle (calcium phosphate) and collecting duct (calcium oxalate) may adhere to collecting duct epithelial surfaces, then by endocytosis/transcytosis the crystals are delivered to the papillary interstitium and accumulate. Dissolution by epithelial cells or by interstitial cells (including macrophages) may provide a clearance mechanism. This figure was originally published in Ref. [6].

shown that these mutations resulted in decreased transporter activity [21], thus confirming their role in the disease. In the SLC26A6 gene has also recently been described a single nucleotide polymorphism associated with increased calcium oxalate kidney stones [22]. **Table 4** summarizes what we know about the genetics of hyperoxaluria.

2.5. Renal citrate handling

Citrate is filtrated freely at the glomerulus. In humans, from 65 to 90% of the filtered citrate is reabsorbed, mainly in the proximal tubule [23]. Urinary citrate is an important calcium chelator, consequently reducing the potential of calcium and oxalate to interact. In addition, citrate binds crystals' surface preventing their adhesion to renal epithelial cells [24]. It is intriguing that oxalate transport by SLC26A6 and citrate transport by the sodium dicarboxyl cotransporter SLC13A2—both located in the apical membrane of the proximal tubules and small intestine—have been found to interact. This was demonstrated in SLC26A6 KO mice, which are not only hyperoxaluric, but also hypocitraturic [25]. Hypocitraturia is a known risk factor for the development

Gene	Protein	Disorder	Mode of inheritance
ATP6V1B1	β 1 Subunit of H ⁺ - ATPase	Distal renal tubular acidosis (dRTA)	AD
ATPV0A4	α 4 Subunit of H ⁺ - ATPase	Distal renal tubular acidosis (dRTA) with neural deafness at birth or late onset	AR
SLC12A1	Sodium-potassium-chloride transporter (NKCC2)	Bartter syndrome type 1	AR
KCNJ1	Potassium channel (ROMK1)	Bartter syndrome type 2	AD
CLCNKB	Chloride channel ClC-Kb	Bartter syndrome type 3	AR
BSND	Barttin	Bartter syndrome type 4	AR
MAGED2	Melanoma associated antigen D2	Bartter syndrome type 5	XLR
CASR	Calcium-sensing receptor (CasR)	Hypercalciuria with hypocalcemia	AD
G6PC	Glucose-6-phosphatase	Glycogen storage disease type 1a	AR
FAH	Fumarylacetoacetase	Tyrosinemia type 1	AR
ATP7B	Copper-transporting ATP-ase	Wilson disease	AR
CLCN5	Chloride channel 5 (ClC-5)	Dent disease 1	XLR
OCRL	Phosphatidylinositol 4,5-bisphosphate 5-phosphatase (OCRL1)	Dent disease 2, Lowe syndrome	XLR
CLDN16	Claudin-16, tight junction	Familial hypomagnesemia with hypercalciuria	AR
CLDN19	Claudin-19, tight junction	Familial hypomagnesemia with hypercalciuria	AR
SLC34A1	Sodium-phosphate transport protein 2A	Fanconi renal tubular syndrome 2	AR
ADCY10	Adenylate Cyclase 10, Soluble	Familial idiopathic hypercalciuria	AD
KLOTHO	Regulator of calcium homeostasis (Klotho)	Tumoral calcinosis, hyperphosphatemic	AR

AD, autosomal dominant; AR, autosomal recessive; XLR, X-linked recessive.

Table 2. Monogenic forms of hypercalciuria: kidney as the primary defect.

Gene	Protein	Disorder	Mode of inheritance
SLC34A1	Sodium-phosphate transport protein 2A	Fanconi renal tubular syndrome 2, Hypophosphatemic nephrolithiasis/osteoporosis-1	AD
SLC34A3	Sodium-phosphate transport protein 2C	Hereditary hypophosphatemic rickets with hypercalciuria (HHRH)	AR
SLC9A3R1	Na ⁺ /H ⁺ exchange regulatory cofactor NHE-RF1	Hypophosphatemic nephrolithiasis/osteoporosis-2	AD
FGF23	Fibroblast growth factor 23	Hypophosphatemic ricket	AD
KLOTHO	Regulator of calcium homeostasis (Klotho)	Tumoral calcinosis, hyperphosphatemic	AR
GALNT3	Polypeptide N-acetylgalactosaminyltransferase 3	Tumoral calcinosis, hyperphosphatemic	AR
PHEX	Phosphate-regulating neutral endopeptidase	Hypophosphatemic rickets	XLD
FAM20C	Extracellular serine/threonine protein kinase	Raine syndrome	AR
FGFR1	Fibroblast growth factor receptor 1	Osteoglophonic dysplasia	AD
DMP1	Dentin matrix acidic phosphoprotein 1	Hypophosphatemic rickets	AR

AD, autosomal dominant; AR, autosomal recessive; XLD, X-linked dominant.

Table 3. Genetic basis of altered renal phosphate handling.

of nephrocalcinosis/nephrolithiasis. No monogenic form of hypocitraturia has been reported so far, whereas genetic associations have been demonstrated between polymorphisms in the VDR and SLC13A2 genes and hypocitraturia [26, 27]. Very recently, Rendina et al. [28] provided evidence of an epistatic interaction between VDR and SLC13A2 in the pathogenesis of hypocitraturia. This may come as no surprise because the active form of vitamin D in the nephron uses VDR to modulate citrate metabolism and transport [26]. Finally, Shah et al. [29] have suggested

Disorder	Gene	Protein	Mode of inheritance
Hyperoxaluria type I	AGXT	Serine-pyruvate aminotransferase	AR
Hyperoxaluria type II	GRHPR	Glyoxylate reductase/hydroxypyruvate reductase	AR
Hyperoxaluria type III	HOGA1	4-hydroxy-2-oxoglutarate aldolase, mitochondria	AR
Calcium oxalate kidney stones	SLC26A1	Sulfate anion transporter 1	AR

PH, primary hyperoxaluria; AR, autosomal recessive.

Table 4. Inherited disorders of renal oxalate handling.

other genetic influences on citrate handling too: they propose a codominant inheritance of alleles at a single locus based on their trimodal frequency distribution of citrate excretion.

2.6. Renal magnesium handling

Hypomagnesuria is the biochemical abnormality found in about 19% of kidney stone patients, alone or in association with other biochemical abnormalities [30]. The kidney has a key role in maintaining a normal magnesium balance. The TAL of the loop of Henle and the DCT are crucially important in regulating serum magnesium levels and body magnesium content (Figure 2). Understanding the molecular defects behind rare genetic magnesium loss disorders has greatly contributed to our understanding of renal magnesium handling. About 80% of all plasma magnesium is filtered through the glomeruli, and 15–20% of it is reabsorbed by the proximal tubules, and 55–70% by the cortical TAL [31]. Magnesium is reabsorbed via a paracellular pathway in this nephron segment. Members of the claudin family of tight junction proteins have been attributed a role in controlling magnesium and calcium permeability of the paracellular pathway (Figure 4) [31]. Although only 5–10% of the filtered magnesium is reabsorbed in the DCT, this process is finely regulated and plays an important part in determining its final urinary excretion [31, 32].

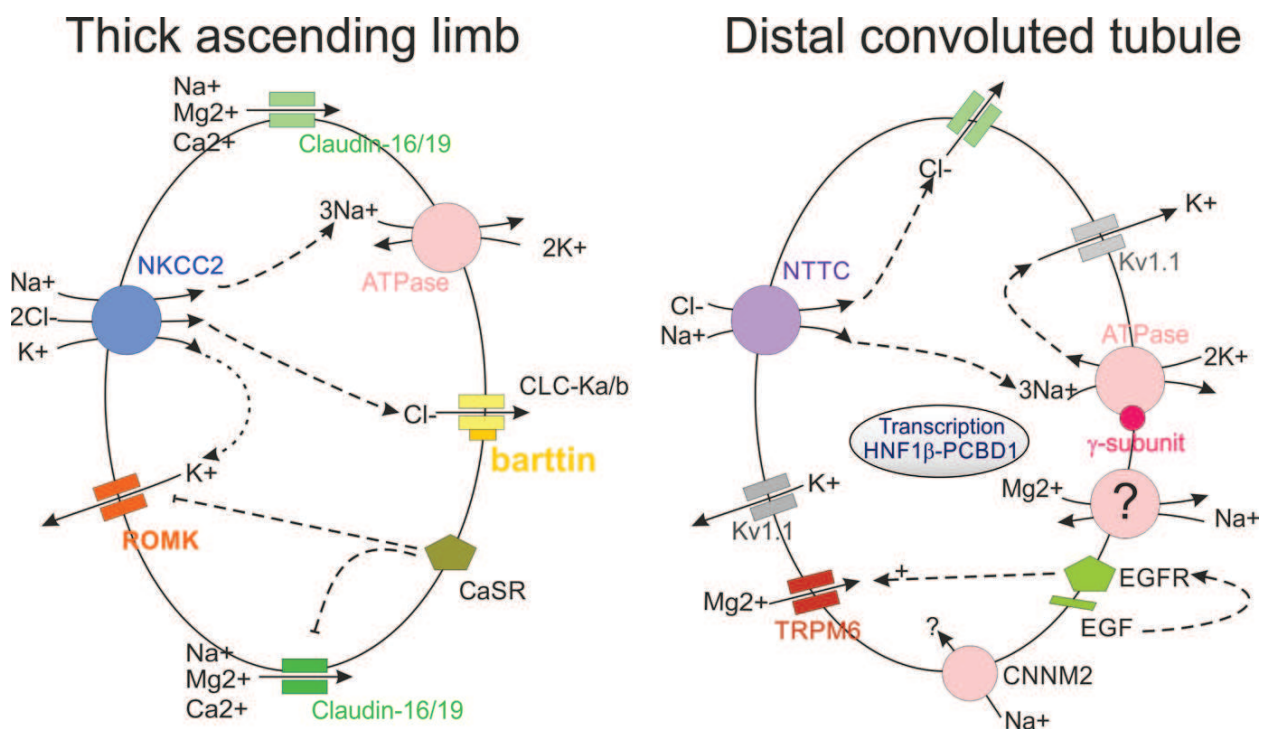


Figure 4. Magnesium reabsorption in the cortical thick ascending limb (TAL) of Henle’s loop and in the distal convoluted tubule (DCT). The key proteins influencing magnesium reabsorption are indicated. Magnesium reabsorption in the TAL is passive and occurs through the paracellular pathway. The driving force is the lumen-positive transcellular voltage, which is generated by the transcellular reabsorption of NaCl and the potassium recycling back to the tubular fluid via ROMK . Magnesium transport through DCT cells is active and depends on the negative membrane plasma potential. This mechanism seems to depend on a sodium gradient that results from the coordinate action of NCCT , Na-K-ATPase and Kir4.1 .

Gene	Protein	Disorder	Mode of inheritance
SLC12A1	Sodium-potassium-chloride transporter (NKCC2)	Bartter syndrome type 1	AR
KCNJ1	Potassium channel (ROMK1)	Bartter syndrome type 2	AR
CLCNKB	Chloride channel ClC-Kb	Bartter syndrome type 3	AR
BSND	Barttin	Bartter syndrome type 4	AR
CLDN16	Claudin-16, tight junction	Familial hypomagnesemia with hypercalciuria and nephrocalcinosis	AR
CLDN19	Claudin-19, tight junction	Familial hypomagnesemia with hypercalciuria and nephrocalcinosis with ocular impairment	AR
CASR	Calcium-sensing receptor (CasR)	Hypercalciuric hypercalcemia	AD
FXYD2	Gamma subunit Na/K/ATPase	Hypomagnesemia 2, renal	AD
TRPM6	TRPM6 cation channel	Hypomagnesemia 1, intestinal with secondary hypocalcemia	AR
SLC12A3	NaCl cotransporter (NCCT)	Gitelman syndrome	AR
EGF	Epidermal growth factor (Pro-EGF)	Hypomagnesemia 4, renal	AR
KCNA1	Kv1.1 Potassium channel	Myokymia 1 with hypomagnesemia	AD
KCNJ10	Kir4.1 potassium channel	SESAME syndrome	AR
HNF1B	HNF1 β transcription factor	HNF1B nephropathy	AD

Note: AD = autosomal dominant; AR = autosomal recessive.

Table 5. Inherited disorders of renal magnesium loss.

Hereditary forms of hypomagnesemia include rare, genetically determined disorders that may affect renal magnesium handling either primarily or secondarily. **Table 5** summarizes the spectrum of underlying genetic defects [31].

2.7. Pyrophosphaturia

Pyrophosphate (PPi) is present in urine and can contribute 50% CaOx monohydrate (COM) crystal growth inhibition in the collecting duct and up to 80% in the urine [33, 34]. It has been postulated that hypopyrophosphaturia is a metabolic risk factor for recurrent stone formers [35]. That the concentration of inorganic PPi is higher in urine than in plasma cannot fully explain the origin of urinary PPi, but does suggest that it is somehow either secreted

into the tubule or generated locally [36]. PPi is generated mostly in the mitochondria, and it is a byproduct of about 190 biochemical reactions. The PPi end product must be promptly removed to ensure irreversible, one-way reactions. PPi may be removed in three ways: by hydrolysis via cytoplasmic phosphatases; by PPi compartmentalization; or by its exportation from the cytoplasm via a transporter such as ANKH protein, which is located in the principal cells of the renal collecting duct (**Figure 2**) [6, 37]. Many authors now assume that PPi is removed from the cell by this third means, although the exact physiological function of the ANKH protein has never been clarified [36]. Underexpression or loss of activity of ANK (the mouse homolog of ANKH) is believed to lead to CaP deposition in numerous tissues, due to loss of PPi's inhibitory effects on CaP formation, and to the ubiquitous nature of CaP mineralization [38]. The majority of known ANKH mutations are assumed to be of the gain-of-function type, however, and are responsible for clinical phenotypes characterized by calcium PPi deposition in the joints, i.e., calcium pyrophosphate deposition disease [39]. Loss-of-function mutations presumably responsible for the loss of ANKH activity and a lower extracellular PPi were detected in patients with craniometaphyseal dysplasia, which is characterized by overgrowth and sclerosis of the facial bones and abnormal long bone modeling. No renal calcification was seen in association with this disease, however. Unlike bone, ion content in the tubular environment varies considerably, and the picture is further complicated by various reabsorption mechanisms, which may in turn be affected by a negative feedback from the tubular ion content [36]. This might explain why ANKH loss of function does not cause nephrocalcinosis or kidney stones.

2.8. Regulation of urinary acidification

One of the main functions of the kidney is to keep the systemic acid-base chemistry constant. The kidney has evolved so that it can regulate blood acidity by means of three key functions: (1) by reabsorbing the HCO_3^- filtered through the glomeruli to prevent its excretion in the urine; (2) by generating a sufficient quantity of new HCO_3^- to compensate for the loss of HCO_3^- due to dietary metabolic H^+ loads and loss of HCO_3^- in the urea cycle; and (3) by excreting HCO_3^- (or metabolizable organic anions) following a systemic base load [40]. For the kidney to be able to perform these functions, various types of cell throughout the nephron have to respond to changes in acid-base chemistry by modulating specific ion transport and/or metabolic processes in a coordinated fashion, such that the urine and renal vein chemistry is adjusted appropriately. The kidney contributes to acid-base homeostasis by recovering filtered bicarbonate in the proximal tubule. Distally, intercalated cells of the collecting duct generate new bicarbonate, which is consumed by the titration of non-volatile acid [41].

The renal tubular acidosis (RTA) syndromes encompass a disparate group of tubular transport defects that share the inability to secrete hydrogen ions (H^+). This inability results in failure to excrete acid in the form of ammonium (NH_4^+) ions and titratable acids or to reabsorb some of the filtered bicarbonate (HCO_3^-). Either situation coincides with a drop in plasma bicarbonate levels, leading to chronic metabolic acidosis. Much of the morbidity of RTA syndromes is attributable to the systemic consequences of chronic metabolic acidosis, including growth retardation, bone disease, and kidney stones [42].

Dysfunction of the proximal tubules, where approximately 90% of the bicarbonate is reabsorbed, leads to proximal RTA [43], whereas malfunctioning of the intercalated cells in the collecting ducts accounts for all known genetic causes of distal RTA (dRTA).

Inherited proximal RTA is a rare disorder that may be inherited as an autosomal recessive or dominant trait [44]. The more common autosomal recessive form has been associated with mutations in the basolateral sodium bicarbonate cotransporter NBCe1, encoded by the SLC4A4 gene. Mutations in this transporter lead to a reduced activity and/or trafficking, thus, disrupting the normal bicarbonate reabsorption process in the proximal tubules [45]. As an isolated defect of bicarbonate transport, proximal RTA is rare. It is more often associated with Fanconi syndrome, which features urinary wastage of solutes such as phosphate, uric acid, glucose, amino acids, and low-molecular-weight proteins, as well as bicarbonate. The distal acidification mechanisms remain intact, however, and acid urine can still be produced. The clinical phenotype is of a metabolic acidosis with hypokalemia; metabolic bone disease is common, but nephrocalcinosis and nephrolithiasis are rare [46].

In contrast, 80% of cases of distal RTA (dRTA) are associated with medullary nephrocalcinosis. The molecular basis underlying primary dRTA is a defective functioning of alpha intercalated cells [41]. The molecular defects behind proximal and distal RTA are listed in **Table 6**.

The clinical signs and symptoms of dRTA can vary, depending on the underlying mutation: patients may reveal a mild metabolic acidosis after the incidental detection of kidney stones, or they may have severe health issues with failure to thrive and growth retardation in children, rickets, severe metabolic acidosis, and nephrocalcinosis. Kidney stones in dRTA consist of CaP due to the release of Ca and Pi from bone to buffer the acidosis, leading to hypercalciuria and consequent CaP precipitation due to an alkaline pH [47].

2.9. Macromoleculuria

The formation of crystal aggregates involves interaction between crystals and urinary macromolecules (UMs) that serve as an adhesive. The number of UMs isolated in urine has been

Disorder	Gene	Protein	Mode of inheritance
Distal RTA type1	SLC4A1	AE1	AR
	SLC4A1	AE1	AD
	ATP6V1B1	β 1 Subunit of H ⁺ - ATPase	AR with early onset hearing loss
	ATPV0A4	α 4 Subunit of H ⁺ - ATPase	AR with later onset hearing loss
Proximal RTA type2	SLC4A4	Sodium bicarbonate cotransporter 1 (NBC1)	AR
Combined Proximal and Distal RTA type3	Ca2	Carbonic anhydrase 2 (CAH2)	AR

PH, primary hyperoxaluria; AR, autosomal recessive.

Table 6. The inherited renal tubular acidoses.

steadily increasing, and they now form a large group of proteins and some glycosaminoglycans [48, 49]. The main macromolecules involved in crystallization are summarized in **Table 7**.

Although the role of these proteins in stone formation is still far from clear, coating of the crystals by the urinary macromolecules seems to prevent crystal aggregation or at least delay it for long enough for the urine to transit through the kidney.

An inhibitory role has repeatedly been confirmed for osteopontin (OPN) [50–54], which is synthesized in the kidney and excreted in the urine in concentrations that suffice to inhibit CaOx crystallization. No naturally occurring mutations in the SSP1 gene encoding OPN have ever been reported in human diseases, but SSP1 polymorphisms have been associated with the risk of nephrolithiasis [55–57].

Tamm-Horsfall protein (THP), also called uromodulin, is a kidney-specific protein synthesized by cells in the TAL of the loop of Henle. It is the most abundant protein in human urine. It is a potent inhibitor of crystal aggregation *in vitro*, and its ablation *in vivo* predisposes one of the two existing mouse models to spontaneous intrarenal calcium crystallization, but there are still some key issues to clarify regarding the role of THP in nephrolithiasis. By conducting a long-range follow-up of more than 250 THP-null mice and their wild-type controls, Liu et al. [58] demonstrated that renal calcification was a highly consistent phenotype of the THP-null mice. The crystals consisted primarily of CaP in the form of hydroxyapatite. They were located in the interstitial space of the renal papillae more frequently than in the tubules (particularly in older animals), and there was no accompanying inflammatory cell infiltration. The interstitial deposits of hydroxyapatite observed in THP-null mice strongly resemble the renal crystals found in human kidneys with idiopathic CaOx stones. In humans, a number of naturally occurring THP mutations are reportedly linked to autosomal dominant medullary

Protein	Role in CaOx crystallization and nephrolithiasis
Tamm-Horsfall	Inhibitor of aggregation
Osteopontin	Free OPN Inhibits crystal nucleation, growth, aggregation and attachment, immobilized OPN promotes crystal attachment
Prethrombin fragment-1	Inhibitor of growth and aggregation
Bikunin and inter- α -inhibitor	Inhibitor of nucleation, growth, aggregation and attachment
α -1-microglobulin	Inhibitor of crystallization
CD-44	Promoter of crystal attachment
Calgranulin	Inhibitor of crystal growth and aggregation
Matrix gla protein	Inhibitor of crystal deposition
Heparan sulfate	Inhibitor of crystal aggregation and attachment
Osteonectin	Calcium binding
Fibronectin	Inhibitor of crystal aggregation, attachment and endocytosis

Table 7. Crystallization-modulating macromolecules (Modified from Khan SR and Canals BK 2009 [13]).

cystic disease and familial juvenile hyperuricemic nephropathy (Uromodulin-related diseases). Mutations lead to a defective intracellular trafficking of THP, and to a reduced THP excretion and secretion. No renal stone disease has been described in patients with any of these mutations to date, however [13].

2.10. Medullary sponge kidney

Medullary nephrocalcinosis is a frequent finding in medullary sponge kidney (MSK), a renal malformation associated with renal stones, urinary acidification and concentration defects, cystic anomalies in the precalyceal ducts, a risk of urinary infections, and renal failure. In a large series of 375 patients with macroscopic nephrocalcinosis, it was found that the clinical diagnoses most frequently associated with MSK were hyperparathyroidism and dRTA [9]. The prevalence of MSK in the general population is not known because no systematic autopsy searches have been performed. In a large series of subjects undergoing iv urography for various reasons, pictures ranging from clearly evident MSK to faint radiological signs of the disease were seen in 0.5–1% of cases [59]. MSK is relatively well represented in renal stone patients, however, and has been found in up to 20% of recurrent renal calcium stone formers [60, 61].

Why this malformative condition may predispose to medullary nephrocalcinosis remains to be established. MSK is considered a rare and sporadic disorder, but a recent study showed that 50% of MSK stone formers had relatives with milder forms of MSK, suggesting that it is relatively common for MSK to be familial, and it may be inherited as an autosomal dominant trait [62]. It has also been reported that 12% of unrelated MSK patients carried in heterozygosity two very rare variants of the glial cell line-derived neurotrophic factor (GDNF) gene, and these variants were inherited and co-segregated with the MSK phenotype in some families [63].

Mezzabotta et al. [64] had the chance to conduct an *in vitro* analysis on the behavior of papillary renal cells coming from the healthy portion of a kidney resected due to renal cancer in a MSK patient with medullary nephrocalcinosis, who harbored one of these rare GDNF gene variants. They found an unexpected and previously never reported phenomenon involving the spontaneous formation of Ca_2PO_4 nodules very similar to those of calcifying vascular cells. They demonstrated that silencing the GDNF gene in a human renal cell line and cultivating the silenced cells in osteogenic conditions triggered the deposition of Ca_2PO_4 . These results demonstrate the functional role of GDNF gene mutation in determining the medullary nephrocalcinosis associated with the MSK phenotype. They also provide the first experimental evidence of human renal tubular cells having a pivotal role in driving a calcification process. The role of renal cells in nephrocalcinosis is discussed in the subsequent paragraphs.

3. Proposed mechanisms of nephrocalcinosis

3.1. Tubular nephrocalcinosis

It is commonly assumed that crystals of CaOx or CaP form in the tubular fluid because of supersaturation and are presumably a renal mechanism for excreting excess waste [65–69]. In physiological conditions, this process is well controlled and lowers the risk of supersaturation [70–72]. When these control mechanisms fail, however, or changing conditions alter the

solubility of the urinary calcium salts, there is a consequent crystal retention and renal calcium deposition. This may involve epithelial crystal adhesion when the crystals are smaller than the diameter of the tubular lumen or lead to crystals obstructing the tubules when crystal formation and/or aggregation becomes excessive (**Figure 5A**).

3.1.1. Adhesion of crystals to the tubular epithelial cells: the fixed particle theory

The first step in crystal formation is nucleation, i.e., the process by which free ions in solution become associated forming microscopic particles. Crystallization can occur in solution micro-environments, such as those potentially existing in certain parts of the nephron [73], as well as on surfaces (like those of cells), and in the extracellular matrix [74]. Nucleation is followed by an aggregation of the crystals forming in the free solution, giving rise to larger particles. Finlayson and Reid [75] postulated that crystals cannot grow large enough during the short time it takes them to transit through the tubules to be retained in the tubules because of their size (“free particle” mechanism). This led to the hypothesis that crystals can only remain in the kidney if they adhere to the tubular epithelium (the ‘fixed particle’ theory) [74–76]. As a general mechanism for the etiology of tubular nephrocalcinosis, it was therefore suggested that crystallization starts at particular sites on the epithelial surface, not

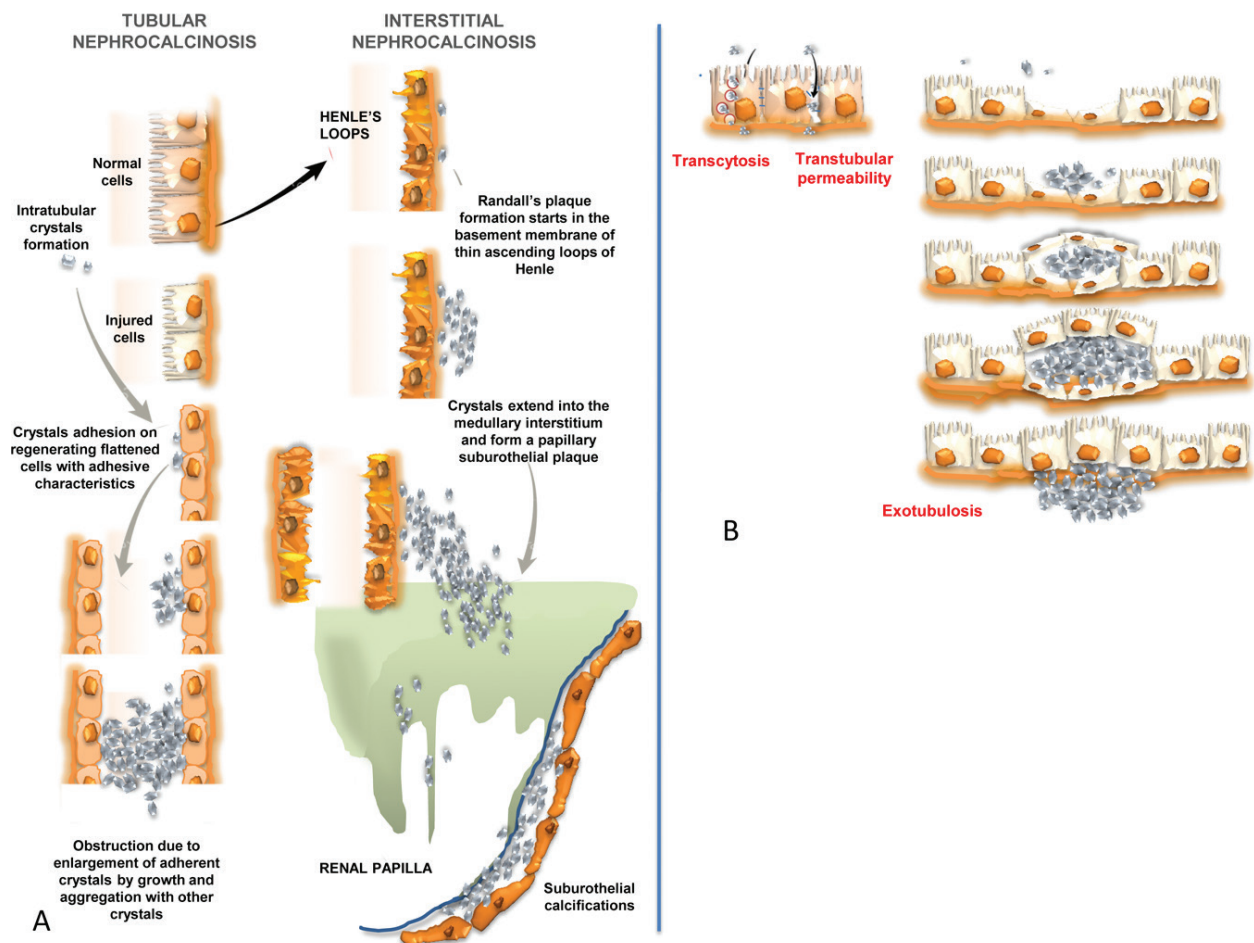


Figure 5. Proposed mechanisms of nephrocalcinosis. (A) Processes of tubular and interstitial calcium crystal deposition. (B) Possible mechanisms of interstitial crystal formation.

freely in the tubular fluid. A nascent crystal then becomes aggregated with other crystals, forming a mass large enough to occlude the nephron, leading to an obstructive tubulopathy (**Figure 5A**).

These crystals can be found in contact with the surface of injured/regenerating epithelial cells, apoptotic and/or necrotic cells, and denuded basement membranes [4, 77–84], giving the impression that the composition of the cell surface is crucial in modulating this process. In fact, it has been demonstrated on primary or immortalized tubular epithelial cells exposed to CaOx crystals that the crystal deposits preferentially adhere to injured, apoptotic, depolarized, immature, migrating, or proliferating tubular epithelial cells, rather than to fully differentiated, normal epithelia [85–88]. In this context, there is interesting evidence to suggest that proximal tubular cells bind crystals regardless of their differentiation status, whereas distal tubular cells (which are physiologically more likely to encounter crystals) only bind crystals when they are dedifferentiated [70], meaning that the distal tubular epithelium is unable to bind crystals when differentiated.

What we know about crystal adhesion in the proximal and distal tubules stems mainly from having identified the characteristics of the luminal membrane and the molecular composition of the crystal-binding epithelia, which led to the discovery of several crystal-binding molecules [4, 80, 89–93]. Importantly, these crystal-binding molecules are upregulated or redistributed to the apical membrane under certain conditions of cellular dedifferentiation, such as injury or repair, or variations in pathophysiological conditions [78, 87, 88, 94–96], which determine whether or not the crystals are retained in the kidney.

The different categories of crystal-binding molecules identified *in vitro* to date include: (i) terminal sialic acid residues [79, 97, 98]; (ii) phospholipids, i.e., phosphatidylserine [78, 84, 99, 100]; (iii) membrane-bound proteins, i.e., collagen IV [101], OPN [102–106], annexin 2 (ANX2) [107, 108] and nucleolin-related protein (NRP) [88, 93, 109]; and (iv) glycosaminoglycans, of which hyaluronan (HA) appears to be the most potent crystal-binding polysaccharide [95, 96, 110]. It has been demonstrated that other proteins, such as matrix Gla protein (MGP), are implicated in this process too [48, 111]. It is intriguing, moreover, that all known crystal-binding molecules contribute to inducing a negative cell-surface charge, a feature that has proved important in crystal adhesion to renal epithelial cells [85, 97, 112]. This would suggest that an array of aberrant phenotypes could bind crystals if there are appropriate amounts of crystals and appropriately oriented negative charges on the luminal membrane.

On the other hand, crystals and/or concomitant high concentrations of calcium, oxalate, or phosphate have been found to induce injury, proliferation, inflammatory mediator production, and oxidative stress on contact with epithelial cells *in vitro*, suggesting that epithelial dedifferentiation could be a consequence rather than a cause of crystal adhesion [113–120].

3.1.2. Is the crystal-binding cell phenotype a cause or a consequence of crystal adhesion?

Crystals do not adhere to normal epithelial cells, so it is highly unlikely that crystal adhesion might be the initial cause of cellular injury and epithelial phenotypic alterations, which are probably triggered instead by forced contact and transient interaction with normal epithelia

during the passage of the crystals/oxalate. Either way, it is evident that the tubular epithelium must have a very important direct role in the initiation of intratubular nephrocalcinosis.

Some reports have suggested that the renal tubular epithelial cell injury in crystal-cell interactions occurs more easily in a setting of prior cell injury [99, 118]. The “incubation” period observed during transient toxic or mechanical crystal-cell interactions capable of affecting the tubular epithelium is consistent with the need for a shift in the epithelial phenotype prior to crystal adhesion [119–121]. This would mean that crystal adhesion is a consequence, not the initial cause of epithelial injury *in vitro*.

The nucleation of ions from the renal tubule and subsequent growth of a calcium crystal cannot usually occur and, even if it does, such processes do not proceed quickly enough to produce particles of sufficient size to be retained in the kidney, and occlude tubules simply because of their bulk [68, 76, 118, 122, 123]. The crystals are not only the outcome of the physicochemical properties and urinary concentrations of the minerals involved. They are also influenced by crystallization regulators that may promote or inhibit crystallization and by signaling pathways triggered by the crystals, thus leading to different types of renal cell injury [8, 71, 124–127]. Urine or, more properly, tubular fluid probably contains inhibitors of crystal formation that specifically prevent their nucleation, growth, or aggregation. It has been claimed that the inhibitors’ role in controlling crystal formation is important in the normal defenses against the development of stones, and that abnormalities of these inhibitors may allow for stone formation and growth.

There are different types of such crystallization inhibitors in the urine, including small organic anions such as citrate, small inorganic anions such as PPis, multivalent metallic cations such as magnesium, and macromolecules such as OPN and Tamm-Horsfall protein, which can take effect on different levels during the crystal formation process (**Table 7**). Citrate lowers the saturation of CaOx by forming complexes with calcium and inhibits the aggregation of preformed crystals and the attachment of crystals to the renal epithelium [97, 128]. PPi is a substance naturally occurring in urine that has been found to inhibit the crystallization of both CaOx and CaP [129]. Magnesium has also been shown to prevent stone formation by inhibiting the growth and aggregation of crystals (and presumably interferes with their nucleation too) [130]. OPN (known to inhibit the spontaneous nucleation of crystals from solutions) was found to prevent the growth of preformed crystals in a seed growth assay [131, 132], but there is also evidence to suggest that OPN bound to the surface of cells may enhance crystal attachment [102, 103]. In addition, the inhibitory effect of OPN on CaOx aggregation *in vitro* can be switched to an aggregation promoting effect if its net negative charge is neutralized by polyarginine [133].

Tamm-Horsfall glycoprotein (THP) is the most abundant of the urinary proteins under normal circumstances [134]. THP coats CaOx crystals and prevents their adhesion to cultured epithelia, but there are few *in vivo* studies on how it would affect their aggregate, once it anchored to the epithelia [134, 135]. Another protein, called urinary prothrombin fragment 1, has been isolated from the matrix of crystals formed by adding oxalate to urine [136]. This is an effective inhibitor of CaOx crystal growth and aggregation, *in vivo* as well as *in vitro* [137].

3.2. Interstitial nephrocalcinosis

The presence of crystals in the renal interstitium is defined as interstitial nephrocalcinosis (**Figure 5A**). Although the causal role of aberrant epithelial tissue in crystal adhesion—demonstrated in renal cell lines *in vitro*, in animal models, in kidney transplant patients, and in neonates—may account for intratubular crystal formation and retention [138–140], the specific pathogenic mechanisms leading to interstitial crystal formation and deposition are still unclear [7].

Translocation of intratubular crystals and/or *de novo* interstitial calcification have been proposed as causative factors (**Figure 5B**).

3.2.1. Translocation of intratubular crystals to the interstitium

Crystal translocation can be induced by transcytosis (**Figure 5B**), a process during which small intraluminal crystals are internalized within apical vesicles (with or without the mediation of a receptor) and transferred across the cell wall to the basolateral side, where they are released into the interstitial extracellular environment [12]. Apical endocytosis of small crystals has been well described [141–144], but there is little evidence of any basolateral release of crystals into the interstitium. It has been suggested that these crystals probably disintegrate into lysosomes [142, 143, 145]. Very recently, however, Chiangjong et al. [146] demonstrated that, after exposure to CaOx crystals, renal tubular epithelial cells secrete more crystal-binding protein (enolase-1 [147]) into the basolateral compartment; the authors suggested that this protein could in turn promote CaOx crystal invasion through the renal interstitium. The translocation of crystals into the interstitium is associated with inflammation, attracting leukocytes, monocytes, and macrophages that—according to some—would then remove the crystalline material [148].

An alternative mechanism of transepithelial crystal translocation was described using the term “exotubulosis” (**Figure 5B**) in an *in vivo* study conducted by De Bruijn et al. [149, 150]. These authors demonstrated that crystals adhering to the inside wall of the tubule can be overgrown by tubular epithelial cells adjacent to the site of adhesion of the crystals. After proliferation and migration, the tubular epithelial 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 toward the lumen, thereby restoring the epithelial integrity of the affected tubule, and translocating the crystals into the interstitium.

For a long time, translocation was the only explanation for the advent of mineral deposits in the interstitium [151, 152], but crystals can also form *de novo* in the interstitium.

3.2.2. *De novo* interstitial crystal formation

It has been claimed that these crystal deposits start in the interstitium around the thin limbs of the loop of Henle (below the basement membrane) and give rise to subepithelial calcifications better known as Randall’s plaques [153].

Nobody knows, however, whether this *de novo* crystal formation is due merely to a chemically driven supersaturation or whether cells are involved too. For some time, the most accredited hypothesis advanced to explain the onset of interstitial nephrocalcinosis was purely physicochemical, relating to spontaneous Ca_2PO_4 crystallization in the interstitium as a result of calcium and phosphate oversaturation in this milieu. Evidence has been produced of a lower expression and defective barrier and fence functions of the tight junction in renal tubular epithelial cells exposed to CaOx crystals. This could lead to intercellular (paracellular) migration of intratubular COM crystals, and of calcium, oxalate, and phosphate ions to the interstitium to initiate tubulointerstitial injury, inflammation, and interstitial nephrocalcinosis [154–157].

Dysregulation of calcium homeostasis in the renal interstitium (and probably on a systemic level too) may have a key role in the pathogenesis of nephrocalcinosis. Bushinsky DA [154] proposed a sequence of events that could lead to an increased supersaturation and subsequent crystal formation. *“Following ingestion and absorption of dietary calcium, the renal-filtered load of calcium would increase, resulting in increased tubular calcium concentration. The medullary countercurrent mechanism would concentrate the calcium extracted from the TAL into the hypertonic papilla. The vasa recta, also with an increased calcium concentration, would fail to readily remove calcium from the interstitium. The increased serum calcium would stimulate the calcium receptor and decrease reabsorption of water in the collecting duct, further concentrating the interstitium. Vectorial proton transport into the collecting duct would alkalinize the interstitium. The pH of the vasa recta would also increase following gastric proton secretion, the so-called alkaline tide, resulting in less bicarbonate removal from the medullary interstitium. The increased pH would decrease the solubility of CaP complexes. Perhaps an extracellular matrix protein, specific to the papillary interstitium, could provide a site promoting heterogeneous nucleation, which occurs with a lower degree of supersaturation than homogeneous nucleation.”*

Estimates of tubular fluid supersaturation based on data obtained in the rat suggest that CaP supersaturation often occurs in the thin limbs of the loop of Henle [158], where tubular fluid is saturated even under normal circumstances. In humans, this condition could drive the precipitation of CaP deposits at interstitial sites, in the inner medulla—known as Randall’s plaques when they become extensive enough to be macroscopically visible [4, 6, 158, 159]. Randall’s plaques have been proposed as a nidus for the development of the most common variety of CaOx stones [4, 160].

3.2.3. Randall’s plaques

Randall demonstrated that interstitial crystals are located at, or adjacent to, the papillary tip [161]. These crystals in the papillary interstitium are composed not of CaOx (the most common solid phase found in patients with nephrolithiasis), but of CaP [162, 163], that then eroded into the urinary space, serving as a heterogeneous nucleation surface for CaOx. Randall concluded that renal stones originated as a slow deposition/crystallization of urinary salts (CaOx, CaP, uric acid) on a lesion of the renal papilla—a picture confirmed and extended in patients with idiopathic CaOx nephrolithiasis [4, 5, 164] (**Figure 6**).

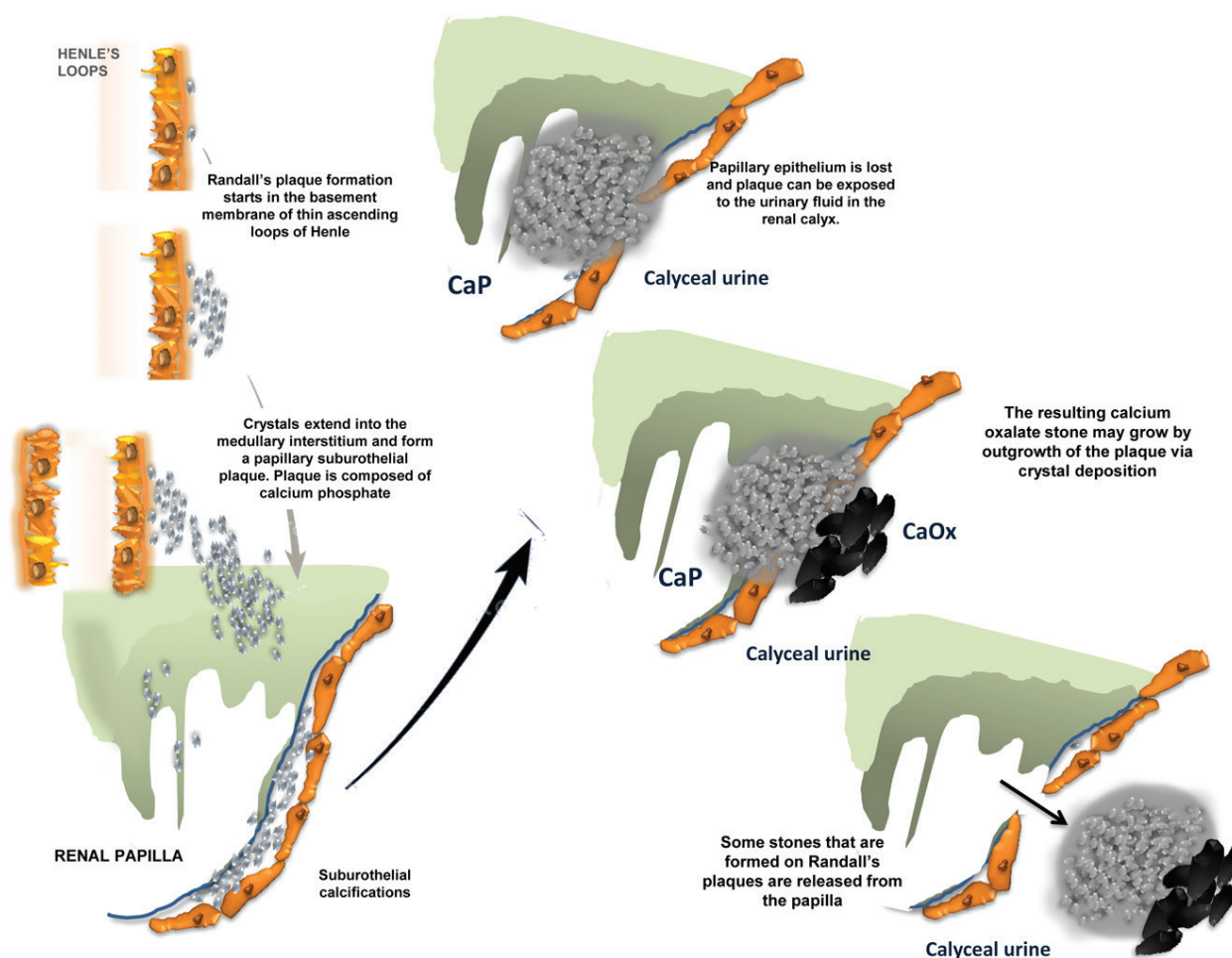


Figure 6. Mechanism of stone growing on Randall's plaque. The plaque appears in the interstitial tissue within the renal papilla, with no crystals present in any tubular lumens. The plaque is composed of calcium phosphate (CaP) in the mineral form of apatite. Papillary epithelium is lost, and the plaque can be exposed to urinary fluid in the renal calyx. The resulting calcium oxalate stone may grow and the plaque keeps the stone from flowing out with the urine, and the insolubility of the calcium oxalate makes the stone quite. Stones that are formed on Randall's plaques are released from the papilla in the renal calyx.

4. Cell-driven calcification: the example of vascular calcification

In the last decade, some researchers have attempted to clarify the effects of high oxalate and crystal concentrations on the biology of renal tubular cells because the exact role of the tubular cells in response to the influx of these potentially precipitating ions is still uncertain.

A role in the pathogenesis of Randall's plaques has also been suggested for interstitial cells capable of transdifferentiating along the bone lineage, leading to the hypothesis that nephrocalcinosis could be an osteogenic cell-driven process, similar to that of vascular calcification [64, 165–168]. Tubular epithelial cells have a well-known ability to differentiate into cells with the mesenchymal phenotype (for instance, renal interstitial myofibroblasts may originate from renal tubular cells undergoing epithelial-mesenchymal transformation) [169]. This capacity for differentiation is not exclusive to renal cells, or epithelial cells. It is shared with

Ito cells in the liver [170], and a subpopulation of smooth muscle cells (SMCs) in the intima of arteries—cell populations that are thought to be pericyte-like. Remarkably, vascular pericytes have the ability to undergo osteoblastic differentiation and mineralization [171, 172], and seem to play a crucial part in ectopic vascular calcification.

The underlying mechanisms that lead to pathological calcification are complex and thought to involve active, strictly regulated processes that are common to bone formation [54, 173, 174]. Cells that may readily undergo osteogenic-like transition include: vascular smooth muscle cells (VSMCs) [171, 175–193] in the media, myofibroblasts in the adventitia, pericytes in the microvessels [171, 172, 194], multipotent vascular mesenchymal progenitors, and valve interstitial cells [195, 196]. Vascular calcification was long thought to result from passive degeneration [197], but actually involves a complex, regulated process of biomineralization similar to osteogenesis, which mediates the deposition of bone matrix in the blood vessels [175–193].

5. Mechanisms of vascular calcification

Calcification may involve both osteogenic and chondrogenic differentiation. In humans, it is primarily osteogenic (with bone tissue formation), whereas in mice it is primarily chondrogenic (with cartilage formation). Although osteoblasts and chondroblasts are distinct cell types, they have substantial similarities in mineralization mechanisms and gene expression, leading to the formation of a complex and highly structured extracellular matrix, which can also be found in the calcified vasculature.

There is evidence to indicate that the proteins controlling bone mineralization are involved in regulating vascular calcification as well. Many key bone formation regulators and bone structural proteins, including pro-osteogenic factors like the bone morphogenetic proteins (BMP) [171–186], and inflammatory mediators such as tumor necrosis factor- α (TNF- α), are expressed in atherosclerotic plaques as well as during the osteogenic differentiation of VSMCs. These factors can induce calcification via *Msx2* and Wnt signaling, which plays a crucial part in the commitment of pluripotent mesenchymal cells, activated during vascular calcification [198–202], and they have been implicated in the regulation of osteoblastic VSMC transdifferentiation [203, 204]. Wnt signaling induces an upregulated expression of the transcription factors *Cfb1/Runx* (core-binding factor subunit1 α /runt-related transcription factor 2) and *osterix* [177, 178, 198–200, 205–207]. In turn, *Runx2* increases the expression of the bone-related proteins osteocalcin (OCN), sclerostin, and receptor activator of nuclear factor-kappa β ligand (RANKL) [208]. Downstream from *Runx2*, *osterix* increases the expression of other bone-related proteins, including bone sialoprotein, alkaline phosphatase (ALP) [206, 209, 210], OPN [211–213], matrix γ -carboxyglutamic acid protein (MGP) [214], and osteoprotegerin (OPG) [215].

The cellular and systemic conditions that permit VSMC differentiation to osteoblast-like cells are multifactorial. At cellular level, procalcifying conditions may occur because of the factors that increase cellular stress responses. Similarly, systemic factors, such as a loss of circulating inhibitors of calcification, or changes in levels of hormonal regulators of calcium and phosphate homeostasis can also facilitate VSMC differentiation and vascular calcification.

Osteogenic differentiation of VSMCs is prevented under normal conditions by physiological inhibitors, such as MGP, OPN, and OPG [216, 217], and regulated by monocyte- and macrophage-osteoclast differentiation within the vascular wall. The growth of crystals is also hindered thermodynamically and inhibited by PPI [183, 218]. Unlike OPN, OPG, and MGP, which function in the vessel wall, fetuin A is a circulating inhibitor of calcification that has a high affinity for hydroxyapatite crystals and is thought to function by binding small CaP particles via a domain particularly rich in acidic residues, stabilizing and clearing them to phagocytes for removal [218]. *In vitro*, fetuin A inhibits the *de novo* formation of hydroxyapatite crystals, but does not affect crystals that have already formed [219]. Fetuin A also has an anti-inflammatory function, dampening the effects of CaP particles in neutrophil stimulation, and is responsible also in macrophage cytokine release and induction of apoptosis. Additionally, fetuin-A has been shown to accumulate in VSMC-derived matrix vesicles, preventing them from initiating and propagating calcification.

Given the complexity of the systems that regulate vascular calcification, it is likely that many of these factors are at work simultaneously, but in some situations the physiological balance is disrupted and vascular calcification can progress (**Figure 7**).

An alternative mechanism for vascular calcification has recently been suggested. The “circulating cell theory” [220] postulates that circulating cells coming from sources such as bone

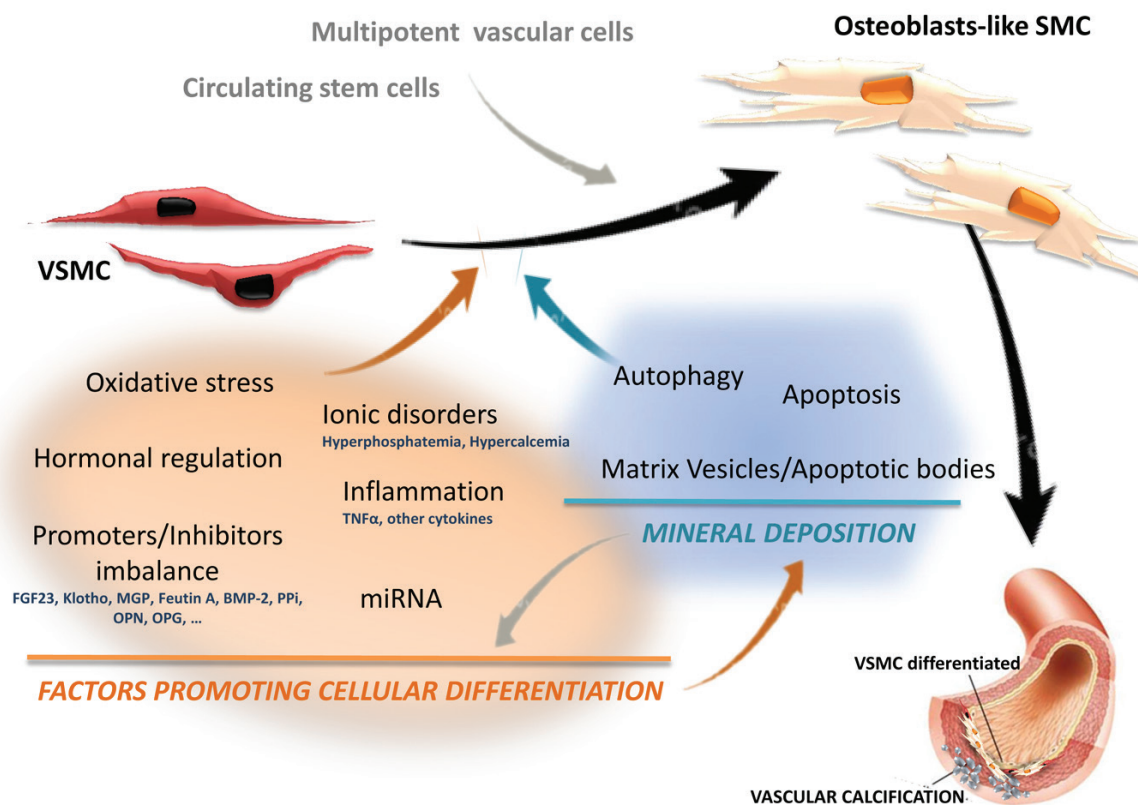


Figure 7. Physiopathological mechanisms promote cellular differentiation and mineral deposition during vascular calcification. Vascular smooth muscle cells undergo differentiation to osteoblast-like cells when exposed to different factors. These osteoblast-like cells participate in vascular calcification.

marrow may have an active role in vascular calcification. A circulating immature bone-marrow-derived cell population has been identified, and a small subset of this bone marrow population reportedly possesses bone-forming properties *in vitro*. Under the influence of chemoattractants (released by damaged endothelium, for instance), these cells may home in on diseased arteries. Under pathological conditions such as an imbalance between promoters and inhibitors of vascular calcification, this population may undergo osteogenic differentiation in the lesions, promoting vessel mineralization [220]. In another study, it has also been claimed that multipotent vascular stem cells (MVSC) in the blood vessel wall might differentiate into osteoblast-like cells [221]—though this theory remains highly controversial for the time being.

6. Factors regulating vascular calcification

6.1. Phosphate

The role of phosphate in the osteoblastic differentiation process is well established [176, 177, 181, 185, 187, 222]. *In vitro*, high extracellular phosphate concentrations induce a rise in intracellular phosphate concentrations actively mediated by three types of sodium-dependent phosphate cotransporter, of which the type III transporters Pit-1 and Pit-2 are ubiquitously expressed and predominant in humans. Only Pit-1 is required for the osteogenic differentiation of VSMCs [177, 223–225]. Increasing phosphate concentrations in the VSMCs induce their phenotypic switch to osteoblast-like cells [177, 178, 184]. In the event of renal failure, phosphate plays a key part in this mechanism [165, 168]. Vascular SMCs exposed to pro-calcifying levels of phosphate (akin to what may happen in patients with chronic kidney disease (CKD)) lose their expression of the smooth muscle contractile proteins, SM22 α and SM α -actin, and express the bone markers Runx2, OPN, OCN, and ALP instead [178].

As well as phosphate, many other factors can influence the osteoblastic-like phenotype. A long-term exposure of VSMCs to a variety of chronic stresses and ionic disorders (especially hyperphosphatemia and hypercalcemia), for example, can override the action of some endogenous inhibitors, such as MGP, OPN, OPG and PPi [217], inducing differentiation [226]. Oxidative stress, inflammation, hormonal perturbations, and metabolic disorders can lead to vascular calcification too.

6.2. Oxidative stress

Oxidative stress and endoplasmic reticulum stress have both been implicated in vascular calcification and shown to promote smooth muscle cell (SMC) differentiation. In particular, oxidative stress generated in VSMCs by hyperlipidemia and oxidized lipoproteins, or a uremic milieu [227] prompts the expression of BMP2, Runx2 [228], and osterix, and governs Wnt signaling [207]. Reactive oxygen species (ROS) signaling can also induce other markers of osteoblastic differentiation. In the vascular wall, the induction of oxidative stress can recapitulate osteogenesis in the VSMC from their undifferentiated state [229]. The role of ROS formation and signaling in vascular calcification may also reveal a link between inflammation and vascular calcification, since inflammatory cytokines induce calcification via the Msx2/Wnt/ β -catenin

pathway [202]. It has also been found that calcium deposits colocalize with inflammatory cells both *in vitro* [230, 231] and *in vivo* [232]. Mineral crystals may therefore be pro-inflammatory per se, prompting and exacerbating the inflammation and calcification [233, 234].

6.3. Hormones

Hormones have pleiotropic effects on calcific vasculopathy. For example, the adipose-derived factor, leptin, promotes vascular cells *in vitro* [235] and *in vivo* [236], while adiponectin-deficient mice have increased levels of vascular calcification [237]. The influence of parathyroid hormone (PTH), which is involved in the bone turnover process, is also well known. PTH has a crucial role in calcium homeostasis, and so does PTH-related peptide (PTHrP), and the two may function as pathological calcification mediators. Both PTH and PTHrP prevent VSMC calcification in a dose-dependent manner by inhibiting ALP activity [238]. In addition, PTHrP is secreted from VSMCs, an action that is impaired by calcitriol (1,25-dihydroxyvitamin D, the active form of vitamin D) [239]. PTH not only promotes the release of calcium from bone but also mobilizes salts, including bicarbonate and phosphate and impairs renal phosphate excretion, leading, for example, to advanced nephron loss in CKD patients, and thus resulting in severe hyperphosphatemia [240]. Accrued high levels of serum phosphate then further stimulate the secretion of PTH, forming a vicious cycle [241]. Hyperphosphatemia increases FGF23 (a protein released by bone), which—together with its co-receptor Klotho (a transmembrane protein expressed by the kidney and blood vessels)—may also be a pathogenic factor in vascular calcification [242, 243]. Klotho maintains the balance of circulating calcium and phosphate [244]. Activation of the vitamin D receptor increases the expression of Klotho and FGF23 to promote renal phosphate excretion by downregulating the sodium phosphate transporters Slc34A1/NaPi-2a and Slc34A3/NaPi-2c. Intriguingly, Klotho inhibits vascular calcification by preventing VSMC differentiation while disrupting Klotho-FGF23 signaling results in hyperphosphatemia with ectopic calcification [244, 245].

Calcitriol may also exacerbate dystrophic calcification. Vitamin D toxicity is a common animal model used to study vascular calcification [246]. Calcitriol dose-dependently increases both calcification and ALP activity in VSMCs [239]. In response to interferon- γ , macrophages express 25-hydroxyvitamin D 1 α -hydroxylase, the enzyme needed to convert 25-hydroxyvitamin D into calcitriol [239]. Once calcitriol binds to its receptor, signaling through this pathway has pleiotropic effects. The vitamin D receptor influences many genes in the vessel wall, including vascular endothelial growth factor (VEGF), matrix metalloproteinase 9, myosin, and structural proteins (including elastin and type I collagen [247–250]), and this explains some of the effects of calcitriol on vascular calcification.

Glucocorticoids, a class of steroid hormones with anti-inflammatory properties, have also been shown to mediate osteoblastic differentiation and thereby promote ectopic calcification. Long-term glucocorticoid use has been associated with osteoporosis, however, and these compounds have been shown to initiate differentiation to an osteochondrogenic phenotype in vascular cells [251, 252]. Similarly, pericytes exposed to dexamethasone exhibit a weaker expression of MGP and OPN, and an increased ALP activity and calcium deposition.

6.4. Matrix vesicles

Bone formation involves hydroxyapatite $[\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2]$ crystals, which begin to develop matrix vesicles that grow out of osteoblasts. VSMC that have undergone osteoblastic differentiation are able to release similar mineralization-competent matrix vesicle-like structures in the extracellular matrix too [176, 180, 215, 226, 253–256]. These matrix vesicles serve as mineral nucleation sites and are responsible for the initial deposition of calcium and phosphate in blood vessels (**Figure 7**). Matrix vesicles contain proteins related to calcification, extracellular matrix and extracellular matrix-modifying enzymes, calcium channels, trafficking and cytoskeletal proteins, oxidant and endoplasmic stress-related proteins, and other serum proteins [226]. All these proteins are involved in the disruption of the normal vessel architecture and thus serve as the nidus for calcification. Matrix vesicles also have an increased expression and activity of transglutaminase 2, a calcium-dependent enzyme that promotes extracellular matrix crosslinking, and matrix metalloproteinase-2 [226, 257]. Matrix vesicles are secreted from multivesicular bodies and are enriched with exosomes found to contain amorphous calcium-phosphate crystals under calcifying conditions, and detected at the site of calcification [258].

Prolonged cellular stress may activate homeostatic repair processes, or cells may undergo apoptosis when overwhelmed by the stress. Apoptosis regulates VSMC calcification *in vitro* and inhibiting apoptosis reduces VSMC calcification [171, 176, 259–261]. In advanced carotid atherosclerotic plaques, the matrix vesicles contain high levels of BAX (a pro-apoptotic member of the BCL2 family), indicating that they may be remnants of apoptotic cells [171, 176, 260]. Apoptotic VSMC-derived matrix vesicle-like structures are also able to concentrate and crystallize calcium, triggering calcification [176, 183, 189, 193, 222]. It has likewise been reported that chondrocyte-derived apoptotic bodies might contribute to the calcification of articular cartilage [262]. All these data support the idea that the formation of apoptotic bodies may be another factor initiating ectopic calcification in cells under certain conditions.

Autophagy—a catabolic process that may be an adaptive response to cell stress—has been found to limit SMC calcification by inhibiting matrix vesicle release. When phosphate levels are high, inhibiting autophagy resulted in an increased VSMC calcium deposition. Downregulating autophagy was also associated with a loss of VSMC contractile proteins, but not with any VSMC differentiation to an osteogenic phenotype. On the other hand, inhibiting autophagy did increase the release of procalcific matrix vesicles with high levels of ALP activity [263]. In short, factors that interfere with autophagy are likely to increase VSMC and vascular calcification.

6.5. MicroRNAs

MicroRNAs (miRs) have emerged as key regulators of cell differentiation to osteoblast-like cells, regulating gene expression under pro-calcifying conditions. Some studies have described a stronger expression of miRs targeting smooth muscle contractile proteins and a weaker expression of miRs targeting osteoblast differentiation markers under these conditions [264]. For example, the miR-143/145 complex, which regulates the expression of VSMC differentiation markers and Kruppel-like factor4 (KLF4), is downregulated; and KLF4 is known

to control bone homeostasis by negatively regulating both osteoclast and osteoblast differentiation [265]. Other studies showed that downregulation of miR-204, miR-205, miR-133a, or miR-30b/c in VSMCs occurs prior to calcification and upregulates Runx2 expression [266, 267]. Micro-RNA-125b, which targets Ets1 and osterix, was found downregulated 21 days after exposing VSMCs to osteogenic medium [268]. Another set of miRs, miR-135a(*n*), miR-762, miR-714, miR-712(*n*), that target the calcium flux proteins NCX1, PMCA1, and NCKX4, have also been implicated in VSMC calcification [266]. It is still not clear, however, whether these miRs are really important in VSMC differentiation to an osteoblast-like phenotype, or whether this process is associated with changes in the expression of a panel of miRs targeting several proteins important for calcification.

7. Evidence of cell-driven renal calcification

It is worth considering the possibility of ectopic renal calcification being an osteogenic-like process. Evidence to support the notion that resident renal cells could be prompted to transdifferentiate, or differentiate along an osteogenic lineage, comes from the following observations. Madin-Darby canine kidney (MDCK) cells grown in monolayers directly on a plastic dish, or a dish coated with collagen gel, developed small blisters/domes/nodules after 21 days that became more prominent after 30 days [269, 270]. Microscopic examination showed that the nodules were CaP crystals. MDCK cells grown in agar produced spherical colonies in which layers of epithelial cells, with their apical surface on the outside, enclosed CaP crystal deposits on the basal side of the epithelium [90, 93, 270–272].

Kumar et al. [71] found that rat inner medullary collecting duct cells grown in a calcifying medium formed calcifying nodules that were positive for typical bone proteins. Miyazawa et al. [273, 274] reported finding that CaOx crystals upregulated vimentin (VIM) in normal rat kidney proximal cells and that other genes, such as OPN, fibronectin (FN), cathepsins B and L, and mitogen-activated protein kinase, related to the pathogenesis of stone formation. Using MDCK cells grown for 28 days in the presence of 10 mM β -glycerophosphate, Azari et al. identified a mineralization process with an increased ALP activity and the presence of small aggregates of hydroxyapatite crystals within membrane-bounded vesicles [275]. Other related osteogenic genes (RUNX1 and 2, osterix, BMP2 and 7, bone morphogenetic protein receptor 2, collagen, OCN, osteonectin (ON), OPN, MGP, OPG, cadherins, FN, and VIM) were found upregulated in the kidney of hyperoxaluric rats [276, 277]. Khan et al., again, showed a pronounced expression of MGP, together with that of collagen, OPN and FN, in renal medullary peritubular vessels of hyperoxaluric rats [111], confirming that the tubular epithelial cells of hyperoxaluric kidneys acquired a number of osteoblastic features, and suggesting a dedifferentiation of epithelial cells to the osteogenic phenotype [278].

Mezzabotta et al. were the first to provide evidence of human renal cells transdifferentiating into an osteogenic-like phenotype, producing CaP deposits [64]. They found spontaneous instances of calcification phenomena in primary papillary renal cells derived from a patient with medullary sponge kidney (MSK) and medullary nephrocalcinosis, who carried a mutation in the GDNF gene. To investigate whether this spontaneous mineralization was merely a

physicochemical phenomenon or a well-organized biomineralization process, they searched for any sign of the bone mineralization machinery being expressed in the cells. They found the cells positive for osteogenic markers such as ON, ALP, collagen I, laminin and Runx2, and weakly positive for OCN, but negative for OPN (a known inhibitor of crystal formation). The upregulation of ON and downregulation of OPN were also demonstrated at mRNA level. Investigating which cells were the main actors behind the phenomenon observed, the authors found that the cells were mesenchymal stroma cells (MSCs), which are very similar to pericytes. The microvasculature of the renal papilla is particularly rich in pericytes, which regulate microvascular integrity in the peritubular capillary network and give the descending vasa recta its contractile function [279]. Thus, like VSMCs, papillary MSCs associated with the perivascular niche may be capable of driving an osteogenic process under certain conditions.

In the same paper, the Authors demonstrated that human renal tubular HK-2 cells exposed to an osteogenic medium displayed the ability to produce Ca_2PO_4 by regulating the ON/OPN ratio in favor of ON.

Overall, all these very interesting data underscore that renal cells may acquire an osteoblast-like phenotype, and that a process very similar to vascular calcification may have a role in the development of human nephrocalcinosis.

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