CHAPTER 2
REVIEW OF LITERATURE

2.1. Kidney stone disease

2.1.1. Overview

Urinary stone disease is the deposition of stones in urinary tract that has afflicted human kind for many centuries and continues to pose a universal health problem even today. Nephrolithiasis is a significant clinical problem in everyday practice with a subsequent burden for the health system and remains a chronic disease and our fundamental understanding of the pathogenesis of stones as well as their prevention and cure still remains rudimentary. Histological and archeological studies have clearly revealed that ancient man suffered from urinary tract stone disease [Eknoyan, 2004]. The incidence of urolithiasis appears to have been generally increasing over the last 100 years, particularly in countries which have either hot climate or which have moved from an agriculture economy to one based on industrial and technological development [Zilberman et al., 2010]. It is estimated that at least 10–12% of the population in the industrialized part of the world is afflicted by urinary tract stone disease [Moe, 2006]. Following the onset of the disease, the average recurrence rate is 31.5–50% within 5 years [Knoll, 2007] and more than 72% after 20 years [Koyuncu et al., 2010]. Regardless of the fact that supersaturation of stone forming salts in urine is essential, abundance of these salts by itself will not always result in stone formation. It is classically explained as the derangement in the process of biomineralization involving the equilibrium between promoters and inhibitors of crystallization: a deficit of one or several inhibitors or an excess of one or several promoters plays a pivotal role in the stone formation [Moro et al., 2005]. Though,
approximately 75% of stones are primarily calcium oxalate, but up to 50% of these include calcium hydroxylphosphate (brushite or calcium hydroxyapatite), in trace or greater amounts; 10–20% of stones are composed of magnesium ammonium phosphate (struvite or triple phosphate); 5% are composed of urate; and 1–2% are composed of cystine [Bihl et al., 2001; Reynolds, 2005]. Our fundamental understanding of the pathogenesis of stones as well as their prevention and cure, remains rudimentary. With its multifactor etiology and high rate of recurrences, urinary tract stone disease provides a medical challenge. It is necessary to identify risk factors that might be of etiological importance and thus get some clues for predicting the further course of the disease.

Figure 2.1 Location of stones in Urolithiasis
2.1.2. History

Kidney stone disease has been a well-known entity for centuries and was first recorded thousands of years ago. This has been markedly established by different archeological findings, as well as by writings about painful stone colic and therapeutic trials for stone removal [Eknoyan, 2004]. In ancient centuries urolithiasis was often a disastrous disease, with a catastrophic outcome all too often leading to the patient’s death. In 1901, a stone discovered in the pelvis of an ancient Egyptian mummy was dated to 4,800 BC. Medical texts from ancient Mesopotamia, India, China, Persia, Greece, and Rome all mentioned about the calculous disease. Part of the Hippocratic Oath suggests there were practicing surgeons in ancient Greece to whom physicians were to defer for lithotomies. The Roman medical treatise De Medicina by Aulus Cornelius Celsus also contained a description of lithotomy [Celsius, 1831], and this work served as the basis for this procedure until the 18th century [Shah and Whitfield, 2002]. Famous people who were kidney stone formers include Napoleon I, Napoleon III, Peter the Great, Louis XIV, George IV, Oliver Cromwell, Lyndon B. Johnson, Benjamin Franklin, Michel de Montaigne, Francis Bacon, Isaac Newton, Samuel Pepys, William Harvey, Herman Boerhaave, and Antonio Scarpa [Ellis, 1979]. Lithotomy for the removal of stones is one of the earliest known surgical procedures [Eknoyan, 2004]. New technology in lithotomy began to emerge starting in 1520 but the operation remained risky. After Henry Jacob Bigelow popularized the technique of litholapaxy in 1878, [Bigelow, 1878] the mortality rate dropped from about 24% to 2.4%. However, other treatment techniques continued to produce a high level of mortality, especially among inexperienced urologists [Shah and Whitfield, 2002; Ellis, 1979].

2.1.3. Prevalence

The overall probability that an individual will form stones varies in different parts of the world. The risk of developing urolithiasis in adults appears to be higher in the western hemisphere than
in the eastern hemisphere, although the highest risks have been reported in some Asian countries such as Saudi Arabia [Ramello et al., 2000; Robertson and Hughes, 1994]. As it is clear from these historical clues, urinary stone has always been a common disease and presently it is the third most common affliction of the urinary tract [Atmani, 2003] with a worldwide prevalence of between 2 – 20% [Johri et al., 2010]. It is well established that calculi in the urinary tract occur more frequently in the natives of certain geographic regions of the world (e.g. Middle East, North Africa, Southern China, Mediterranean regions, North-western state of India and Southern states of US of Turkey and Egypt, the Volga Valley in U.S.S.R., East coast of U.K. and South-Eastern States of U.S.A. [Straffon and Higgins 1970]. Since considerable variations exist in these so called stone belts regarding the mineral content of the water and soil, dietary habits of their natives, and environmental conditions, it is safe to conclude that factors other than these may be important in the etiology of urolithiasis. Depending on the socio-economic conditions and subsequent changes in the dietary habits, the overall probability of stone formers differs in various part of world: 1-5% Asia, 5-9% Europe, 13-15% USA, 12% Canada and 20% Saudi Arabia. The so called stone belts of the world are located in the countries of the Middle East, North Africa, Mediterranean regions, North-western state of India and Southern state of USA [Lopez and Hoppe, 2010]. In India, with a prevalence rate of 15% (Rizvi et al, 2002), two high incidence stone belts have been found to occur. The first belt starts from Amritsar in North and while passing through Delhi and Agra ends up in U.P. The other belt which starts from Jamnagar in west coast extends inwards towards Jabalpur in central India. Very low incidence areas have been in West Bengal and coastal areas of Maharashtra, Karnataka, Kerala, Tamil Nadu, Andhra Pradesh [Tandon et al., 1999]. The incidence of urinary stones has been increasing over the last few years while the age of onset is decreasing [Devuyst and Pirson, 2007]. With the prevalence rate of >10% and an expected recurrence rate of ~50%, stone disease has an important effect on health care system [Knoll, 2007].
2.2. Types of stones

Urolithiasis is not a single disease; approximately 75% of stones are primarily calcium oxalate, but up to 50% of these include calcium hydroxyl phosphate (brushite or calcium hydroxyapatite), in trace or greater amounts; 10–15% of stones are composed of magnesium ammonium phosphate (struvite or triple phosphate); 5% are composed of urate; and 1–2% are composed of cystine (Table 2.1 A pictorial view of the different types of kidney stones is depicted in figure 2.2.

<table>
<thead>
<tr>
<th>Type of stones</th>
<th>Percentage Prevalence</th>
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<tbody>
<tr>
<td>Calcareous stones (Calcium oxalate stones &amp; Calcium phosphate stones)</td>
<td>75-90%</td>
</tr>
<tr>
<td>Magnesium Ammonium Phosphate (Struvite stones)</td>
<td>10-15%</td>
</tr>
<tr>
<td>Uric acid stones</td>
<td>3-10%</td>
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<tr>
<td>Cystine and other stones</td>
<td>1-2%</td>
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Table 2.1 Percentage prevalence of different types of kidney stones.

2.2.1 Calcium oxalate stones (monohydrate or dihydrate)

Calcium oxalate stones are the most common type of urinary calculi and can exist in monohydrate and dihydrate forms, with or without phosphate. High phosphate content may be associated with higher recurrence rates. Calcium oxalate stones are radiopaque and usually visible on plain film radiography or noncontrast CT (Computed Tomography). Hypercalciuria (i.e., more than 250 mg per 24 hours) [Menon and Koul, 1992] is the most common metabolic abnormality associated with these calculi. Other causes of calcium oxalate stones include hyperoxaluria (i.e., more than 45 mg per 24 hours), hypocitraturia (i.e., less than 450 mg per 24 hours), which involves a deficiency of the naturally occurring stone inhibitor citrate, and hyperuricosuria (i.e., more than 800 mg per 24 hours) [Pietrow and Karellas, 2006]. Hypercalciuria is heterogeneous in origin and three types existed: 1) absorptive hypercalciuria, in which the primary abnormality is an increased intestinal absorption of calcium;
2) renal hypercalciuria, characterized by a primary renal wasting of calcium; and 3) resorptive hypercalciuria, characterized by increased bone demineralization [Menon and Koul, 1992]. Oxalate is an unavoidable component of the human diet as it is a ubiquitous component of plants and plant-derived foods. Endogenous oxalate synthesis primarily occurs in the liver with glyoxylate as an immediate oxalate precursor. Hyperoxaluria can be generally divided into two categories: primary and secondary hyperoxaluria. Primary hyperoxaluria is the result of inherited (mostly) hepatic enzyme deficiencies leading to increased endogenous oxalate synthesis. Secondary hyperoxaluria results from conditions underlying increased intestinal oxalate absorption, such as (1) a high-oxalate diet, (2) fat malabsorption (enteric hyperoxaluria), (3) alterations in intestinal oxalate degrading microorganisms, Oxalobacter formigenes and (4) genetic variations of intestinal oxalate transporters [Robijn et al., 2011]. The cause of hypocitraturia often is idiopathic, although high dietary acid loads (e.g., from excessive meat intake) and dehydration can exacerbate this condition [Pietrow and Karellas, 2006]. Hyperuricosuria, being a risk factor for calcium oxalate stone disease can be attributed to several factors, including promotion of heterogeneous nucleation by uric acid or monosodium urate, blocking of some of the inhibitory effects of urine macromolecules on calcium oxalate crystal formation, or a lowering of the limit of metastability for calcium oxalate by uric acid.

2.2.2 Calcium phosphate stones
Calculi that consist predominantly of calcium phosphate occur more often in women than in men. Reports indicate a higher rate of recurrence in stones with a greater fraction of calcium phosphate. They often are associated with acidification disorders such as renal tubular acidosis; less common etiologies include primary hyperparathyroidism, excessive alkalization, and sarcoidosis. Renal tubular acidosis is associated with hypercalciuria and hypocitraturia. Medical treatment of these stones consists of replenishing urinary citrate to prevent new stone formation and delay growth of existing stones [Pietrow and Karellas, 2006].
Patients with stone disease often manifest high levels of urinary phosphate excretion, either as a result of excessive dietary intake or due to a renal phosphate leak as a component of the syndrome of absorptive hypercalciuria [Williams et al., 1996].

2.2.3 Struvite stones
Struvite stones, also known triple-phosphate stones consist of magnesium, ammonium, and calcium phosphate. They occur more often in women than in men and are the leading cause of staghorn calculi. Neurogenic bladders and foreign bodies in the urinary tract also predispose patients to struvite calculi. Recurrent urinary tract infections with urea-splitting organisms result in alkalization of urine and the addition of ammonium to the milieu. The prerequisites are thereby fulfilled for the precipitation of both magnesium ammonium phosphate and carbonate apatite. The formation product of magnesium ammonium phosphate is \( 2.5 \times 10^{-13} \text{(mol/L)}^3 \). Struvite stones are usually radiopaque on standard radiographic imaging but may be quite faint [Tiselius, 2003; Pietrow and Karellas, 2006].

2.2.4 Uric acid stones
Uric acid stones may consist of uric acid only, or they also may contain calcium [Moe et al., 2002]. Uric acid is a by-product of ingested or endogenous purine metabolism and is excreted in the urine primarily in insoluble form. The primary cause of uric acid stones is a urinary pH below the pK\(a\) for uric acid (5.5) along with high excretion of urate and small urine volume. Uric acid can precipitate at normal urate concentrations if the pH is sufficiently low; the formation product of uric acid is \( 5.0 \times 10^{-9} \text{(mol/L)}^2 \) [Tiselius, 2003]. Radiographic imaging can be difficult because pure uric acid calculi typically are radiolucent. They are, however, readily apparent on non-contrast CT.
2.2.5 Cystine stones
Patients with cystine calculi have an autosomal recessive disorder of dibasic amino acid transport leading to decreased cystine resorption in the kidney. Only homozygote patients form cystine calculi and often present with stones during childhood. Calculi may be pure cystine or may be mixed with calcium oxalate. Cystine is poorly soluble at normal urinary pH and will readily form stones when levels rise above a concentration of 250 mg/L or at an ion-activity product of $>1.3 \times 10^{-20}$ (mol/L)$^3$. Pure cystine stones are yellow and radiolucent [Tiselius, 2003; Pietrow and Karelis, 2006].

2.2.6 Other types
People afflicted with xanthinuria often produce stones composed of xanthine. People afflicted with adenine phosphoribosyltransferase deficiency may produce 2, 8-dihydroxyadenine stones [Kamatani, 1996] alkaptonurics produce homogentisic acid stones, and iminoglycinurics produce stones of glycine, proline and hydroxyproline. Urolithiasis has also been noted to occur in the setting of therapeutic drug use, with crystals of drug forming within the renal tract in some people currently being treated with agents such as indinavir, sulfadiazine [Schlossberg and Samuel, 2011] and triamterene [Carr et al., 1990].
Figure 2.2 A pictorial view of different types of kidney stone
2.3. Etiology of Urolithiasis

The risk factors involved in kidney stone formation might influence the clinical course of the disease. Various risk factors are involved in development of urinary calculi along with their mechanism of action [Pietrow and Karellas, 2006]. Risk factors are generally divided into urinary factors and prurinary factors.

2.3.1. Urinary factors

a) Effect of pH: Uric acid stones occur in patients with very low urine pH (below pH 5) and in those with hyperuricosuria [Sakhee et al., 2002]. In some patients low urine pH is caused by a defect in renal ammonia secretion that results in less buffering of secreted hydrogen ion and lower urine pH.

b) Concentration of salts in urine: The key process in the development of kidney stones is supersaturation. Salts such as calcium oxalate, uric acid, cystine, or xanthine can become extremely concentrated under certain circumstances. If the volume of the urine is significantly reduced, or if abnormally high amounts of crystal forming salts are present, they precipitate out and form crystals.

c) Volume of urine: Increasing urinary volume is an important tool in the prevention of calcium renal stones. Urine dilution considerably reduces crystallization phenomena induced in vitro by an oxalate load in both calcium stone-formers and normal subjects [Guerra et al., 2005].

d) Enzymes: The initial step in the pathogenesis of urolithiasis must be the precipitation of an organic matrix of mucoproteins. An important factor in this process may be the activity of and/or concentration of the urinary enzyme, urokinase, which would affect the level of urinary mucoproteins. A decrease has been observed in urinary urokinase concentration of renal stone patients which, once again, underlines the possible involvement of urokinase in renal stone formation [Toit et al., 1997].
2.3.2. Preurinary risk factors

a) Heredity: Several workers have given evidence to support the concept that the familial incidence of urinary calculi is related to heredity. Now it is known to be a polygenic defect influencing various enzymes of oxalate, uric acid metabolism [Khan and Canales, 2009; Koyuncu, 2010]. Ethnicity has also been shown to be an important factor for calculosis. For example, a low incidence of calculi has been found in Negroes of Africa and America, North American Indians, natives of Israel, South Indians and South Americans.

b) Age: The maximum incidence of idiopathic calcium stone disease has been shown to occur between the third and the fifth decade of life. It has been found to be quite uncommon in children and elderly people [Soucie et al., 1994; Hiatt and Friedman, 1982].

c) Sex: Males are generally known to be more prone to calcium stone disease as compared to females. This could be due to the fact that as compared to females, males are known to excrete more calcium, oxalate, uric acid in their urine leading to its higher saturation. The anatomical structure of the female urogenital tract also facilitates easy passage of initially formed crystallloid. Moreover, effect of estrogens in increasing the urinary citrate excretion which have a solubilising effect on calcium could be responsible for the low incidence of urinary calculi in females. The incidence of renal calculi is four times higher in males as compared to females [Davidson et al., 2009; Hiatt and Friedman, 1982].

d) Geographical distribution: The incidence pattern of renal stones in India has been determined and two high incidence stone belts have been found to occur [Coblabawala, 1971]. The first belt starts from Amritsar in North and while passing through Delhi and Agra ends up in U. P. The other belt which starts from Jamnagar in the West Coast extends inward towards in Jabalpur in Central India. Very low incidence has been found in West Bengal and coastal areas of Maharashtra, Karnataka, Kerala, Tamilnadu and Andhra Pradesh. The Northern part of the United States (except in Negroes) has also been shown to have a relatively high incidence of urinary calculi. Other geographic areas surveyed and reported to have high incidence include Central Europe, Japan, North India, Pakistan, Northern Australia, parts of Malaysia and China [Zerwekh et al., 1983].
e) **Climatic factors**: The high summer temperatures in South Eastern United States have been shown to be responsible for high incidence of urinary calculi. Reasons for higher incidence in summers could be an increased conversion of vitamin D to its active metabolites resulting in increased calcium absorption from intestines. Decrease in urine production due to greater loss of water from sweat causes an increase in the concentration of stone constituents in urine and hence supersaturation of urine with stone forming constituents leading to calculosis [Milliner, 1995; Maalouf, 2012].

f) **Dietary factors**: The dietary content of animal proteins and fats has been found to be approximately 5 times higher and that of sugar 10 times greater in developed countries [Milliner, 1995; Grases, 2006] than in Africa (South of Sahara) where occurrence of Ca-stones is very rare. Use of refined carbohydrates and animal proteins has further been shown to increase urinary calcium and oxalate excretion which could be responsible for high incidence of renal calculi. Sucrose has been observed to cause a significant increase in intestinal calcium absorption in idiopathic stone formers as well as in normal subjects. The effect of nutrient rich and fiber depleted diets on absorptive and excretory mechanism is likely to increase urinary supersaturation with respect to CaOx [Taylor et al., 2009]. Vitamin K deficiency is known to be associated with stones of renal origin. Vitamin K is known to promote the formation of gamma-carboxy glutamic acid which has high affinity for Calcium. If the renal carboxy glutamic proteins were to play a role in tubular handling then a decrease in their formation as possibly reflected by the decrease in urinary gamma-carboxy glutamic acid excretion in vitamin K depleted animals should lead to an altered tubular handling at calcium [Angayarkanni and Selvam 1998]. Thiamine deficiency has also been reported to increase the risk of calcium lithiasis by causing hyperoxaluria and/or decreasing the citrate excretion in urine.
g) Occupation: It has been found that urinary calculi are more likely to be found in individuals having sedentary occupations as compared to individuals doing manual work [Taylor et al., 2009]. The possibility exists that the physically strenuous nature of the manual work leads to the disruption of the crystal aggregation phenomenon in the urinary tract.

h) Water intake: A number of investigators have shown that increased water intake and increased urinary output decreases the incidence of urinary calculi [Miggiano and Migneco, 2007]. A small urinary volume, whether caused by low fluid intake or increased fluid loss through other routes has been shown to result in increased stone formation.

2.4. Pathophysiology of Urolithiasis

2.4.1. Supersaturation

During the process of water conservation, kidneys supersaturate urine [Carvalho and Nakagawa, 1999; Evan, 2010]. The formation of renal stones is a consequence of increased urinary supersaturation with subsequent formation of crystalline particles. Supersaturation (SS) is the driving force for crystallization in solutions like urine, which means that it will contain crystals that are formed spontaneously [Tsujihata, 2008]. The concentration at which saturation is reached and crystallization begins is called the thermodynamic solubility product (Ksp). Urine is thus metastable with respect to calcium salts. Indeed, stone formers tend to excrete urine that is more supersaturated than that of non-stone formers [Robertson et al., 1968]. However, supersaturation values overlap widely [Kok and Papapoulos, 1993], and people who have never formed a stone may nonetheless pass highly supersaturated urine [Robertson et al., 1968]. Humans normally excrete millions of urinary crystals daily, indicating at least transient development of supersaturation. Since most of the solid particles crystallizing within the urinary tract will be excreted freely, particle formation is by no means equivalent to symptomatic stone disease. It has been suggested that with a transit time across the kidney of 5 to 10 min, residence
time is too short for crystals to nucleate and grow large enough to be trapped [Finlayson and Reid, 1978]. In tubular fluid and urine, crystallization processes are largely dependent on solution composition. A variety of urinary constituents may affect solution supersaturation because of their activity as chelaters. For instance, by forming soluble complexes with calcium and oxalate, respectively, citrate and magnesium reduce free ion activity and the relative supersaturation of calcium oxalate [Robertson et al., 1981]. If inhibitors of crystal formation were not able to act and control their size, the final result will be nephrolithiasis and/or nephrocalcinosis [Carvalho et al., 2002; Carvaho et al., 1999]. Inhibitors allow higher concentration of calcium salts to be held in solution than in pure solvents. In as much as recurrence preventive treatment ideally should be as selective as possible, there are two steps that are necessary to enable such an action. First, to decide whether the patient has a high supersaturation level or may be at risk of forming critically supersaturated urine. Thereby the supersaturation or the ion-activity product is the effective or secondary risk factor. Second, it is necessary to identify those factors that in an important way contribute to the supersaturation and the risk of crystallization. These latter variables can thus be considered as primary risk factors.

2.4.2. Calcium Oxalate Crystallization

The understanding of crystalluria further requires some knowledge of crystal nucleation, growth and aggregation, all of which depend greatly on solution concentration. Both the monohydrate and dihydrate species of calcium oxalate (CaOx) crystals are present in kidney stones [Wesson et al., 1998]. The various parts of calcium oxalate crystallization are schematically summarized in figure 2.3.
Figure 2.3 Schematic representation of various cellular and extracellular events during stone formation. *(OPN - Osteopontin, HA - Hyaluronic acid, SA - Sialic acid, MCP-1 - Monocyte chemoattractant protein-1)*
2.4.2.1. Nucleation

Nucleation is the formation of a solid crystal phase in a solution. It is an essential step in renal stone formation [Finlayson and Reid, 1978; Kok, 1997; Khan et al., 1999]. Nucleation of calcium oxalate is assumed to be induced by one or several promoters. Growth and aggregation of calcium oxalate crystals can proceed as long as the ion-activity product of calcium oxalate exceeds the solubility product (SP). Urine contains inhibitors of crystallisation and can hold large concentrations of solute above the Ksp, a metastable state. If the concentration of solute increases further and a point is reached where it cannot be held in solution, this concentration is known as KF, which is the point of formation of product in urine. The process of nucleation in a pure solution is known as homogeneous nucleation [Finlayson and Reid, 1978]. In secondary nucleation, new crystals deposit on pre-existing crystal surfaces of similar type. Secondary nucleation results in the 'mass production' of crystals. Epitaxy is a process whereby material of one crystal type is precipitated upon the surface of another whose lattice dimensions are almost identical [Lonsdale, 1968]. Epitaxy is clinically important in the formation calcium oxalate stones; the presence of uric acid crystals may promote formation of calcium oxalate stones. These 2 processes are closely related to heterogeneous nucleation. Urine is not a pure solution and nucleation in urine often occurs over an existing surface, or an alternative structure. This process is called heterogeneous nucleation. Heterogeneous nucleation sites in urine can be epithelial cells, red blood cells, cell debris, urinary casts, other crystals and bacteria. Hyperuricosuria can promote calcium oxalate crystallisation without epitaxy, so-called salting out effect. The formation product of heterogeneous nucleation is the ion-activity product at which facilitated crystal formation occurs and the formation product of homogeneous nucleation is the ion-activity product needed for spontaneous formation of crystals. Although crystallogenesis is essential to stone formation, CaOx crystal growth rates are sluggish, to the extent that during typical urine transit times single crystals will not grow large enough to become lodged in the terminal collecting duct of the kidney [Finlayson and Reid, 1978].
Crystallisation represents the first phase of urinary stone formation. Stones result from a phase change in which dissolved salts condense into solids and this transformation is influenced by supersaturation (SS). If SS is <1, crystals of substance will dissolve but crystals can form and grow if SS >1 and urine SS >1 is metastable and excess dissolved substance will precipitate. Standard laws of physical chemistry can explain the occurrence of crystals in a static solution but urine in the kidney is not static. In a landmark study kidney biopsies from area adjacent to Randall’s plaque on stone forming patients [Evan et al., 2003; Matlaga, et al., 2007]. In hypercalciuric calcium oxalate stone formers, they observed initial calcium phosphate deposition in the basement membrane of the thin limbs of the loop of Henle. They also found further extension to the vasa recta, then to the interstitial tissue surrounding the ducts of Bellini, and finally proceeding to the urothelium of the papillary tip. In patients with hyperoxaluria resulting from intestinal bypass initial crystal deposition was found in the lumens of a few collecting ducts and crystals were hydroxyapatite. No crystal deposits were seen in the interstitium or around the thin loop of Henle. These findings raised further questions; why does the initial crystallisation occur in these sites and why initial crystals are composed of calcium phosphate. Bushinsky hypothesied that physiological changes within the interstitium and vasa recta following ingestion and absorption of dietary calcium, may decrease bicarbonate removal from the medullary interstitium [Bushinsky, 2003]. The resultant increased pH would reduce the solubility of calcium phosphate complexes and probably an extracellular matrix protein may promote heterogeneous nucleation. Stoller [Stoller et al., 2004] suggested a possible intravascular phenomenon in the vasa recta may affect the adjacent urinary collecting system and may promote initial solid phase. There is evidence that the process of calcium stone formation starts as a precipitation of calcium phosphate either in the loop of Henle or in the distal part of the distal tubule [Luptak et al., 1994; Tiselius, 1996; Asplin et al., 1996; Hojgaard and Tiselius, 1999]. Although the urine at these levels of the nephron might be critically supersaturated with calcium oxalate in patients with
hyperoxaluria and in experimental animals following administration of ethylene glycol, the ion-activity product of calcium oxalate is usually too low to result in calcium oxalate crystal formation [Luptak et al., 1994]. There are also other less specific constituents of nephron urine that have the capacity to induce nucleation of calcium salts, such as, for instance, blood cells, crystals of sodium urate, cholesterol, or other intratubular particles. Crystals of calcium phosphate that form at a high nephron level [Tiselius, 1997] might be dissolved when they are exposed to the acid urine in the collecting duct [Hojgaard, 1999]. Dissolution of calcium phosphate causes a high level of supersaturation with calcium oxalate by increased urine concentration of calcium. Nucleation of calcium oxalate can thus take place either by epitaxis on the surface of the dissolving calcium phosphate crystal or by nucleation, with or without the contribution of a promoter or by nucleation in the macromolecular environment that surrounds the calcium phosphate crystals. The latter process might take place either freely in the tubular lumen or more likely at tubular wall.

2.2.4.2 Crystal growth

After nucleation, crystal growth is the next major step of stone formation. What causes crystals to grow? The driving force for crystallization is a reduction in the potential energy of the atoms or molecules when they form bonds to each other. The crystal growth process starts with the nucleation stage. Several atoms or molecules in a supersaturated liquid start forming clusters; the bulk free energy of the cluster is less than that of the liquid. The total free energy of the cluster is increased by the surface energy (surface tension), however, this is significant only when the cluster is small. Crystal growth is determined by the molecular size and shape of the molecule, the physical properties of the material, SS levels, pH, and defects that may form in the crystal’s structure. Crystal growth is one of the prerequisites for particle formation. Using the powerful atomic-force microscope (AFM), Laboratory researchers are discovering complex growth mechanisms and three-dimensional structures of solution-based crystals [Qiu et al., 2004].
Under normal conditions, the crystals of calcium oxalate and calcium phosphate that form are small and well protected from crystal growth and crystal aggregation by a cover of inhibitory macromolecules. The negatively charged macromolecules have a high affinity to the positively charged surface of calcium salt crystals. The aggregation inhibiting properties of small as well as of large molecules are related to their electronegativity which establishes repulsive forces between adjacent crystals and between the crystals and the similarly negatively charged macromolecular layer on the surface of tubular cells (Figure 2.5). In this way, it is likely that small crystals are fast and can easily move through the tubular system and be excreted with urine. It is possible that small calcium phosphate crystals are completely dissolved during their transport through the collecting duct. Under appropriate conditions primary nucleation of calcium oxalate might occur in collecting duct urine. As long as these crystals remain small and are well protected from growth and aggregation they leave the tubular system without problems.

Low concentrations or structural abnormalities of crystallization modifying macromolecules or small molecules will cause increased growth and aggregation of crystals so that large crystal masses form either of calcium phosphate or of calcium oxalate [Coe and Parks, 1990]. Large crystal masses with or without insufficient protection by macromolecules will adhere to the tubular cell. The crystals might alter the plasma membrane so that endocytosis occurs whereas crystals of reasonable size can be taken care of and destroyed by the cell, larger crystal agglomerates might cause cell death [Khan et al., 2000; Koul et al., 1996]. When the crystals that are bound to the basolateral membrane or deposited interstitially are too large, the capacity of macrophages and inflammatory cells will probably be insufficient to cope with the crystals. Such crystal material might accordingly be transported within the interstitial tissue down to the papilla where it can provide a basis for crystal deposition and growth following erosion of the epithelial surface. It is understood that the insufficient or defect control of the crystallization process also will result in development of large crystals or crystal agglomerates that remain within the tubular lumen.
Under such conditions crystals of calcium oxalate or calcium phosphate might be trapped at the lower and narrow end of the collecting duct and thereby serve as a nidus for further crystal deposition in the supersaturated urine. Figure 2.6 summarizes the various possibilities of calcium salt crystalluria and calcium stone formation. Small crystals of calcium oxalate or calcium phosphate that might have formed in the nephron can disappear either by intraluminal dissolution or by cellular action. These crystals can also be excreted with urine as microscopic crystalluria, which is a common finding in both stone formers and normal subjects.

2.2.4.3 Cell crystal interaction/crystal adherence
Finlayson and Ried calculated that free particle stone formation [Finlayson and Reid, 1978] was mathematically impossible and proposed that stone disease required the adherence of crystals to the renal epithelium (fixed particle nucleation) but Kok and Khan using modern computational methods have proposed that aggregation of free crystals can result in urinary microliths large enough to occlude collecting ducts (free particle nucleation). Recalculation of old data has, however, indicated that free particles of calcium phosphate as well as of calcium oxalate might form at the levels of supersaturation that occasionally are built up in nephron urine [Kok and Khan, 1994]. In this way crystals might become large enough to be trapped intra-tubularly. Any crystallization that occurs in this part of the nephron most certainly is facilitated by promoters (Figure 2.4) and it has been suggested that lipoprotein membranes from the brush border of proximal tubular cells might serve this purpose [Khan, 2000]. Experimental research has shown that the brush border membrane might be injured by free radicals formed as the result of toxic effects on the cell. This might lead to lipid peroxidation and cell death [Thamilselvan and Khan, 1998]. The released membrane fragments that are transported down the nephron thereby can supply a suitable surface for deposition of both calcium oxalate and calcium phosphate. The interaction between the tubular cells and crystals are modified by several macromolecules excreted with urine or secreted by the tubular cells [Verkoelen et al., 1997; Lieske et al., 1997a].
Experimental studies with cell cultures have shown that calcium oxalate monohydrate crystals adhere to tubular cells in a specific and rapid way [Lieske and Coe 1996]. It has moreover, been shown that a number of polyanions might prevent the adherence of crystals to cells. Such an effect accordingly was recorded for glycosaminoglycans (heparin, heparan sulfate, hyaluronic acid, and chondroitin sulfate), citrate, nephrocalcin, and uropontin. Tamm–Horsfall protein (THP) on the other hand, did not counteract the adherence of crystals to the cells, but inhibited crystal endocytosis. It was concluded that crystals of calcium oxalate binds to sialic acid residues on cell surface glycoproteins. Also, the lipids of the plasma membrane appear to be of great importance for the adherence of crystals [Bigelow et al., 1997].

Normally, cells lining the tubular system are well protected from crystal adherence, but the situation alters dramatically following cell injury. The same principle for crystal attachment as for calcium oxalate is applicable to crystals of hydroxyapatite (HAP) [Lieske et al., 1997b]. Crystals of calcium oxalate monohydrate (COM), calcium oxalate dihydrate (COD), and HAP that have adhered to the cell surface might be internalized by endocytosis [Lieske et al., 1992; Lieske et al., 1999]. Some of these crystals might be dissolved by the action of lysosomal enzymes within the tubular cell, whereas others might be transported to the basolateral membrane and expelled into the interstitial tissue where macrophages and other inflammatory cells can take care of the crystals and destroy them [Khan, 1996]. In this way a substantial amount of crystalline material might be removed from the tubular system and it is likely that this is one of the defense systems that the kidney has developed to protect from intratubular crystallization and obstruction. In response to high concentrations of oxalate or calcium oxalate crystals tubular cells might proliferate and thus increase the capacity to eliminate crystals. The risk of crystal adherence is certainly greatest for calcium phosphate and calcium oxalate crystals that are large and thus move slowly through the nephron. For very large crystals and crystal masses the repulsive forces described above are probably insufficient to counteract both crystal aggregation and crystal–cell adherence.
It is also well recognized that patients with calcium stone disease excrete in their urine larger crystals and crystal aggregates than normal subjects. Given these principles for the normal crystallization in the nephron, a pathological crystallization leading to stone formation might be the net result of one or several abnormalities or defects in the control of this process. A primary precipitation of calcium phosphate and subsequent dissolution in acid collecting duct urine can give rise to crystal aggregates containing calcium oxalate and calcium phosphate or, in case of complete dissolution, pure calcium oxalate. Pure calcium oxalate aggregates also might form when calcium oxalate is primarily precipitated in the nephron unrelated to a calcium phosphate crystal phase [Lieske et al., 1998]. In patients with a constantly high pH, calcium phosphate crystals will not dissolve and inasmuch as calcium phosphate is the favoured crystal phase in alkaline urine, pure calcium phosphate crystals and stones will be the result. Such crystals most commonly consist of HAP, but might occasionally be composed of Bru, particularly at a lower pH.

2.2.4.4 Crystal aggregation

Crystal aggregation and attachment of crystals or aggregates to an alternative nidus such as renal epithelial cells are critical processes in stone formation (crystal agglomeration). In this process crystals in solution stick together and form a larger particle. Aggregation of particles in solution is determined by a balance of forces, some with aggregating effects and some with disaggregating effects. A small interparticle distance increases attractive force and favours particle aggregation. In addition, Tamm-Horsfall glycoprotein and other molecule may act as glue and increase viscous binding [Hess et al., 1993]. Furthermore, aggregate may be stabilised by solid bridges formed by crystalline material connecting two particles. The main force that inhibits aggregation is the repulsive electrostatic surface charge, known as Zeta potential. In various steps of stone formation, crystal aggregation is a more important factor than nucleation and growth because aggregation occurs within seconds [Blomen, 1982].
It is a widely held belief that the process of calcium stone formation starts as a precipitation of calcium phosphate (CaP) in the loop of Henle or the distal part of the distal tubule [Kok, 1997; Hojgaard et al., 1999]. In normal physiоchemical condition repulsion occurs between the CaP crystals and tubular cells and may result in elimination of small CaP crystal by dissolution or spontaneous passage in urine. Primary nucleation of calcium oxalate (CaOx) crystal is induced by CaP, small crystals are then excreted in the urine. In addition, internalisation and macrophage destruction of CaOx crystals occurs in the interstitial tissue. Pathological crystallisation results from the effects of secreted macromolecules secreted in the urine and secreted by the brush border of proximal tubular cells on the interaction between the tubular cells and crystals [Verkoelen, 1997]. Experimental studies have demonstrated that injury from free radicals may result in sloughed membrane fragments in the tubular lumen and this may providing a suitable surface for nucleation of CaP and oxalate [Khan et al., 1999]. This event may cause formation of masses of crystals by growth and aggregation followed by adherence of CaP crystals aggregates to the tubular surface. Newly formed crystals adhere to the tubular cell surface and the cellular responses that follow could result in crystal retention and thereby set in motion a series of events that lead to pathologic renal calcification. Dissolution of CaP in acid urine causes a high level of supersaturation (SS) with CaOx [Hojgaard et al., 1999] and nucleation of CaOx facilitates the formation of a large mass of CaOx and phosphate attached to the tubular cell wall.

2.4.2.5 Crystal retention
Irrespective of whether the initial crystallization is the result of free or fixed particles, stone formation cannot occur unless crystal material is retained in the renal collecting system. Retention of crystal material can, however, also be the result of interaction between the crystals and the cells and such a mechanism is assumed to play an important role [Mandel, 1994; Khan, 1996; Verkoelen et al., 1995; Verkoelen et al., 1997; Lieske and Deganello, 1999]. Urolithiasis requires formation of crystals followed by their retention and accumulation in the kidney.
Crystal retention can be caused by the association of crystals with the epithelial cells lining the renal tubules. Crystal formation predominantly depends on the composition of the tubular fluid; crystal retention might depend on the composition of the renal tubular epithelial cell surface [Verkoelen et al., 2000; Schepers, 2002]. A non-adherent surface of the distal tubules, collecting ducts, ureters, bladder, and the urethra may provide a natural defence mechanism against crystal retention, and may become defective when the anti-adherence properties are compromised. In a cell culture model, Verhulst et al observed upregulated cell surface expression of hyaluronic acid, osteopontin, and their receptor CD44, as well as the formation of a hyaluronic acid-dependent cell coat, and suggested that it may play a crucial role in the process of crystal retention [Verhulst et al., 2003].
Figure 2.4 Overview of the various possible steps in calcium stone formation. (SS=supersaturation)
Figure 2.5 (a) Hypothetical interpretation of the possible series of events of the normal crystallization in urine: (1) brush-border membrane of proximal tubular cells; (2) repulsion between small calcium phosphate crystals; (3) between calcium phosphate crystals and tubular cells; (4) elimination of small calcium phosphate crystals by dissolution or passage with urine; (5) internalization and intracellular dissolution of calcium phosphate crystals; (6) primary nucleation of calcium oxalate; (7) calcium oxalate nucleation induced by calcium phosphate; (8) attachment of small calcium oxalate crystals to the tubular cell; (9) internalization and dissolution; (10) macrophage destruction of calcium oxalate crystals in the interstitial tissue, small intraluminal crystals of calcium oxalate are excreted with urine.
Figure 2.6 (b) Hypothetical interpretation of the possible series of events of the pathological crystallization in urine: (1) destruction of the brush-border membrane of proximal tubular cells; (2) nucleation of calcium phosphate crystals promoted by membrane fragments; (3) formation of large masses of calcium phosphate crystals by growth and aggregation; (4) adherence of crystal aggregates to the tubular surface; (5) dissolution of calcium phosphate in acid urine and nucleation of calcium oxalate; (6) formation of a large mass of calcium oxalate and calcium phosphate crystals attached to the tubular wall; (7) primary nucleation of calcium oxalate induced by membrane fragments with or without participation of urinary macromolecules; (8) attachment to the tubular cell of large calcium oxalate aggregates; (9) partial dissolution of internalized calcium oxalate crystal material; (10) migration of crystals to the interstitial tissue where the capacity of macrophages is insufficient to cope with the large crystal masses; (11) destruction of tubular cells with binding of crystals to the basolateral membrane; (12) formation of an intratubular stone nidus by an assembling and trapping of crystal aggregates; (13) interstitial migration of crystalline material to the papillary tip.
2.5. STONE & STONE MATRIX

Like other products of crystallization in biological systems [Lowenstam and Weiner, 1989] stones are a composite of crystals and organic material, often referred to as matrix [Boyce, 1972; Iwata et al., 1988; Khan and Hackett 1993a]. A variety of crystals including CaOx, CaP, uric acid, struvite, and cystine can be present in stone [Finlayson, 1978; Finlayson, 1977; Khan and Hackett 1987]. CaP crystals appear most frequently in both the urine and stones however, CaOx is the major crystal in most stones. Stones, particularly those containing CaOx or uric acid have a compact structure. Their outer surfaces appear smooth at low magnification but reveal the presence of individual tabular or plate-like CaOx monohydrate (COM) crystals at higher magnifications. Crystal habits are generally not evident on surfaces exposed by cutting or fragmenting the stone. Such surfaces are typically stratified with radial striations and concentric laminations or layers, with radial striations being the predominant feature. Some of the striations run through many laminations while others are limited to only one. Many converge to a point at the base of a lamination mimicking the arrangement of petals in a flower. These points are suggested to be the nucleation sites of crystals.

The laminations are approximately 50-60 μm thick and in many stones can be easily separated from each other exposing the underlying surfaces. The latter show the same structure as the outer stone surface, with protruding tips of the tabular COM crystals frequently covered with amorphous to flaky matrix material. Overall it is a highly ordered structure. Many stones have a well-defined nucleus that is less ordered, with a granular and non-stratified appearance. It is generally occupied by spherulitic or amorphous CaP and/or aggregates of dumbbell shaped twinned COM crystals. CaP frequently fills the space between CaOx crystals as well as that in the concentric laminations.

The organic matrix of most urinary stones accounts for 2-3% of their total dry weight. Matrix consists of macromolecules generally present in the urine. These molecules play a significant role in the development of kidney stones. Some of them promote crystal formation, growth,
aggregation and retention, while others inhibit these processes. Their activity is often complex and depends on the urine conditions prevailing at the time of crystallization or retention. The same macromolecule can both promote and inhibit a process. For example macromolecules behave differently in solution than when they are attached or adsorbed to a surface. It may well be, that compounds free in solution cover a crystal surface and inhibit its growth or ability to aggregate while the same compound bound to a surface acts to accumulate salt ions and forms a template for the first nucleus. The latter will play a role when stone formation involves processes at cell surfaces and in the sub-epithelial space [Kok and Schell-Feith, 1999]. Boyce et al., defined and established the importance of stone matrix in urolithiasis, proposing that the matrix actively participates in the assembly of kidney stones [Xie et al., 2001]. In their view, the matrix acts as a template and controls crystallization within its bounds. An opposite hypothesis was advanced by Vermeulen et al., who viewed the matrix and its ubiquitous presence as merely coincidental because stones form by crystallization in urine in the presence of large macromolecules [Vermeulen and Lyon, 1968; Finlayson et al., 1961]. According to them the matrix is adventitiously acquired, primarily by physical adsorption of urinary mucoproteins on crystal surfaces. Another hypothesis proposed by Khan et al explains that the role of matrix compounds is different in the formation of the stone center and in the subsequent build-up of the stone. The first is a short-term event involving crystal formation and retention. The second is a long term event occurring after a stone nidus has been formed and retained. Both events do not necessarily take place at the same site. Solution depletion [Leal and Finlayson, 1977] and examination of crystals incubated in protein solutions by transmission electron microscopy tested the theory of physical adsorption of urine proteins on surfaces of CaOx crystals. Results showed proteins have a strong affinity for CaOx crystals. Adsorption of anionic proteins was sensitive to calcium ion concentration, whereas cationic protein adsorption depended upon the oxalate ion concentration with temperature and pH playing only a minor role.
Proteins formed a discontinuous coat around the crystals ranging in thickness from 10 to 20nm. It has been suggested that newly formed crystals with a macromolecular coat are less likely to dissolve during the routine urinary ionic and pH changes and therein may lay the importance of matrix in stone formation [Khan and Hackett, 1993b]. The organic matrix of urinary stones contains lipids, GAG's (20%), carbohydrates and proteins. Proteins comprise approximately 64% of the matrix. Table 2.1 lists the compounds, which have been identified in matrices of urinary stones. Most of them are proteinaceous in nature. A number of other proteins have also been detected but not identified. Initially lipids were not recognized as constituent of stone matrix [Boyce, 1962] even though detected as an osmiophilic substance during histochemical examination of decalcified stones. All urine macromolecules can become part of stone matrix but only some are there because they have participated in crystallization and stone formation. This appreciation led investigators to study crystallization in vitro; using freshly collected urine to determine the macromolecules that become a part of the crystal matrix [Morse and Resnick, 1989].

2.6. MODULATORS OF CRYSTAL FORMATION AND RETENTION

In urine, three classes of modulators are recognized; low molecular weight (MW) compounds like citrate and pyrophosphate, glyco-proteins, high MW non-protein compounds like acid mucopolysaccharides, glycosaminoglycans and various types of lipids. They modulate crystal formation and retention in the urinary tract either directly by interacting with the crystal or indirectly by influencing the urinary environment. From crystallization experiments with urine, it appears that in non-stone formers the concerted actions of these compounds ensure that:

1. The crystals formed remain unaggregated and small enough to be excreted [Kok et al., 1986; Kok et al., 1990]
2. The crystals have a reduced affinity for epithelial cells [Verkoelen et al., 1996; Schepers et al., 2002; Lieske et al., 1995] and;

3. The crystals if needed are easily recognized and removed by macrophages [Water et al., 2001].

Crystallization in confined spaces, e.g. simulating Randall's plaque formation at the basal membrane below the tubular epithelium has been less studied but even here, inhibitors can decrease crystal growth rates [Achilles et al., 1991; Achilles et al., 1995]. Which inhibitors are the most effective: The first approach to answering this question has been to identify individual urine compounds and test their "inhibitory" potency in crystallization and cell-culture systems. The next problem has been to translate these data to the whole urine situation where singular inhibitors may also co-operate or compete with each other and where restrictions posed by the kidney and urinary tract itself (flow-rates, residence time and changing urine composition) affect their inhibitory and stimulatory powers. Undiluted whole urine strongly affects calcium salt nucleation, crystal growth and crystal aggregation. When preformed CaOx crystals were added to supersaturated whole undiluted urine their growth was almost completely stopped. Crystal growth only occurred when the supersaturation was drastically increased by adding extra oxalate. Urine has an overabundance of inhibitors. Tested in vitro as single compounds some are clearly more effective than the others, however, experimental data suggest that when the most efficient compounds are lacking, others readily take over. For instance, the low MW compound citrate can inhibit crystal growth very effectively at concentrations between 0.1 mM and 1 mM. When citrate was added at these concentrations to urine, however, it did not change the growth inhibitory action of that urine [Kok et al., 1986b]. In studies of large groups of stone formers and healthy controls where urine was tested in a 1:5 dilution, approximating the degree of dilution existing in the collecting ducts, both urine from stone formers and normal subjects strongly inhibited CaOx crystal growth [Kok et al., 1986a; Kok et al., 1990; Erwin et al., 1994]. When all macromolecules were removed by ultrafiltration, the degree of crystal growth inhibition was only slightly reduced [Drach et al., 1979]. In vitro tests have confirmed that macromolecules are the most effective inhibitors of crystal growth. Apparently the low MW compounds take over the
inhibitory function when the high MW compounds are gone. An additional effect of crystal growth inhibition may be that supersaturation will persist longer and the process of nucleation will have more time to proceed [Erwin et al., 1994]. How relevant this is, in view of the short transit times of urine through the nephron (a few minutes) [Kok and Khan, 1994] is not clear. Normal urine can also strongly inhibit crystal aggregation. This function is reduced in single stone former urine and severely reduced in recurrent stone former urine [Kok et al., 1986b; Kok et al., 1990]. Aggregation is important as it can lead to particle retention, just like crystal cell interactions and disturbed flow conditions [Kok and Khan, 1994]. The inhibition of aggregation in urine is correlated to the citrate concentration [Kok et al., 1990]. However, in ultrafiltered urine this relationship is gone [Koide et al., 1981]. Apparently citrate modulates the effect that high MW compounds have w.r.t. crystal aggregation. In addition, it was found that the urinary macromolecular fraction (>10 kDa MW) of single stone formers inhibited crystal aggregation less than that of normals and even less by those from recurrent stone formers [Erwin et al., 1994]. In this study 70-90% of the inhibitory activity was destroyed by proteinase treatment. Citrate has been shown to improve the inhibitory effect of Tamm Horseyall protein (THP) on crystal aggregation [Hess et al., 2000]. Overall it appears that urine contains numerous components, both small and large that competes and cooperates in modulating crystallization and inhibiting stone formation.

2.6.1. Low Molecular Weight Compounds

2.6.1.1. Pyrophosphate and Bisphosphonate

Pyrophosphate is present in urine at concentrations of 15-100μM. In a seeded crystal growth system, it inhibits COM crystal growth by 50% at 16-20 μM [Schwille et al., 1988; Kok et al., 1988; Ryall et al., 1988; Sidhu et al., 1986]. It can also inhibit COM crystal growth inside a gel matrix [Achilles et al., 1989] and effectively inhibits the growth of CaPs [Grases et al., 2000; Robertson, 1975]. If it is equally efficient in urine it can contribute 50% crystal COM growth.
inhibition in the collecting ducts (5 times dilution) and up to 80% in the urine. This efficacy prompted interest in therapies that raise the urine output of pyrophosphate and in non-biodegradable pyrophosphate analogues, bisphosphonates. These inhibit COM crystal growth at least as good as pyrophosphate, 50% inhibition at 1-20 μM concentrations [Kok, 1995]. Another feature of pyrophosphate, 2.0 10-4 mol/l, which it shares with citrate, 1.0 10-3 mol/l, is that COD is preferentially formed in its presence. The critical pyrophosphate concentration above which COD formation is prevented may be lowered to the physiological range by adding citrate [Yuzawa, 1998]. This is of interest as COD is the major crystal phase in normal crystalluria [Cerini et al., 1999] while there is more COM in stone former crystalluria and COM predominates in stones. The effects of pyrophosphate and bisphosphonates on crystal aggregation are more complex. Pyrophosphate increasingly inhibits crystal aggregation at increasing concentrations [Kok et al., 1988]. At its concentration range in the collecting ducts it could contribute to the whole urine effect on aggregation. While some bisphosphonates have a comparable effect, others show no effect, a stimulatory effect on aggregation or even a biphasic effect, inhibiting aggregation at low concentrations and stimulating it at higher concentrations [Kok, 1995]. From experiments with a series of bisphosphonates where slight variations were made in their structure it was concluded that bisphosphonates bind to the crystal surface by a combined action of the two phosphonate groups and side chains in close proximity. Increasing the affinity for calcium of these side groups increased the capacity to inhibit crystal growth. The presence of two calcium binding phosphate groups makes bisphosphonates to likely form large polymolecular complexes with calcium ions acting as a bridge [Bone et al., 1979]. These complexes act as one macromolecular structure and inhibition of aggregation is reversed to stimulation [Kok, 1995]. The complexes bind to more than one crystal at the same time and act as a bridge (viscous binding). Viscous binding can also explain why some macromolecules may at the same time strongly inhibit crystal growth and strongly stimulate crystal aggregation [Kok et al., 1986b].
Bisphosphonates with a large side chain (steric hindrance) do not form such large complexes and do not show stimulation of crystal aggregation. Growth inhibition by bisphosphonates also depends on their protonation state, thus on the pH and its pKa-values. The triply deprotonated form, present when the pH surpasses the pKa3-value, is the most effective in inhibiting crystal growth. A pH-dependency is also found for pyrophosphate and citrate [Caudarella and Vescini, 2009]. In the urine pH range ionic species of pyrophosphate are $\text{PP}^4^+\text{, HPP}^3^-\text{ and H}_2\text{PP}^2^-$. The first two adsorb onto COM crystals [Wagner and Finlayson, 1978] and will predominate at higher pH values. Variation of the pKa3 value of a bisphosphonate increases its activity at low urine pH values and might reduce its anti bone resorptive capacity. It may be possible to construct a bisphosphonate that strongly inhibits CaOx crystal growth and crystal aggregation at the urine pH levels and does not interfere with bone resorption activity at the low pH levels existing under active osteoclasts. Since pyrophosphate is an effective inhibitor under non-urine conditions, several groups have investigated if stone formers have a low urine pyrophosphate excretion. Pyrophosphate enters the urine in the glomerular filtrate. The plasma concentration is 2-3 $\mu$M, of which 70-80% is ultrafiltrable. The urine excretion rate is variable. In male non-stone formers the concentration averages 20-40 $\mu$M, the 24hr excretion rate is 30-60 $\mu$moles (range 15-98 $\mu$moles). It is nevertheless possible that increasing pyrophosphate excretion raises the growth inhibitory power of urine and as such is beneficial.

2.6.1.2. Citrate

Citrate inhibits COM crystal growth at concentrations above 0.1 mM [Hess et al., 2000], which is in the range of its concentration in the loop of Henle [Kok, 1996b]. It is also inhibits crystal growth in a gel matrix [Achilles et al., 1989]. Citrate may contribute to crystal growth inhibition at sites where other, macromolecular, modulators have not entered the fluid yet [Caudarella and Vescini, 2009]. Citrate also affects crystal aggregation, both in solution [Kok et al., 1988] and in a matrix situation [Achilles et al., 1997].
Tested as single modulator present, citrate inhibits crystal aggregation at concentrations above 0.1mM [Kok et al., 1988] and thus could be active up to the loop of Henle. However, this data cannot be directly extrapolated to the whole urine situation. When whole urine is tested in 1:5 dilution (the dilution State in the collecting duct) urine is found to strongly inhibit crystal aggregation, and there is a strong correlation with the urine citrate concentration [Kok et al., 1986b; Kok et al., 1990]. But when all macromolecules are removed and citrate remains, the urine loses most of its capacity to inhibit crystal aggregation and the relationship between crystal aggregation inhibition and citrate concentration is lost.

2.6.2. High Molecular Weight Compounds
2.6.2.1. Glycosaminoglycans (GAGS)
In 1684 Anton von Heyde discovered the presence of a mucoprotein matrix in stone. Later, urine was found to contain many different anionic proteins and non-protein anions like (GAGS), RNA and acid mucopolysaccharides. Most prominent are the GAGS, polyanionic compounds with varying MW of usually 18-40 kDa but up to 100 Da. GAGS can enter the urine by filtration, by release from the glomerular basement membrane [Pitcock et al., 1988] from the surface of the tubular epithelial lining and the urothelium further down the urinary tract, including the bladder [Edyvane et al., 1983]. Well-known GAGS include heparin (not present in urine) and the urinary GAGS heparan sulfate (HS), chondroitin sulfate A B and C (CS-A, CS-B, CS-C) dermatan sulfate (DS), keratan sulfate (KS) and the non-sulfated hyaluronic acid (HA). Some, but not all urinary GAGS are found in crystals and stones [Morse and Resnick 1989; Nishio et al. 1985; Roberts and Resnick 1986; Yamaguchi et al., 1993; Suzuki et al., 1994a]. Although quantity does not appear to play a role, some data indicate that the quality of GAGS may vary. Urinary macromolecules and urine from children inhibit crystal aggregation better than urine of adults. The macromolecule fraction of pediatric urine contained more GAGs [Teller et al., 1962].
GAGs from stone formers had an increased nucleation promoting activity but similar crystal growth inhibitory activity [Erturk et al., 2002]. The first appeared related to a changed action of HA in stone formers [Gohel et al., 1992]. However, CS of healthy individuals also showed a basal crystallization-promoting property [Shum et al., 1999]. Under inorganic solutions and urine conditions the non-urine GAG heparin is the most effective on a molar basis. Of the GAGs present in urine HS is most effective followed at a distance by CS and HA. The heparin analog pentosan polysulfate has an efficacy between heparin and CS. With respect to crystal-cell interactions, coating of crystals by GAGs decreased the binding of crystals to renal epithelial cells in culture, but did not completely abolish it [Schepers et al., 2002; Lieske et al., 1995; de Water et al., 2001].

2.6.2.2. Lipids

Even though lipids account for a small proportion of the matrix; 7-14% in bone, 2-6% in dentin, 12-22% in newly mineralised enamel, 9.6% in submandibular salivary gland calculi and 10.2% in supragingival calculus, they play a significant role in calcification. They promote crystal nucleation, modulate growth and aggregation and become incorporated in growing calcifications. The matrix of all stones examined to date, including struvite, uric acid, CaOx and CaP contains lipids [Khan et al. 1988; Khan et al. 1996]. The protein to lipid ratio is, however, higher in the matrix of struvite and uric acid stones than in CaOx and CaP stone matrices. Even though there are no significant differences in types of lipid, the matrix of struvite stones contains more cholesterol, cholesterol ester and triglycerides than the other three stone types. One dimensional thin layer chromatography separated and identified various phospholipids and glycolipids including sphingomyelin (SM), phosphatidyl choline (PC), phosphatidyl ethanolamine (PE), cardiolipin (CL) and trace amounts of phosphatidylserine (PS) in all stone matrices. Occasionally, the stone matrix also contains phosphatidyl inositol (PI), lyso-PC, lyso-phosphatidic acid (PA) and lyso-PE.
In all stones glycolipids include gangliosides, D-sphingosine, and glucocerebrosides. In addition, the struvite stone matrix contains sulfatides and digalacto diglycerides while CaOx and CaP stone matrix contains cerebrosides 1 and 2 and digalacto-diglycerides. All stone matrices contain both complexed and non-complexed lipids. The amount of complexed lipids is highest in CaP and lowest in uric acid stones. Both complexed and noncomplexed lipids contain cholesterol, triglycerides, phospholipids and gangliosides. Both CaOx and CaP crystals induced in the urine contain lipids [Khan et al., 1996]. There are no significant differences in either the nature of lipid constituents or the amounts of lipid per gram of crystal between the two types of calcific crystals. Glucocerebrosides are the most common glycolipids and SM the most common phospholipid. Gangliosides are the second most common glycolipid, PC and PE the most common phospholipids. Determinations of lipids in the urine before and after experimental induction of CaOx crystals show that the formation of crystals depletes the urine of its phospholipids indicating its incorporation in the crystal matrix. Almost all the urinary phospholipids become incorporated during the formation of crystals [Khan et al., 1996].

2.6.3. Proteins

More than twenty individual proteins have been detected in the matrix of various types of stones. While most of them have been identified (Table 2.2), few are fully characterized and some still remain nameless [Binette et al., 1996] and a few await confirmation of their identity [Tang et al., 1995]. There are Human serum albumin (HAS), α and γ-globulin and Tamm-Horsfall Protein (THP) were the first proteins identified in stone matrix [Boyce et al., 1962]. Albumin is a major component of the matrix of all stone types including CaOx, uric acid, struvite and cystine. It is also found in the matrix of CaOx and CaP crystals precipitated from human urine and it is more pronounced in crystals induced in stone formers urine [Atmani et al., 1998]. Both CaOx and CaP crystals are known to adsorb HAS. THP is not always detected in stones and even then in only minor quantities, 0.002-1.04 mg/g (w/w) of stone [Grant et al., 1973].
THP associated with CaOx crystals is easily removed by washing the crystals with sodium hydroxide solution [Maslamani et al., 2000; Gokhale et al., 1996a] indicating THP’s loose interaction with the CaOx crystal surfaces. Ultrastructural investigations of human CaOx urinary stones and CaOx nephroliths induced in an animal model supported the hypothesis that THP is not included in the crystals [Gokhale et al., 1996a; Gokhale et al., 1996b]. This may explain THP’s scanty presence in the stone matrix. Of the other proteins listed in Table 2.1, osteopontin (OPN), α-1- microglobulin, urinary prothrombin fragment-1 (UPFT-1), and light and heavy chains of inter-α-inhibitor have been identified in the matrix of CaOx and CaP crystals precipitated from the urine of normal and stone forming individuals. Ultrastructural examination reveals OPN to be pervasive in the crystals and stones and a key component of the matrix of CaOx stones [Mckee et al., 1995; Hoyer, 1994]. More OPN is present in CaOx monohydrate stones (800 μg/100mg stone) than in COD stones (10 μg/100mg stone).
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<tr>
<th>S.No</th>
<th>Name of Protein</th>
<th>Mol. Wt. (kDa)</th>
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<th>Role in crystallization (I- Inhibitor/P - Promoter)</th>
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<td>Monocyte chemoattractant protein-1 (MCP 1)</td>
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2.6.3.1. Tamm-Horsfall Protein

THP was first isolated from the urine by Tamm and Horsfall and characterised as a glycoprotein [Tamm and Horsfall, 1950]. It is one of the most abundant proteins in normal human urine and the major constituent of urinary casts. Muchmore and Decker isolated a protein called uromodulin from the urine of pregnant women [Muchmore and Decker, 1985]. Based on amino acid and carbohydrate analysis THP and uromodulin were shown to be identical. THP has a molecular weight of approximately 80 kDa with a tendency to aggregate to the polymeric form. Polymerisation is increased in the presence of free calcium ions, at high ionic strength, osmolality and at low pH. THP has been the subject of extensive research for its implication in stone formation. However, its exact contribution to urolithiasis remains unclear and the results of various studies have been controversial [Hess, 1992]. Some studies indicated that THP promoted CaOx and CaP crystallization [Hallson and Rose, 1976; Yoshioka et al., 1989]. Other studies demonstrated that the macromolecule does not support CaOx crystallization and has no effect on spontaneous precipitation [Yoshioka et al., 1989; Weaver et al., 2009]. Still other studies indicated that THP has no effect on nucleation or growth, but is a potent inhibitor of CaOx crystal aggregation [Hess et al., 1991; Hess et al., 1993]. Hess et al. found that the addition of citrate reduced CaOx crystal aggregation by reducing the self aggregation of THP isolated from stone formers urine. It is important to point out that low citrate or hypocitraturia is common in stone formers and can contribute to crystal aggregation and stone formation in this fashion. THP

![Table 2.2 Urinary and stone matrix protein modulators of crystallisation](#)
activity is controlled by its concentration, urinary osmolality and physicochemical environment of the urine [Scurr and Robertson, 1986]. For example, at low concentrations, THP has a minor effect on CaOx crystallization yet promotes it at higher concentrations. Also, when ionic strength was increased or the pH lowered the inhibition of CaOx monohydrate crystal aggregation by THP was decreased [Hess et al., 1991]. Apparently, at high ionic strength, high THP concentration and low pH, the viscosity of THP increases due to its polymerisation. Several studies have shown that there is no significant difference in the daily urinary excretion of THP between normal subjects and CaOx stone formers [Bichler et al., 1976]. This fact led Hess et al. to hypothesize that THP of stone formers is structurally different from that of the healthy subjects [Hess et al., 1991]. They showed that THP isolated from the urine of stone formers contained less carbohydrate (mainly sialic acid) than the THP obtained from control subjects [Hess et al., 1995]. It has been suggested that the abnormality may be inherited, but sufficient evidence to support this concept is not available at this time.

Studies have also shown differences in sialic acid contents and surface charge between THP from stone formers and normal individuals. Isoelectric focussing (IEF) studies have shown that THP from healthy individuals has a pI value of approximately 3.5, while THP from recurrent stone formers has pI values between 4.5 and 6 and the two exhibit completely different IEF patterns [Schnierle, 1995]. THP is exclusively produced in the kidneys. Based primarily on studies in rat kidneys, it is agreed that THP is specifically localized in epithelial cells of the thick ascending limbs of the loops of Henle [Hoyer et al., 1979; Bachmann et al., 1990] and is generally not seen in the papillary tubules. When CaOx crystal deposits, the nephroliths, are experimentally induced in rat kidneys, THP is seen in close association with the crystals, both in the renal cortex as well as papillae [Gokhale et al., 1996a; Gokhale et al., 1996b]. However, THP is not seen occluded inside the crystals nor produced by cells other than those lining the limbs of the Henle’s loop [Gokhale et al., 2001]. There is no significant biochemical differences in the
THP between one secreted by normal rats or rats with CaOx nephroliths. They have similar amino acid composition, carbohydrate contents, molecular weights and rates of urinary excretion. However, THP from nephrolithic rats has slightly less sialic acid contents, 20% of the total carbohydrate in nephrolithic rats vs. 26% in normal rats. In an aggregation assay, both the normal rat THP and nephrolithic rat THP reduced CaOx crystal aggregation in vitro by approximately 47%. Results of these rat model studies led to the conclusions that THP is most likely involved in controlling aggregation and that the major difference between normal and stone formers THP may be their sialic acid contents. However animal studies cannot rule out THP’s role in modulating crystal nucleation or growth. Another rat model study has shown increased expression of THP in kidneys following unilateral ureteric ligation, which caused tubular dilatation [Miyake et al., 1998]. The results indicated that THP expression in kidneys may be increased without crystal deposition and that increased expression in nephrolithic kidneys may be a result of crystal associated injury to the renal epithelial cells. Even rat model studies have provided controversial results for THP. One study shows decreased renal expression of THP during CaOx crystal deposition [Marengo et al., 2002] while results of another study show upregulation of the THP gene [Katsuma et al., 2002].

2.6.3.2. Nephrocalcin (NC)

NC is a glycoprotein with a monomeric molecular weight of approximately 14 kDa and has a tendency to self-aggregate into a larger macromolecule and thus can exist as a dimer, trimer or tetramer with molecular weights of 23-30, 45-48 or 60-68 kDa respectively [Worcester et al., 1992; Nakagawa et al., 1983; Nakagawa et al., 1985; Nakagawa et al., 1987; Nakagawa et al., 1981]. NC can also bind to THP in the presence of calcium and magnesium ions. NC can be reversibly dissociated into its monomeric form with incubation in ethylenediaminetetraacetic acid (EDTA) for several days. NC is composed of 110 amino acid residues of which 25% are
glutamic and aspartic acid. It contains 2 cysteine and 2 or 3 \( \gamma \)-carboxyglutamic acid (Gla) residues which are suggested to play a significant part in its ability to inhibit CaOx crystallization. Carbohydrate content represents about 10.3% of its weight, with no glucuronic acid and 0.4% sialic acid. Originally purified from human urine, NC has subsequently been isolated from human kidney tissue culture medium, human renal cell carcinoma, kidneys of many vertebrates, mouse renal proximal tubular cells in culture and rat kidney and urine [Coe et al., 1994; Nakagawa et al., 1989]. Immunohistochemical techniques have localised NC in the renal epithelial cells of proximal tubules and thick ascending limb of Henle’s loop [Nakagawa et al., 1990]. The site of its synthesis has not yet been confirmed by localization of NC mRNA. Daily excretion of NC in human urine is about 5-16 mg [Worcester et al., 1992; Nakagawa et al., 1983]. NC was originally regarded as the principal inhibitor of CaOx monohydrate (COM) crystallization in the urine, accounting for approximately 90% of the total urinary crystallization inhibitory activity [Worcester et al., 1992]. According to the recent results however, the contribution of this inhibitor is suggested to be limited to only 16% [Worcester et al., 1993]. NC is suggested to inhibit nucleation, growth, and aggregation of COM crystals. The fractional inhibition of nucleation due to the presence of NC was shown to be equal to that of urine [Asplin et al., 1991] suggesting that this inhibitor accounts for the total nucleation inhibitory activity of urine. However, the amino acid composition and carbohydrate contents of NCs from both the stone formers and normals appeared similar.

2.6.3.3. Inter-\( \alpha \)-Inhibitor

Inter alpha inhibitor (I\( \alpha \)I) are composed of a combination of heavy chains, H1 (60 kDa), H2 (70 kDa), H3 (90 kDa) covalently linked via a CS bridge to a light chain called bikunin (35-45 kDa). The heavy and light chains also exist independently as single molecules. I\( \alpha \)I (180-240 kDa) is a heterotrimer consisting of bikunin linked to heavy chains H1 and H2. The macromolecule consisting of bikunin linked to heavy chain H2 is called I\( \alpha \)I-like inhibitor (I\( \alpha \)LI). Bikunin is a
broad-spectrum protease inhibitor and an acute-phase reactant. tOIt and related proteins have been linked to various pathological conditions such as inflammatory diseases (Witte et al., 1982; Franck and Pederson 1983), cancer [Chawla et al., 1984; Yoshida et al., 1994], renal failure [Toki and Sumi, 1982] and more recently the urinary stone disease. Both heavy and light chains have been identified in the urine [Atmani et al., 1993a; Atmani et al., 1993b; Atmani et al., 1996a; Atmani et al., 1994; Suzuki et al., 2001]. The average concentration of tOIt in the plasma of healthy human subjects is approximately 450 mg/l. It was shown that bikunin isolated from the patients, contained less sialic acid and exhibited less crystallization inhibitory activity than that purified from the urine of healthy subject [Atmani et al., 1994]. In a separate study mean urinary bikunin to creatinin ratio was found to be significantly higher in stone formers than in non-stone forming healthy male and female controls [Suzuki et al., 2001]. Western analysis showed that a significantly higher proportion of stone patients had a 25kDa bikunin in their urine in addition to the normal 40kDa species. 25kDa bikunin was similar to the deglycosylated bikunin and was less inhibitory.

With respect to kidney stone formation, Atmani et al. isolated a 35kDa urinary protein, which inhibited growth of CaOx crystals. They named the protein uronic acid rich protein (UAP), because of the high uronic acid content with D-glucuronic and L-iduronic acids being the major constituents [Atmani et al., 1993a]. Amino acid composition revealed it to be rich in aspartic and glutamic acid residues, which account for 24% of the total amino acids. No Gla residues were detected. Basic and aromatic amino acids represented 10% and 13%. Carbohydrates accounted for 8.5% of its weight. N-terminal amino acid sequence analysis of human protein demonstrated the homology with tOIt, specifically with bikunin [Atmani et al., 1993b]. Later UAP was isolated the from the rat urine and showed it to have characteristics similar to the human UAP in molecular weight, amino acid composition as well as the crystallization inhibitory activity. In addition, on Western blot analysis, both reacted with an inter-α-trypsin inhibitor antibody. Later, on the basis of bikunin antibody reaction with the UAP in western blot analysis and similarity of
the sequence of first 25 N-terminal amino acid residues of UAP being identical to that of bikunin UAP was identified as bikunin [Atmani et al., 1996a]. Iα1 proteins have been shown to inhibit CaOx crystallization in vitro [Atmani et al., 1993a; Atmani et al., 1993b; Atmani et al. 1996; Medetognon-Benissan et al., 1999; Kobayashi et al., 1998]. The inhibitory activity is confined to the carboxy terminal of the bikunin fragment of Iα1 [Kobayashi et al., 1998].

2.6.3.4. Osteopontin

Its apparent molecular weight has been estimated from 44 to 75 kDa depending on the percentage of polyacrylamide gel used. This anomalous migration is assumed to be due to differences in glycosylation and phosphorylation. In addition to its existence as a monomeric form, the protein may also aggregate to form a higher molecular weight entity. Amino acid analysis of rat OP revealed that it contains 319 residues of which 36% are aspartic and glutamic acid [Denhardt and Guo 1993]. It also contains 30 serine, 12 phosphoserine and one phosphothreonine residues. Osteopontin from all species has high aspartate/asparagine contents accounting for as much as 16-20% of all amino acid residues in the molecule. In addition to bone cells, OPN is present in many epithelial tissues in kidneys, gastrointestinal tract, gall bladder, pancreas, lung, salivary gland and inner ear [Brown et al., 1992]. It is also expressed in a variety of other cell types including macrophages [Pollack et al., 1994; Murry et al., 1994] activated T-cells, smooth muscle cells and endothelial cells.

The significantly higher incidence of a single base mutation in the OPN gene has been found in the patients with recurrent or familial nephrolithiasis [Yamate et al., 2000]. OPN is intimately involved in both the physiological and pathological mineralisation processes including crystallization in the urine and development of calcific kidney stones support for the CaOx crystallization inhibitory actions of OPN [Langdon et al., 2009] is further strengthened by studies in OPN knockout mice [Wesson et al., 2003]. When comparable hyperoxaluria is induced
in OPN knockout and wild type mice, knockout mice developed significant intratubular deposition of CaOx crystals while wild type remained free of any crystals. In addition wild type hyperoxaluric mice showed significant increase in OPN expression in their kidneys, indicating a renoprotective role for OPN. Results of one study show OPN favouring crystallization of COD over COM which may influence the development of kidney stones because renal epithelium is more likely to bind COM crystals than the COD crystals. It appears that structural defects and various post-translational modifications, such as glycosylation and phosphorylation may influence the effect of OPN on crystallization in urine.

2.6.3.5. Urinary Prothrombin Fragment –1 (UPTF-1)

This protein is also known as crystal matrix protein (CMP) because it was found selectively associated with CaOx crystals experimentally induced in human urine [Doyle et al., 1996]. Molecular weight of this protein was found to be 31 kDa. The amino acid sequence analysis of CMP showed an identity with prothrombin [Stapleton et al., 1993; Stapleton and Ryall 1994; Suzuki et al., 1994], a plasma protein involved in coagulation cascade. In the first 34 amino acid residues, 10 of the glutamic acids are γ-carboxylated. The carbohydrate contents represent 17% of its molecular weight. Suzuki et al. proposed that CMP is the activation peptide of human prothrombin [Suzuki et al., 1994]. By using specific antibodies for prothrombin and F1+2 fragment, Stapleton and Ryall demonstrated [Stapleton and Ryall, 1994] that CMP is prothrombin fragment F1 (UPTF-1).

Recent studies have provided evidence that PT gene is expressed in both the human and rat kidneys indicating the possibility of PT biosynthesis in both human and rat kidneys [Grover et al., 2000; Suzuki et al., 1999; Grover et al., 1999]. Recent studies using purified urinary proteins have confirmed earlier results and have demonstrated UPTF-1 to be an inhibitor of both crystal growth and aggregation [Ryall et al., 1989]. Results of another study where a comparison was
being made between the white and black South Africans with regard to urinary crystallization inhibition showed that UPTF-1 is a strong inhibitor of crystal nucleation [Durrbaum et al., 2001]. UPTF-1 from normal black males reduced crystal nucleation by 63.6% as compared to the protein from normal white males that reduced the nucleation by 23.4%.

2.6.3.6. Calgranulin (Calprotectin)

Calgranulin is a 28 kDa member of S100 family of calcium binding proteins, which are small, ubiquitous, and acidic proteins involved in normal developmental and structural activities [Zimmer et al., 1995]. However, they are also implicated in a number of diseases [Kahn et al., 1983]. The protein was recently isolated from human urine at a concentration of 3.5-10 nM. Purified urinary calgranulin inhibited both CaOx crystal growth (44%) and aggregation (50%) in nanomolar range. 28kDa calgranulin was cloned from the human kidney expression library. Western analysis of rat and human kidneys as well as renal epithelial cell lines, BSC-1 and MDCK confirmed its renal presence. Calgranulin is also known as leukocyte antigen L1 and has been identified in circulating neutrophils and 22 monocytes and has bacteriostatic antifungal activites [Steinback et al., 1990]. It has also been identified in matrix of infectious or struvite stones and in CaP deposits formed by MDCK cells [Naito et al., 1997].

2.6.3.7. Albumin

Albumin is one of the most abundant proteins in the urine [Maslamani et al., 2000; Fraij, 1989; Boyce and Garvey, 1956] and has been detected in the matrix of both urinary stones [Fraij, 1989; Boyce and Garvey, 1956; Boyce, 1968] as well as crystals [Atmani et al., 1998; Atmani et al., 1996a] made in the whole human urine. It is known to bind to CaOx as well as uric acid crystals [Worcester, 1994; Dussol et al., 1995] but does not inhibit their growth [Worcester, 1994]. However, it has been shown to inhibit CaOx crystal aggregation in concentration dependent manner [Edyvane et al., 1986; Hess et al., 1995; Grover et al., 1998]. When immobilized to
surfaces and exposed to metastable solutions albumin promotes crystal nucleation [Cerini et al., 1999, Ebrahimpour et al., 1991]. When dissolved in solution albumin exists either in monomeric or and polymeric form [Cerini et al., 1999]. In metastable CaOx solutions both monomeric and polymeric forms promote nucleation of CaOx. In addition, nucleation by albumin leads exclusively to the formation of COD crystals. Urinary albumin purified from healthy subjects contained significantly more polymeric forms and was a stronger promoter of CaOx nucleation than albumin from idiopathic calcium stone formers. Promotion of CaOx nucleation and formation of large number of COD crystals might be protective. Nucleation of large number of small crystals would allow their easy elimination and decrease CaOx saturation preventing crystal growth and aggregation and subsequent stone formation. COD crystals are more common than COM crystals in non-stone formers urine and are generally found in lesser quantities in stones than COM crystals. In addition crystals present in the urine from non-stone formers are significantly smaller than those in stone formers urine. Albumin also exhibits the capacity to bind some of the urinary proteins. Interestingly, urinary proteins that show great affinity for albumin are also those that are included in the stone matrix. It is suggested that proteins become a part of stone matrix by binding to the albumin coating CaOx crystals. It is also suggested that unlike other calcium binding urinary proteins, albumin promotes nucleation by interacting with calcium through the carboxyl group. Strong nucleation activity was observed at pH 7 but was totally eliminated at pH 4 when carboxyl groups are no longer ionized. In addition, morphological studies showed CaOx crystals to nucleate through calcium rich face [Cerini et al., 1999].

2.6.3.8. Renal Lithostathine

Lithostathine is a glycoprotein synthesized by acinar cells and secreted in pancreatic juice. Pancreatic juice is naturally supersaturated in calcium and bicarbonate ions and lithostathine plays an important role in inhibiting calcium carbonate crystal growth. A protein immunologically
related to lithostathine is actually present in urine of healthy subjects and in renal stones, renal lithostathine (RL) [Verdier et al., 1992]. Western blot analysis of proteins extracted from concentrated normal urine or kidney stones demonstrated the presence of a protein molecular weight of 23 kDa [Verdier et al., 1993]. Because of its structural and functional similarities with pancreatic lithostathine, it was called renal lithostathine. Renal Lithostathine seems to control growth of calcium carbonate crystals. Several reports showing the presence of calcium carbonate (CaCO3) in renal stones suggested that crystals of CaCO3 might be present in the early steps of stone formation. Such crystals therefore might promote CaOx crystallization from supersaturated urine by providing an appropriate substrate for heterogeneous nucleation [Geider et al., 1996, Grover et al., 2002].

2.6.3.9. CD44

CD44 is a transmembrane protein and the main cell surface receptor for hyaluronan or hyaluronic acid (HA) as well as OPN [Weber et al., 1996]. Both CD44 and HA are upregulated during injury and inflammation and are involved in the formation of a cell coat or pericellular matrix on surfaces of proliferating and migrating cells. HA is restricted to the inner medullary interstitium of the normal kidneys. Distal collecting duct cells express both CD44 and HA on apical cell surfaces of the proliferating cells. At confluence however, CD44 is expressed at the basolateral membrane while HA is undetectable. Proliferating cells are receptive to adhesion of CaOx crystals, a property lost when cells become confluent. In addition removal of pericellular matrix by hyaluronidase treatment also results in loss of crystal adhesion property of the proliferating cells [Verhulst et al., 2003; Asselman et al., 2003]. Based on these observations it has been proposed that intact epithelium does not bind crystals because of the absence of a pericellular matrix and crystal attachment depends upon the expression of CD44, OPN and HA by the damaged renal epithelial cells [Verkoelen et al., 2000].
2.6.3.10. Trefoil Factor 1

Chutipongtanate et al. have reported human trefoil factor 1 (TFF1) as CaOx crystal growth inhibitor [Chutipongtanate et al., 2005]. It belongs to the trefoil factor family of proteins, is expressed predominately in gastric mucosa, and is synthesized by mucosal epithelial cells. It has antiapoptotic and motogenic activities, and its main functions in the gastrointestinal tract are thought to involve maintenance of mucosal integrity and mucosal repair in response to inflammation or injury. TFF1 has been identified previously in human urine using radioimmunoassay and Western blotting. Urinary TFF1 at the concentration of 7 ng/ml inhibited CaOx crystal growth. The significant inhibitory effect was demonstrated after 10 minutes’ incubation and remained significant through the end of the assay (1 hour).

2.6.3.11. Hyaluronic Acid (HA)

HA is a linear glycosaminoglycan (GAG) that is composed of multiple units of glucouronic acid and N-acetylg glucosamine (1, 4-GlcUA-1, 3-GlcNAc)n. It is an extremely large and high molecular weight GAG i.e. \( >10^6 \) Da, one disaccharide is approximately 400 Da, high molecular weight HA is a chain of >2500 disaccharide repeats. HA chains occupy huge tissue domains and can entrap large amounts of solvent due to its expanded random coil structure [Lee and Spicer, 2000]. HA is produced by HA synthase (HAS) proteins that are located at the inner face of the plasma membrane, where it is extruded across the membrane into the extracellular space. HA is a major component of the extracellular matrix (ECM) in the renal medullary interstitium and the pericellular matrix (PCM) of mitogen/stress-activated renal tubular cells. Hydrated PCM provide the microenvironment that is conducive to adjustments in cell shape and epithelial architecture during dynamic morphogenetic processes like wound healing, embryonic development, inflammation and cancer [Toole, 1997]. The size, negative ionic charge, and ability to form hydrated gel-like matrices make HA an excellent crystal-binding molecule. Crystal binding to
HA leads to crystal retention in the renal tubules and to the formation of calcified plaques in the renal interstitium (Randall’s plaques). HA also directly influences cell behavior through its ability to communicate with the cell interior via cell-surface receptors, such as CD44 and CD168 [Toole, 1997; Toole, 1982; Lee and Spicer, 2000].

In a healthy kidney, HA is abundant in the renal medullary interstitium but in the cortex it is almost undetectable, and renal tubular cells normally do not express HA. HA should be considered an enormous inhibitor of crystallization that efficiently prevents papillary calcification for the reason that interstitial HA is low during antidiuresis, the highest risk for crystal formation most likely occurs during periods of water deprivation. On the other hand high fluid intake leads to high interstitial HA which protects against crystallization. Ca$^{2+}$ also becomes associated with the COO$^-$ groups in the HA matrix, thereby preventing CaP precipitation. Precipitated calcium crystals (CaP) may bind to the HA matrix, which could play an important role in interstitial plaque formation (Randall’s plaques) [Verkoelen, 2006]. Therefore, it was speculated that HA could be an inhibitor of crystallization as long as calcium salts are in solution because the carboxyl groups of the HA chains race with anions such as phosphate and oxalate for binding to calcium, whereas HA may serve as binding substance for precipitated calcium salts because of the affinity of these crystals for high molecular weight HA. In case of renal cell injury, HA may act as promoter for crystal adhesion to the cell surface, which will ultimately result into stone formation. Crystal retention in the human kidney may depend on the expression on damaged distal tubular epithelium of CD44, OPN and HA rich cell coats [Verhulst et al., 2003]. HA becomes expressed in areas of the kidney where it is absent under normally conditions, such as in the cortico-interstitium and on the luminal surface of renal tubular cells [Asselman et al., 2003]. HA is up-regulated in the kidney during inflammatory renal disease states such as interstitial nephritis [Sibalic et al., 1997], acute ischemic injury [Goransson et al., 2004], autoimmune renal injury [Feusi et al., 1999], acutely rejecting human kidney grafts [190],
acute tubular necrosis [Verhulst et al., 2003], and obstructed kidneys and EG poisoning [Asselman et al., 2003]. HA-expressing renal tubular cells invariably also express the HA receptor CD44 [Verhulst et al., 2003; Wuthrich, 1999; Jones et al., 2003]. HA is also one of the major constituents of the organic matrix of renal stones. IIA is present in kidney stones in fractions that are disproportionate to its urinary concentrations.

2.6.3.12. Annexin II

Annexin II protein is a 37 kDa, member of the annexin family i.e. calcium-dependent phospholipid-binding protein family. These family members play a role in the regulation of cellular growth and in signal transduction pathways. As a family, annexins are characterized by a conserved COOH-terminal protein “core” that mediates their membrane and calcium-binding properties. The conserved COOH-terminal core is 70 amino acid long chains and contains four repeats, each of which consist of a calcium-binding motif G-X-G-T [Gerke and Moss, 2002]. Members of the annexin family differ in binding specificity of the core for phospholipid headgroups like PS, phosphatidic acid or phosphatidylinositol, as well as the binding specificity of the NH2-terminal tail.

Annexin II is a calcium-dependent phospholipid-binding protein whose function is to help organize exocytosis of intracellular proteins to the extracellular domain. Annexin II is involved in diverse cellular processes like cell motility, linkage of membrane-associated protein complexes to the actin cytoskeleton, endocytosis, fibrinolysis, ion channel formation and cell matrix interactions. Ax II is a pleiotropic protein meaning that its function is dependent on place and time in the body. Ax-II has been demonstrated on the surface of many cell types, including keratinocytes [Ma and Ozers, 1996], endothelial cells, glioma cells, and smooth muscles cells [Hajjar et al., 1996]; where it can serve receptor-like functions for molecules including lipid A [Eberhard and Vandenberg, 1998], cytomegalovirus [Raynor et al., 1999], 1,25(OH)2D3 [Baran et al., 2000], and 2-glycoprotein I [Ma et al., 2000], tissue plasminogen activator [Hajjar et al.,
Annexin II can bind to the crystals while anchored on the cell surface. Under certain circumstances Ax-II is known to interact with membranes via cholesterol molecules in a calcium-independent mechanism, possibly leaving the calcium-binding motifs available to bind calcium oxalate crystals. The NH2-terminus of Ax-II can bind a p11 dimer and become linked to another Ax-II molecule via its NH2 tail; a process that can mediate aggregation of membrane vesicles. Ax-II-p11 tetramer might link a crystal to a membrane instead of linking two membranes [Gerke and Moss, 1993].

Ax-II mediates the adhesion of COM crystals to renal cells and it may also play a role in their subsequent internalization. Kumar et al. in 2003 used COM affinity to identify apical membrane proteins that might mediate crystal adhesion to renal epithelial cells. They found annexin II to be the major COM crystal-binding protein and it was identified on the apical surface of intact MDCK-I cells. The interaction between crystals and renal tubular cells has been proposed to be a crucial event that elicits subsequent cellular responses, leading to kidney stone formation. Ax-II has a role as a COM crystal-binding molecule on the surface of intact cells as COM crystal adhesion decreased significantly after MDCK-I cells were pre-treated with a monoclonal antibody against Ax-II. Studies suggest that Ax-II may be one of several cell surface crystal-binding molecules that might modulate crystal retention [Kumar et al., 2003].

2.6.3.13. Matrix Gla Protein

Matrix Gla protein (MGP) is a natural inhibitor of vascular calcification. MGP, a vitamin K-dependent extracellular matrix protein was initially isolated from the bone and also expressed in lung, heart, vascular smooth muscle cells of the blood vessel wall and kidney [Fraser and Price, 1988]. MGP is an 84-amino-acid protein that contains five γ-carboxyglutamic acid (Gla) residues which has a high affinity for calcium and phosphate ions, and hydroxyapatite crystals [Proudfoot and Shanahan, 2006]. The pathological mechanism of kidney stone formation was partly similar to vascular calcification: forming calcific plaques, increasing expression of calcification inhibitors and regulating actively calcification process. MGP genetic single nucleotide polymorphism was associated with the individual susceptibility of nephrolithiasis [Gao et al.,
2007]. MGP is involved in cell growth, differentiation and regulation of apoptosis and increase cell density in normal kidney cells [Cancela et al., 1997]. Matrix Gla protein (MGP) is a molecular determinant regulating vascular calcification of the extracellular matrix. MGP is polarly distributed, on the apical membrane of renal tubular epithelial cells and binds directly to crystals, it is also found in the ascending thick limbs of Henle's loop and the distal convoluted tubule in hyperoxaluric rats, its expression was present in the medullary collecting duct in stone-forming rats. Crystals with multilaminated structure were formed in the injurious renal tubules with lack of MGP expression [Lu et al., 2013]. Lu et al. also reported that there was no crystal formation in renal tubules with MGP expression, and crystals only deposit in the damaged renal tubules with lack of MGP expression. MGP mRNA expression was found to be upregulated in renal tubular epithelial cells following exposure to calcium oxalate monohydrate (COM) and oxalate [Lu et al., 2013; Gao et al., 2010]. Luo et al. found that homozygous MGP-deficient mice died within 8 weeks as a result of arterial calcification that led to blood vessel rupture [Luo et al., 1997]. Vascular calcification in MGP-deficient mice was reversed by over expressing MGP in vascular smooth muscle cells [Murshed et al., 2004]. These findings imply that MGP may play a cytoprotective role in maintaining cells survival and inhibiting crystal retention under oxalate and crystal exposure.

2.6.3.14. Monocyte Chemo-attractant Protein-1 (MCP-1)
Monocyte chemoattractant protein-1 (MCP-1/CCL2) is a member of the CC chemokine family and belongs to the group of inflammatory chemokines. MCP-1 is a potent chemotactic factor for monocytes [Gu et al., 1999]. Chemokines are also grouped into two main functional subfamilies: inflammatory and homeostatic. Inflammatory chemokines control the recruitment of leukocytes in inflammation and tissue injury. Homeostatic chemokines fulfill housekeeping functions such as navigating leukocytes to and within secondary lymphoid organs, in addition to in the bone marrow and the thymus during hematopoiesis [Wagner et al., 2007]. Chemokine activation of cell surface G-protein coupled receptors results in directed cell migration, i.e., chemotaxis [Hyduk et al., 2007].
The development of kidney disease involves a complex interplay between neurohormonal, inflammatory and biochemical changes which act on either intrinsic or extrinsic renal cells, or both. This can lead to the development of an innate immune response predominantly characterized by the accumulation and activation of leukocytes, particularly monocytes/macrophages, in the kidney. Chemokine-induced recruitment of peripheral leukocytes into tissues is a critical step in the development of inflammatory responses [Duffield, 2010]. MCP-1 plays an important role in various pathophysiological conditions in many organ systems [Yadav et al., 2010]. Even though MCP-1 is involved in various inflammatory diseases and may not be specific for a particular disease, this should not necessarily preclude its use as a biomarker. MCP-1 plays a critical role in the development of kidney diseases. It has been investigated as a potential urinary biomarker in several renal diseases in a number of studies [Kim and Tam, 2011]. However, the exact role of MCP-1 in stone formation is still not clear but its expression in renal epithelial cells on exposure to oxalate and CaOx crystals indicate a close relationship between inflammation and nephrolithiasis [Umekawa et al., 2002].