

# Nanobacteria: controversial pathogens in nephrolithiasis and polycystic kidney disease

E. Olavi Kajander<sup>a</sup>, Neva Ciftcioglu<sup>a</sup>, Marcia A. Miller-Hjelle<sup>b</sup> and J. Thomas Hjelle<sup>b</sup>

Nanobacteria are unconventional agents 100-fold smaller than common bacteria that can replicate apatite-forming units. Nanobacteria are powerful mediators of biogenic apatite nucleation (crystal form of calcium phosphate) and crystal growth under conditions simulating blood and urine. Apatite is found in the central nidus of most kidney stones and in mineral plaques (Randall's plaques) in renal papilla. The direct injection of nanobacteria into rat kidneys resulted in stone formation in the nanobacteria-injected kidney during one month follow-up, but not in the control kidney injected with vehicle. After intravenous administration in rats and rabbits, nanobacteria are rapidly excreted from the blood into the urine, as a major elimination route, and damage renal collecting tubuli.

Nanobacteria are cytotoxic to fibroblasts *in vitro*. Human kidney cyst fluids contain nanobacteria. Nanobacteria thus appear to be potential provocateurs and initiators of kidney stones, tubular damage, and kidney cyst formation. It is hypothesized that nanobacteria are the initial nidi on which kidney stone is built up, at a rate dependent on the supersaturation status of the urine. Those individuals having both nanobacteria and diminished defences against stone formation (i.e. genetic factors, diet and drinking habits) could be at high risk. Kidney cyst formation is hypothesized to involve nanobacteria-induced tubular damage and defective tissue regeneration yielding cyst formation, the extent of which is dependent on genetic vulnerability. *Curr Opin Nephrol Hypertens* 10:000–000. © 2001 Lippincott Williams & Wilkins.

<sup>a</sup>Department of Biochemistry, University of Kuopio, Kuopio, Finland; and

<sup>b</sup>Departments of Biomedical and Therapeutic Sciences, University of Illinois College of Medicine at Peoria, Peoria, Illinois, USA

Correspondence to Dr E. Olavi Kajander, Department of Biochemistry, University of Kuopio, Savilahdentie 9F, POB 1627, FIN-70211, Kuopio, Finland  
Tel: +358 17 163054; fax: +358 17 2811510; e-mail: olavi.kajander@uku.fi

**Current Opinion in Nephrology and Hypertension** 2001, 10:000–000

## Abbreviation

**PKD** polycystic kidney disease

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1062-4821

## Introduction

Nanobacteria were discovered over 10 years ago as autonomously replicating biological particles causing cytotoxicity in mammalian cell cultures [1]. This agent mediates apatitic calcification in culture. The apatite produced is biogenic because it is formed in a carbon-containing biomatrix around the agent, forms small spherical units of apatite in nanoscale crystal size that are very resistant to acid hydrolysis, and can be formed at non-saturating concentrations of calcium and phosphate [2,3]. Such spherical units were identified in most human kidney stones examined, were cultured from these kidney stones, and produced new stones in culture [4,5]. Calcified and non-calcified forms of nanobacteria have been observed free in culture and intracellularly in mammalian cells *in vitro* [4–6]. Calcified nanobacteria are very resistant to chemical disinfection [7], heat, ultraviolet and high gamma irradiation, and can survive in a lyophilized state for extended periods of time [8].

Methods to diagnose nanobacteria in biological substances, cells, tissues, blood and urine include immunodetection with nano-specific monoclonal antibodies, electron microscopy and culture [6,9]. Susceptibility tests can be used to test the effects of antibiotics and other chemotherapeutic agents [10,11]. The eradication of nanobacteria from serum and culture media has been obtained using high gamma irradiation or filtration, and nanobacteria-free culture media are commercially available.

The concept that nanobacteria are living organisms is controversial. The size of nanobacteria is typically only 0.1–0.2 microns, making them 100-fold smaller than common bacteria. We have explored the concept that, although relatively small, such particles could be primitive life forms [1]. In addition, we have sought alternative concepts, such as nanobacteria or fragments of nanobacteria being non-living biomineralization products that are somehow able to multiply with kinetics similar to life forms [12]. The general debate continues over the existence of relatively tiny microbes, which are sometimes generically called nanobacteria [13,14]. However, our fundamental findings of nanobacteria detection and culture have been reproduced all or partly by several groups [15,16,17•18••]. Although the issue of organism status is important, more relevant to medicine is the following question: are nanobacteria pathogenic?

### How is apatite formed by nanobacteria?

The mechanism(s) by which apatite is formed around nanobacteria is unknown. However, the effectiveness of its biomineralization is remarkable: apatite formation *in vitro* stopped only when the calcium level decreased by 50% from 1.8 to 0.9 mM and the phosphate levels fell to near zero [19\*\*]. Nanobacteria could feed on dolomite [ $\text{CaMg}(\text{CO}_3)_2$ ] as a calcium source [20]. Furthermore, synthetic apatite added to nanobacteria cultures dissolved while nanoparticles were accumulating apatite (our unpublished observation). Nanobacteria-induced apatitic biofilm formation is dependent on the presence of oxygen [1,5], can be prevented with several antibiotics and antimetabolites, and by high gamma irradiation at sterilizing doses [10,11\*].

The mechanism might be similar to bone formation, which is also not fully understood. Models for bone formation, which use metastable concentrations of calcium and phosphate, involve gels that include nidi, such as matrix vesicles, apoptotic vesicles or collagen, but exclude the known proteinaceous inhibitors for crystal formation. Such systems have not been tested with nanobacteria. Vali *et al.* [16] demonstrated that nanoforms contain apatite–protein complexes and immuno-electron microscopy revealed protein antigens in proximity to apatite, suggesting a novel form of protein-associated mineralization. Recently, Cisar *et al.* [17\*] were able to repeat the propagation of nanobacteria-like particles, observed similar kinetics for apatite mineralization, but could not extract DNA or proteins from the particles. Their interpretation was that the particles were self-replicating inorganic apatite. Unfortunately, their work was confounded by the lack of positive and negative controls, surveillance for nanobacterial contamination, metabolic studies, the use of monoclonal antibodies to identify nanobacteria, and the contamination of polymerase chain reaction reagents by bacterial DNA. It has been our experience that nanobacteria actually inhibit the amplification of exogenous classical bacterial DNA by polymerase chain reaction methods.

Cisar *et al.* [17\*] did not analyse the antigenicity and infectivity of the particles they claimed were nanobacteria. Our studies have revealed specific nanobacteria (protein) antigens that result in an immune response in animals and humans. There is one verifiable case of human seroconversion after laboratory exposure to nanobacteria. Rats housed in the same cage became infected when only one animal was inoculated with nanobacteria. Apatite is a normal body constituent known to be non-immunogenic and non-infectious, which we have verified in rats and rabbits (unpublished results). Our ongoing studies indicate that approximately 10% of adult healthy people in Scandinavia have

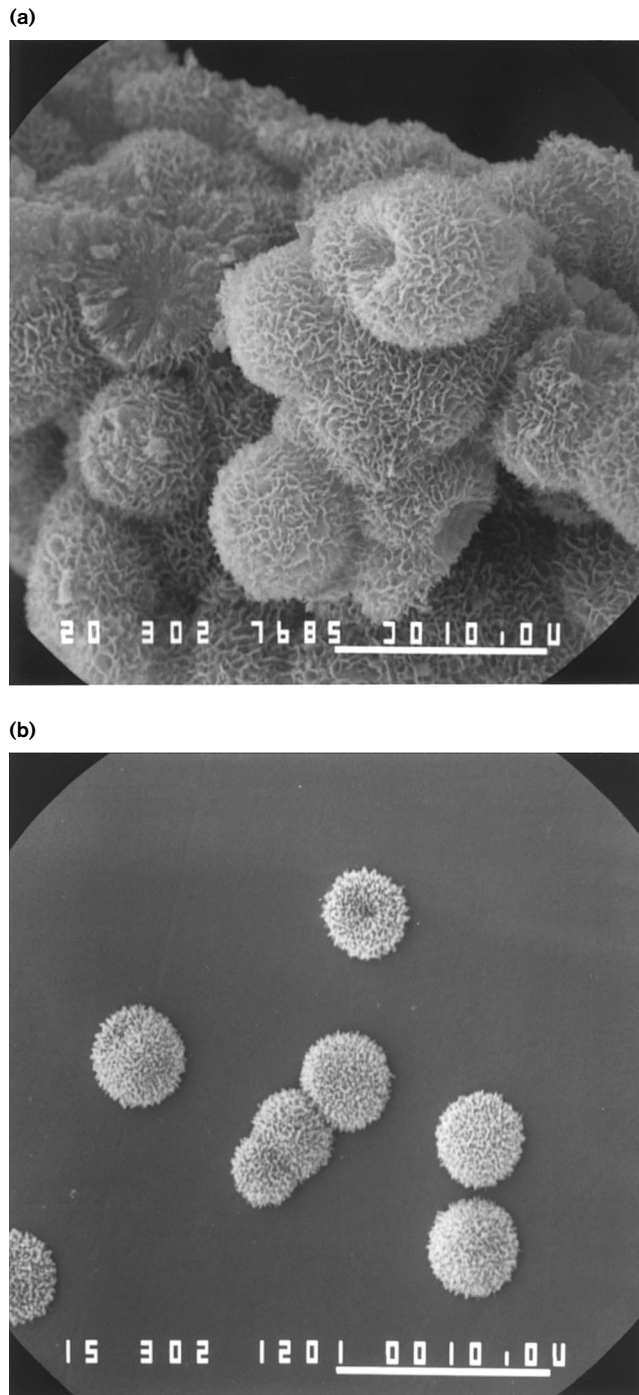
antinobacteria antibodies, whereas patient groups with kidney diseases and atherosclerosis have a much higher incidence of antibodies against nanobacteria. It is thus plausible that nanobacteria are human pathogens.

### Nanobacteria and pathogenesis of kidney stones

Figure 1 shows similar apatite units produced in nanobacterial cultures and in human apatitic kidney stones. Both grow as layers of mineral and matrix [5]. Figure 2 shows a simplified contemporary scheme for stone formation. The following observations support a role for nanobacteria in nephrolithiasis. Nanobacteria are renotropic: Tc-labelled nanobacteria were eliminated from the body via uptake to kidney tissue from the blood, and were transported to urine in a process lasting approximately 15 min [22]. They retained their Tc label and culturability during excretion. Nanobacteria can be cultured in urine or artificial urine [15], and look like Carr's concretions (see legend to Fig. 2). Apatitic particles are the predominant crystal type in the urine of both controls and recurrent stone-formers. However, crystalluria appears to form at a lower urinary ionic concentration in stone-formers [23], suggesting a higher crystallization potency in stone-formers, i.e. active nidi or weaker crystallization inhibitor activity. Nanobacteria may thus represent transportable apatitic nidi from blood to kidney tissue and tubuli.

Collections of Carr's concretions probably adhere to epithelia in the collecting tubuli or near papilla, as observed with Randall's plaques. Adhesion may require previous cellular injury [24]. Phosphatidylserine is ordinarily confined to the inner leaflet of the plasma membrane, but is relocated in apoptotic cells to the cell surface, where it may act as a binding site for nanobacteria. Nanobacteria do adhere to cells and can cause apoptotic injury [6], especially in collecting tubuli [22]. Calcium oxalate monohydrate and hydroxyapatite crystals rapidly adhere to anionic sites on the surface of cultured renal epithelial cells, but this process is inhibited by specific urinary anions, such as citrate, glycosaminoglycans, uropontin, bikunin [25\*] or nephrocalcin, each of which coat the crystals. Competition for the crystal surface could thus determine if a crystal binds to a tubular cell [26]. A novel protein closely related to nucleolin was recently identified as a possible receptor for the binding of apatite crystals [27]. Once present on the cell surface, crystals are internalized by renal cells [28\*]. Crystals may be dissolved slowly or may infest cells and tissue as Randall's plaques. Randall's plaques are found most frequently in patients with calcium salt stones and abnormalities in their urinary milieu [29\*]. Randall's plaques may start stone formation as a nucleus for deposits of calcium salts, depending on supersaturations in urine.

Figure 1. Scanning electron microscope images of human kidney stone and nanobacteria



(a) Human kidney stone composed of carbonate apatite units, and (b) cultured nanobacteria showing similar apatite units. Bar=10  $\mu$ m. Similar pictures have been published by Kajander and Ciftcioglu [5] and Ciftcioglu *et al.* [19\*\*].

### Hypothesis of nanobacterial kidney stone formation

Figure 3 places nanobacteria in the array of overlapping factors known either to inhibit or promote kidney stone formation. In the context of these factors, nanobacteria would add novel aspects to kidney calcification. First, a robust apatite nucleator, such as a nanobacterium, may overwhelm (either chronically or episodically) otherwise marginally effective anti-nucleation factors in vulnerable individuals. Second, nucleating particles (nanobacteria) may be transported from the blood into the urine. Any infection by nanobacteria at any place in the body might thus provoke kidney stones or even calcification at the original site of infection. Third, anti-nanobacterial treatment strategies may be explored in patients susceptible to kidney stones. Nanobacteria are sensitive to tetracycline class antibiotics *in vitro*, but clinical trials are necessary to clarify treatment regimens and outcome [10]. Some nucleoside analogues, such as 5-fluorouracil, are effective *in vitro* against nanobacteria (our unpublished data) suggesting that antiviral, antimycotic and antimetabolite agents should be screened as drugs or drug combinations.

Important evidence for the hypothesis comes from two recent papers. An association with kidney stone formation was established by detecting nanobacteria in 70 out of 72 kidney stones [19\*\*]. Interestingly, apatite stones gave the highest immunopositivity for nanobacteria, but overall nanobacteria positivity did not depend on the stone type. Garcia Cuerpo *et al.* [18\*\*] developed a translumbar, percutaneous renal puncture method for the injection of materials into the kidney without antibiotic coverage. Using this technique, rats developed kidney stones in a nanobacteria inoculum-dependent manner. Such findings are required to prove Koch's postulates linking nanobacteria to kidney stone formation. Larger scale animal studies using this elegant model are required to confirm causality and to serve as a basis for tests of anti-nanobacterial therapies.

The life-long prevalence of kidney stones appears to have increased throughout the whole 20th century, and occurs in up to 15% of the population [30]. Treatment of kidney stones has been estimated to cost over US\$3000 per patient per year [31]. The incidence of new cases and recurrences may continue to rise. Therefore, new approaches in treatment and prevention could have a huge economic effect, apart from benefits in terms of reduced morbidity. Interestingly, a high prevalence of kidney calcifications is observed in polycystic kidney disease (PKD) [32\*]. Could nanobacteria also contribute to the pathology of this disease?

Figure 2. Formation of kidney stone in a renal papilla

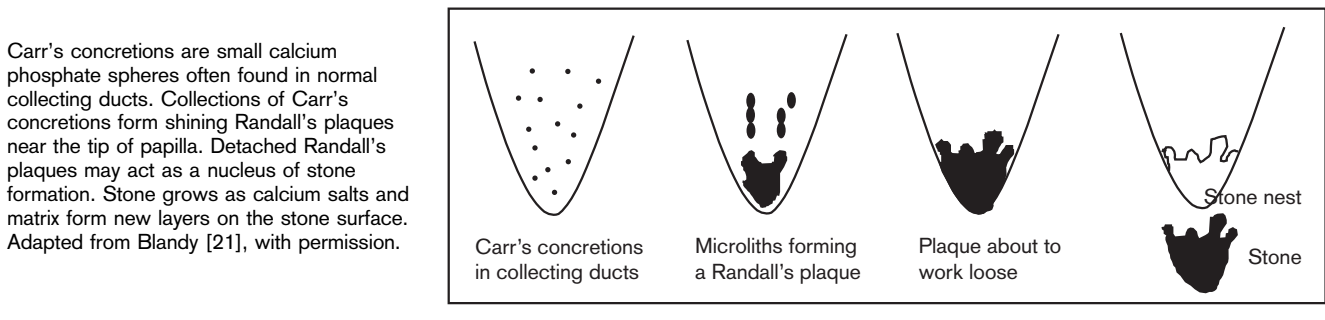
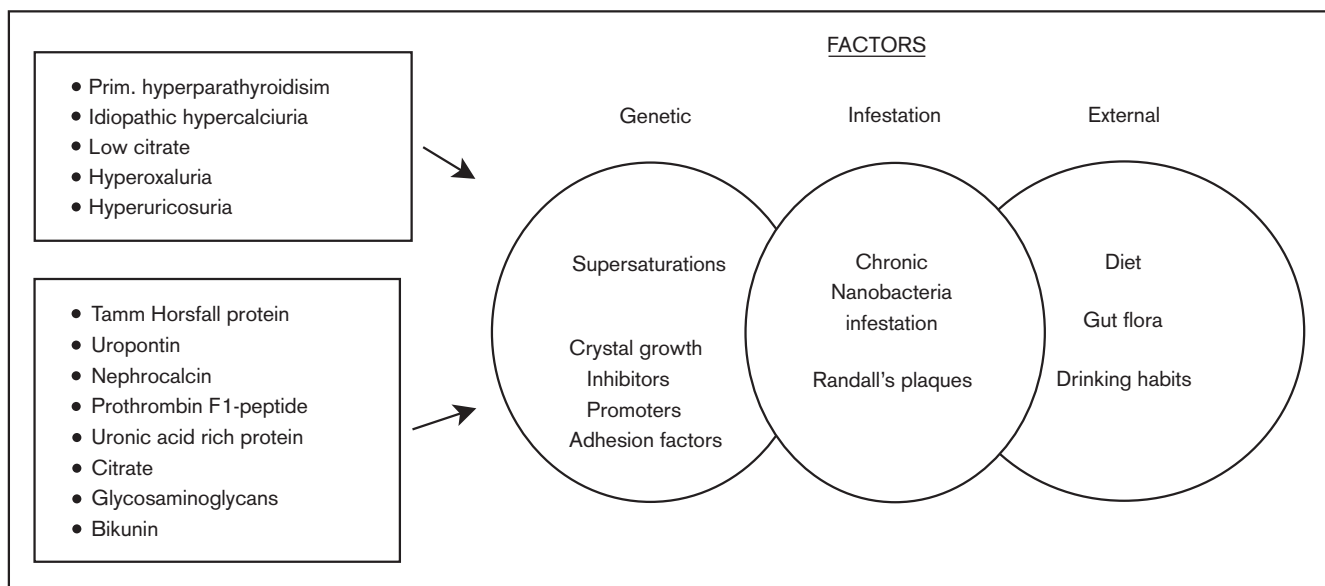


Figure 3. Hypothesis for kidney stone formation by nanobacteria



**Infectious agents/microbial toxins as contributors to renal cyst formation**

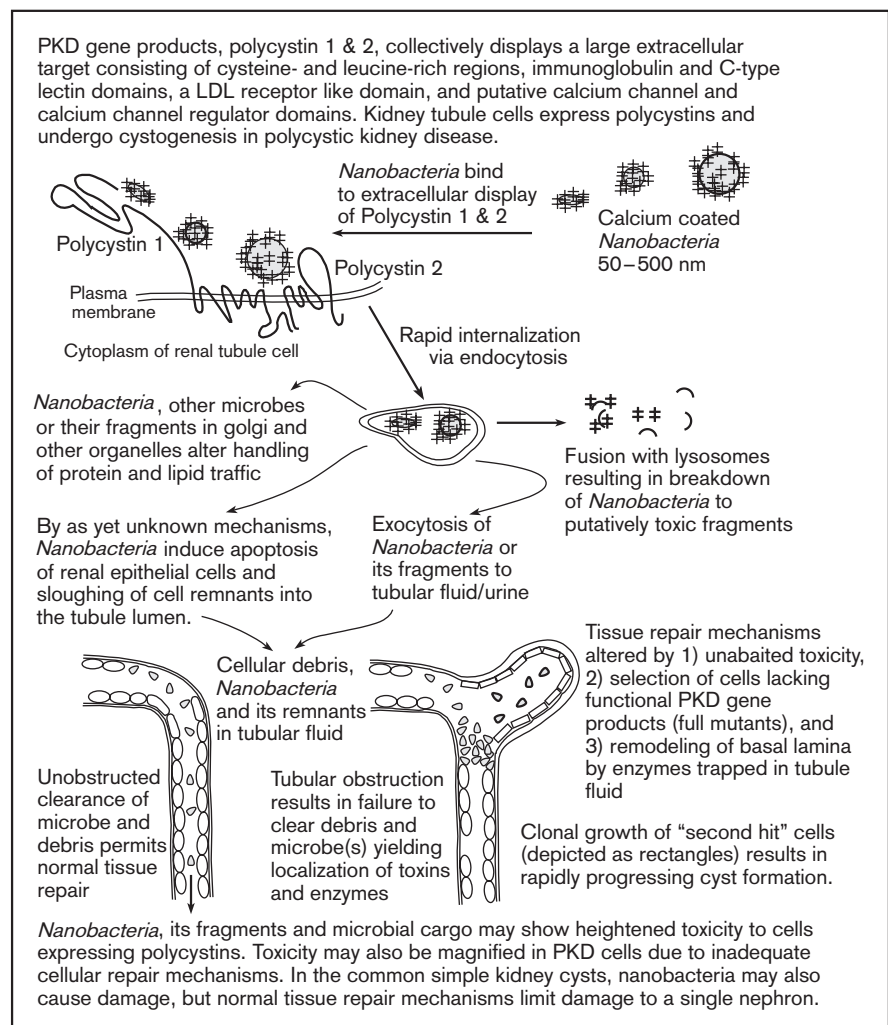
Modern methods and new concepts of pathogenic mechanisms for infectious agents and microbial toxins, released during infection or present in food, reveal a greater than anticipated role of such agents in causing chronic disease [14,33–35,36\*,37\*]. PKD, the most common autosomal dominant disease in humans, can be viewed simultaneously as a genetic disease with variable expression [38,39\*,40–42], a neoplastic process [43], and an infectious disease or microbial toxicosis [44\*\*,45,46,47\*\*]. There are reports of endotoxin [44\*\*,45], nanobacteria [44\*\*], and fungal antigens and antibodies [46] in human kidney cyst fluids. The presence of such microbiological material in human cyst fluids cannot be dismissed as innocuous, because the perceived anomalies of PKD are plausibly related to the

reported biological effects of endotoxins and mycotoxins found or logically inferred from findings in PKD kidneys [47\*\*,48,49\*,50–53] (Fig. 4). However, plausibility does not prove causality, even when yet to be defined environmental factors are thought to alter the progression of PKD [38,41,42]. Future research will need to address the issue of exposure to infectious agents/toxins relative to the onset of cystogenesis.

Infectious agents or microbial toxins could influence the expression of PKD in two ways: by causing the first or second mutations in PKD genes or by establishing a tissue environment in which cells with one or more PKD gene mutations cannot adequately repair tissue damage [54–57]. In the first case, PKD cysts have been described as ‘fluid-filled tumors’ [41,43] associated with a ‘two-hit model’ for PKD mutagenesis [58]. Mutagenesis in PKD

Figure 4. Hypothesis for nanobacteria provocation of kidney cystogenesis

PKD, Polycystic kidney disease.



has been speculated to involve such concepts as an intrinsic instability of PKD genes [59,60], oxidative damage to DNA caused by tissue inflammation [60], and a functional deficiency of folate caused by the actions of microbial toxins on the sphingolipid-associated, glycosylphosphatidylinositol-linked folate binding protein [47••]. On this last point, the disruption of sphingolipid biology may be a central phenomenon linking together PKD [47••], the protection of kidney tubule cells during stress [61,62], and mycotoxin-induced pathology of the liver, kidney, gastrointestinal tract, and altered embryogenesis [49•,63,64••,65]. Folate deficiency is a contributor to birth defects and cancer [66]. Maternal infection during fetal development is linked to several birth defects [67]. Infectious etiologies for many cancers are considered likely [36•].

If a mutation of a PKD allele occurs during spermatogenesis, oogenesis, or blastocyst formation, the mutated PKD

allele will be present in all or some cells, respectively, although not all cell types express PKD gene products [60]. Such heterozygous autosomal dominant PKD individuals could, later in life, develop a mutation of the remaining normal copy of the PKD allele. This can occur in one or more tissues, leading to the complete loss of functional PKD gene products (polycystins 1 and 2 [57,60]) and a derangement of those cellular functions dependent on these proteins [68,69]. The bioavailability and distribution of infectious agent(s)/toxin(s) would impact both the degree and site of PKD lesions. Much is yet to be learned of the effect of heterozygosity on (i) the mutability of the remaining normal PKD allele; (ii) the apparent vulnerability of PKD patients to infection [70–72]; (iii) the integrity of the colon [73]; and (iv) the ability to repair tissue damage caused by infectious agents/toxins [74,75]. Some PKD gene mutations may yield polycystins that retain a degree of functionality, thereby influencing phenotypic expression [39•,40].

The endotoxins and fungal antigens present in the human PKD kidney are plausibly derived from the gastrointestinal tract [44\*\*]. A preliminary report of nanobacteria in marine, pond, and potable water [15] makes ingestion a possible route of exposure. Anomalies of the colon referred to as diverticula are reported to occur in 80% of PKD patients [73]. The PKD colon may be the initial domino in the progression of events leading to cystogenesis: (i) damage to gut barriers; (ii) enhanced absorption of colonic microbial and diet-derived cystogenic materials; (iii) actions of absorbed microbial materials on kidney and liver cyst formation and vasculature aneurysms. Although widely thought to be abnormal [41], a clear demonstration that the PKD colon is continuously or episodically 'leaky' has yet to be provided.

What cellular toxicity caused by microbial agents/toxins might account for renal cystogenesis? Apatite-coated nanobacteria are renotropic, and cause damage to the renal collecting tubules [22]. Although nanobacteria from human PKD cysts give a positive differential Limulus amoebocyte lysate test for endotoxin and immunoblot assays for chlamydial lipopolysaccharide (LPS: endotoxin) and *Bartonella henselae* antigens, it is not known whether these constituents are products of nanobacterial metabolism [44\*\*]. Microbial components are known to bind to apatite, a property useful in assays applicable to the detection of microbial contamination of meat [76]. Nanobacteria with toxins are presented to the kidney, either as products of nanobacterial metabolism or absorbance of microbial material present in saliva, the colon, and the environment (Fig. 2). The extraction and carriage of toxins in foods (e.g. fumonisin [49\*,64\*\*]) by nanobacteria or other particles to the kidney represents a potential new link between diet and kidney diseases.

In PKD, the tissue distribution of lesions follows the expression of polycystins. Kidney tubule epithelium, vasculature, and gut epithelium express polycystin 1 [57,60], a transmembrane protein that exhibits domains (e.g. C-type lectins), which in other proteins (e.g. C-type collectins) mediate the binding of microbes. On the basis of these descriptions of polycystin 1, we posited that microbial components may bind to polycystin 1 and thereby localize to tissues expressing polycystin [46], ultimately causing cysts, aneurysms, or diverticula. The relative efficiency of normal versus aberrant polycystins in binding nanobacteria or microbial toxins is unknown. Microbe-induced damage to PKD cells results in cells that poorly conduct tissue repair and yield altered tissue structure [74].

Tubule obstruction is proposed by Tanner *et al.* [77] to be an early event in cystogenesis. In Fig. 2, the trapping of nanobacteria in a tubule obstructed as a result of

nanobacterial cytotoxicity may provide selection pressure for the emergence of progressively more cystic cell types: cytoresistant [61,62] PKD cells intrinsically defective in repairing tissue [74]. A similar model might also explain simple kidney cysts, in which a lack of genetic vulnerability limits cyst formation to an individual nephron. Evidence of nanobacteria or antigens has been found in simple and PKD kidney cysts, PKD liver cysts, and human pineal cyst fluids [44\*\*,78].

Nanobacteria bind to and are internalized by human PKD cells *in vitro* [44\*\*]. The release of apatite-bound toxins would occur after internalization in endocytic vesicles as a result of the acidic pH that would etch apatite. In fibroblasts, a cell type that expresses polycystin [57], internalization is required before cytotoxicity is observed [6]. In models of nephrolithiasis and cystogenesis, cellular digestion of oxalate stones is reported to cause the release of cytotoxic free radicals, leading to cytotoxicity [79,80]. Therefore, continued research into nanobacteria and related concepts of 'particulate vectors for toxin absorption, concentration, and delivery' may yield new insights into an array of diseases in which tissue calcification, mutagenesis, or cystogenesis are important [81].

## Conclusion

Nanobacteria remain controversial agents that mediate apatite nucleation and crystal growth. They are renotropic, cause apoptotic cell death, are present in human kidney stones and kidney cyst fluids. They may trigger renal pathology involving damage to tubular epithelium, biomineralization, and perhaps tubule obstruction and chronic infection, resulting in defective tissue repair.

## Acknowledgements

The authors acknowledge and appreciate support from TEKES (Technology Development Centre, Finland), the Finnish Academy, and 'X-Bugs: Friends of Microbes in Chronic Disease Research' (Peoria), which includes Heart Care Midwest, Peoria Radiology Associates, Robert and Eleanor Flinn, and donations to the University of Illinois Foundation for PKD Research.

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