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URINARY TRACT INFECTION

PATHOGENESIS AND COMPLICATIONS

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ABSTRACT

Urinary tract infections (UTI) are one of the most common infections in women and children worldwide. If not diagnosed and treated appropriately, it may lead to severe illness and long-term complications. The economic impact caused by UTI on society is significant. In addition, bacterial resistance to common antibiotics is spreading at a high rate. Recent years, the understanding of the host-pathogen interaction and activation of the immune response in the urinary tract has increased considerably. However, there is still a lack of understanding how these basic molecular events can be translated into clinical practise.

In this thesis, the complex interaction between the uropathogenic *E. coli* and the urinary bladder and the kidney epithelium is described in the context of novel findings about virulence factors, immune response and complications following UTI.

In the first study, we investigated the chemokine profile in the kidneys of mice with pyelonephritis and the impact of IL-1 β by using an IL-1 β knockout mouse and renal cell lines. We could show a robust induction of IL-8/MIP-2 and of the chemokines MCP-1 and RANTES, not previously associated with bacterial infection.

In the second study, we described a new set of molecules involved in UTI pathogenicity, the metalloproteinase MMP-9 and its natural inhibitor TIMP-1. They increased in experimental pyelonephritis in mice and were found to be expressed by immune cells and resident renal cells. In children with pyelonephritis, we could link TIMP-1 to an increased risk of renal scarring.

In the third study, we investigated the virulence factor cytotoxic necrotizing factor (CNF1), commonly found in uropathogenic *E. coli*. Increased inflammatory reaction could be attributed to CNF1 *in vitro*; however, urine from patients with UTI caused by CNF1 positive or negative *E. coli* showed no difference in inflammatory response. Thus, the difference seen *in vitro* is probably of minor importance *in vivo* in comparison to other virulence factors of the uropathogenic *E. coli*.

In the last paper, we demonstrated that women supplemented with vitamin D had a stronger response of the antimicrobial peptide cathelicidin when biopsies from the urinary bladder were infected *ex vivo*. We also showed that normal urinary bladder cells were able to activate 25 hydroxyvitamin D₃ to its functional form 1,25 dihydroxyvitamin D₃, indicating a role for locally produced vitamin D in the urinary bladder. In addition, bladder cells increased their cathelicidin stores after vitamin D treatment and exerted antibacterial properties against uropathogenic *E. coli*.

In conclusion, this thesis presents new findings about host-microbe interaction in the urinary tract, the immune response and post-infectious complications of UTI. MMP-9, TIMP-1 and the chemokines IL-8/MIP-2, MCP-1 and RANTES were produced in the kidneys during pyelonephritis. IL-1 β and TIMP-1 affected the severity of infection and renal scarring. The *E. coli* toxin CNF1 influenced the immune response only *in vitro*, but did not seem to influence inflammation *in vivo*. Finally, treatment with vitamin D increased the antibacterial properties of the uroepithelium by induction of the antimicrobial peptide cathelicidin.

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- I. Enhanced chemokine response in experimental acute *Escherichia coli* pyelonephritis in IL-1 β -deficient mice
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- II. Matrix metalloproteinase-9 and tissue inhibitor of metalloproteinase-1 in acute pyelonephritis and renal scarring
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- III. Cytotoxic necrotizing factor 1 (CNF1) induces an inflammatory response in the urinary tract in vitro but not in vivo
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LIST OF ABBREVIATIONS

1,25D3	1,25 dihydroxyvitamin D3
25D3	25 hydroxyvitamin D3
ANOVA	Analysis of variance
C3	Complement factor 3
CAMP	Cathelicidin antimicrobial peptide
CD14	Cluster of differentiation 14
CFU	Colony forming units
CNF1	Cytotoxic necrotizing factor 1
CRP	C-reactive protein
CYP24A1	Cytochrome P450, family 24, subfamily a, polypeptide
CYP27B1	Cytochrome P450, family 27, subfamily b, polypeptide
DAPI	4'-6-Diamidino-2-phenylindole
DBP	Vitamin D binding protein
ESR	Erythrocyte sedimentation rate
FBS	Fetal bovine serum
G-CSF	Granulocyte Colony-stimulating factor
GSL	Glycosphingolipid
HBD	Human beta defensin
hCAP18	Human cathelicidin antimicrobial peptide
IL-1 β	Interleukin 1 beta
IL-8	Interleukin 8
LL-37	Amino acids 134-170 of hCAP18
LPS	Lipopolysaccharide
MCP-1	Monocyte Chemotactic protein 1
MD-2	Myeloid differentiation factor 2
MMP-9	Matrix metalloproteinase 9
PAMPs	Pathogen-associated molecular patterns
PCR	Polymerase chain reaction
PFGE	Pulse-field gel electrophoresis
RANTES	Regulated upon activation, normal T-cell expressed and secreted
RXR	Retinoid X receptor
TGF- β	Transforming growth factor beta
TIMP-1	Tissue inhibitor of metalloproteinase
TLR	Toll-like receptor
UTI	Urinary tract infection
VDR	Vitamin D receptor
VDRE	Vitamin D responsive element

1 INTRODUCTION

The Austrian paediatrician Theodor Escherich (1857–1911) discovered the pathogen later to be known as *Escherichia coli* (*E. coli*) in 1885. He isolated it from urine samples of young girls with symptoms from the lower urinary tract, thus recognizing the significance of urinary tract infections (UTI) in children (Shulman, Friedmann et al. 2007). Since then, much effort has been made to understand the signs and symptoms of UTI, and to understand the pathophysiology behind them. Nevertheless, diagnosing UTI is associated with considerable difficulties especially in infants and young children (Tullus 2011). Still it is important since extensive and sometimes painful radiological investigations will follow and medication for a prolonged period of time may be initiated. As the diagnosis is confirmed, the rapidly increasing resistance to urinary tract antibiotics challenges clinicians. Thus, new methods for early detection and treatment are paramount. Although much of this thesis has been based on laboratory investigations and findings, the aim has always been to link our findings to the patients, young or old, suffering from UTI.

1.1 URINARY TRACT INFECTION

UTI remains an important cause of serious bacterial infections especially in children but also in women. It accounts for significant morbidity and medical costs with an estimated 3 billion dollars being spent annually only in the United States (Litwin, Saigal et al. 2005).

Except for early infancy, girls and women are more likely to experience a UTI than boys and men. Half of all women will have at least one UTI during their lifetime (Fihn 2003) and almost one in three women will have suffered from a UTI requiring treatment by the age of 24 (Foxman 2002). In Swedish children, the cumulative UTI incidence rate is reported to be 7.8% for girls and 1.7% for boys (Hellstrom, Hanson et al. 1991) by the age of 7 years which is considerably higher than the 3.3% reported by Winberg in 1974 (Winberg, Andersen et al. 1974).

If the infection is confined to the urinary bladder the term cystitis is used whereas if involvement of the pelvis and parenchyma of the kidney is established, the term acute pyelonephritis is used. In children, pyelonephritis may result in chronic kidney disease due to tissue remodelling resulting in scar formation following the acute infection. In severe cases, bacteria may spread further to the blood to cause septicaemia. Thus, prompt diagnosis and initiation of treatment are important. New guidelines on paediatric UTI emphasize the need to direct resources towards early suspicion and diagnosis of UTI (Mori, Lakhanpaul et al. 2007).

Due to the high incidence of UTI, it is one of the most common reasons for antibiotic prescription. With the high rate of recurrences, frequent courses of antibiotics in the same individual are not uncommon. In addition, for children, low-dose antibiotics are

often prescribed as prophylaxis during extended periods of time. This combination of low dose and long duration favours the development of resistance to common urinary tract antibiotics (Williams and Craig 2011). Indeed, ever-increasing resistance rates are worrisome and the World Health Organization (WHO) calls antibiotic resistance one of the three greatest threats to human health (Wise 2011). Importantly, the efficiency of prophylactic antibiotics is also questioned, as their use may lead to breakthrough infections with resistant organisms while not actually preventing renal damage (Conway, Cnaan et al. 2007). Judicious use of antibiotics and development of novel therapeutic methods for prevention and treatment of UTI are important elements to help slow this progression.

Until present, manipulating the immune response for use in clinical practise has proved difficult and has been largely a disappointment in the fight against infectious diseases (Dinarello 2001). On the other hand, treating inflammatory conditions like rheumatoid arthritis and inflammatory bowel disease with biological therapy targeting vital parts of the immune system has proved successful (Feldmann and Maini 2010). With more basic knowledge about host-pathogen interaction, new treatment strategies could be developed also for UTI and the complications associated to it. These would be aimed at reducing adverse effects of innate immunity and boosting the favourable ones, thereby using the innate immunity to fight infections.

1.2 CLINICAL PRESENTATION

The clinical symptoms of UTI are dependent on age, stage of infection, host response and type of bacteria causing the infection. Young infants often present with unspecific symptoms like fever, irritability, vomiting, lethargy, or poor feeding. As the children grow older, and in adults, more explicit symptoms like pain upon voiding and increased frequency are present in lower UTI. Upper UTI on the other hand is associated with flank pain and fever. Recently, a meta-analysis evaluated the diagnostic accuracy of UTI signs and symptoms in children. For infants, a history of previous UTI, fever more than 24 hours, suprapubic tenderness and absence of circumcision all increased the likelihood of UTI. For older children, abdominal pain and fever, back pain, new onset of urinary incontinence and dysuria, were most reliable (Shaikh, Morone et al. 2007). In a proposed algorithm for evaluating women with symptoms of UTI, a combination of several clinical symptoms reached a probability of UTI of 90% without laboratory testing (Bent, Nallamotheu et al. 2002). The clinical presentations in postmenopausal women were more severe than in premenopausal women with more generalized unspecific symptoms and urinary incontinence (Arinzon, Shabat et al. 2011).

2 PATHOGENESIS OF UTI

UTI results from bacteria entering the urinary tract from the nearby vagina and perineum. Since these areas are normally heavily colonized with bacteria and the urethral opening is located here, the urinary tract is vulnerable to infection. The common pathogens causing UTI are residents of the enteric or vaginal flora. *E. coli* is by far the most common cause of UTI, causing about 80 % of infections in otherwise healthy women (Stamm 2002) and girls (Gaspari, Dickson et al. 2005), followed by *Klebsiella*, *Enterococcus*, group B streptococcus, *Proteus* and *S. Saprophyticus*.

2.1 UROPATHOGENIC *E. COLI*

E. coli is a Gram-negative rod that normally colonizes the term infant within hours after birth and form an important part of the normal human gut flora (Kaper, Nataro et al. 2004). Certain *E. coli* isolates can cause extraintestinal disease, hence termed ExPEC (Croxen and Finlay 2010). Organs targeted are diverse, for example the urinary tract, the central nervous system and the lungs (Kaper, Nataro et al. 2004). The mechanisms by which *E. coli* gain access to the urinary tract reflect an exceptional ability to adapt to an environment very different from the gut. They need to alter their metabolism (Alteri, Smith et al. 2009), ascend against the flow of urine and adhere to the epithelial layer. The *E. coli* that successfully invade the urinary tract harbour specific factors that enables them to survive. These strains of *E. coli* are commonly named uropathogenic *E. coli* or UPEC.

The virulence of UPEC compared to non-pathogenic *E. coli* results from specific virulence genes in the bacterial chromosome. These vary considerably between different isolates and no single gene has been solely implicated in uropathogenesis (Wiles, Kulesus et al. 2008). Hence, UPEC is not a homogenous group but rather isolates of *E. coli* with different subsets of virulence factors enabling adherence, invasion and survival in the urinary tract. In line with this, there are currently no tests that can determine whether an *E. coli* strain is uropathogenic or not, unless it has been appropriately isolated from the urine of a patient with symptoms of UTI.

Many bacterial factors contribute to the complex pathogenesis of UTI. In fact, 131 UPEC-specific genes were reported, many of which may contribute to virulence (Lloyd, Rasko et al. 2007). Flagellae are thread-like structures that provide *E. coli* with the ability to move. It has been found to bind to TLR5 (Hayashi, Smith et al. 2001) and is of importance for the immune response to *E. coli* in UTI in mice (Andersen-Nissen, Hawn et al. 2007). Adhesion is a critical step for UPEC to avoid being washed out with the urine and the first step in a series of events leading to infection. The type-1 fimbriae are adhesion factors studied in great detail and are critical for adhesion and invasion of UPEC into bladder cells (Connell, Agace et al. 1996; Mulvey, Lopez-Boado et al. 1998). They are equipped with a protein on the tip called FimH, which is responsible for the interaction with the host cell (Jones, Pinkner et al. 1995). It binds to several

structures on uroepithelial cells, the most important being uroplakin IA that coats the facet cells of the bladder (Zhou, Mo et al. 2001). They also bind to β -integrin, which triggers cytoskeleton rearrangement leading to bacterial internalization (Eto, Jones et al. 2007). In renal epithelial cells, complement factor 3, which is secreted by epithelial cells during infection, can bind to type 1 fimbriae to form a complex that interacts with CD46 to promote internalization (Springall, Sheerin et al. 2001; Li, Zhou et al. 2009). Other fimbriae like P fimbriae are associated with kidney infection since they bind to glycosphingolipids on kidney epithelial cells (Eden and Leffler 1980).

Interestingly, colonization of the upper urinary tract requires a tightly regulated interplay between virulence factors, so-called phase variation (Casadesus and Low 2006). Flagellae are activated when the bacteria ascend towards the kidneys. To attach to the renal epithelial cells, P fimbriae are turned on and type 1 fimbriae turned off. Complex interactions between P and type 1 fimbriae may be seen when P fimbriae attach to kidney epithelial cells at the same time as type 1 fimbriae are responsible for inter-bacterial binding in the renal tubule, causing tubular obstruction (Melican, Sandoval et al. 2011).

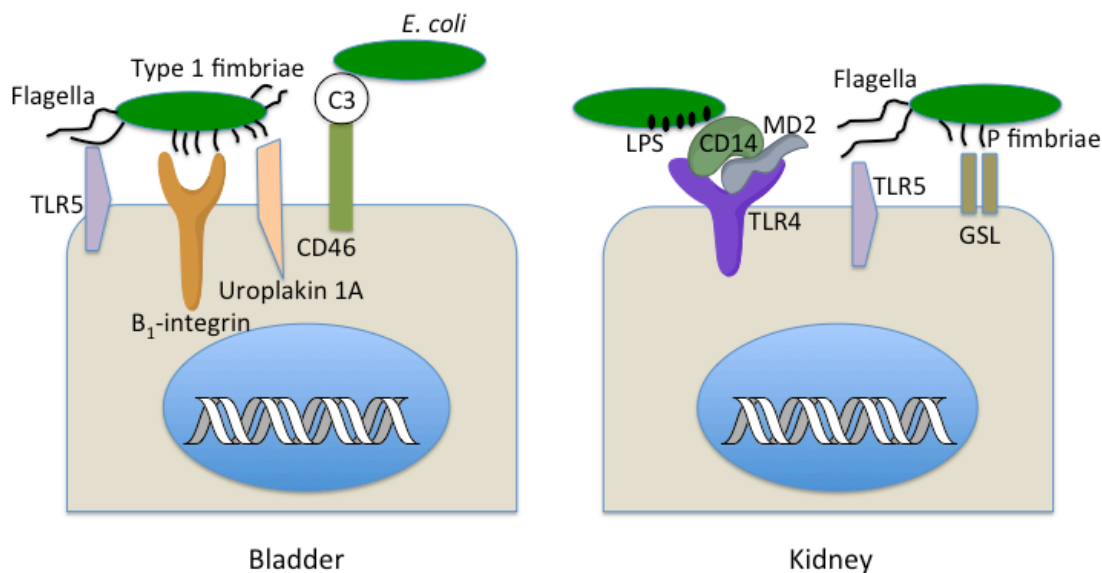


Figure 1. Attachment to urinary bladder cells (left) and renal epithelial cells (right) by uropathogenic *E. coli*.

Flagella provide the bacteria with mobility and may interact with the superficial bladder cell through TLR5. Further adhesion is provided by type 1 fimbriae binding to uroplakin 1A or β_1 -integrin, which also promote internalization into the cell. Complement secreted upon infection bind to the bacteria and promotes interaction with the bladder through CD46. In the kidney, P fimbriae of the bacteria bind to glycosphingolipids on the surface of renal epithelial cells. Bacterial invasion is further promoted by TLR4 and TLR5.

Once bacteria have attached they need mechanisms to survive in the new environment. Since iron is an essential nutrient for UPEC viability and the urinary tract is a low-

iron environment, they exhibit multiple mechanisms for iron extraction. In fact, a large part of the UPEC genome is devoted to iron scavenging genes. For UPECs to gain access to the host tissue cytotoxic proteins are produced. One example is alpha-hemolysin which is a well characterized virulence factor frequently expressed by *E. coli* causing UTI (Smith, Rasmussen et al. 2008). In the early stage of acute pyelonephritis, when bacteria attach to the kidney epithelial cells, alpha hemolysin induces calcium oscillations intracellularly which activates the proinflammatory cytokines IL-6 and IL-8 (Uhlen, Laestadius et al. 2000) and in the bladder, sloughing of the uroepithelium and bladder haemorrhage is initiated (Smith, Rasmussen et al. 2008).

Another virulence factor that causes cytoskeletal rearrangements is cytotoxic necrotizing factor 1 (CNF1). CNF1 is a member of the Rho family of GTPase activating toxins and is often genetically linked to alpha-hemolysin (Blanco, Alonso et al. 1990). It can induce an inflammatory reaction and apoptosis in uroepithelial cells (Mills, Meysick et al. 2000). Animal studies however, have shown conflicting results from different research groups regarding its role *in vivo*. Bacteria can also produce biofilm to protect it from immune defense mechanisms. Curli fimbriae and cellulose are key components in biofilm formation (Kai-Larsen, Luthje et al. 2010).

Table 1. Some common virulence factors to UPEC and their major functions.

Virulence factor	Major function
Flagella	Ascension of <i>E. coli</i> in the urinary tract
Type 1 fimbriae	Adhesion to bladder epithelial cells, interbacterial adhesion
P fimbriae	Adhesion to kidney epithelial cells
Dr fimbriae	Cell invasion
F1C fimbriae	Unknown
Cytotoxic Necrotizing Factor 1	Adhesion, invasion, apoptosis of host cells
Hemolysin	Invasion, tissue damage
Secreted autotransporter toxin	Tissue damage
LPS	Immune response activator
Curli fimbria	Adhesion, biofilm formation, invasion
Cellulose	Biofilm formation
Iron and zinc acquisition (several)	Nutrition
Capsule	Resistance to phagocytosis

Bacteria also carry pathogen associated molecular patterns (PAMPs) that are structures on the bacterial surface that the host cells can recognize and mount an appropriate response against. Lipopolysaccharide (LPS) is the classic gram negative PAMP and is situated on the outer membrane. The contribution of LPS to UTI pathogenesis is debated. Unlike myeloid cells that respond promptly to LPS, uroepithelial cells respond weakly. One reason is the lack of membrane-associated CD14 which is an important LPS receptor in complex with toll-like receptor 4 (TLR4), which has been identified in

biopsies from the epithelium of the entire urinary tract (Samuelsson, Hang et al. 2004). The capsule of the UPEC is important for protection against the host response for example complement-mediated killing (Buckles, Wang et al. 2009). In the kidney, there are also ways for non-cytolytic bacteria to enter the kidney epithelial cells however. While cytolytic UPEC like the prototypic strain CFT073 used in this thesis can invade the renal epithelium to reach the underlying tissue, non-cytolytic UPEC use a lipid-raft mediated transport in order to translocate through the epithelium (Chassin, Vimont et al. 2008).

Taken together, the virulence factors of UPEC are diverse and complex and no specific combination of presence or absence of virulence factor genes can serve as a marker to predict recurrent UTIs. It seems however as if *E. coli* causing recurrent UTI have more virulence factor genes than *E. coli* causing sporadic UTI (Ejrnaes, Stegger et al. 2011).

2.2 HOST FACTORS

Apart from the distal one-third of the urethra, where a mix of commensal bacteria can be found, the urinary tract is normally sterile. This is in spite of the close proximity to the gastrointestinal tract. Thus, there must be ways for the urinary tract epithelium to efficiently defend itself from invading pathogens. The first-line defence against microorganisms in the urinary tract consists of the mechanical properties of the highly specialized transitional uroepithelium, the unidirectional flow of urine, anatomical structures like the flap-valves of the vesicoureteral junction and biochemical factors like neutralizing peptides for example mucin and Tamm Horsfall protein, immunoglobulin and antimicrobial peptides (Chromek and Brauner 2008). Since white blood cells are not normally found in the urine, the sterility is rather a result of action of the epithelial cell layer. Recent research from our group suggests that the fast innate immune response of the epithelial lining is highly important (Chromek and Brauner 2008; Hertting, Holm et al. 2010). Although a combination of mechanical and epithelial factors is probably involved, little focus has been attributed to the biological defence of the uroepithelium.

Importantly, the outcome of the action of virulence factors mentioned above may be different in the lower and upper urinary tract. One example is apoptosis. Several virulence factors of UPEC seem to have pro-apoptotic properties, including type 1 fimbriae (Klumpp, Weiser et al. 2001), alpha-hemolysin (Chen, Tofighi et al. 2006) and CNF1 (Mills, Meysick et al. 2000). Apoptosis is probably a beneficial process for the host in early lower UTI. It results in exfoliation of the superficial cells of the multi-layered epithelium and thus also eradication of the bacteria attached to and invaded into the cells. In the kidney however, where the epithelium is single-layered and close to the underlying kidney tissue and blood vessels, apoptosis is more likely to be part of a deleterious circle of tubular atrophy, cytolytic events and renal scarring (Serlachius, Sundelin et al. 1997; Yang, Johnson et al. 2001). For the bacteria however, it provides access to the kidney tissue.

2.2.1 Mechanical properties

2.2.1.1 Urine flow

The urine flow contributes to the sterility of the urine by making it difficult for bacteria to enter the urinary bladder. If they enter and attach to the uroepithelium, the flow of urine flushes the exfoliated cells out and thus also clearing the bacteria bound or invaded into them. As a result, voiding dysfunction or residual urine in the bladder lead to reduced flow of urine and in turn risk of bacterial growth. Also, the flap-like valves of the vesicoureteral junction prevent retrograde flow of urine into the ureters and kidneys. As mentioned, children with vesicoureteral reflux may therefore be of greater risk of infection.

2.2.1.2 Uroepithelium

The uroepithelium itself is interesting because it is unique to the body by being flexible to allow filling and emptying of the bladder and at the same time impermeable to fluid and able to cope with the varying pH, osmolality and toxicity, for example high ammonium concentration (Apodaca 2004). It has a distinct composition of different layers of cells with the umbrella or facet cells lining the lumen. They are multinuclear, large cells with uroplakin facing the urine. Uroplakins are proteins contributing to the impermeability of the epithelium but can also act as a receptor for type 1 fimbriae on the uropathogenic *E. coli* (Wu, Sun et al. 1996).

2.2.2 Innate immune response

The innate immune system is our first line of defence against invading organisms and does not depend on prior exposure to a specific pathogen (Chaplin 2010). It must therefore be rapid and cannot rely on recruitment of immune cells or the production of antibodies. The uroepithelial cells are the first to encounter invading UPECs. Consequently, they are equipped with receptors that sense microbes and initiate a response in order to limit the infection process. However, the host must be able to distinguish between pathogenic and non-pathogenic bacteria in order to avoid constant activation of the immune system. LPS is a classic PAMP but is present in both non-pathogenic and pathogenic *E. coli*. It has therefore been proposed that the lack of the LPS-receptor CD14 on uroepithelial cells prevents activation by non-pathogenic *E. coli* whereas TLR4 together with specific co-receptors and several adaptor proteins activates the cells (Fischer, Yamamoto et al. 2006). TLR4 downstream signalling eventually results in the production of proinflammatory cytokines (Fischer, Ellstrom et al. 2007). Type 1 fimbriae or P fimbriae can activate the innate immune response through interaction with TLR4 by their respective receptors whereas commensals, which normally lack P fimbriae, do not. Consequently, children who developed UTI have increased tendency to carry *E. coli* strains with P fimbriae in their faecal flora and these strains also cause their infection (Plos, Connell et al. 1995).

If UPEC come in contact with the epithelium, within minutes, the antimicrobial peptide cathelicidin is secreted (Chromek, Slamova et al. 2006) and acts on the bacteria. Within hours, cytokines and chemokines are produced (Hertting, Khalil et al. 2003) and their signalling will start to recruit professional immune cells to the site of infection (Connell, Agace et al. 1996). The bacteria on the other hand, will try to circumvent the immune defence in different ways. One is to enter the cell cytoplasm and form intracellular bacterial communities (IBCs) in order to “hide” from the immune response (Hunstad and Justice 2010); another is to down regulate the immune response with different modes of signalling (Hunstad, Justice et al. 2005). Depending on the number of bacteria, the virulence factors they carry and the host status, the bacteria will either persist in the urinary tract or be eliminated and washed out with the urine.

2.2.3 Cytokines and chemokines in UTI

If this first line of defence against pathogens entering the urinary tract fails, an inflammatory response is initiated. Attachment to the bladder uroepithelial cells by bacterial fimbriae allows for close contact between host and pathogen. Transmembrane signalling through TLRs leads to the production of inflammatory mediators such as chemokines with subsequent recruitment of professional immune cells to the infectious focus. Chemokines are divided into different groups depending on cysteine arrangement, hence named CC, CXC, C or CX₃C chemokines. Specifically, the CXC-chemokine IL-8 is needed for neutrophil recruitment and activation in the urinary tract (Agace, Hedges et al. 1993). Now, a second wave of neutrophil-derived antimicrobial peptides can act to eliminate invading bacteria (Chromek, Slamova et al. 2006). Other toxic components, like lysozyme, elastase and myeloperoxidase are also released by the neutrophils, and bacteria are cleared by phagocytosis. Neutrophils are crucial for UPEC clearance in UTI, as shown by depletion of neutrophils in a UTI model, resulting in impaired bacterial clearance (Haraoka, Hang et al. 1999). The neutrophils may however also cause unwanted effects for the host such as tissue destruction (Jahnukainen, Chen et al. 2005).

When the inflammatory response subsides, bacteria may still be left in the bladder epithelium. Bacteria that form IBCs can escape the different steps in host defence (Anderson, Palermo et al. 2003) and treatment with antibiotics will be less efficient because of poor antibiotic penetration into the IBCs. From the IBC, bacteria can be expelled from the cells by a TLR4 mediated mechanism (Song, Bishop et al. 2009) or in mature IBCs, bacteria form filamentous structures, then detach from the cell to colonize adjacent cells (Justice, Hunstad et al. 2006). The cells may also be exfoliated allowing the underlying immature cells to be exposed to further UPEC invasion. Here, they can turn into quiescent intracellular reservoirs (QIRs) for weeks, only to re-emerge to cause recurrent infections (Mysorekar and Hultgren 2006).

If the bacteria ascend further in the urinary tract, pyelonephritis may develop. In the kidney bacteria may cause tissue damage and reach the blood circulation, causing

septicaemia, commonly called urosepsis. This increases the mortality from 0.3% in pyelonephritis to 7.5-30% in urosepsis (Foxman, Klemstine et al. 2003; Brown, Ki et al. 2005). Furthermore, destruction of the kidney tissue may cause renal fibrosis, scarring, which in the end may affect the renal function in a deleterious way. Unlike the process of P fimbriae mediated attachment, the process of bacteria entering the kidney tissue is less well understood but seems to involve a TLR4-facilitated lipid raft mediated translocation across renal collecting duct cells (Chassin, Goujon et al. 2006). Collecting duct cells are the primary site of UPEC adherence and have abundant TLR4 expression. For clearance of bacteria from kidney tissue, chemokines and the chemokine receptor CXCR1 are pivotal. Lack of CXCR1 receptor in mice leads to neutrophil entrapment and renal scarring (Hang, Frendeus et al. 2000) and may possibly have a human counterpart in patients susceptible to pyelonephritis where low CXCR1 expression was found (Frendeus, Godaly et al. 2000).

2.2.4 Matrix metalloproteinases

Successful eradication of uropathogenic *E. coli* by the host requires initiation of chemokine release and influx of immune cells. However there must also be a resolution of inflammation and finally remodelling of the extracellular matrix (ECM). Matrix metalloproteinases (MMPs) are zinc-containing enzymes and, as a group, with the ability to degrade all components of the ECM (Sternlicht and Werb 2001). MMPs are not expressed in healthy tissue but can be detected in any remodelling process in virtually all cells in the body (Parks, Lopez-Boado et al. 2001). Different MMPs have different substrates however and they are the only known enzymes to be able to digest collagen. They are tightly regulated on a transcriptional level as well as on the activating level (Hadler-Olsen, Fadnes et al. 2011). They are produced as pro-forms and cleavage to its active form is carried out by other MMPs and other proteinases. MMPs are additionally regulated by the tissue inhibitors of metalloproteinases (TIMPs), which bind to the catalytic site of MMPs, thereby inhibiting uncontrolled digestion of ECM components (Bode, Fernandez-Catalan et al. 1999). MMP-2 and MMP-9 are so called gelatinases with gelatin-binding domains that enable enhanced interaction with collagen (Parks, Wilson et al. 2004). They are typically anchored to the cell membrane, thus achieving high enzymatic activity locally. Today, MMP functions have expanded beyond ECM remodelling to include multiple steps of immune activation. In the early response, MMPs activate antimicrobial peptides and chemokines. For example, MMPs may cleave cytokines or chemokines either to augment or to inhibit their functional activity (Parks, Wilson et al. 2004). MMPs are involved in the local tissue damage that facilitates migration of leukocytes towards a chemokine gradient. In the brain, they are produced by leucocytes in order to break down the blood-brain barrier (Yong, Power et al. 2001) and cause vascular leakage in sepsis (Tressel, Kaneider et al. 2011). In kidney, MMP-9 has been linked to fibrosis in obstructive nephropathy in mice (Wang, Zhou et al. 2010).

2.2.5 Antimicrobial peptides

In 1981, Hans Boman and co-workers isolated the antimicrobial peptide (AMP) cecropin from the moth cecropia (Steiner, Hultmark et al. 1981). Today, more than 1000 peptides with antimicrobial properties have been identified. AMPs are peptides that participate in the early innate immune response. They are exceptionally well conserved evolutionary, which imply that they have been of crucial importance for survival in plants, insects and mammals. In insects, systemic levels of AMPs are very high whereas in mammals they are barely detectable in the resting state but induction is seen upon microbial stimulation. AMPs are positively charged peptides with broad-spectrum antimicrobial activity; bacteria, viruses, parasites and fungus are all potential targets. They are multifunctional with chemotactic, angiogenetic and wound healing properties (Lai and Gallo 2009). In humans, defensins and cathelicidin are the most important. They are produced as precursor proteins, which then are activated to form the active peptides. The antibacterial activity is targeted at the bacterial membrane and involves peptide-pathogen interaction by electrostatic attraction between the positively charged peptides and the negatively charged bacteria. This leads to membrane damage through a number of complex mechanisms (Toke 2005).

2.2.5.1 *Defensins*

In the urinary tract, human beta defensin-1 (HBD-1) is found in the distal nephron of human kidney and is thought to be the primary source of urine HBD-1. In contrast to cathelicidin, defensins seem to be expressed constitutively to a higher degree and reach much higher concentrations in the urine. Interestingly, it was shown that pregnant women have higher HBD-1 levels in the urine than both non-pregnant women and men (Valore, Park et al. 1998) suggesting hormonal regulation of defensins and an increased need for local protection during pregnancy. In pyelonephritic kidneys both HBD-1 and HDB-2 have been found (Lehmann, Retz et al. 2002) and in cultured human kidney cells, HDB-2 was produced in response to synthetic TLR2, TLR3, TLR4, TLR7, NOD1 and NOD2 agonists (Uehara, Fujimoto et al. 2007).

2.2.5.2 *Cathelicidin*

Only one cathelicidin is known in man. It is encoded by the *CAMP* gene and is expressed as a precursor protein hCAP18. This protein is later cleaved to LL37, a peptide of 37 amino acids with potent antimicrobial activity. It was initially shown that hCAP18 cleavage was performed by proteinase 3 present in neutrophils (Sorensen, Follin et al. 2001). The precursor hCAP18 can also be processed to peptides other than LL37, which also display antimicrobial activity (Murakami, Lopez-Garcia et al. 2004). In neutrophils, LL37 is stored in granules but the storage and cleavage in epithelial cells is less well understood. In this text we use the word cathelicidin to describe hCAP18 and LL37 together. Cathelicidin is expressed and secreted in response to hostile microbial stimuli in both immune cells and epithelial cells. Apart from its well-recognized antibacterial effects, cathelicidin has additional

immunoregulating properties. It can neutralize LPS, activate epithelial cells and it has chemotactic activity towards neutrophils, monocytes, T cells (De, Chen et al. 2000), mast cells (Niyonsaba, Iwabuchi et al. 2002) and eosinophils (Tjabringa, Ninaber et al. 2006). The chemotactic activity is achieved through interaction with formyl peptide receptor-like 1 (FPR1) (De, Chen et al. 2000). Recently, cathelicidin was also reported to inhibit biofilm formation by interacting with the curli fimbria of uropathogenic *E. coli* (Kai-Larsen, Luthje et al. 2010).

In urinary tract cells, cathelicidin seems to react very rapidly in response to bacterial stimuli (Chromek, Slamova et al. 2006). This probably stops bacterial division, which provides an efficient way for the host to halt an impending bacterial infection at an early stage (Chromek and Brauner 2008). This is of importance since uropathogenic *E. coli* replicates rapidly in the urine during UTI. In uropathogenic *E. coli* taken directly from the urine of women with UTI, the most highly expressed genes were those encoding ribosomal proteins (Hagan, Lloyd et al. 2010), which are known to increase proportionally to growth rate (Gausling 1977).

The mechanism of activation of cathelicidin seems to differ between organ and cell type. In contrast to defensins, cathelicidin seems only partly to be dependent on TLR or cytokine signalling. Rather, histone acetylation and vitamin D signalling seems important. Interestingly, butyric acid can increase histone acetylation. In a model of *Shigella* enteritis in rabbit, oral butyrate treatment reduced clinical illness, inflammation and bacterial load (White 2008).

2.2.5.3 Vitamin D

Vitamin D is a steroid hormone that has multiple effects on human health, the most important being the regulation of calcium homeostasis. Consequently, hypercalcaemia is the most important side effect of vitamin D treatment. Vitamin D is produced from precursors originating in the skin under the influence of UVB light or from digestion of animal products, mainly fatty fish or fortified dairy products (Ross 2011). Since activation in the skin is dependent on the intensity of UVB light, people in the Nordic hemisphere will produce little vitamin D during winter months but also protection from the sun by clothing or sunscreen in summertime will reduce vitamin D activation. Two enzymatic steps activate Vitamin D before it can bind with high affinity to the vitamin D receptor (VDR). Activation normally starts in the skin, where UVB radiation converts 7-dehydrocholesterol to cholecalciferol. In the next step, the hepatic 25-hydroxylase CYP27A1 produces 25-hydroxycholecalciferol (25D₃), which relocates to the circulation, bound to vitamin D binding protein (DBP). The last step is when the renal 1 α -hydroxylase CYP27B1 converts 25D₃ to the active 1,25D₃ or calcitriol. 1,25D₃ binds to VDR in target cells, until the 24-hydroxylase (CYP24A1) inactivates it (Prosser and Jones 2004).

In recent years, the presence of CYP27B1 in peripheral tissue other than the kidney has led to a completely new way of looking at vitamin D metabolism since it allows local vitamin D activation in response to different stimuli to take place (Zehnder, Bland et al.

2001). There seems to be only one type of vitamin D receptor in humans and this receptor mediates all vitamin D effects. The number of VDR binding sites on the genome is cell type-specific however, making different cell types or tissue respond differently to vitamin D (Pike, Meyer et al. 2011).

The connection between vitamin D and infectious diseases is as much medical history as it is modern molecular medicine. The notion that being outdoor was beneficial for patients with infectious diseases dates back to the late 1800s when nobody knew why, but tuberculosis patients sent to rest in sanatoria at sunny places and with nourishing diet often experienced some degree of remission (Campbell 2005). Later, the connection between UVB light from the sun and the production of vitamin D in the skin became evident and in 1983 the presence of vitamin D receptors in human leucocytes indicated that non-classical vitamin D activity could also include the immune system (Provvedini, Tsoukas et al. 1983).

The discovery that vitamin D directly induces production of the antimicrobial peptide cathelicidin (Wang, Nestel et al. 2004; Gombart, Borregaard et al. 2005) further strengthen the link between vitamin D and innate immunity. In 2006, the mechanism behind this upregulation in immune cells was demonstrated. It included TLR activation of human macrophages, which increased expression of the VDR and the converting enzyme CYP27B1 genes. This in turn led to induction of cathelicidin and killing of intracellular *M. tuberculosis* (Liu, Stenger et al. 2006). As a consequence, many studies on vitamin D and its effect on innate immunity were initiated. In fact, in the 2007 Time magazine list of the top medical breakthroughs of the year, the benefits of vitamin D was number 10 (Guthrie 2007). The link between vitamin D and cathelicidin is possible because of a vitamin D responsive element (VDRE) located upstream in the cathelicidin gene (*CAMP*) promoter (Wang, Nestel et al. 2004). The VDRE in the *CAMP* gene originated in the lineage leading to humans, apes, old world monkey and new world monkeys and it has been estimated that this happened about 50 million years ago (Gombart, Saito et al. 2009). As a result, rodent models of infection cannot be used for studying vitamin D induction of *CAMP*.

3 RISK FACTORS

General risk factors of UTI and especially recurrences are for example urinary catheter carriage or poorly controlled diabetes mellitus (Muller, Gorter et al. 2005). In men presenting with UTI, which will not be covered here, obstruction of the lower urinary tract should always be suspected since the most common cause, next to urolithiasis, is prostate hyperplasia (Heyns 2011).

3.1 CHILDREN

Specific risk factors in children include infrequent voiding, poor fluid intake and functional stool retention, which were more frequent in girls with recurrent urinary tract infections than in control girls (Stauffer, van der Weg et al. 2004). If the urinary bladder is not emptied sufficiently, as is the case in neurogenic bladder and other conditions of dysregulated bladder emptying, the residual urine may be colonized with bacteria. In a Swedish study of adolescents with myelomeningocele, 15% had recurrent UTI but only 1.7% had decreased renal function (Olsson, Dahl et al. 2007). In children, congenital hydronephrosis, vesicoureteral reflux, ureteropelvic junction obstruction or ureterovesical junction obstruction are all anatomic anomalies associated with UTI (Figure 3). These structural risk factors highlight the importance of regular emptying of the bladder to avoid bacteria to multiply and gain access to the urinary tract. In other words, the urinary tract depends partly on the unidirectional flow of urine for its sterility to be maintained. The link between anatomical anomalies in the urinary tract and its role in UTI and renal scarring is under debate. For example, combined surgical and antibiotic treatment for vesicoureteral reflux caused a 57% reduction in febrile UTIs by five years of age but did not decrease the risk of progressive renal damage (Nagler, Williams et al. 2011)

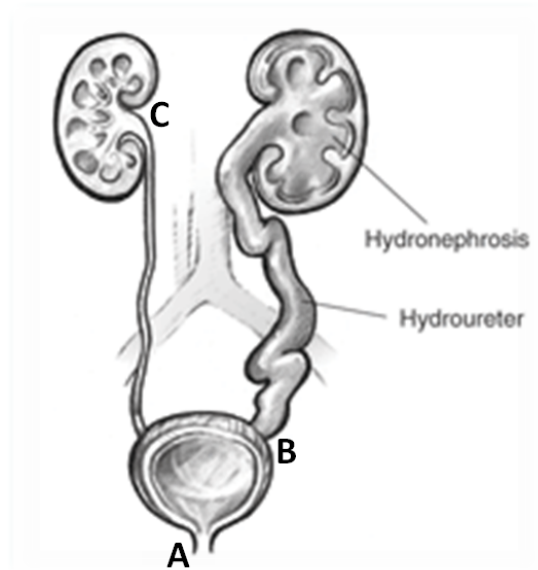


Figure 2. Anatomical abnormalities may predispose to UTI.

A. Posterior urethral valve
B. Vesicoureteral valves
C. Ureteropelvic obstruction
Left-sided vesicoureteral reflux is seen.

Image courtesy of the National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health.

3.2 WOMEN

Known risk factors in pre-menopausal women include sexual intercourse and pregnancy (Foxman 2002). Asymptomatic bacteriuria occurs in 2% to 10% of pregnancies and, if untreated, around 30% of these women will develop acute pyelonephritis (Smaill and Vazquez 2007). UTI in pregnancy is correlated to intra-uterine growth restriction, pre-eclampsia, caesarean sections and pre-term deliveries (Mazor-Dray, Levy et al. 2009). Using certain types of birth control may also increase the risk for UTI (Foxman and Chi 1990). After menopause, UTI may become more common because of lower estrogen levels, which may cause mucosal vulnerability and influence the production of the defensin HBD-2 in the vagina (Han, Kim et al. 2010). In contrast to children and men, anatomical abnormalities are rarely found in women, even if they have recurrent infections (Finer and Landau 2004).

4 COMPLICATIONS

Normally, UTIs treated accurately resolve quickly and are not associated with severe complications. In a minority, however, UTI may cause severe infection and long-term disabilities. Infants younger than 3 months are at increased risk of bacteraemia (Richardson and Lakhanpaul 2007) and pregnant women are as mentioned at high risk of UTI. The most common complications and the ones covered in this thesis are recurrent infections and renal scarring following UTI in children.

4.1 RECURRENT URINARY TRACT INFECTIONS

25% of women will have a recurrent UTI following their primary infection although they do not have obvious urinary tract anatomical abnormalities (Finer and Landau 2004). These recurrences are often caused by the same bacterial strain, suggesting incomplete resolution of the primary infection rather than a new infection (Brauner, Jacobson et al. 1992; Russo, Stapleton et al. 1995). The new findings regarding the intracellular life-style of UPECs have introduced new aspects of the pathology behind recurrent UTIs. This may explain why some patients develop UTI very frequently despite antibiotic treatment.

4.2 RENAL SCARRING

If the host response fails, bacteria may ascend to the upper urinary tract and cause pyelonephritis. The inflammation caused by the infection can lead to renal scarring, especially in children. Renal scarring is a process involving a number of actors. A crucial event is the influx of neutrophils. As a consequence, granulocytic cytotoxic products including lysozyme, elastase and myeloperoxidase are released and oxidative bursts occur (Heinzelmann, Mercer-Jones et al. 1999). These processes, aimed at killing and removing the pathogens may paradoxically be deleterious to the host with tissue destruction and fibrosis to follow (Jahnukainen, Chen et al. 2005). As a result of more widely available antenatal ultrasound imaging, scarring of the kidney has also been attributed to congenital abnormalities (Wennerstrom, Hansson et al. 2000).

5 AIMS OF THE THESIS

The overall aim of this thesis was to provide a greater insight to the host-pathogen interaction in urinary tract infection. This is important for several reasons. New diagnostic techniques are needed for early recognition of UTI, for distinguishing patients with upper or lower UTI and for risk assessment for recurrent UTIs or chronic kidney damage following pyelonephritis. Data about the pathophysiological basis of UTI are also needed to understand the role of the innate immunity in order to develop new treatments.

Specific aims for the included papers were:

- I. To study the effect of *E. coli* and IL-1 β on the expression of IL-8/MIP-2, MCP-1 and RANTES in an experimental mouse model of pyelonephritis.
- II. To investigate MMP9 and TIMP-1 induction and its relation to renal scarring in children with pyelonephritis.
- III. To study the contribution of the *E. coli* virulence factor CNF1 to the immune response in the urinary tract in children and adults with urinary tract infection.
- IV. To investigate if vitamin D treatment can induce the antimicrobial peptide cathelicidin in the urinary tract of postmenopausal women.

6 MATERIALS AND METHODS

6.1 CLINICAL STUDIES

Three of the papers presented in this thesis include clinical material from human subjects. Paper II and III involve children with UTI, paper III also adults with UTI and paper IV includes adult volunteers. All studies were approved by the local ethics committees as described in each separate publication. All episodes of UTI were caused by *E. coli* and verified by a positive bacterial culture with bacterial growth of $> 10^5$ CFU/mL in the urine. For acute pyelonephritis, additional criteria of rectal temperature $> 38^\circ\text{C}$, CRP $> 20\text{ mg/L}$ or ESR $> 20\text{ mm/hr}$ were used. In paper IV, women post menopause (at least 12 months since a menstrual period), were included. Exclusion criteria were urological malformations or other kidney diseases, UTI or recent supplementation with vitamin D. Oral supplementation with 2000 IU of 25D₃ was given daily for 12 weeks. Before treatment started, blood was collected for analyses and urinary bladder biopsies were taken for *in vitro* infection. The study is schematically outlined in figure 3.

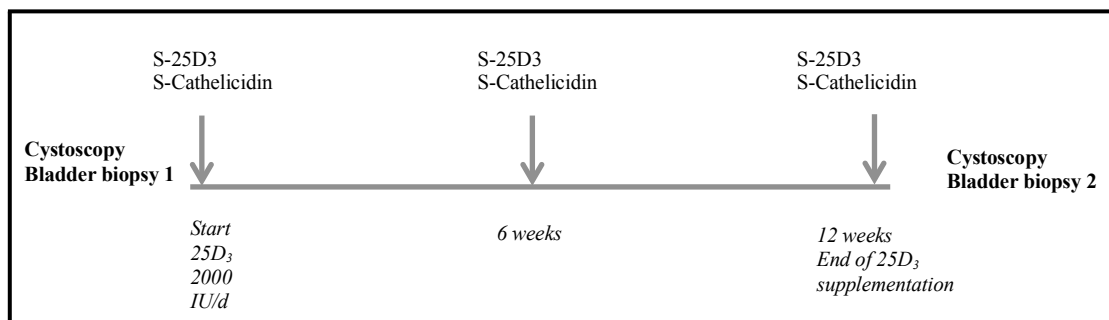


Figure 3. Schedule for vitamin D supplementation study presented in paper IV.

Bladder biopsies were taken from healthy postmenopausal women and supplementation with 2000 IU of vitamin D₃ daily was initiated. Blood sampling was performed at baseline, at six weeks and at twelve weeks when follow-up biopsies also were taken.

6.1.1 DMSA scan

DMSA scan is performed by injecting radio labelled dimercaptosuccinic acid (DMSA) intravenously and then visualising kidney uptake using a gamma camera. In paper II, data from children with DMSA scan performed within 10 days after acute infection and at 1-year follow-up were used. DMSA scan is a sensitive method of detecting acute pyelonephritis (Jaksic, Bogdanovic et al. 2011) and renal uptake is normally a good way of measuring renal function (Quinn and Elder 1991). It is also the preferred method of assessing renal scars in the kidneys (Moorthy, Wheat et al. 2004). In order to

standardize the interpretation of the DMSA scans, a score has been calculated by determining uptake defects in the kidneys (Tullus, Fituri et al. 1994).

6.1.2 Serum analyses

In papers and II, III and IV, serum analyses of CRP, ESR, creatinine, calcium, glucose, parathyroid hormone and phosphate were analysed using routine hospital methods. Serum cathelicidin in study IV was analysed with ELISA. An antibody-based chemiluminescence assay was used for total serum 25D₃ analyses.

6.2 MOUSE MODEL OF UTI

An experimental model of acute pyelonephritis in mouse was used in paper I and II. B6 NMRI outbred mice or C5BL/6 inbred mice were used between 8 and 10 weeks of age. To establish UTI, a thin catheter was introduced into the urethral meatus under general anaesthesia (Hung, Dodson et al. 2009) followed by inoculation of 50 µl of either *E. coli* CFT073 (10⁹ CFU/mL) or normal saline in control mice. In order to avoid immediate voiding, the meatus was obstructed for 6 hours with collodion. In paper I, IL-1β deficient mice on C5BL/6 background was used in addition to its wild type counterpart. It was created by gene targeting in embryonic stem cells to introduce an IL-1β null allele. The phenotype is healthy and fertile but resistant to fever (Zheng, Fletcher et al. 1995). At 0, 24, 48 hours or 6 days, mice were sacrificed and organs were aseptically collected for further analyses. Homogenized kidneys were used for bacterial count and protein measurement and whole kidneys were prepared for immunohistochemistry or in situ hybridization.

6.3 IN VITRO EXPERIMENTS

6.3.1 Bacteria

The prototypic uropathogenic *E. coli* CFT073 was used in paper I, II and IV. It was isolated from the urine and blood of a patient with pyelonephritis (Mobley, Green et al. 1990). CFT073 has been fully sequenced and encodes several fimbrial genes, including type 1, P, F1C, Dr, Auf, S fimbriae (Welch, Burland et al. 2002). It has been widely used to induce urinary tract infection in mice (Johnson, Russell et al. 1993; Mobley, Jarvis et al. 1993; Cirl, Wieser et al. 2008; Cirl, Wieser et al. 2008).

In paper III, uropathogenic *E. coli* CP9 were used. It is derived from a CNF1 positive human urosepsis isolate. CP9_{cnf1} carries an in-frame deletion mutation in the CNF1 gene and CP9_{lacZ} is the wild-type counterpart. In paper IV, a GFP-labelled CFT073 was used to detect CFT073 intracellularly (Mansson, Melican et al. 2007).

6.3.1.1 Pulse-field gel electrophoresis

Pulse-field gel electrophoresis (PFGE) is used for determining genetic similarity between different bacterial strains. In paper III, we used PFGE to determine if UTIs in children with repeated infections were caused by the same bacterial strain. Colonies from overnight cultures were washed in water and Tris-EDTA buffer. Chromosomal DNA was prepared in agarose plugs and digested with restriction enzymes. Gel electrophoresis was then performed and a photo was taken. Strains with the identical PFGE profile were defined as representing the same strain.

6.3.1.2 Studies on multinucleation and bacterial adhesion

In paper III, the presence of CNF1 in clinical strains was determined by investigating multinucleation in HeLa cells (Island, Cui et al. 1998). Sonicated bacterial strains were added to confluent HeLa cells for 24 hours. Cells were fixed, stained with Giemsa reagent and assessed under light microscope. For attachment assay in paper III, cells were pre-stimulated with sonicated bacteria and then infected with live CP9_{cnf1} or CP9_{lacZ} bacteria for 30 minutes. Cells were washed and then lysed with triton X-100 and plated on blood agar plates overnight for bacterial CFU counting.

6.3.2 Cells

Different urinary tract and monocytic cell lines as well as normal bladder and mesangial cells were used. T24 and 5637 cells are derived from patients with bladder cancer and A498 from renal cell cancer. THP-1 monocytic cells are leukaemia derived and HeLa cells are from a patient with cervical cancer. TERT-NHUC cell line is immortalized with human telomerase reverse transcriptase (hTERT). They resemble normal bladder epithelial cells to high degree (Chapman, Hurst et al. 2006). Normal human primary mesangial cells (NHMC) used in paper I and II and normal human primary bladder epithelial cells (HBEP) in paper IV were both from commercial vendors. 5637, A498, THP-1 and HeLa cells were grown in medium recommended from ATCC. TERT-NHUC and normal human bladder cells were grown in serum-free conditions. NHMC was cultured in NHMC medium (paper I) or RPMI supplemented with insulin-transferrin-selenium and FBS (paper I and II). TERT-NHUC requires a modified polystyrene surface for optimal growth and were therefore grown in BD Primaria treated flasks and multi-well plates.

For the vitamin D studies, all cell experiments were kept away from direct light since UV-light rapidly degrades vitamin D compounds (Lebwohl, Quijije et al. 2003). Furthermore, instead of normal FBS, charcoal-treated FBS was used to remove liposoluble hormones, which may interfere with the biochemical reactions (Cao, West et al. 2009).

6.3.2.1 Cell treatment and infections

In paper I and II, A498 and NHMC were grown to form a confluent layer while THP-1 cells in paper II were seeded 10^6 cells per well. Infections were carried out in serum-free medium with *E. coli* CFT073 in the presence of gentamycin 40 µg/mL to prevent bacterial overgrowth and decreased cell viability. Cells incubated with medium only served as controls. In paper III, T24 bladder cells and renal A498 cells were grown to confluency and then infected with CP9_{cnfl} or CP9_{lacZ}. Cell supernatants were assayed for IL-8 and TIMP-1. In paper IV, confluent T24, 5637, TERT-HNOC and HBEP cells were treated with 25D₃ or 1,25D₃ and in selected experiments infected with *E. coli* CFT073 10^7 .

6.3.2.2 Antibacterial assay

To test the antibacterial effect induced by vitamin D treatment of urinary bladder cells, T24, 5637 and TERT-NHUC cells were seeded in 6-well plates until nearly confluent. 1,25D₃ or vehicle (ethanol) was added for 48 hours in a thin layer of medium. Bacteria were then incubated in the conditioned medium for 90 minutes and the suspension was plated on blood agar plates for bacterial count.

6.3.2.3 Cell viability testing

For different treatment and infections, cell viability testing was done using trypan blue staining or the MTT method. Trypan blue staining was performed in cell suspension with trypan blue reagent and assessed under light microscope. Dead cells take up the trypan blue and are stained blue. Measurement with MTT method was performed after cell treatment or infection. Medium was aspirated and MTT solution was added. After incubation for one hour the MTT solution was removed and the cells were solubilised in DMSO. Absorbance at 540 nm was measured in plate reader (Mosmann 1983).

6.3.3 PCR

Total RNA extraction was performed using phenol–chloroform technique in paper I and II and with spin column-based technique in paper IV. cDNA was synthesized and polymerase chain reaction (PCR) was performed to multiply the targets of interest. In paper I, the traditional mode of PCR was used where the specific DNA is amplified after which it is separated in a gel and DNA is stained with ethidium bromide to confirm the amplicon size (Paper I). For paper IV, real-time PCR with fluorescent probes was used. In order to determine changes in mRNA due to specific stimuli, the sample was normalized to a housekeeping gene. We used GAPDH (Paper I and II) for conventional PCR and 18s (paper IV) for real-time PCR. Changes in mRNA expression were calculated by measuring light intensity in the gel (Paper I) or by the $2^{-\Delta\Delta CT}$ method for real-time PCR (Paper IV) (Schmittgen and Livak 2008).

6.3.4 ELISA

Urine levels of MMP-9, TIMP-1 and IL-8 as well as mouse MIP-2, MCP-1 and RANTES in kidney homogenates were measured using ELISA. MMP-9 in kidney homogenates was analyzed with a mouse MMP-9 activity assay. An antibody-based chemiluminescence assay was used for total serum 25D₃ analyses. Urinary creatinine levels were detected using Jaffés method and all human urine protein levels were expressed as protein/creatinine ratios.

6.3.5 Histopathology and immunohistochemistry

Histopathology was examined by staining sections of kidney tissue with haematoxylin and eosin. The inflammatory changes in mouse kidneys were summed following a semi-quantitative blinded scoring system. Immunohistochemistry for MMP-9 and TIMP-1 was performed in paper II. Following deparaffinization, rehydration, antigen retrieval and blocking, sections were incubated with primary anti-MMP-9 or anti-TIMP-1 antibodies. A goat anti-rabbit biotinylated secondary antibody was used and avidin/biotin complex was formed. Finally, nuclei were stained with DAB and sections were mounted in mounting medium.

In paper IV, urinary bladder biopsies were immediately placed in cell culture medium and infected with *E. coli* in a test tube for 90 minutes. After infection, biopsies were fixed for immunohistochemistry or processed for RNA extraction (see PCR section).

Fluorescence immunohistochemistry was used to detect cathelicidin, VDR, CYP27B1 and CYP24A1 in bladder tissue and in bladder cells in culture. Following deparaffinization, rehydration and antigen retrieval, blocking with serum and signal enhancer, primary anti-cathelicidin, anti-VDR, anti-CYP27B1 and anti-CYP24A1 were used with matching secondary AlexaFluor antibodies. Nuclei were stained with DAPI and sections were mounted in mounting medium. Cells cultured on cover slips were stained in a similar way except for antigen retrieval. Confocal microscopy was performed on a Leica SP5 system. Fluorescence intensity was quantified using the ImageJ image processing software (Abramoff, Magelhaes et al. 2004).

6.3.6 In situ hybridization

In situ hybridization was used to localize MIP-2, RANTES and MCP-1 mRNA in kidney sections of mice with acute pyelonephritis in paper II. Synthetic oligonucleotide probes were labelled using ³⁵S dATP. A mixture of two to three different probes was used and controls were obtained by parallel hybridization of adjacent sections with the ³⁵S-labelled sense probe for each chemokine. Emulsion autoradiography was performed and cells expressing more than 15 grains over the cytoplasm were counted by dark field microscopy at 100x magnification. Accuracy was checked at higher magnification and light microscopy. The tissue section areas

were measured by graded mesh and results were expressed as numbers of mRNA expressing cells per 100mm² tissue section.

6.3.7 Flow cytometry

Flow cytometry measures protein on a single cell level, thus making it possible to find out how a cell population is distributed with regards to the presence of the peptide or protein of interest. We used flow cytometry in paper IV to measure intracellular cathelicidin in vitamin D treated and untreated urinary bladder cells. Since they are adherent epithelial cells they were first trypsinized in low-concentration trypsin. Cells were simultaneously fixed and permeabilized and then labelled with a primary anti-cathelicidin monoclonal antibody and a secondary Alexa Fluor antibody. Cells were analysed on a Becton Dickinson FACScalibur™.

7 RESULTS

7.1 CHEMOKINE RESPONSE IN ACUTE PYELONEPHRITIS (PAPER I)

Pyelonephritis is a severe bacterial infection involving inflammation of the renal tissue, which may lead to renal scarring if not treated promptly. Inflammatory mediators such as cytokines and chemokines elicited upon bacterial infection are thought to induce the transition from acute infection to renal scarring. In the first paper, we studied the chemokine response in *E. coli*-induced pyelonephritis in normal and IL-1 β -deficient mice. Overall inflammatory changes were more pronounced and more often found in IL-1 β deficient mice as compared to wild-type mice. In addition, they were more likely to have extensive destructive inflammation with large numbers of neutrophils and renal cortical abscesses. It is well known that IL-8 is important for the pathogenesis of UTI but we show here for the first time that RANTES and MCP-1 are also produced. This was seen both in mice with pyelonephritis and in human kidney cells infected with the same uropathogenic *E. coli* strain. IL-1 β deficient mice produced more MIP-2 upon infection compared to wild-type mice. In summary, in addition to IL-8/MIP-2, the CC chemokines MCP-1 and RANTES are produced during experimental pyelonephritis and IL-1 β deficiency promotes renal inflammation after pyelonephritis.

7.2 MMP-1 AND TIMP-1 IN ACUTE PYELONEPHRITIS (PAPER II)

For chemokines to be activated and for neutrophils to infiltrate the area of infection, the proteolytic activities of matrix metalloproteinases (MMPs) are important (Page-McCaw, Ewald et al. 2007). In paper II, we sought to elucidate the role of MMP-9 and its inhibitor TIMP-1 in pyelonephritis and renal scarring in children and in an experimental mouse model of pyelonephritis. In children with acute pyelonephritis, urine levels of MMP-9 and TIMP-1 were significantly higher compared to 6-week follow-ups and to samples from children with non-renal fever. In accordance, TIMP-1 was expressed in response to *E. coli* infection in human renal cells. We further demonstrated that TIMP-1 levels in children correlated to renal scarring as demonstrated by DMSA scan. In children with urinary TIMP-1 levels higher than MMP-9, more severe changes were observed in acute and follow-up DMSA scan. MMP-9 seemed to be produced mainly in leukocytes whereas TIMP-1 was seen both in leukocytes and in renal epithelial cells. Thus, MMP-9 and TIMP-1 are produced as a result of ascending *E. coli* infection in the urinary tract. TIMP-1 rather than MMP-9 seems to be involved in the process leading to renal scarring following pyelonephritis in children and may therefore be used as a marker for risk of developing scarring after acute pyelonephritis.

7.3 CNF1 AND ITS IMPORTANCE IN URINARY TRACT INFECTION (PAPER III)

The host response to *E. coli* pyelonephritis was studied in papers I and II and implied the role of chemokines, cytokines and the MMP inhibitor TIMP-1. In the third paper, we wanted to describe the contribution of bacterial virulence factors to the immune response and persistence in the urinary tract. While *E. coli* P fimbriae have been shown to induce inflammation in bladder and kidney (Wullt 2003), the role of bacterial toxins is not yet clear. Because of contradictory results regarding the effect of the toxin CNF1 in UTI mouse models, we decided to take a more clinical approach. We hypothesised that patients infected with CNF1-positive strains would have higher inflammatory markers in the urine and that CNF1-positive strains would be overrepresented in children with recurrent UTIs as a result of increased persistence in the urinary tract. By infecting uroepithelial and kidney cells with CNF1-negative or CNF1-positive bacteria, we could investigate the effect on adhesion and invasion into cells. No difference was found when only live bacteria were used. When the cells were pre-treated with sonicated bacteria and then infected with live bacteria, CNF1-positive bacteria attached more efficiently than CNF1 negative bacteria. Since persistence in the urinary tract is an important feature of the pathogenesis of UTI, we then correlated CNF1 expression to the likelihood of causing recurrent UTI. However, CNF1 frequency did not differ between children with sporadic or recurrent UTI. Although CNF1-expressing *E. coli* and purified CNF1 toxin increased IL-8 in uroepithelial cells and monocytes, children and adults with UTI showed no correlation between CNF1 expression and IL-8 levels.

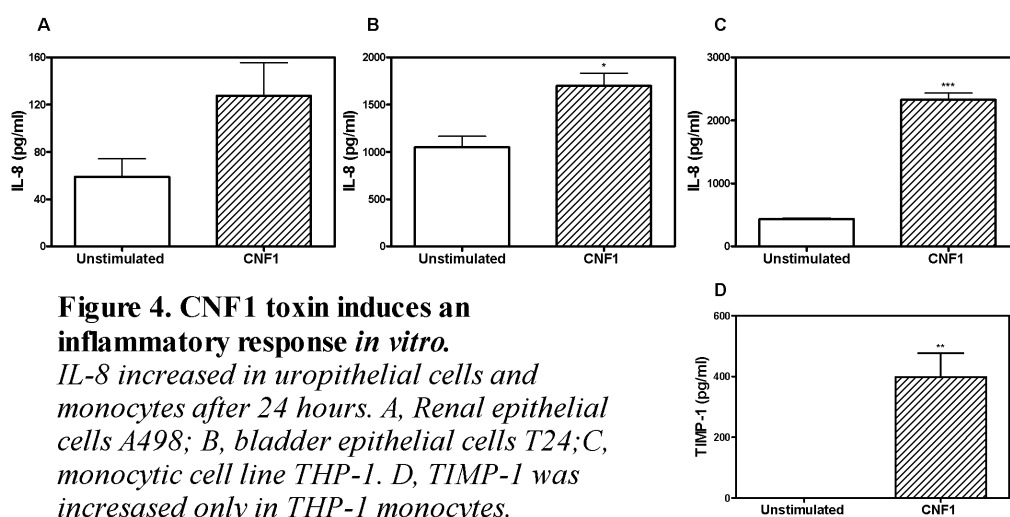


Figure 4. CNF1 toxin induces an inflammatory response *in vitro*. *IL-8 increased in uroepithelial cells and monocytes after 24 hours. A, Renal epithelial cells A498; B, bladder epithelial cells T24; C, monocytic cell line THP-1. D, TIMP-1 was increased only in THP-1 monocytes.*

Since TIMP-1 levels correlated to renal scarring in our previous study, we investigated the effect of CNF1 on TIMP-1 expression as well. A rapid increase was observed in kidney epithelial cells, but not in bladder cells. Our results suggest that although CNF1 induces IL-8 and TIMP-1 in uroepithelial and renal epithelial cells in culture, it seems more likely that other virulence factors contribute more to inflammation and persistence in the urinary tract *in vivo*.

7.4 VITAMIN D INDUCTION OF CATHELICIDIN IN THE HUMAN URINARY BLADDER (PAPER IV)

After studying the inflammatory response in acute pyelonephritis we were interested in finding out how to increase endogenous defence mechanisms in the urinary tract. Our group previously showed that the antimicrobial peptide cathelicidin protects the urinary tract from infection (Chromek, Slamova et al. 2006). Since it was also shown that vitamin D could induce cathelicidin in monocytes (Liu, Stenger et al. 2006), we investigated if vitamin D would have a similar effect in the urinary bladder. Postmenopausal women were supplemented with cholecalciferol (vitamin D₃) for 12 weeks. Serum-25D₃ increased by 41% without affecting calcium, phosphate, parathyroid hormone or serum cathelicidin. Before and after the three months of vitamin D₃ supplementation, biopsies were taken from the urinary bladder mucosa. Cathelicidin increased significantly in *E. coli* infected biopsies after the supplementation period compared to before. In addition, several different bladder cell lines and primary bladder cell were treated with vitamin D. Induction of cathelicidin was seen on both mRNA and protein level.

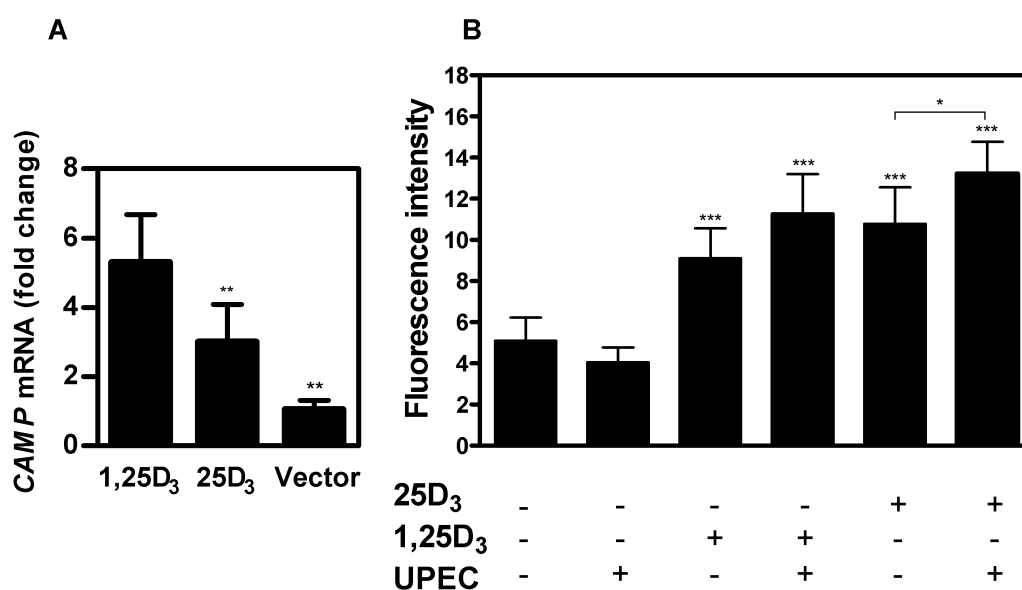


Figure 5. Vitamin D induction of cathelicidin.

Bladder epithelial cells were treated with 1,25D₃ or 25D₃. Gene expression of CAMP (A) as well as cathelicidin peptide (B) increased. Uropathogenic *E. coli* (UPEC) increased the cathelicidin when combined with vitamin D treatment.

To be functional, 25D₃ needs to be converted to 1,25D₃. We showed that the converting enzyme, CYP27B1 is present and active in the urinary bladder. Increased cathelicidin was seen on mRNA and protein levels after 25D₃ or 1,25D₃ treatment and with additional increase if the cells were infected with *E. coli*.

We also show that uroepithelial cells facilitate intracellular killing of *E. coli* and that bacteria and cathelicidin co-localised in the bladder cells. With these findings, we further investigated the functional role of vitamin D-induced cathelicidin in urinary tract cells. After treating uroepithelial cells with vitamin D or vector only, we incubated the conditioned medium with *E. coli*. Significantly more bacteria recovered from the vector-treated medium compared to the vitamin D treated. The result was similar when we lysed the cells to evaluate intracellular antibacterial properties. Finally, a neutralizing antibody against cathelicidin blocked the antibacterial effect in vitamin D treated medium (Figure 5).

These results show that the bladder is equipped with the enzymes and receptors necessary for vitamin D activation and that vitamin D treatment increases the antibacterial effect by induction of the antimicrobial peptide cathelicidin.

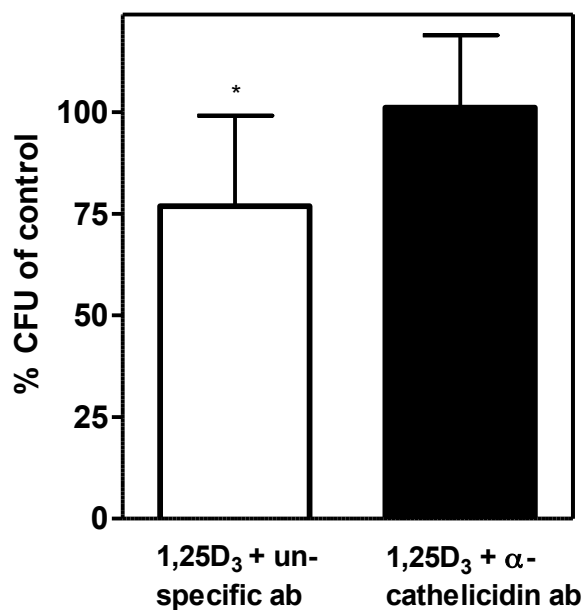


Figure 6. Antibacterial effect of vitamin D treated bladder cells.

Bladder epithelial cells (TERT-NHUC) were treated with 1,25D₃. An anti-cathelicidin neutralizing antibody abrogated the antibacterial effect of the resulting conditioned medium against uropathogenic E. coli.

8 DISCUSSION

Urinary tract infection remains one of the most common bacterial infections despite widely accessible diagnostic aids, antibiotics and increased knowledge about its pathogenesis. The first line of defence in the lower urinary tract will in most cases succeed in preventing bacterial attachment and establishment. However, sometimes, and more often in certain risk groups, bacteria will establish themselves in the urinary tract to cause infection. Less commonly, bacteria spread to the upper urinary tract causing pyelonephritis. Children and women with recurrent UTI are often prescribed antibiotics repeatedly or as prophylaxis for longer periods of time. The problem of bacterial resistance to antibiotics used in UTI is increasing at high speed and new treatment strategies are therefore needed. In order to be able to therapeutically intervene in the host-pathogen interaction in the urinary tract, better understanding of the pathophysiology is needed. In this thesis we studied the immune response in human UTI as well as in a mouse model and cell lines. We describe new findings about chemokine production and proteinase release in pyelonephritis *in vitro* as well as *in vivo*. We also present a new way of increasing the endogenous antimicrobial defence in the urinary bladder.

In paper I, we investigated the role of IL-1 β in the pathogenesis of pyelonephritis and renal scarring as well as the expression of the CXC chemokine IL-8 and the CC chemokines MCP-1 and RANTES. Since IL-1 β genotype influences the severity of meningococcal disease (Read, Camp et al. 2000) and IL-1 β deficiency is associated with delayed clearance of Chlamydia in salpingitis in mice (Prantner, Darville et al. 2009), we were interested in its role in acute pyelonephritis. Accordingly, the histological kidney changes suggested increased inflammation and renal scarring in the IL-1 β -deficient mice. However, the total bacterial count in the kidney did not differ in the IL-1 β -deficient and wild type mice, implying that the histological differences rather reflect an altered immune response to bacteria. Indeed, the higher MIP-2 levels found in IL-1 β -deficient mice support this. *In vitro* on the other hand, IL-1 β treatment led to increased cytokines in renal cells, something that is supported by a recent gene array study (Thorburn, Kolesar et al. 2009). This discrepancy in the animal model and in cell culture is probably a result of complex cross talk between different cells *in vivo*.

The high MIP-2 levels found in IL-1 β -deficient mice may provide an explanation for the increased renal scarring seen in IL-1 β -deficient mice. With more neutrophils infiltrating the tissue more cytotoxic compounds are subsequently released. In fact, neutralizing MIP-2 has successfully decreased neutrophil influx and tissue damage in haemolytic uremic syndrome (Roche, Keepers et al. 2007) as well as renal ischemia/reperfusion injury (Miura, Fu et al. 2001). In pyelonephritis on the other hand, MIP-2 seems to have dual functions. Initially, it is responsible for attracting neutrophils from the circulation; next, MIP-2 directs neutrophils across the epithelium and into the urine. At this latter stage, blocking MIP-2 prevents this transmigration and leads to entrapment of neutrophils in the sub-epithelial tissue (Hang, Haraoka et al. 1999).

With the extensive histo-pathological changes seen in the IL-1 β -deficient mice, they are more likely to have a systemic inflammation. Recently, it was demonstrated that systemic IL-1 β can induce endogenous cortisol increase (Tkachenko, Jaaskelainen et al. 2011; Vukelic, Stojadinovic et al. 2011) and in LPS-challenged mice, serum cortisol elevation was impaired in IL-1 α / IL-1 β -deficient mice (Oguri, Motegi et al. 2002). IL-1 β -deficiency in our study may therefore predispose to increased proinflammatory signalling through impaired cortisol production. Children with acute pyelonephritis treated with cortisone have earlier normalization of DMSA scan abnormalities (Huang, Chen et al. 2011), highlighting the importance of the cortisol system in renal inflammation.

In comparison to IL-8, not much attention has been given to the CC chemokines in UTI. Since MCP-1 and RANTES have both been associated with the development of interstitial fibrosis in kidneys (Lloyd, Minto et al. 1997; Schneider, Panzer et al. 1999) they are also of interest to study in post-infectious renal scarring. Bacteria and IL-1 β induced IL-8/MIP-2, RANTES and MCP-1 *in vitro* as well as *in vivo*. However, in IL-1 β -deficient mice where profound MIP-2 accumulation was observed, there was no RANTES and MCP-1 increase. These findings point toward individual differences in activation of different chemokines, something that may prove important in attempts to modulate their actions. In parallel with our study, Tsuboi *et al* found similar results in mouse renal epithelial cells treated with LPS (Tsuboi, Yoshikai et al. 2002). Interestingly, they showed that the RANTES increase, unlike MCP-1, was dependent on *de novo* protein synthesis, which could explain the delay we saw in RANTES induction after treatment with *E. coli* or IL-1 β . Unlike Tsuboi *et al*, who studied LPS-stimulated mouse renal cells, we used whole uropathogenic *E. coli* to mimic the natural situation when infecting human cells and mice. These differences aside, distinct concordance is found in the two studies, emphasizing the similarities of activation of cells of different origin in response to bacteria or bacterial products.

For chemokines to be activated and to recruit neutrophils, and for neutrophils to travel through tissue to infiltrate the area of infection, the matrix metalloproteinases are important (Page-McCaw, Ewald et al. 2007). In paper II, we found that MMP-9 and TIMP-1 increased in the urine of children with UTI and we localised the production to infiltrating leucocytes and renal epithelial cells by using our mouse model of UTI. Interestingly, in children, elevated TIMP-1 but not MMP-9, was associated with renal scarring. This suggests that TIMP-1 rather than MMP-9 contributes to the pathogenesis of renal scarring following acute pyelonephritis. These findings were further supported in later studies demonstrating that TIMP-1 may cause entrapment of neutrophils and destruction of renal parenchyma (Chromek, Tullus et al. 2004).

It is also well known that MMP-9 can activate important mediators in the early innate immune cascade. Hence, inhibiting MMP-9 may affect this process and thereby accelerating the infectious process. Following our study, several others have showed increased MMP-9 and TIMP-1 in bacterial infection such as meningitis (Tsai, Shi et al.

2011), septicaemia (Lauhio, Hastbacka et al. 2011) and in *S. pneumoniae* infection in the lung (Hong, Greenlee et al. 2011). In the later study, MMP-9 was found to be important in the early defence against bacteria by cleavage of IL-17, which is required to avoid excessive accumulation of neutrophils in the lung. MMP-9 also increased reactive oxygen mediated bacterial killing by neutrophils. Furthermore, MMP-9 can activate TGF- β , which seems to be protective against post-infectious renal scarring (Farmaki, Papachristou et al. 2005) but also to have pro-fibrotic properties in obstructive kidney diseases (Duymelinck, Dauwe et al. 2000). TIMP-1, which we could correlate to renal scarring, has been shown to be down-regulated by IL-1 β in renal proximal tubular cells (Nee, McMorro et al. 2004). Hence, in mice lacking IL-1 β , one would expect TIMP-1 levels to be higher, leading to an increased risk of renal scarring.

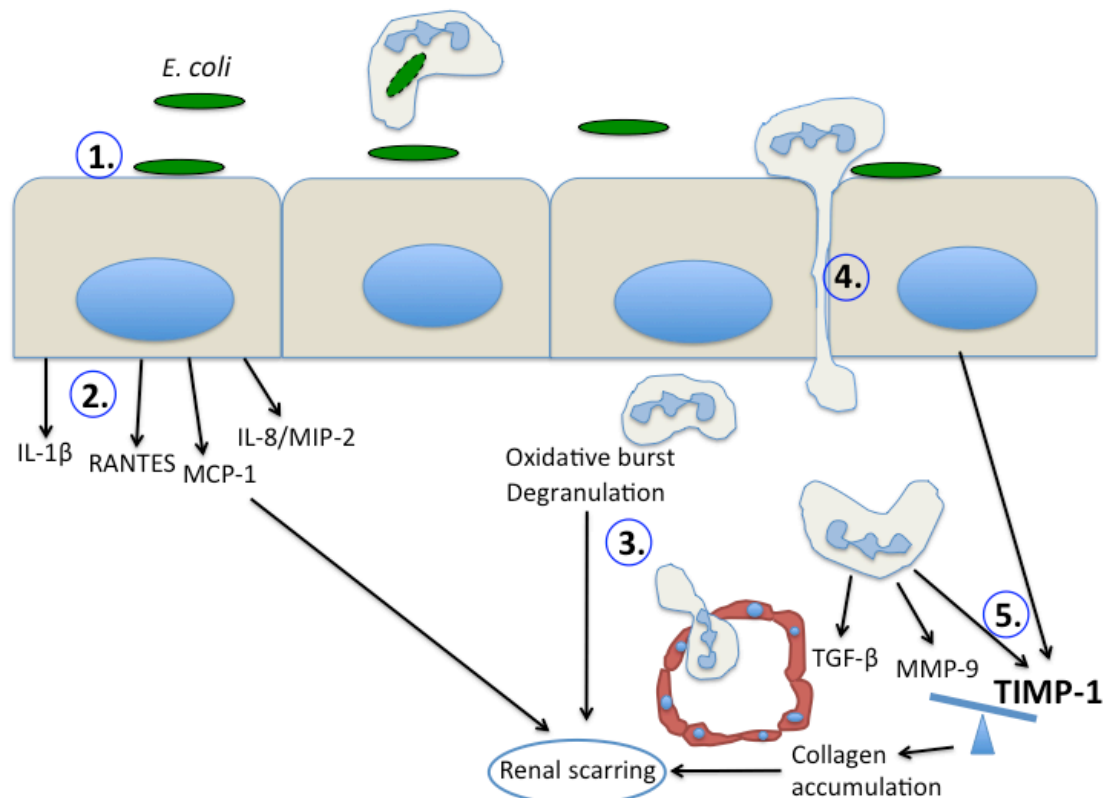


Figure 7. Pathogenesis of renal scarring.

E. coli (green) attach to the epithelial cell (1) resulting in cytokine and chemokine secretion (2). The chemotactic gradient of MIP-2 directs the neutrophils from the circulation (3) into the tissue where an excess of reactive oxygen species are formed (oxidative burst) and cytotoxic granular products released (degranulation). Neutrophils further transverse the epithelium into the urine (4). An increased production of TIMP-1 leads to imbalance between MMP-9 and TIMP-1, which contributes to fibrosis development as a result of increased collagen accumulation (5).

In studies I and II, we examined the tissue inflammation in acute pyelonephritis and renal scarring. Renal scarring is dependent on the degree of inflammation as found in many studies where high acute-phase protein and cytokines levels predict renal scarring

(Tullus, Fituri et al. 1994; Bressan, Andreola et al. 2009). Since TIMP-1 correlated with renal scarring, we wanted to study if specific bacterial factors contributed to the initial inflammation.

P-fimbriae and type-1 fimbriae are well-studied bacterial factors in the uropathogenic *E. coli*. P-fimbriae initiate inflammation in the urinary tract (Linder, Engberg et al. 1991; Bergsten, Samuelsson et al. 2004). Although type-1 fimbriae facilitate attachment (Zhou, Mo et al. 2001), invasion (Martinez, Mulvey et al. 2000) and the formation of intracellular bacterial communities (Wright, Seed et al. 2007) in the urothelium, inflammation in the bladder *in vivo* has not been demonstrated (Bergsten, Wullt et al. 2007). This appears to be logical since half of ABU strains express type-1 fimbriae (Roos, Nielsen et al. 2006) and should by definition not elicit inflammation.

Bacterial toxins like hemolysin and CNF1 and their role in UTI are less well understood. Although hemolysin was shown to induce calcium oscillation-induced cytokine release *in vitro* (Uhlen, Laestadius et al. 2000), it was not associated with acute inflammation in UTI (Connell, de Man et al. 1993). CNF1 seems to be important for UTI pathogenesis since it is expressed in a majority of UTI strains (Brauner, Katouli et al. 1990). We demonstrate that CNF1 promotes bacterial attachment and invasion to uroepithelial cells and that it can induce an inflammatory response with IL-8 and TIMP-1 production. However, as we studied the urine of children and adults with UTI, no correlation between CNF1 expression and inflammatory response or recurrent infections were found. Thus, although CNF1 contributes *in vitro*, it seems to be less important *in vivo*.

Previous studies in mice have shown conflicting results regarding immune response to CNF1 in the urinary tract. Johnson and colleagues demonstrated that CNF1 does not contribute to colonization, persistence or inflammation in the murine urinary tract (Johnson, Drachenberg et al. 2000) or damage to human bladder cells *in vitro* (Island, Cui et al. 1998). Others on the contrary, found that CNF1-positive strains caused deeper infection in murine UTI (Rippere-Lampe, O'Brien et al. 2001) and increased cytotoxicity in bladder cells (Mills, Meysick et al. 2000). Real et al. used a similar, clinical approach in a study parallel to ours. They showed that patients with haemorrhagic UTI more often were infected with *E. coli* carrying the *cnf1* gene and that MCP-1 and IL-8 were significantly higher in the urine of patients with *cnf1* gene positive *E. coli* (Real, Munro et al. 2007). The main differences compared to our study are that we made a phenotypic rather than genotypic estimate of CNF1 expression and that we included the outcome of recurrences as a measurement of persistence in the urinary tract.

One reason for CNF1 not to be associated with more inflammation or recurrent UTIs in our study could be that CNF1 also induces apoptosis (Mills, Meysick et al. 2000). Apoptosis in the bladder leads to shedding of the superficial uroepithelial cells, thus clearance of bacteria attached and invaded into the shedded cells. This is a beneficial host response although there is a risk that shedding will expose underlying cells to

infection. In the kidneys, the single-layered epithelium is vulnerable to bacterial toxins and apoptosis is not a protective response. Therefore, if the bacteria reach the kidneys, CNF1 probably induce kidney tissue damage. Another reason could be the stronger IL-8 response with CNF1 leading to efficient clearance of the bacteria by infiltrating neutrophils. If the host fails to induce IL-8, or the mouse equivalent MIP-2, the bacterial burden increases (Haraoka, Hang et al. 1999).

There is still uncertainty to what extent CNF1 contributes to the immune response in UTI *in vivo*. We were not able to show any adverse consequences correlated to *E. coli* equipped with CNF1. There are problems however when interpreting studies like this. Since the *cnf1* gene almost always is co-expressed with *hly* it is difficult to evaluate the impact of *cnf1* versus *hly*. An *E. coli* knockout strain specifically lacking *cnf1* is therefore a very useful tool. We and others have used this approach with conflicting results as discussed above. The strengths in our study are the clinical outcome and that both live bacteria and purified CNF1 toxin were used.

Although UTIs are common, it is surprising how well the urinary tract can withstand bacterial infection taking into account the anatomical location. It is also well known that bacteria frequently enter the urinary tract without causing symptoms of UTI. Several factors are involved in keeping the urine free from bacteria or preventing the bacteria from invading the uroepithelium. Apart from the mechanical forces of the urine flow and the neutralizing secreted proteins, antimicrobial peptides are produced both in the kidney and in the bladder. Human beta defensin-1 is constitutively produced mainly in the renal epithelial cells and cathelicidin in the kidney and in the bladder. Both possess antimicrobial activity against *E. coli* (Valore, Park et al. 1998; Chromek, Slamova et al. 2006; Hertting, Holm et al. 2010). Cathelicidin seems to be produced in low concentrations constitutively but is rapidly released as bacteria come in contact with the epithelium (Chromek, Slamova et al. 2006).

Vitamin D induces cathelicidin in skin (Peric, Koglin et al. 2009), respiratory epithelium (Yim, Dhawan et al. 2007), white blood cells (Wang, Nestel et al. 2004) and placenta (Liu, Kaplan et al. 2009) and in study IV we show for the first time production in the urinary bladder. Postmenopausal women were treated with vitamin D for 12 weeks. Cathelicidin expression increased in *E. coli*-infected bladder biopsies taken after vitamin D supplementation. Vitamin D alone was not enough to cause cathelicidin increase in the biopsies. This is in contrast to what we found in urinary bladder cells in culture and may merely reflect differences in intracellular vitamin D levels *in vivo* compared to *in vitro*. Prolonged elevated levels of cathelicidin in the urinary bladder may cause inflammation (Oottamasathien, Jia et al. 2011). Therefore, activation of cathelicidin only when bacteria are present may be beneficial to the host.

Both 25D₃ and 1,25D₃ increased cathelicidin in cultured cells. Similarly, respiratory epithelial cells reacted with increased cathelicidin production after 25D₃ and 1,25D₃ treatment (Hansdottir, Monick et al. 2008). Monocytes however, seem not to induce cathelicidin in response to 25D₃ unless also treated with a TLR ligand (Liu, Stenger et

al. 2006). $1,25D_3$ on the other hand, activated cathelicidin also in the absence of additional stimuli. $25D_3$ is inactive unless converted to $1,25D_3$, which means that the difference between monocytes and epithelial cells seems to be the conversion of $25D_3$ to $1,25D_3$. We found that the converting enzyme CYP27B1 is present in the human bladder and that it functionally converts $25D_3$ to $1,25D_3$. This is important since it means that the production of active vitamin D can occur locally and that this locally converted vitamin D leads to production of cathelicidin.

With these findings, we have introduced a potentially new concept for the treatment of UTIs. By supplementing with vitamin D, the preparedness of the epithelium for bacterial invasion is increased through the production of cathelicidin. Indeed, vitamin D status influences the risk of obtaining infectious diseases. People with low levels of vitamin D run increased risk of acquiring tuberculosis (Gibney, MacGregor et al. 2008; Hansdottir, Monick et al. 2008; Williams, Williams et al. 2008) and cord blood vitamin D deficiency is associated with respiratory syncytial virus bronchiolitis in the first year of life (Belderbos, Houben et al. 2011). In both of these infections, at least *in vitro*, cathelicidin has been shown to be of importance (Hansdottir, Monick et al. 2008; Sonawane, Santos et al. 2011). Although it remains to be shown whether vitamin D supplementation can prevent UTI in clinical studies, we here show that vitamin D functionally upregulates cathelicidin in the urinary bladder.

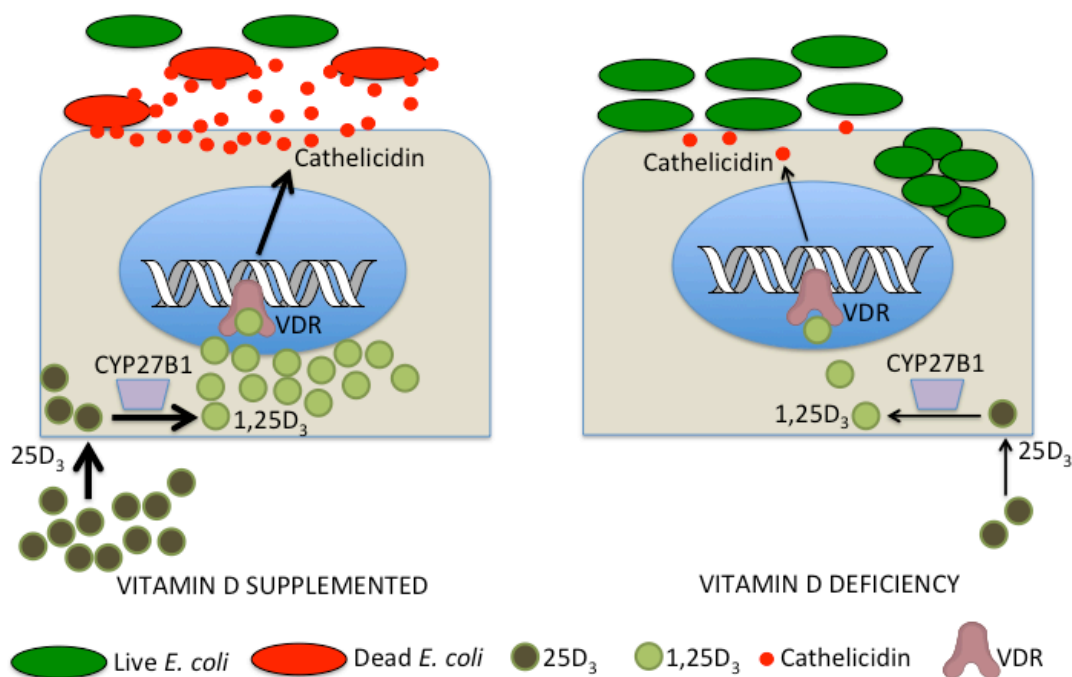


Figure 8. Schematic figure of the vitamin D supplemented (left) and vitamin D deficient (right) state in the urinary bladder.

*Insufficient vitamin D levels lead to lower substrate for the converting enzyme CYP27B1. In turn, less of the active $1,25D_3$ is produced and less cathelicidin can be produced. This may lead to increased susceptibility to *E. coli* infection.*

In postmenopausal women, the conversion of 25D₃ to 1,25 D₃ depends on the substrate concentration (Rejnmark, Vestergaard et al. 2008), which emphasizes the need for sufficient levels of serum 25D₃. Skin exposure to UVB light is by far the most efficient way of increasing serum levels and compared with ingested vitamin D, skin derived vitamin D may last at least twice as long in the blood (Haddad, Matsuoka et al. 1993). Lowest serum 25D₃ are found in the end of winter worldwide (Holick 2011) but with the limited amount of sunlight at the northern latitude, there is an increased risk of vitamin D deficiency here. Under these circumstances, it is important with a sufficient daily intake of vitamin D. However, food sources of vitamin D are limited (Holick 2006). Fatty fish such as salmon, tuna and mackerel are exceptions and smaller amounts of vitamin D levels are also found in egg yolk and cheese. Therefore, supplementation of dairy products and infant formula with vitamin D is a common practice. However, recent recommendations from leading researchers in the field suggest levels far higher than what could be achieved from the diet alone. This year, the Institute of Medicine of the National Academies, USA increased the recommended daily intake of vitamin D to 600 IU (Ross, Manson et al. 2011), which reflects the growing belief that higher daily doses than previously recommended are needed. Recent data from the United States suggest that 90% of women older than 70 years did not meet the recommended daily intake of vitamin D (Ross 2011) . The Swedish National Food Agency recommends 400 IU for children under 2 years and 300 IU for children above two years and for adults (Becker W 2005) and a tolerable upper intake level of 2000 IU per day.

Table 2. Recommended dietary allowance of vitamin D from American and Swedish guidelines

<i>Age (years)</i>	<i>IOM 1997 (IU/day)</i>	<i>IOM 2011 (IU/day)</i>	<i>SWE (IU/day)</i>
0-1		400	400*
1-50	200	600	300
50-70	400	600	300
>70	600	800	300
Pregnancy & Lactation		600	400

IOM = Institute of Medicine of National Academies (USA)

SWE=Swedish recommendations. *0-2 years.

25D₃ can be given in high doses without adverse events as seen in a recent study where pregnant and nursing women were given up to 6000 IU daily (Hollis, Johnson et al. 2011) and as expected, with 2000 IU daily we found no effect on calcium or cathelicidin in serum. 1,25D₃ however, should be used with caution due to the risk of hypercalcaemia. It is mainly used as a therapeutic agent for treating vitamin D deficiency if the converting activity is severely compromised as in chronic kidney disease (Zehnder, Quinkler et al. 2008). Because of its calcaemic properties,

structurally modified analogues have been developed. Although these have been subjected to trials in patients with chronic kidney disease, no clinical trials on antimicrobial peptide induction have been published. Our data support that increasing serum vitamin D levels also increases the antimicrobial properties of the urinary tract. Vitamin D insufficiency should be considered in patients with recurrent urinary tract infections and, if needed, appropriate supplementation should be started.

Taking advantage of the immune response both for diagnostic and therapeutic purposes is one of the great challenges for researchers in the field of infectious diseases. In the future, diagnostic tools may include a number of pathogen and host response markers, which may aid the clinician to the right diagnosis and to find the patients at risk of severe disease or renal scarring. In this thesis, TIMP-1 is proposed as a marker for renal scarring and perhaps also to distinguish upper from lower UTI, a difficult but important task especially in young children (Pecile, Miorin et al. 2004). Larger clinical studies to evaluate TIMP-1 and other inflammatory markers in UTI could prove important.

A major obstacle for scientists today is the increasingly problematic situation with bacterial resistance against conventional antibiotics. As indicated by the WHO and because UTI is such a common infection, this may very well constitute an impending public health crisis. New antibiotics and altogether new treatment strategies are therefore needed. Although the number of antibiotics in clinical trials is now increasing for the first time in 30 years, the pipeline of antibiotics with entirely new mechanisms of action is still close to empty (Payne, Gwynn et al. 2007). Therefore, antimicrobial peptides constitute a promising new treatment group and little resistance has been observed. However, the nature of these peptides makes them difficult to administer and expensive to synthesize. There are also adverse effects of AMPs reported like bladder inflammation (Oottamasathien, Jia et al. 2011) and tumour progression (Coffelt, Marini et al. 2009). Based on the findings in this thesis, we believe that therapeutic methods to induce endogenous AMPs are the most promising way forward. Patients suffering from recurrent urinary tract infections are especially well suited for clinical trials of vitamin D treatment.

We also show intracellular killing taking place in the bladder mucosa. This is important since UPECs enter the urothelial cells to form intracellular bacterial communities as a strategy to escape the immune response and antibiotics. This has entirely changed the old concept of uropathogenic *E. coli* being an obligate extracellular pathogen and therefore, new pharmacological treatment must have the capacity of killing bacteria also intracellularly. Upon vitamin D treatment of bladder epithelial cells *in vitro*, we showed that the antibacterial effect increased both extracellularly and intracellularly, which may indicate that vitamin D is also efficient against the intracellular niche of bacteria. Randomized, double-blind, placebo-controlled studies are of importance in order to evaluate the role of vitamin D in UTI and to clinically evaluate the findings presented here.

9 FUTURE PERSPECTIVES

Modern medicine faces enormous challenges with an increasing population and more expensive and advanced treatments. Therefore, the future of medicine is dependent on finding individuals at risk so that disease can be avoided. Individual assessment is also important because it enables treatment to be adjusted around the specific set of biological characteristics for that patient. Apart from the potential clinical benefit of our findings already discussed, future perspectives include increased awareness of genetic predisposition to UTI and tailored treatment. Genetic variation in genes that regulate the innate immune response in UTI may influence the susceptibility to infection. In the activation of vitamin D, the converting enzyme CYP27B1, the 1,25D₃ catabolic enzyme CYP24A1 and the VDR have been associated with gene polymorphism in different studies ranging from diabetes, cancer, neurological disorders but also infectious diseases. It would be of great interest to study alterations in the genetic vitamin D metabolism pathways of patients with recurrent UTIs. This would provide information that could help us identify individuals at risk of recurrent or severe UTI but also to provide these patients with prompt and correct evaluation and treatment.

10 CONCLUSION

From the results presented in this thesis the following specific conclusions were drawn:

- I. The chemokines MIP-2, MCP-1 and RANTES are expressed in a mouse model of acute *E. coli* pyelonephritis. IL-1 β deficient mice showed an increased MIP-2 production and more severe renal scarring than wild type mice.
- II. MMP-9 and TIMP-1 are produced in acute *E. coli* pyelonephritis in children. TIMP-1 correlates with renal scarring following acute pyelonephritis.
- III. *In vitro*, CNF1 contributes to the inflammatory response in urinary bladder cells. *In vivo* however, the presence of CNF1 seems to be of subordinate importance.
- IV. Vitamin D induces the expression of cathelicidin in *E. coli* infected urinary bladder biopsies of postmenopausal women and in human urinary bladder cells, which has an *E. coli*-cidal effect.

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