Urinary tract infections in pregnancy - studies in vivo and in vitro

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By

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LIST OF PUBLICATIONS

I.   Uropathogenic Escherichia coli isolates from pregnant women in different countries.
        Ramos NL*, Sekikubo M*, Dzung DT, Kosnopfel C, Kironde F, Mirembe F, Brauner A.
        J Clin Microbiol. 2012 Nov; 50(11):3569-74.
        * Shared first authors

II.   Antibiotic overconsumption in pregnant women with urinary tract symptoms in Uganda

        Sekikubo M, Hedman K, Brandkvist C, Mirembe F, and Brauner A

        Manuscript

III.   The impact of vitamin D on the innate immune response to uropathogenic Escherichia
        coli during pregnancy.
        Ramos NL*, Sekikubo M*, Kironde F, Mirembe F, Sääf M, Brauner A.
        Clin Microbiol Infect. 2015 May; 21(5):482.e1-7.
        * Shared first authors

IV.   Labisia pumila var. alata reduces bacterial load by inducing uroepithelial cell apoptosis.
        Fazliana M, Ramos NL, Lüthje P, Sekikubo M, Holm A, Wan Nazaimoon WM, Brauner
        A.
        J Ethnopharmacol. 2011 Jun 14; 136(1):111-6.

Dedicated to my wife – Tahiya – who I would have described in superlatives but she detests them.

ABSTRACT

The risk of urinary tract infection (UTI) in pregnant women is increased and predisposes them to acute pyelonephritis together with poor pregnancy outcomes. Increased frequency of micturition, nocturia and lower abdominal discomfort are common non-specific complaints during pregnancy, which render clinical diagnosis of UTI inaccurate. To prevent undesirable effects on the growing fetus, antimicrobial agents used in management of UTI during pregnancy should be carefully selected. In this thesis, we investigated virulence determinants and susceptibility of *E. coli* to antimicrobial agents and evaluated the possibility for medical staff - with limited training in microbiology - to evaluate easy-to-use tests for the diagnoses of UTI. The easy-to-use tests included nitrite, leucocyte esterase, urine microscopy and dipslide. Further, we evaluated the impact of vitamin D on innate immunity during pregnancy as well as the *in vitro* effects of Labisia pumila var. alata - a medicinal herb - on *E. coli* infected uroepithelial cells.

E. coli resistance to antimicrobial agents differs by region and is influenced to some extent by virulence characteristics of the bacteria. We hypothesized that antibiotic resistance and virulence characteristics of *E. coli* differ depending on the use of antibiotics. We report significantly higher prevalence of antibiotic resistance among isolates from Uganda and Vietnam compared to those from Sweden. Presence of the *flu* gene was associated with increased risk of antibiotic resistance.

The high prevalence of antibiotic resistance seen in Uganda is partly due to limited access to microbiology services. In comparison to urine culture, 96% of pregnant women diagnosed based on symptoms mimicking those of urinary tract infection freely received unnecessary antibiotics. We therefore evaluated nitrite, leucocyte esterase, leucocyturia and dipslide in outpatient settings as a way of reducing antibiotic misuse. We demonstrated high specificity, but low sensitivity of the combined nitrite and leucocyte esterase tests. There was poor correlation between leucocyte esterase and leucocyturia as analyzed by microscopy. Nurses and gynaecologist with limited knowledge of microbiology correctly diagnosed *E. coli* with a simplified culture method – the dipslide test.

During pregnancy, vitamin D is required for development of the fetal skeleton, but it is also important for innate immunity. The major source of vitamin D is sunlight. Although Uganda has high sun exposure the whole year, persons with dark pigmented skin may not be able to optimally synthesize vitamin D. We found vitamin D as well as the antimicrobial peptide LL-37 - that is induced by vitamin D, increased with advancing gestational age. There were significantly higher levels in the third compared to the first trimester. Furthermore, serum had increased bactericidal activity with increasing vitamin D levels. Conversely, IL-8 decreased with advancing gestational age. In *vitro*, vitamin D decreased expression of IL-8 in a dose-dependent manner potentially modifying the inflammatory response to infection.

The breakaway resistance to antibiotics recommended in management of UTI calls for alternative intervention that could be used alone or in combination with antibiotic therapy. The mechanisms of action of many medicinal herbs with potential disease-modifying effects have not been elucidated. We evaluated the medicinal herb Labisia pumila var. alata (LPva) – a herb whose effects on female genital conditions are widely reported but whose mode of action in UTI is not properly understood. LPva was not bactericidal but prevented bacterial invasion by down regulating β1 integrin and it also induced apoptosis in a dose-dependent manner.

In conclusion, management of UTI in pregnancy will be improved by using effective antibiotics coupled with improved diagnostics. More vigorous interrogation of medicinal herbs hold promise for alternative therapies. We demonstrate for the first time the variation of LL-37 during pregnancy; given impaired vitamin D synthesis among dark-skinned persons, fortification of food especially for pregnant women needs to be considered.

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LIST OF ABBREVIATIONS

ABU   Asymptomatic bacteriuria

AMP   Antimicrobial peptide

ATCC   American type culture collection

BCA   Bicinchoninic acid

CFU   Colony forming unit

CHROM agar   Chromogenic agar

CLED   Cysteine lactose electrolyte deficient agar

CNF-1   Cytotoxic necrotizing factor 1

E. coli   *Escherichia coli*

ESBL   Extended spectrum beta-lactamase

ExPEC   Extra-intestinal pathogenic *E. coli*

FBS   Fetal blood serum

GAPDH   Glyceraldehyde-3-phosphate dehydrogenase

hBD   Human beta-defensin

HRP   Horse radish peroxidase

LB   Luria-Bertani

LPS   Lipopolysaccharide

LPva   *Labisia pumila* var *alata*

MALDI-TOF   Matrix-assisted laser desorption ionization - time of flight mass spectrometry

MDR   Multiple drug resistant

PAMP   Pathogen-associated molecular pattern

PBS   Phosphate buffered saline

PCR   Polymerase chain reaction

PVDF   Polyvinylidene di-fluoride

RT-PCR   Real-time polymerase chain reaction

SDS-PAGE   Sodium dodecyl sulphate polyacrylamide gel electrophoresis

TdT   Terminal deoxynucleotidyl Transferase

TLR   Toll-like receptor

UPEC   Uropathogenic *E. coli*

UTI   Urinary tract infection

VDRE   Vitamin D response elements

1 INTRODUCTION

Urinary tract infections are the most common bacterial infection in women (Foxman 2010). This is mainly attributed to the short urethra and colonization of the peri-urethral area by pathogens from the gastro-intestinal tract. From the peri-urethral area, pathogens ascend to colonize the urinary bladder or kidneys (Bacheller and Bernstein 1997). In pregnancy, acute pyelonephritis is associated with spontaneous preterm birth and septicemia (Wing, Fassett et al. 2013). With advancing gestational age, increased frequency of micturition and lower abdominal discomfort are common complaints. It is therefore likely that clinical diagnosis of UTI maybe less accurate in pregnant compared to non-pregnant women. The higher prevalence of UTI in women compared to men led to description of honeymoon cystitis among newly married women (Buckley, McGuckin et al. 1978). A surgical procedure was designed for the management of recurrent symptoms of post-coital cystitis (Smith, Roberts et al. 1982).

In this thesis, the prevalence of antimicrobial resistance and diagnosis of UTI in settings with limited access to microbiological services were investigated. In addition, the roles of vitamin D, antimicrobial peptides and LPva – a phytoestrogenic herb – in the pathogenesis of UTI were explored.

1.1 URINARY TRACT INFECTIONS IN WOMEN

Women between 16 and 35 years of age are 40 times more likely than men to develop UTI and about 25% of them suffer a recurrent infection within 6 months (Foxman 2002). Among women of reproductive age, asymptomatic bacteriuria (ABU) is commonly transient and requires no antibiotic therapy (Hooton, Scholes et al. 2000). However, during pregnancy, 20% to 30% of women with ABU develop acute pyelonephritis (Little 1966). Screening and treating ABU during pregnancy to reduce the risk of acute pyelonephritis is recommended and widely practiced in developed countries (Smaill 2001). However, with more rigorous study designs to establish the association between ABU and acute pyelonephritis in pregnancy, the beneficial effects of screening for ABU in pregnancy have been questioned (Kazemier, Koningstein et al. 2015). The main causative organism of UTI in women is *E. coli* accounting for up to 85% of infections (Salvatore, Cattoni et al. 2011). The rest of the infections are attributed to other Enterobacteriaceae and Gram positive bacteria (Ronald 2002).

2 PATHOGENESIS OF UTI

In the host-pathogen interaction during UTI, the pathogen adheres, invades and survives in the uroepithelium (Figure 1), acquires essential nutrients and may deploy toxins to disrupt host cell function (Mulvey, Lopez-Boado et al. 1998, Justice, Hung et al. 2004). The host response includes production of antimicrobial peptides (Chromek, Slamova et al. 2006), chemokines that recruit neutrophils (Hedges and Svanborg 1994) and apoptosis of umbrella cells.
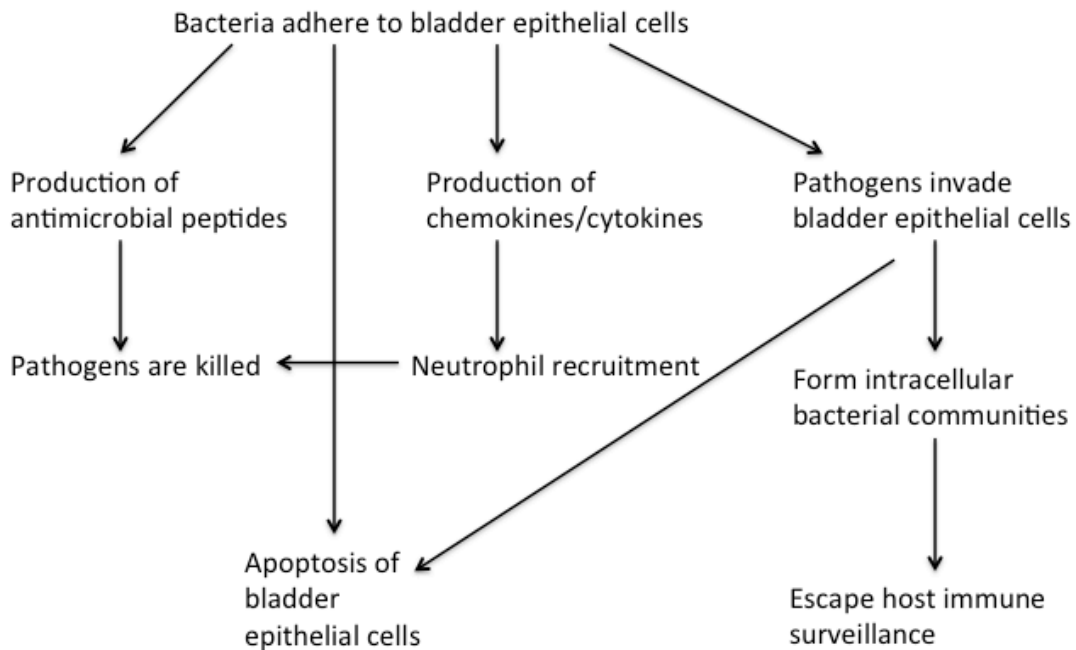
Figure 1: Bacteria adhere to bladder epithelial cells leading to production of antimicrobial peptides and chemokines/cytokines subsequently recruiting neutrophils. The combined effects of antimicrobial peptides and neutrophils are killing of pathogens. However, some pathogens invade bladder epithelial cells and form intracellular bacterial communities thus evading the host immune response. Both extra- and intra-cellular bacteria induce apoptosis of bladder epithelial cells.

2.1 Uropathogenic *E.coli*

Uropathogenic *E. coli* (UPEC) is a subset of extra-intestinal pathogenic *E. coli* associated with urinary tract infections (Dale and Woodford 2015) and may colonize the host gastro-intestinal tract from which it spreads to cause UTI (Nielsen, Dynesen et al. 2014). UPEC possess flagella that facilitate propulsion against urine flow, adhere and invade epithelial cells using fimbrial and afimbrial adhesive organelles, are able to multiply in terminally differentiated and undifferentiated bladder epithelial cells leading to formation of intracellular bacterial communities and quiescent intracellular reservoirs respectively. Secrete toxins that disrupt host

cell function, can be enclosed in polymeric material that protects them against host defenses (Figure 2). Collectively, these adaptations of UPEC are referred to as virulence factors.
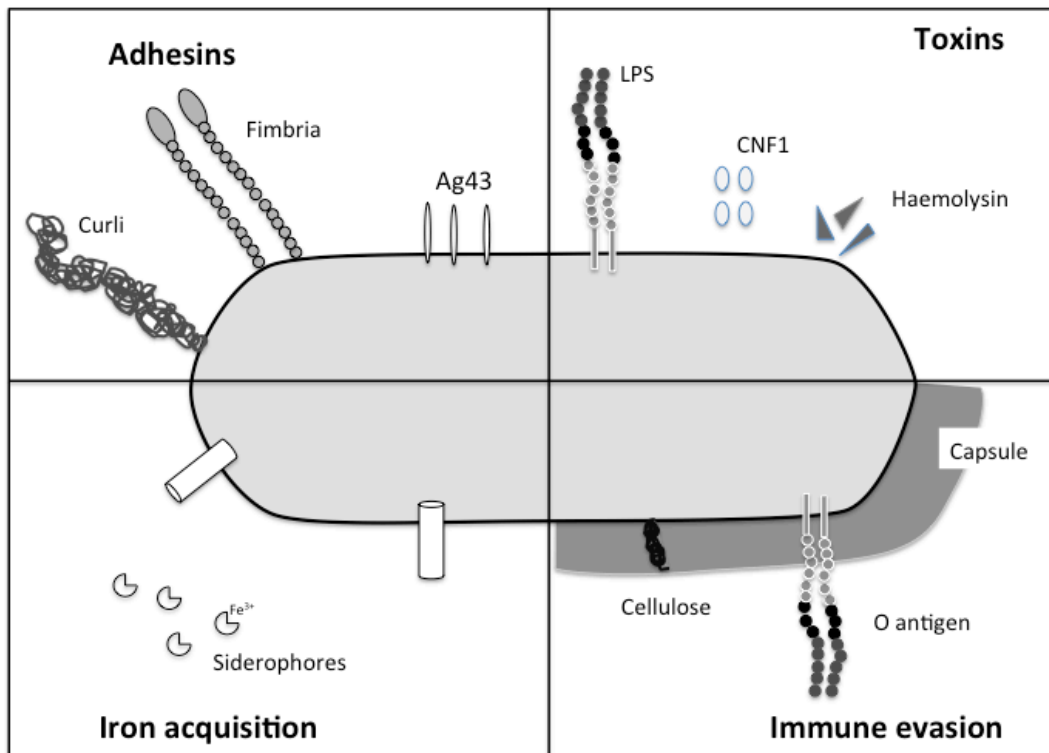

Figure 2: *E. coli* express virulence and fitness factors that facilitate its ability to colonize; evade host immune responses and persist in the urinary tract. Ag43 – Antigen 43, CNF1 – cytotoxic necrotizing factor 1, LPS – lipopolysaccharides. (Adopted from Luthje and Brauner 2014, Adv Microb Physiol.)

There is no specific combination of virulence factors that characterize UPEC but they broadly express genes that mediate adhesion, invasion, immune evasion, promote acquisition of iron and secretion of toxins (Kaper, Nataro et al. 2004). These factors are mostly found on mobile genetic elements referred to as pathogenicity islands (Gal-Mor and Finlay 2006). *E. coli* are generally classified into four phylogenetic groups (Clermont, Bonacorsi et al. 2000). Majority of UPEC belong to phylogenetic group B2 and to some extent group D while most commensal isolates belong to phylogenetic group B1 and A (Ewers, Li et al. 2007). Correspondingly, isolates belonging to phylogenetic group B2 have higher virulence factor scores compared to those of phylogenetic groups B1 and A (Moreno, Andreu et al. 2008). However, apart from phylogenetic group and virulence factor score, there are pathogen and host factors that influence the ability of a given strain to cause UTI (Moreno, Andreu et al. 2008).

2.1.1 Adhesion and invasion of uroepithelial cells
UPEC express fimbrial and afimbrial adhesins (Luthje and Brauner 2014). Type 1 fimbriae are one of the main adhesins that mediate irreversible binding to uroepithelial cells (Bahrani-

Mougeot, Buckles et al. 2002). Expression of type 1 fimbria is not exclusive to ExPEC, however, its deletion among pathogenic *E. coli* severely attenuates adhesion, invasion and the host inflammatory response (Connell, Agace et al. 1996).

Type 1 fimbriae are synthesized through the chaperone-usher pathway (Lillington, Geibel et al. 2014). The tip adhesin on type 1 fimbria irreversibly binds uroplakins 1a on bladder epithelial cells that mediates adhesion (Zhou, Mo et al. 2001). Conversely, interaction between fimH and host β-1 integrin leads to internalization of UPEC by a zippering mechanism (Eto, Jones et al. 2007).

For long, *E. coli* were considered extracellular pathogens, however, their discovery intracellularly has since changed this norm (Martinez, Mulvey et al. 2000). However, *E. coli* escape into the cell cytoplasm and rapidly multiply to form multicellular communities referred to as intracellular bacterial communities (Wright, Seed et al. 2007). Invasion promotes bacterial survival in the host through evasion of immune defenses and resistance to antimicrobial agents (Anderson, Martin et al. 2004).

Similar to type 1 fimbriae, P fimbriae are synthesized through the chaperon-usher pathway (Sauer, Knight et al. 2000). They are more common among isolates that cause acute pyelonephritis and those that persist in the urinary tract leading to recurrent infections (Norinder, Luthje et al. 2011). P fimbriae bind glycosphingolipid residues commonly found on renal epithelial cells (Korhonen, Virkola et al. 1986). Given the coordinated expression of type 1 and P fimbria (Snyder, Haugen et al. 2005) and multi-step pathogenesis approach that bacteria employ in colonization of the urinary tract (Kaper, Nataro et al. 2004); it is likely that pyelonephritis strains express type 1 fimbria to colonize the urinary bladder and switch on P fimbria as they ascend and colonize the kidney (Blomfield 2001).

2.1.2 Iron acquisition

Iron is essential for bacterial growth (Andrews, Robinson et al. 2003) but free iron concentrations are remarkably low in the host. Therefore, bacteria secret proteins that chelate iron with stronger affinity compared to host proteins (Fischbach, Lin et al. 2006). These iron-chelating proteins are referred to as siderophores and include enterobactin, aerobactin, salmochelin, and yersiniabactin. The siderophore-iron complexes anchor on outer membrane receptors like ChuA, IroN, IutA, Hma. From the surface, the complexes are actively transported to the cell cytoplasm and metabolized to release elemental iron (Braun and Braun 2002). There is redundancy in carriage of different siderophore receptors by pathogenic *E. coli* since triple catecholate receptor knock-out mutants (*fep*A, *ih*a and *iro*N) are not out-competed by wild type isolates (Garcia, Brumbaugh

et al. 2011). However, presence of *iroN* promotes biofilm formation (Magistro, Hoffmann et al. 2015).

2.1.3 Biofilm formation

Formation of multicellular bacterial communities (biofilm) is commonly found in persistent and chronic human infections (Costerton, Stewart et al. 1999). Biofilm can be found on both abiotic and biotic surfaces (Flemming and Wingender 2010). In the urinary tract, biofilm formation has been reported on catheters and epithelial cells (Anderson, Palermo et al. 2003, Wang, Lunsdorf et al. 2010). The process of biofilm formation involves reversible and irreversible binding to surfaces, production of an extracellular polymeric substance; formation of micro-colonies within the matrix and dispersal of filamentous bacteria from mature biofilms (Van Houdt and Michiels 2005). Bacteria in a biofilm evade host immune responses, exchange genetic material and are more resistant to antimicrobial agents compared to planktonic bacteria (Hall-Stoodley, Costerton et al. 2004). Flagella, type 1 fimbriae, curli, cellulose, and Ag43 are involved in biofilm formation.

Curli facilitates adhesion of *E. coli* to bladder epithelial cells and inter-bacterial adhesion (Kikuchi, Mizunoe et al. 2005). Equally, curli is a component of the extracellular polymeric substance in biofilm that protects them against LL-37-mediated killing of pathogenic bacteria (Kai-Larsen, Luthje et al. 2010). Cellulose is an inert component of the extracellular polymeric matrix that protects bacteria against host immune cells (Serra, Richter et al. 2013). The *csgD* gene regulates expression of both curli and cellulose but duo expression of curli and cellulose occurs in one third of UTI isolates (Brombacher, Baratto et al. 2006). However, *E. coli* that expresses both curli and cellulose form more biofilm compared to those expressing either curli or cellulose (Bokranz, Wang et al. 2005).

Ag43 is a self-recognizing surface protein commonly found among *E. coli* of phylogenetic group B2 and D (Restieri, Garriss et al. 2007). It mediates auto-aggregation leading to micro-colony formation in bacterial biofilms (Schembri, Kjaergaard et al. 2003). *Ag43* has five allelic variants; the pyelonephritis strain CFT073 carries two alleles (*Ag43*a *and Ag43*b) with 98% homology (Roche, McFadden et al. 2001). However, of the two alleles, *Ag43a*-expressing strains produce more biofilm compared to those expressing *Ag43b* (Ulett, Valle et al. 2007).

2.2 Host defense factors

The urinary tract remains sterile despite its proximity to the highly colonized gastro-intestinal tract (Chromek and Brauner 2008). Sterility is maintained by a combination of mechanical and chemical protective functions (Figure 3). In case pathogens overcome the mechanical barriers protecting the urinary tract, the host innate response broadens to include production of

antimicrobial peptides, chemokines and induction of apoptosis. Although these responses occur in parallel, they converge in function to eliminate invading pathogens. In addition, there are intricate mechanisms that prevent exaggeration of the inflammatory response and limit tissue damage (Abraham and Miao 2015).
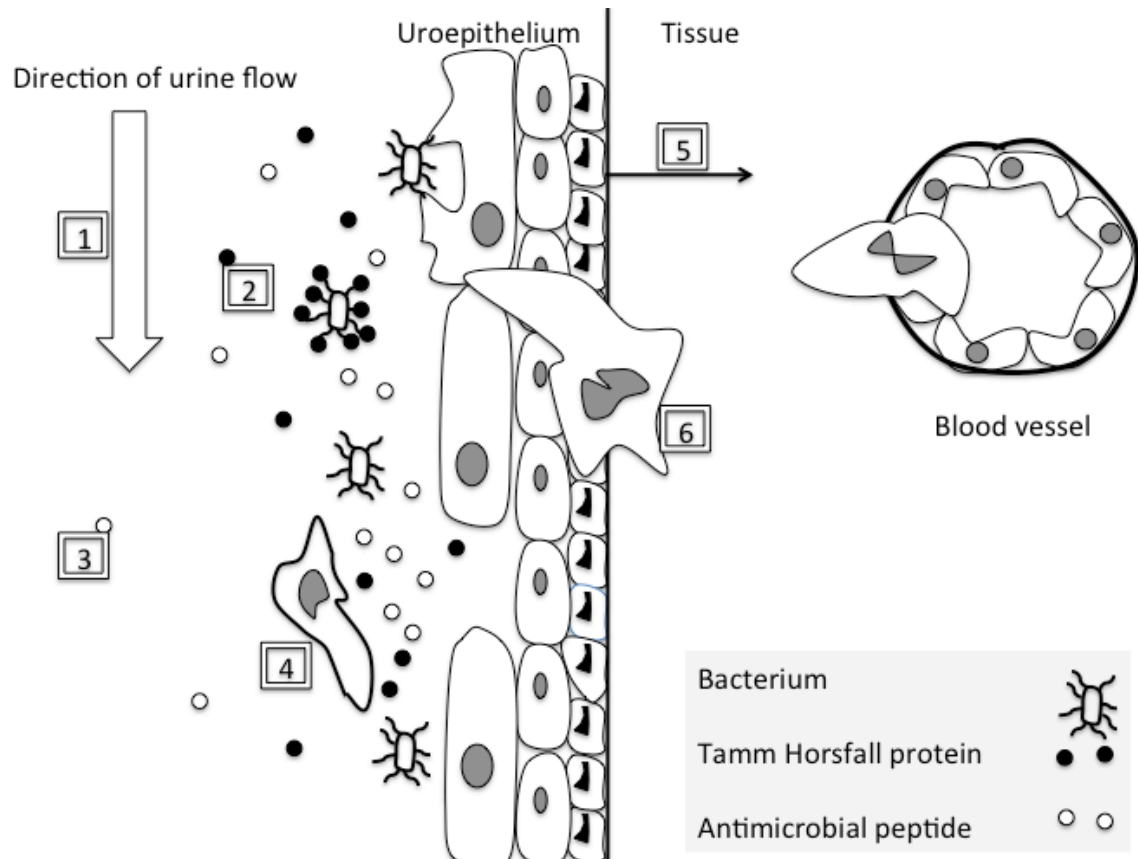
Figure 3: Host factors in the protection of the urinary tract against bacteria during infection: 1 - Urine flow, 2 – Anti-adherence factors [e.g. Tamm–Horsfall protein (THP)], 3 - Antimicrobial factors (e.g. nitric oxide, cathelicidin, defensins), 4 - Exfoliation of cells, 5 - Production of chemokines and cytokines, and 6 - Neutrophils. (Adopted from Chromek and Brauner 2008, Journal of Molecular Medicine)

2.2.1 Urine flow and antibacterial proteins

The bladder epithelium is adapted to regular filling and emptying and is impermeable to urine solutes (Walz, Haner et al. 1995). Urination washes out non-adherent bacteria and apoptotic cells. Voiding dysfunction like vesico-ureteric reflux increases the risk of developing UTI and may lead to renal damage thus further demonstrating the importance of bladder emptying (Swerkersson, Jodal et al. 2007).

Antimicrobial proteins that protect the urinary tract include Tamm Horsfall protein (THP) and lipocalin 2. THP is constitutively produced by the ascending loop of Henle (Bachmann, Metzger

et al. 1990) and neutralizes *E. coli* type 1 fimbriae (Pak, Pu et al. 2001). Conversely, bacterial activation of α-intercalated cells in the kidney induces production of lipocalin 2 (Paragas, Kulkarni et al. 2014). Lipocalin-2 sequesters enterochelin – a bacterial siderophore and delivers the siderophore-iron complex to host cells for degradation (Miao and Abraham 2014).

2.2.2 Host recognition of bacterial surface antigens

Toll-like receptors (TLR) are proteins that recognize conserved pathogen-associated molecular patterns (PAMPs) (Fischer, Yamamoto et al. 2006). In humans, there are eleven TLRs of which TLR2, TLR4, TLR5 and TLR11 are present in the urinary tract (Kawai and Akira 2010). TLR 4 in co-operation with CD14 recognizes LPS (Fischer, Yamamoto et al. 2006). Presence or absence of CD14 on uroepithelial cells is still debatable (Hedlund, Frendeus et al. 2001, Chromek, Stankowska et al. 2005), however, in cells that lack membrane-bound CD14, soluble CD14 plays a complementary role (Brenner, Zacheja et al. 2014).

2.2.3 Antimicrobial peptides

In the last quarter of the 19th century, human tissues and body fluids were reported to have antimicrobial properties as reviewed by Skarnes (Skarnes and Watson 1957). However, the chemical mediators of antimicrobial action remained elusive until the 1990s (Agerberth, Gunne et al. 1995, Boman 1995). There are around a thousand antimicrobial peptides (AMP) in plants, animals and humans. Broadly classified into cathelicidin, defensins, S100 family of protein and RNase A superfamily (Wiesner and Vilcinskas 2010).

Generally, epithelial and immune cells produce antimicrobial peptides that protect epithelial surfaces against microbial infections (Zasloff 2002). In pregnancy, antimicrobial peptides are abundant in amnion, vernix caseosa, vaginal secretions and the cervical mucus plug that separates the vaginal environment from the otherwise sterile uterine compartment (Kai-Larsen, Gudmundsson et al. 2014).

2.2.3.1 Cathelicidin

LL-37 is the only cathelicidin antimicrobial peptide identified in humans. Apart from its antimicrobial properties, it is chemoattractant for neutrophils, monocytes and CD4 T cells (Tjabringa, Ninaber et al. 2006). Bladder epithelial cells constitutively produce LL-37 and its production markedly increases following a bacterial challenge (Chromek, Slamova et al. 2006). The antimicrobial properties of LL-37 include forming pores in the bacterial cell wall (Burton and Steel 2009) and inhibition of CsgA polymerization preventing curli formation and by extension biofilm (Kai-Larsen, Luthje et al. 2010).

2.2.3.2 Defensins

In humans, defensins are classified as α and β defensins (Selsted, Tang et al. 1993, Lehrer and Lu 2012). Alpha defensins are predominantly found in neutrophil azurophilic granules however, during acute inflammation, they may appear at epithelial surfaces possibly from infiltrating neutrophils (Doss, White et al. 2010). Conversely, β defensins are constitutively secreted by uroepithelial cells and their production increases following bacterial challenge (Hazlett and Wu 2011). Although α defensins have definitive antimicrobial properties including pore-formation in bacterial cell walls, human β defensing 1 (HBD-1) has weak antimicrobial properties (Ganz and Lehrer 1995). It is likely that defensins act synergistically with LL-37 to protect the uroepithelium.

2.2.3.3 Other important antimicrobial peptides in the urinary tract

Apart from cathelicidin and defensins, antimicrobial peptides from both the RNase and S100 families have been reported in the urinary tract. RNase 7 is produced by immune and epithelial cells including those lining the urinary tract (Spencer, Schwaderer et al. 2011). Its production increases during acute pyelonephritis (Spencer, Schwaderer et al. 2013). Psoriasin – a member of the S100 family of antimicrobial peptides – is produced by bladder epithelial cells (Celis, Rasmussen et al. 1996). It is bactericidal to *E. coli* and potentially protects the female genital tract from contamination by gastro-intestinal flora (Mildner, Stichenwirth et al. 2010). However, their role in the pathogenesis of UTI is still being explored.

2.2.4 Chemokines and cytokines

Cytokines are multifunctional modulators of the host immune response functionally classified into pro-inflammatory, immune-regulatory and growth factors. They act through cognate receptors in a paracrine, autocrine and to some extent, in an endocrine manner (Spencer, Schwaderer et al. 2014). In the urinary tract, chemokines and cytokines are produced by epithelial and immune cells and mediate recruitment of neutrophils to the site of infection, induction of pyrexia, secretion of IgA and release of neutrophils from the bone marrow (Ragnarsdottir and Svanborg 2012).

IL-8 is produced by uroepithelial cells in response to bacterial challenge and is necessary in neutrophil recruitment (Baggiolini, Walz et al. 1989, Godaly, Proudfoot et al. 1997). At the uroepithelial surface, neutrophils engulf and kill bacteria in phagosomes with reactive oxygen species, antimicrobial peptides or elastases (Witko-Sarsat, Rieu et al. 2000, Nauseef and Borregaard 2014).

2.2.5 Apoptosis

Apoptosis is programmed cell death that maintains tissues homeostasis; it can be initiated by pathological and physiological environmental factors (Kerr, Wyllie et al. 1972). Apoptosis of bladder epithelial cells markedly increases during urinary tract infections and *E. coli* may activate either the intrinsic or extrinsic pathways depending on the presence of virulence factors (Klumpp, Weiser et al. 2001, Klumpp, Rycyk et al. 2006). As part of the host innate response, apoptosis reduces bacterial load when detached cells are washed out during urination. However, it exposes deeper layers of bladder epithelial cells in which *E. coli* can establish quiescent intracellular communities. Quiescent intracellular bacterial communities are associated with recurrent urinary tract infection (Schwartz, Chen et al. 2011).

2.2.6 Vitamin D

Vitamin D is a fat-soluble vitamin that is synthesized under the skin. Photolytic conversion of 7-dehydrocholestrol leads to formation of previtamin D_3 that is transported to the liver. Cytochrome P450 enzymes hydroxylate previtamin D_3 to $25OHD_3$, circulating $25OHD_3$ can be converted to the active hormone $1,25OH_2D_3$ by renal 1α-hydroxylase (Figure 4). However, at epithelial surfaces and in immune cells, 25OHD3 can be converted into the active hormone and activates vitamin D-regulated genes (Adams and Hewison 2010). Most of the vitamin D is synthesized from the skin, alternative dietary sources include fatty fish or fortified foods. Optimal vitamin D during pregnancy is required in the deposition of the fetal skeleton while its deficiency is associated with low birth weight, small for gestational age and to some extent pregnancy-induced hypertension (Wei 2014, Chen, Fu et al. 2015).

Sun

UVB

7-dehydrocholesterol → Vitamin D3   Skin

Vitamin D3
↓ 25 hydroxylase
25 OHD3

Liver

25 OHD3
↓ 1α hydroxylase
1,25 $(OH)_2D3$

Kidney

Calcium metabolism
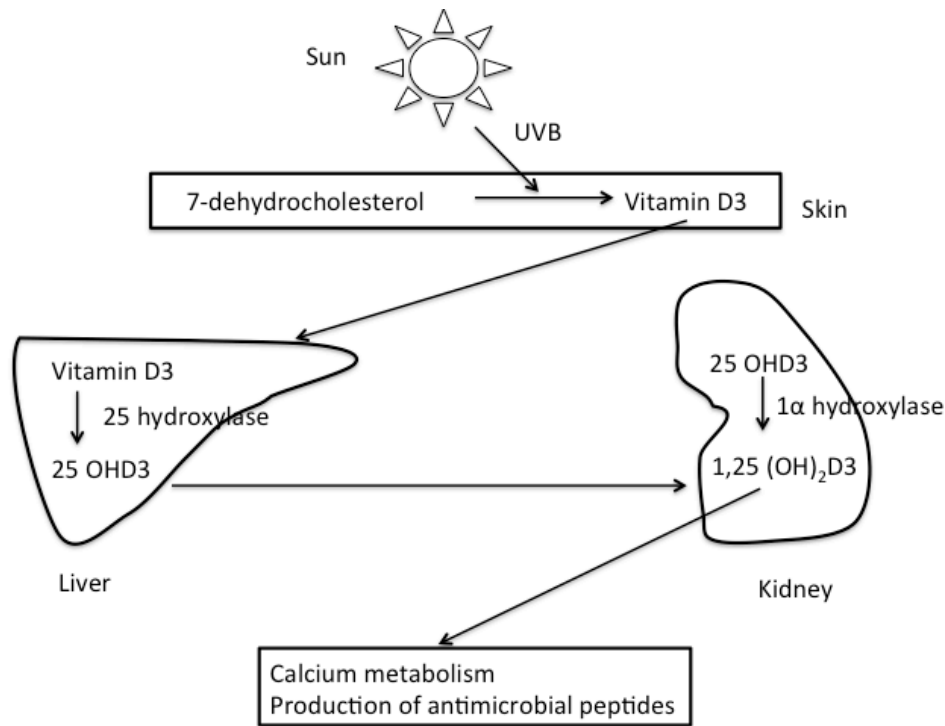Production of antimicrobial peptides

Figure 4: Photolytic conversion of 7-dehydrocholesterol occurs under the skin leading to formation of vitamin D3 – a substrate of 25-hydroxylase enzymes in the liver. It is converted into 25-OHD3 (the main circulating form of vitamin D). Apart from renal 1α-hydroxylase, bladder and innate immune cells are able to convert 25-OHD3 into 1,25 $(OH)_2D3$ the active form of vitamin D (Hertting, Holm et al. 2010, Moran-Auth, Penna-Martinez et al. 2013). 1,25 $(OH)_2D3$ induces production of antimicrobial peptides when coupled with vitamin D receptor.

Therapeutic effects of vitamin D were recognized in the late 19th Century leading to a Nobel prize in Medicine award to Niels Ryberg Finsen in 1903 (nobelprize.org). Recently, vitamin D was reported to modulate innate and adaptive immune responses and influence disease outcomes (Olliver, Spelmink et al. 2013, Chun, Liu et al. 2014). Vitamin D deficiency rickets has rebounded even in areas where vitamin D supplementation is routinely done (Singleton, Lescher et al. 2015, Wheeler, Dickson et al. 2015). Globally, dark-skinned people living in temperate climates (van der Meer, Karamali et al. 2006), the elderly and those living in the tropics but extensively cover their bodies to allow adequate synthesis of vitamin D (Lips 2010) are at increased of vitamin D deficiency.

Vitamin D induces expression of both *CAMP* and *DEFB*4 genes that have vitamin D response elements in their promoter regions (Gombart, Borregaard et al. 2005, Liu, Schenk et al. 2009). In the urinary tract, vitamin D supplementation increases production of LL-37 during an infection challenge, potentially defending the uroepithelium against pathogenic bacteria (Hertting, Holm et al. 2010). Therefore, pregnant women together with their unborn babies could potentially benefit

from optimal serum vitamin D levels. Where vitamin D deficiency is likely to occur due to inadequate skin exposure or lack of vitamin D fortified foods or skin pigmentation; supplementation with vitamin D could be an alternative.

2.2.7 Diagnosis of UTI in pregnancy

Clinical diagnosis of UTI and initiation of antimicrobial therapy is cost-effective among adult non-pregnant women where the causative organisms and their antimicrobial susceptibility pattern are known (Fenwick, Briggs et al. 2000, Bent, Nallamothu et al. 2002). However, during pregnancy, symptoms are largely non-specific for UTI therefore, clinical diagnosis may be overestimating the prevalence. Bacteriological culture is the golden standard in the diagnosis of UTI but is not universally available. Low-cost alternative diagnostic tests like nitrite, leucocyte esterase, microscopy and dipslide were evaluated in this thesis.

2.2.8 Management of UTI in pregnancy

Antibiotic use during pregnancy may have side effects on the fetus (Bisschop, Merkus et al. 1985, Kenyon, Pike et al. 2008). In Uganda, cotrimoxazole and amoxicillin are recommended in the management of lower UTI while ciprofloxacin is reserved for upper UTI (Uganda 2012). However, there is increasing resistance of uropathogens to cotrimoxazole and amoxicillin (Ramos, Dzung et al. 2011). Additionally, cotrimoxazole use is contra-indicated in the first trimester of gestation because it doubles the risk for neural tube defects in the newborn (Hernandez-Diaz, Werler et al. 2001). Ciprofloxacin crosses the placenta and is associated with arthropathy, restricting its use during pregnancy (Giamarellou, Kolokythas et al. 1989).

3 AIMS OF THE THESIS

The overall aim of this project was to investigate UTI in pregnancy with special emphases on diagnoses, bacterial virulence factors and prevention of infection.

The specific aims were:

1. To investigate the susceptibility pattern and virulence factors of uropathogenic *E. coli* isolates from pregnant women in Uganda and in comparison Vietnam and Sweden.

2. To evaluate low-cost, simple diagnostic approaches to diagnose UTI in pregnancy.

3. To determine the impact of vitamin D on innate immune responses to *E. coli* during UTI in pregnancy.

4. To evaluate the effect of the phyto-estrogenic herb *Labisia pumila* var. *alata* (LPva) on the host-pathogen interaction *in vitro*.

4 PARTICIPANTS AND METHODS

4.1 PARTICIPANTS

The first three papers of this thesis used material collected from human participants and were approved by the local ethics committees in Kampala, Uganda, Hanoi, Vietnam, and Stockholm, Sweden.

In paper I, 148 pregnant women with *E.coli* UTI were recruited. Study participants were attending antenatal clinics at Mulago Hospital, Kampala (n = 56), the National Hospital of Obstetrics and Gynecology, Hanoi, (n = 42), and Karolinska University Hospital, Stockholm (n = 50). They were between 17 and 48 years old and their gestational ages ranged from 4 to 40 weeks. Only those who had not used antibiotics two weeks prior to screening were included in the study. Age-matched non-pregnant women with *E.coli* UTI attending clinics in the Stockholm area, Sweden served as controls (n =50). The antibiotic susceptibility pattern and virulence factors of all isolates were investigated.

In paper II, 2 562 participants were altogether recruited; 1 621 were pregnant women with or without UTI symptoms attending routine antenatal care in Mulago hospital. The rest of the participants (n=941) were outpatients, 472 general outpatients from Mulago and 469 were non-pregnant women who were attending clinics in the Stockholm area.

In paper III, variation of serum vitamin D levels in pregnancy and its impact on innate immune responses during UPEC infection were investigated. Blood samples were collected from 32 pregnant women attending the antenatal clinic at Mulago Hospital, Kampala, during the 1st, 2nd and 3rd trimester; further, from 16 of these women, samples were collected after delivery. Age-matched never pregnant women (n = 25) served as controls. The median age of pregnant and never pregnant women was 23 years. In an interviewer-administered questionnaire during their first visit, pregnant women were asked about consumption of vitamin D rich or vitamin D-fortified foods over the previous 24 hours.

4.2 SAMPLE COLLECTION

In paper I, and II, midstream urine was collected in sterile containers and either analyzed at the point-of-care or delivered to the microbiology laboratory within one hour.

In paper III, blood was collected in vacutainer tubes loaded with a gel separator (Greiner bio-one) and centrifuged at 1500 × *g* for 15 minutes to separate serum that was aliquoted and stored in light-protected containers at − 80°C until analysis

4.2.1 Urine analyses

In paper I, urine cultures were done following protocols at the respective sample collection sites. Overall, primary cultures were done on Uriselect agar (Bio-Rad Laboratories) in Hanoi, blood and MacConkey agar (Bio-Rad Laboratories) in Kampala, while in Stockholm, primary cultures were done on blood and CHROMagar (bioMérieux, Marcy l'Etoile). They were incubated at 37°C overnight. Significant bacteriuria for women with cystitis was $> 10^3$ CFU/ml of *E. coli*, $> 10^4$ CFU/ml of Gram negative or $>10^5$ CFU of Gram positive bacteria while $> 10^5$ CFU/ml of urine in the absence of symptoms was classified as ABU.

Species identification was done at the respective sites as well as at Karolinska University Hospital.

In paper II, urine samples were analyzed using Multistix 10SG® (Siemens, Munich, Germany), microscopy, Uricult Trio® (Orion Diagnostica, Espoo, Finland) and urine culture. Multistix 10SG were used to detect presence of leucocytes and nitrite following the manufacturer's instructions. To enumerate microscopic leucocyturia, approximately 3-5 milliliters aliquot of well mixed urine were centrifuged at $450 \times$ g for 5 minutes, the supernatant was decanted and the pellet was suspended in about 1 ml of urine. Fifteen microliters were transferred on a glass slide and covered with a coverslip. Ten fields were examined at 40x magnification under a microscope (Olympus Ch20, Olympus, USA) and > 10 leucocytes per high power field was considered significant pyuria.

Uricult Trio® (Orion Diagnostica, Espoo, Finland) is a dipslide method that has CLED agar covering one side of the slide and half of both MacConkey agar and *E. coli*-specific agar on the other side. It was inoculated with midstream urine and incubated overnight to determine bacterial growth together with lactose fermentation. CFU count and lactose fermentation were done on CLED agar, while detection of beta-glucuronidase-producing bacteria was done on the *E. coli*-specific agar.

4.2.2 Protein assay by ELISA

In paper III, serum was analyzed for LL-37 (Hycult), hBD-2 (Alpha Diagnostics), IL-8 (R&D systems) and soluble CD14 (R&D systems) using ELISA following the respective manufacturer's protocols. Serum 25 OHD was determined using an antibody-based chemiluminescence assay (Diasorin).

4.3 *IN VITRO* EXPERIMENTS

4.3.1 *Labisia pumila* var *alata*

LPva is a medicinal herb that belongs to the family Myrsinaceae. It is widely used by women in Malaysia (Stone 1988) and was found to have phyto-estrogenic effects (Fazliana, Gu et al. 2012).

In our experiments, a water extract (Yusoff and Mohamud 2011) from a certified Good Manufacturing Practices herbal facility was used. The powder was re-constituted in deionized water and filter-sterilized to make a stock solution. It was diluted in cell culture medium to 10 µg/ml, 100 µg/ml and 1000 µg/ml for *in vitro* experiments (paper IV).

4.3.2 Bacteria

Species identification of clinical isolates

Clinical *E. coli* isolates were evaluated for their antimicrobial susceptibility and virulence factors. In paper I, and paper II, species confirmation of clinical *E. coli* isolates was performed using Vitek 2 Gram negative identification card for fermenting and non-fermenting bacteria (bioMérieux, Marcy l'Etoile, France). Pure colonies were prepared in 0.5% NaCl and the turbidity was adjusted between 0.50 and 0.63 McFarland units.

In paper II, in addition to Vitek 2 identification of clinical *E. coli* isolates, MALDI-TOF mass spectrometry (Bruker, Daltonik, GmbH, Bremen, Germany) was used following the manufacturer's instructions. Briefly, pure colonies were inoculated on the metallic MALDI target plates and overlaid with MALDI matrix; they were left to dry at room temperature and loaded for identification.

Antibiotic susceptibility testing

Antimicrobial susceptibility of clinical isolates to ampicillin, amoxicillin-clavulanic acid, ciprofloxacin, gentamicin, trimethoprim and cephalexin was also done using Vitek 2 (AST-N106). Further, the disc-diffusion method was used to test susceptibility to cefotaxime, ceftibutin, ceftazidime, piperacillin, imipenem and meropenem.

***E. coli* CFT073**

In paper I, III & IV, the pyelonephritis *E. coli* strain CFT073 was used either in infection experiments or as a positive control for virulence factors. CFT073 was originally isolated from urine and blood of a patient with pyelonephritis (Mobley, Green et al. 1990) and expresses both type 1 and P fimbriae (Mobley, Jarvis et al. 1993). It also expresses toxins like secreted autotransporter toxin (Nichols, Totsika et al. 2016) and hemolysin (Mobley, Green et al. 1990), iron acquisition systems like *iroN, chuA, iutA* (Mills, Meysick et al. 2000, Alteri and Mobley 2007), and forms biofilm (Ulett, Valle et al. 2007).

4.3.3 Cell lines

To mimic the host response to *E. coli* infection, human bladder cancer (T24, 5637) and telomerase-immortalized urothelial cells (TERT-NHUC) were used. T24 and 5637 cells were

grown in McCoy's and RPMI 1640 medium respectively, supplemented with 10% fetal bovine serum while TERT-NHUC cells were maintained in Epilife medium with 60μM calcium and human keratinocyte growth supplement (Gibco, Carlsbad, CA, USA). In all cell culture experiments, incubation was done at 37°C in a humidified chamber with 5% CO_2. T24 cells were used in paper I, III and IV; TERT-NHUC in paper III and 5637 in paper IV.

6.3.2.1 Studies on type 1 fimbriae (paper I and IV)

To investigate the expression of type 1 fimbriae, baker´s yeast, *Saccharomyces cerevisiae* was used to test the specificity of the interaction (Korhonen, Leffler et al. 1981). Bacteria were grown overnight on LB agar without salt and suspended in PBS to a final concentration of 10^{10} CFU/ml. Equal parts of bacteria and 3% *Saccharomyces cerevisiae* in PBS were mixed and observed for agglutination. The specificity of the test was confirmed by adding 5% mannose in PBS. In Paper I, expression of type 1 fimbriae by clinical *E. coli* isolates was investigated and in paper IV the interaction of LPva extract with *E. coli* CFT073 type 1 fimbriae was determined.

4.3.2.2 Curli, cellulose and detection of biofilm

Curli and/or cellulose expressing *E. coli* have characteristic morphotypes on Congo red and Calcofluor agar (Bokranz, Wang et al. 2005). Bacteria were streaked on the respective plates and incubated at 37°C; the morphotypes were determined at 24 h and 48 h on Congo red and Calcoflour agar respectively. On Congo red plates, colonies that are curli and cellulose positive appear red; curli negative, cellulose positive bacteria appear pink while curli positive, cellulose negative bacteria appear brown and colonies of bacteria that are negative for both curli and cellulose appear white. On Calcofluor agar, colonies of cellulose expressing *E. coli* fluoresce on exposure to UV light. In paper I, curli and cellulose expression by clinical isolates was determined using Congo red and Calcofluor agar with appropriate controls.

Biofilm formation was determined by the microtiter plate method (Wakimoto, Nishi et al. 2004) in paper I. Briefly; bacteria were grown overnight on LB agar without salt. Colonies were suspended in PBS and centrifuged at $300 \times g$ for 10 minutes to remove aggregates. The supernatants were adjusted to 10^8 CFU/ml by spectrophotometry; a 1:100 dilution was made in LB broth without salt and 200 μl aliquots were pipetted into a 96-well plate (Costar, Corning). The plate was incubated at 37°C for 24 h, the supernatant containing planktonic bacteria was aspirated and discarded, wells were washed with PBS, air dried and stained with crystal violet. The crystal violet was solubilized in acetone-alcohol and the optical density determined at 550 nm by spectrophotometry to quantify the amount of biofilm formed.

4.3.2.3 Inhibition of bacterial growth

The agar diffusion sensitivity assay was used to test the antimicrobial activity of human serum (paper III) and LPva (paper IV). Bacteria were grown to mid logarithmic phase and embedded in a thin layer of LB broth with 1% agarose. The molten agar was poured into petri dishes and left to solidify at room temperature for 30 minutes; 3 mm diameter wells were made in the agar to which 3 µl of test substance were added and incubated overnight. Antimicrobial potential of the tested substance was determined by measuring the diameter of the inhibition zone around the well.

In paper III, inhibition of UPEC CFT073 growth was tested against serum containing low (<50 nmol/L) or high (>80 nmol/L) 25(OH) D.

In paper IV, the antimicrobial potential of LPva was determined against *E.coli* 25922, *P. mirabilis* 29906, *P. aeruginosa* 27853, *S. aureus* 29213, *S. saprophyticus* 15305, and *C. albicans* 90028.

4.3.2.4 Infection experiments

Urothelial cells were infected with 10^6 CFU/ml of either clinical *E. coli* isolates (paper I) or *E. coli* CFT073 (paper III & IV) for different time periods. Total mRNA was extracted for gene expression studies (paper IV), protein quantification was done in cell lysates by Western blot (paper IV) and in supernatants by ELISA (paper III).

4.3.2.5 Bacterial adhesion and invasion of urothelial cells

In paper I, different clinical *E. coli* isolates were tested for adhesion and invasion of T24 cells while in paper IV, the influence of LPva on adhesion and invasion of T24 and 5637 cells by *E. coli* CFT073 was studied.

Cells were infected and incubated for 30 minutes, non-adherent bacteria were washed away with PBS. The cells were lysed with 1%Triton X-100 in PBS. The lysates were serially diluted in PBS and plated on blood agar and incubated overnight at 37^oC to determine the CFU of cell-associated bacteria.

To determine the concentration of intracellular bacteria, the process was as described above followed with a gentamycin protection assay for additional 90 minutes to allow bacteria to invade cells. Thereafter, cells were lysed, serially diluted, incubated on blood agar overnight and CFU count was used to quantify intracellular bacteria.

4.3.2.6 Gene amplification by PCR

Gene amplification was done for bacterial DNA to study virulence factors and human urothelial cells for expression of innate response genes. Bacterial DNA was extracted using a modified boiling method (paper I). It was amplified by multiplex PCR to determine phylogenetic groups

(Clermont, Bonacorsi et al. 2000) and uniplex PCR was done for virulence factor genes. Both multiplex and uniplex PCR products were separated by gel electrophoresis; amplicons were stained with ethidium bromide or GelRed to visualize specific sizes corresponding to the respective virulence factor genes (Mothershed and Whitney 2006). In paper I, phylogenetic groups of clinical isolates and *E. coli* virulence factors that include *flu*, *fluA*$_{CFT073}$, *fluB*$_{CFT073,}$ *iroN*$_{E.coli,}$ and *tcpC* were investigated.

In paper III & IV, total RNA was extracted from urothelial cells using a spin column technique. Complementary DNA (cDNA) was synthesized using standard procedures and following manufacturer's instructions for the respective kits. CAMP, caveolin-1 and β-1 integrin were amplified by RT-PCR. GAPDH was used as the internal reference and relative change in gene expression were measured using the $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak 2008). In paper III, the effect of human serum on IL-8, CD14 mRNA; in paper IV the effect of LPva on LL-37, hBD-2, caveolin-1 and β-1 integrin mRNA expression were determined.

4.3.2.7 Protein assay by Western blot

After infecting cells following LPva treatment (paper IV), they were washed with ice cold PBS and lysed with lysis buffer that includes protease inhibitors (Sigma-Aldrich) and sonicated. The protein concentration was determined using the bicinchoninic acid (BCA) protein assay kit and samples were adjusted to equal concentrations and boiled in Laemmli buffer with or without β-mercaptoethanol for caveolin-1 and β-1 integrin respectively.

Samples were run on SDS-PAGE from Bio-Rad Laboratories and transferred to a 0.45 μm Invitrolon PVDF membrane – Invitrogen, that was then blocked for one hour with 5% milk in Tris-buffered saline with 0.05% Tween 20 at room temperature. The membrane was incubated with primary antibodies goat anti-caveolin-1 and mouse anti-β-1 integrin at 4°C overnight; followed with HRP-labelled secondary anti-goat antibody for caveolin-1 and anti-mouse antibody for β-1 integrin. Super signal Pico Kit (Pierce, Thermo Scientific, Waltham, MA, USA) was used to visualize the bands and relative protein quantities were determined by measuring band intensities with Image J. As controls, membranes were stripped and re-probed with anti-GAPDH antibodies.

4.3.2.8 Apoptosis assay

To assess the effect of LPva on apoptosis of infected and non-infected urothelial cells, T24 cells were grown on coverslips for 24 h and treated with LPva for 6, 24 or 72 h. They were infected with *E. coli* CFT073 for 60 minutes and stained for apoptotic changes at 6 or 24 h (paper IV). Non-treated non-infected cells served as controls. Briefly, cells were stained with Annexin V Fluos and *In Situ* Cell Death Detection Kit (Roche Applied Science, Penzberg, Germany); 4', 6-diamidine-2 phenylindole dihydrochloride (DAPI, Sigma Aldrich) was used to stain the nucleus.

Annexin V stains exteriorized phosphatidylserine on the cytoplasmic membrane – a marker of early apoptotic changes. Propidium iodide stains DNA where cytoplasmic membrane integrity is compromised – as may occur during necrosis. Apoptosis was also investigated using TUNEL assay; cells were fixed with 2% formaldehyde in PBS, permeabilised with ice-cold 70% ethanol and then incubated with TdT buffers. Cells were mounted in Prolong Gold anti-fade reagent (Invitrogen, Carlsbad, CA, USA) and visualized under Leitz-Leica DMRB fluorescent microscope.

5 RESULTS

5.1 UROPATHOGENIC *E. COLI* ISOLATES FROM PREGNANT WOMEN IN DIFFERENT COUNTRIES (PAPER I)

We sought to compare the virulence factors and antimicrobial susceptibility pattern of *E. coli* causing UTI in low, middle and high-income countries. Isolates were collected from pregnant women in Uganda, Vietnam and Sweden to represent the different income categories and where the accessibility of antibiotic differs. From Sweden, *E. coli* from non-pregnant women of reproductive age with UTI served as controls.

E. coli isolates from pregnant and non-pregnant women from Sweden and those from Vietnam belonged to phylogenetic group B2 while more Ugandan isolates belonged to phylogenetic group B1. This finding prompted us to investigate differences in the ability of B1 and B2 isolates to cause infection. We therefore investigated the initial step of the infection process and evaluated the bacteria´s ability to adhere and invade urothelial cells and form biofilm. Isolates belonging to phylogenetic group B1 adhered to and invaded urothelial cells as efficiently as those of phylogenetic group B2. However, those belonging to phylogenetic group B2 produced more biofilm.

The *flu*A gene is implicated in bacterial auto-aggregation leading to formation of biofilm (Ulett, Valle et al. 2007). It was more prevalent among isolates belonging to phylogenetic groups B2 and D compared to B1 and A in Vietnam *(P=0.0004)* and Uganda *(P=0.0001)*; overall, more Ugandan isolates carried the *flu*A gene.

We further observed a strong correlation between the genes *tcpC* and *iroN$_{E\ coli}$* and the phylogenetic group B2. While *tcpC* mediates the ability to modulate host innate immune response, *iroN$_{E.coli}$* encodes a catecholate siderophore. The prevalence of both genes was lower among Ugandan isolates compared to those from Sweden and Vietnam.

Resistance to ampicillin in Uganda, Vietnam and Sweden was 75%, 83% and 22% Sweden respectively. Correspondingly, we observed a high prevalence of ESBL-producing *E. coli* in Uganda (14%) and Vietnam (7%) compared to Sweden (2%). There was a two-fold high prevalence of MDR compared to ESBL.

5.2 ANTIBIOTIC OVERCONSUMPTION IN PREGNANT WOMEN WITH URINARY TRACT SYMPTOMS IN UGANDA (PAPER II)

Clinical diagnosis and antibiotic treatment of patients, including pregnant women, with symptoms of UTI is common practice in developing countries. This is largely due to limited access to diagnostic services. However, during pregnancy, UTI symptoms especially at advanced gestational age are largely unreliable indicators of infection. But that only 4 % of pregnant women with UTI symptoms had culture verified bacteriuria and 96% received unnecessary antibiotics was a surprise. We therefore evaluated simple diagnostic tests with potential for use by midlevel health workers in outpatient clinics to reduce antibiotic consumption. The diagnostic tests included nitrite, leucocyte esterase, microscopy and dipslide.

Nitrite, leucocyte esterase and leucocyturia when used alone had low accuracy in diagnosis of UTI and ABU during pregnancy. The sensitivity of the combined nitrite and leucocyte esterase was poor. Urine microscopy was equally of low diagnostic value. We found low correlation between leucocyturia and leucocyte esterase (38%). Overall, nitrite and leucocyte esterase had low sensitivity and high specificity.

Dipslide, a simplified culture method mainly used in the diagnosis *E. coli* UTI was evaluated. Microbiologically non-trained medical staff, like gynaecologist and nurses could accurately detect *E. coli*; accuracy improved cumulatively. Although, *E. coli* was accurately detected, growth of other Gram-negative bacteria was overestimated.

5.3 THE IMPACT OF VITAMIN D ON THE INNATE IMMUNE RESPONSE TO UROPATHOGENIC *ESCHERICHIA COLI* DURING PREGNANCY (PAPER III)

Vitamin D is important during pregnancy both to the mother and the baby, with possible long-term effects on the child among pregnant women with vitamin D deficiency. Further it has been shown that it also plays a role in adaptive as well as innate immunity. The major source of vitamin D is UV radiation-mediated synthesis from the skin. However, this effect is attenuated by dark skin pigmentation and in persons who are covered. We investigated the role of vitamin D, vitamin D-regulated antimicrobial peptides - LL-37, hBD-2; sCD14 and IL-8 in the pathogenesis of UTI in vivo and in vitro.

Serum vitamin D levels increased with gestational age and were higher in the third compared to the first trimester *(P<0.01)* and compared to never pregnant women. Further, multigravidae had higher vitamin D levels compared to prime gravidae.

Vitamin D can induce cathelicidin, LL-37 and hBD2 by binding to the vitamin D responsive element (VDRE) of the promoter region (Gombart, Borregaard et al. 2005). The human

cathelicidin, LL-37 increased significantly (*P<0.001)* with advancing gestational age. Moreover, in line with the increase of LL-37, the antimicrobial activity of serum increased with gestational age. In the current study, we did not observe any increase of hBD2.

We observed lower IL-8 levels in the third compared to first trimester and also versus after delivery and in serum from never pregnant women, sCD14 did not change. In an *in vitro* model to mimic UTI, uroepithelial cells treated with serum containing >80 nmol/L of vitamin D expressed significantly lower levels of IL-8 at mRNA (*P<0.05*) and protein (*P<0.01*) levels compared to those treated with serum containing <50 nmol/L of vitamin D. To rule out the possibility of other serum factors influencing our results, uroepithelial cells were treated with synthetic vitamin D. Interestingly; vitamin D suppressed IL-8 production in a dose-dependent manner.

5.4 LABISIA PUMILA VAR. ALATA REDUCES BACTERIAL LOAD BY INDUCING UROEPITHELIAL CELL APOPOTOSIS (PAPER IV)

Labisia pumila var. alata (LPva) is a herb commonly used in Malaysia to treat urogenital diseases. Due to its phytoestrogenic effects we assumed that it also had effect against UTI and therefore investigated its *in vitro* effect on *E. coli* infected uroepithelial cells.

We demonstrate that LPva induced apoptosis in a dose-dependent manner and that apoptosis was associated with increased caveolin-1 expression both on the mRNA and protein levels. Further, we demonstrate that β-1 integrin decreased and although bacterial adhesion was not affected a clear down-regulation of invasion was noted. No direct antibacterial effect was observed, nor did LPva induce antimicrobial peptides.

LPva seems to have both an apoptotic activity, clearing infecting bacteria at the same time protecting the deeper tissue layers by down-regulating β-1 integrin and bacterial invasion.

6 DISCUSSION

Pregnant women are more prone to UTI compared to their non-pregnant counterparts and kidney involvement may be associated with poor-pregnancy outcomes. Due to limited access to microbiological services in developing countries, antibiotics are commonly dispensed for UTI based on clinical symptoms. Given the rising burden of antimicrobial resistance globally, alternatives to antimicrobial agents require further inquiry. This thesis compared the virulence characteristics and antimicrobial susceptibility of *E. coli* causing UTI in pregnant women in Uganda, Vietnam to those from Sweden (paper I), evaluated leucocyte esterase, nitrite, dipslide and microscopy against urine culture in the diagnosis of UTI (paper II). The roles of IL-8, sCD14, vitamin D, vitamin D-regulated antimicrobial peptides (paper III) and LPva on the pathogenesis of UTI (paper IV) were investigated.

Pathogenic *E. coli* compared to commensals express more adhesins, toxins and immune evasion mechanisms (Welch, Burland et al. 2002). It is postulated that presence of virulence factors enable ExPEC to harmlessly colonize the gut of warm-blooded animals from which it causes disease in the urinary tract, lungs or meninges (Diard, Garry et al. 2010). There is a preponderance of capsule, iron-acquisition and pathogenicity islands in *E. coli* belonging to phylogenetic group B2 (Johnson and Stell 2000). However, presence of a high number of virulence factors comes with a fitness cost since there is an inverse relationship between virulence factor score and antibiotic resistance (Moreno, Prats et al. 2006). Nonetheless, a proportion of isolates from phylogenetic groups D, B1 and A that possess the requisite virulent factors are able to cause disease (Kudinha, Johnson et al. 2013). Among patients with voiding dysfunction or vesico-ureteric reflux, less virulent strains of *E. coli* efficiently colonize the urinary tract as would virulent strains in patients without abnormalities (Johnson, Roberts et al. 1987). Pregnancy-related voiding dysfunction and vesico-ureteric reflux is partly due to the increasing size of the uterus (Law and Fiadjoe 2012). Similar to other investigators, in paper I, majority of UPEC in pregnant women from Vietnam and Sweden belonged to phylogenetic group B2 and D (Picard, Garcia et al. 1999, Clermont, Bonacorsi et al. 2000). Although more isolates from Uganda belonged to phylogenetic group B1, they didn't differ in their virulence compared to those of phylogenetic group B2 in terms of adhesion and invasion but formed less biofilm. UPEC form biofilm, modify host response following TLR4 activation and sequester iron at low concentrations as part of the survival mechanisms in the host (Cirl, Wieser et al. 2008). *TcpC* hinders recruitment of adaptor proteins to the cytoplasmic domain of Toll/Interleukin 1 receptor potentially modifying host cytokine response and promoting pathogen survival. $IroN_{E\,coli}$ apart from being a siderophore receptor, it also promotes biofilm formation (Magistro, Hoffmann et al. 2015). Given the dominant presence of virulence factors among *E. coli* isolates of

phylogenetic group B2 (Johnson, Owens et al. 2005), we found a strong correlation between presence of both *tcp*C and *iro*N$_{E\ coli}$ with phylogenetic group B2.

The high prevalence of antibiotic resistance observed among Ugandan isolates compared to those from Sweden may in part be explained by the high frequency of phylogenetic group B1 (Cooke, Smith et al. 2010). Conversely, antibiotic consumption exerts selective pressure on bacteria leading to emergence of resistant strains (Andersson and Hughes 2010). A correlation between antibiotic consumption and prevalence of drug resistance has been established (Bergman, Nyberg et al. 2009). Moreover, in both Uganda and Vietnam antibiotics are overused, occasionally sub-optimally and can be dispensed over-the-counter (Mukonzo, Namuwenge et al. 2013, Nguyen, Thi Do et al. 2013). Antibiotic overconsumption may contribute to the differences in drug resistance between Sweden and Uganda or Vietnam.

UTI is the most common indication for antibiotic prescription during pregnancy (Petersen, Gilbert et al. 2010). Notwithstanding the therapeutic effects of antibiotics in UTI, their use in pregnancy is associated with untoward long-term effects in childhood like cerebral palsy and asthma (Kenyon, Pike et al. 2008, Metsala, Lundqvist et al. 2015). Generally, 4% - 10% of pregnant women develop UTI (Foxman 2002) while a slightly higher prevalence is reported in some parts of Africa (Hamdan, Ziad et al. 2011, Alemu, Moges et al. 2012), in paper II, the prevalence of significant bacteriuria among pregnant women with UTI symptoms was 4%. Because antibiotics were dispensed in lieu of presence of symptoms, 96% of pregnant women with UTI symptoms received them unnecessarily. We report massive antibiotic misuse where clinical rather than bacteriological culture is used in the diagnosis of UTI.
Nitrofurantoin remains largely effective against uropathogens and recommended as alternative first line therapy where cotrimoxazole resistance is high (Nicolle 2003, Komp Lindgren, Klockars et al. 2015). Its use is associated with heamolytic anaemia in patients with glucose-6-phosphate dehydrogenase (G6PD) deficiency (Youngster, Arcavi et al. 2010).
Cephalosporin antibiotics are safe in pregnancy but the current magnitude of antimicrobial resistance and its potential to increase poses serious global health challenges (Levy and Marshall 2004).

Antibiotic resistance is driven by over-use, procuring antibiotics over-the-counter and contamination of water sources (Nordmann, Naas et al. 2011). The factors that drive antimicrobial resistance are rife in Africa and Asia; correspondingly, they carry a higher burden of antibiotic resistance compared to developed countries (Rosenthal, Bijie et al. 2012).

Empiric management of uncomplicated UTI in women is a cost-effective intervention (Barry, Ebell et al. 1997, Le and Miller 2001). Especially when more than 80% of the most prevalent uropathogen is susceptible to the recommended antibiotic. However, in Uganda, 72% and 66% of *E. coli* were resistant to trimethoprim and amoxicillin respectively. Therefore, pregnant women with UTI symptoms suffer a double tragedy; 96% receive antibiotics unnecessarily while 4% with UTI get ineffective antimicrobial agents.

In paper II, we investigated the diagnostic accuracy of nitrite, leucocyte esterase, dipslide and microscopy among participants with or without lower UTI symptoms. Nurses, gynaecologist and clinical microbiologist evaluated nitrite, leucocyte esterase and dipslide in different study settings and participants. Negative nitrite and leucocyte esterase have high accuracy in ruling out UTI among pregnant women (Deville, Yzermans et al. 2004) however, their sensitivity and specificity are variable in different patient groups (Williams, Macaskill et al. 2010). It is therefore likely that they may have limited application in general outpatient settings. Surprisingly, the nitrite test was negative among pregnant women with or without UTI symptoms while the sensitivity of the leucocyte esterase was very low to be clinically useful. Microscopic pyuria has been traditionally used in urinalysis to diagnose UTI. Despite improvements like automation (Sterry-Blunt, K et al. 2015), urine microscopy remains a poor surrogate marker of UTI (Kupelian, Horsley et al. 2013).

There are conflicting reports about the clinical usefulness of dipslide in screening for ABU and diagnosis of UTI (Greeff, Jeffery et al. 2002, Anacleto, Resontoc et al. 2009). However, in 87% and 79% of urine samples read by the gynaecologist and nurses respectively, *E. coli* was correctly identified. The duration of training and expertise accumulated by readers trained-on-job influenced the accuracy of dipslide. Dipslide could potentially bridge the diagnostic gap in resource-limited settings but requires training and supervision of frontline medical workers to ensure accurate diagnosis.

Vitamin D is one of the main regulators of calcium homeostasis but it also modulates both innate and adaptive immune responses (Hewison 2010). There are few dietary sources of vitamin D with majority being synthesized by the skin after exposure to UVB light (Holick 1996). Therefore, skin pigmentation, use of sunscreens, residence in temperate climates and consumption of vitamin D-fortified foods are factors that influence serum vitamin D levels (Webb, Kline et al. 1988, Martin, Gowda et al. 2016). During pregnancy, the fetus entirely relies on the mother for both calcium and vitamin D and there is strong correlation between maternal and cord vitamin D levels (Hanson, Anderson-Berry et al. 2016). Like other authors (Cross,

Hillman et al. 1995, Ardawi, Nasrat et al. 1997), we found progressive increase in serum vitamin D with advancing gestational age and serum vitamin D levels were significantly higher in the third compared to the first trimester. Maternal and fetal serum vitamin D correlate and fetal serum vitamin D correlates with calcium (Hashemipour, Lalooha et al. 2013). Although, absorption of calcium from the gastro-intestinal tract increases throughout pregnancy, 80% of calcium in the fetal skeleton is deposited during the third trimester (Kovacs 2001). It is therefore postulated that the progressive increase of vitamin D with advancing gestational age possibly exerts anti-inflammatory effects to sustain pregnancy and increases production of antimicrobial peptides. Indeed, low serum vitamin D in pregnancy is associated with increased risk of preterm birth (Bodnar, Platt et al. 2015).

Cervical ripening that occurs before the initiation of labour is an inflammatory process (Sennstrom, Ekman et al. 2000). It involves degradation of collagen fibres by the neutrophil-derived matrix metalloproteinase 8 (Sennstrom, Brauner et al. 2003). Serum and cervical IL-8 levels are elevated before cervical ripening and potentially predict preterm birth (Dowd, Laham et al. 2001, Tornblom, Klimaviciute et al. 2005, Shahshahan and Hashemi 2014).
In paper III, we found significantly lower levels of IL-8 in the third compared to the first trimester, the low serum IL-8 levels in the third trimester may protect against preterm birth. We demonstrate for the first time that elevated vitamin D levels significantly decrease IL-8 in uroepithelial cells (paper III). In line with our results, vitamin D was reported to decrease IL-8 secretion also in enterocytes (Hidaka, Wakabayashi et al. 2013).

In addition to their barrier function, bladder epithelial cells mediate innate immune responses. They recognize bacterial LPS using TLR4 eventually leading to IL-8 production. CD14 is necessary in recognition of bacterial LPS in macrophages (Jiang, Georgel et al. 2005), however; its role at the bladder epithelium remains ambivalent. It is claimed that bladder epithelial cells don't endogenously produce CD14 (Backhed, Meijer et al. 2002) and that type 1 fimbriae are essential in recognition of LPS among CD14 negative cells (Hedlund, Frendeus et al. 2001) while membrane-bound CD14 has been identified on different bladder epithelial cell lines (Schilling, Martin et al. 2003, Chromek, Stankowska et al. 2005). Despite disagreement on the isoform of CD14 present on bladder epithelial cells, it remains essential in the host response to bacterial LPS. CD14 lacks VDRE in its promoter region but vitamin D modulates its transcription through transcription factor Sp-1 (Moeenrezakhanlou, Nandan et al. 2008). In paper III, sCD14 didn't increase with gestational age and serum vitamin D had no effect on CD14 expression neither on mRNA nor protein levels. This could be protective against preterm birth

since CD14 facilitates LPS recognition leading to elevated levels of IL-8 – a mediator of cervical ripening.

Close proximity of the urinary tract to the highly colonized gastro-intestinal tract in women notwithstanding, the prevalence of UTI remains low. Largely due to mechanical flow of urine, presence of type 1 fimbriae-neutralizing proteins likes Tamm Horsfall and antimicrobial peptides. Antimicrobial peptides including LL-37 and hBD-2 are constitutively produced at epithelial surfaces and upon stimulation in response to bacterial infection. In pregnancy, maternal and fetal LL-37 levels are positively correlated, however, serum levels LL-37 are higher among newborn babies delivered vaginally compared to those delivered by elective caesarean section (Mandic Havelka, Yektaei-Karin et al. 2010). LL-37 is also present in vernix caseosa potentially protecting the newborn against skin infections (Akinbi, Narendran et al. 2004). Moreover, antimicrobial peptides act synergistically to guard against infectious pathogens (Chen, Niyonsaba et al. 2005). We found significantly higher serum LL-37 but not hBD-2 in the third compared to the first trimester among pregnant women. This is in keeping with correspondingly high levels of vitamin D in the third trimester, however, it may indicate that LL-37 is more important in protection against infections or modulating the immune response during parturition.

The dearth in discovery of new antimicrobial agents in the face of a worsening global epidemic of antibiotic resistance calls for critical evaluation of medicinal herbs for their disease-modifying potential. Herbs have long been used for their medicinal value however; their mechanisms of action remain poorly understood. Recently, medicinal herbs have been found to exhibit antimicrobial, anti-inflammatory and biofilm inhibiting properties (Cheong, Lee et al. 2011, Thompson, Meah et al. 2013, Samoilova, Muzyka et al. 2014). Candidate medicinal herbs include those that have a long history of use in various communities, anecdotal reports of their effectiveness or continued use despite advent of modern therapeutic agents. In paper IV, we evaluated the effects of LPva on the pathogenesis of *E. coli* UTI using an *in vitro* model.

As part of the pathogenesis of UTI, UPEC invade bladder epithelial cells through the interaction of bacterial type 1 fimbriae and host β-1 integrin (Martinez, Mulvey et al. 2000, Eto, Jones et al. 2007). Intracellularly, they form intracellular bacterial communities (IBC) that are resistant to antimicrobial agents and are shielded from innate effector molecules. In cells deeper to the terminally differentiated bladder epithelium, non-replicating *E. coli* are enclosed in vesicles. These are referred to as quiescent intracellular reservoirs (QIR), however, with epithelial turnover, bacteria emerge to infect the bladder (Mysorekar and Hultgren 2006). QIR are therefore thought to be the hallmark of recurrent infections that are common in women (Robino, Scavone

et al. 2014). Conversely, as part of the host response to *E. coli* adhesion, apoptotic shedding of bladder epithelial cells occurs to reduce the bacterial load as cell-associated bacteria are washed out during micturition (Mulvey, Lopez-Boado et al. 1998). LPva had no antibacterial effect nor interacted with type 1 fimbriae. Nonetheless, it significantly reduced invasion of bladder epithelial cells and induced apoptosis in *vitro*. Similar effects were reported for *Citrus reticulata* (Vollmerhausen, Ramos et al. 2013). We report a potential mechanism of action that could be beneficial in UTI however, the application LPva in *vivo* requires further evaluation.

UTI in pregnant women is of great public health importance since it can be complicated by involvement of the kidneys. This exposes the mother to not only poor pregnancy outcomes but life-threatening septicemia (Wing, Fassett et al. 2014). In pregnancy, UTI-like symptoms are common non-specific complaints. Therefore, clinical diagnosis of UTI is largely inaccurate and could lead to antibiotic misuse. Host factors like vitamin D and antimicrobial peptides potentially modulate the host response to infection. However, optimization of antimicrobial peptides in the management of infectious diseases is still challenging because they also modulate other cellular responses (Fjell, Hiss et al. 2012). Medicinal herbs have variously been found to modify infectious disease processes (Mwitari, Ayeka et al. 2013) but their toxicity profiles and interaction with prescription medicines remains to be elucidated (Alissa 2014).

7 CONCLUSION

The following conclusions are drawn from our results:-

1. There is a high prevalence of antibiotic resistance among *E. coli* isolates from Uganda that manifests as ESBL and MDR.

2. Limited access to or affordability of diagnostic services leads to massive antibiotic misuse in pregnant women.

3. Vitamin D and LL-37 increased while IL-8 decreased with advancing gestational age

4. LPva has beneficial effect against *E. coli* infection by preventing bacterial invasion and inducing apoptosis in uroepithelial cells in *vitro*.

8 FUTURE PERSPECTIVES

The antimicrobial agents that are recommended for empiric management of UTI have limited effectiveness. Although the national clinical guidelines are regularly revised, there is need to have sentinel surveillance of antimicrobial resistance at regional hospitals to inform policy. This could be achieved by bacterial culture of urine specimens, identification of causative organisms and antimicrobial susceptibility testing. Additionally, isolates should be stored in both regional and national centers for evaluation of the genetic basis of their resistance phenotype.

Maternal sepsis is among the leading causes of mortality and *E. coli* is the main causative organism especially among women with urinary tract infection. However, in low and middle-income countries, characterization of the risk factors of maternal sepsis, causative organisms and their susceptibility to antimicrobial agents has not attracted much attention. Moreover, little is known about the sources of these microbes and their relationship to the host and environment.

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