



**Are Beta Defensin 1 and Beta Defensin 2 Key Innate Immune Effector  
Peptides against Urinary Tract Infection in Women?**

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# Abstract

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## Introduction & Hypothesis

Recurrent urinary tract infection (rUTI) is a debilitating problem affecting 5% of women. Current treatment using intermittent or long-term antibiotics gives limited symptomatic benefit and encourages bacterial resistance. The aetiology of rUTI is unclear but may involve altered innate defence mechanisms in susceptible individuals.

Colonisation of vaginal mucosa by uropathogenic *Escherichia coli* (UPEC) is the initiating event for UTI, with subsequent migration up the urethra and attachment to bladder epithelium. Protective innate immunological mechanisms include epithelial synthesis of cationic antimicrobial peptides (AMPs) such as the beta defensins. These may be expressed constitutively or induced via Toll-like-receptor (TLR) activation by pathogen associated molecular patterns (PAMPs).

This study investigates the hypothesis that women suffering from rUTI have altered tolerance to infecting bacteria related to differences in expression of endogenous AMPs and that identification of such deficiency gives a potentially useful opportunity for novel preventive therapy.

## Methods and Patients

A synergistic methodological approach of *in vitro* modelling with validation in *clinical* samples from the relevant patient group and controls was used.

*In vitro*, cell culture was carried out using RT4 immortalised urothelial-cells, VK2 E6/E7 immortalised vaginal cells and finite primary culture of normal human urothelium. Cells were challenged with *E. coli* and PAMPs. Assays for Beta defensin AMP gene expression, secretion and antimicrobial activity were carried out.

*Clinically*, 98 women (60 rUTI, 38 controls) were recruited with ethical approval. All subjects provided symptom and health state questionnaires; blood for single nucleotide polymorphism (SNP) analysis; vaginal and bladder biopsies for AMP gene expression analysis; plus vaginal washings and overnight urine samples for AMP peptide secretion assays.

## Results

*In vitro*, cell culture experiments demonstrated that beta defensin 1 (BD1) was constitutively expressed and secreted in urothelial and vaginal cells. Beta defensin 2 (BD2) expression and secretion was induced by *E. coli* flagellin and is a potent antimicrobial against UPEC. In vaginal cells, BD2 expression and secretion was enhanced by estrogen.

*Clinically*, women with rUTI were identified as having significantly lower basal levels of vaginal BD2 expression and secretion than controls but no difference in BD1 expression. Postmenopausal women had significantly lower BD2 levels than pre-menopausal women. During active UTI, women with history of rUTI and the TLR5<sup>392Stop</sup> SNP showed significantly lower BD2 expression and secretion in both the bladder and vagina than women with wild type TLR5 gene and rUTI.

## Discussion

This study identifies flagellin induced BD2 expression as a novel and important urogenital innate immune response against invading *E. coli*, which is reduced in a significant proportion of women with rUTI particularly those with the TLR5<sup>392Stop</sup> SNP. Observations *in vitro* on the BD2 inducing effect of estrogen, and *clinically* in pre- and post-menopausal women, raise the possibility that BD2 expression can be modulated by exogenous factors.

بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ

This doctorate thesis is dedicated to:

My parents, for their patience while rearing me.

My supervisors, for their patience while teaching me.

My wife, for her patience while sharing everything with me.

My children, for their patience when work separates them from me.

“Chance favours the prepared mind.”

**Louis Pasteur**

(1822-1892)

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# Expression of Gratitude

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# Abbreviations:

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A	adenine
Ab	antibody
AMA	anti-microbial assay
AMP	anti-microbial peptide
AP1	activator protein 1
AUA	American Urology Association
BAL	bronchoalveolar lavage
BAUS	British Association of Urological Surgeons
BD	beta defensin
bp	base pairs
BSA	bovine serum albumin
C	cytosine
cDNA	complementary DNA
CF	cystic fibrosis
CFU	colony forming units
Cys	cysteine
Da	dalton
DNA	Deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
ds	double stranded
EAU	European Association of Urology
EC	<i>Escherichia coli</i>
EDTA	ethylenediaminetetracetate
ELISA	enzyme-linked immunosorbent assay
EQ-5D	EuroQol 5-domain
ER	estrogen receptor
F	forward
g	grams
G	guanine
GAPDH	glyceraldehydes-3-phosphate dehydrogenase
gDNA	genomic DNA
HD5	human alpha defensin 5

hr	hour
HNP	human neutrophil peptide
HKG	house keeping gene
HPLC	high pressure liquid chromatography
IPTG	isopropyl $\beta$ -D-1-thiogalactopyranoside
Kb	kilobase
kDa	kilodalton
L	litre
LB	Luria Bertani
LCMS	liquid chromatography mass spectrometry
LL37	cathelicidin
LPS	lipopolysaccharide
M	molar
mg	milligram
min	minute
ml	milliliter
mM	milimolar
mRNA	messenger RNA
MSSU	Mid-stream sample of urine
NCBI	National Centre for Biotechnology Information
NF $\kappa$ B	nuclear factor kappa-light-chain-enhancer of activated B cells
NHU	normal human urothelium
NHS	National Health Service
nM	nanometers
NUTH	Newcastle upon Tyne Hospitals NHS Trust
OD	optical density
PAMP	pathogen associated molecular pattern
PBS	phosphate buffer saline
PCR	polymerase chain reaction
PRR	pathogen recognition receptor
qRT-PCR	quantitative real-time PCR
R	reverse
RNA	ribonucleic acid
RT	reverse transcription
rUTI	recurrent urinary tract infection

SD	standard deviation
sec	seconds
SEM	standard error of the mean
SNP	single nucleotide polymorphism
ST	<i>Salmonella typhimurium</i>
T	thymine
TBE	Tris-borate-EDTA
TLR	toll-like-receptor
Tm	annealing temperature
Tris	tris(hydroxymethyl)methylamine
UK	United Kingdom
UPEC	Uropathogenic <i>Escherichia coli</i>
USA	United States of America
USS	Ultrasound scan
UTI	urinary tract infection
UTISA	UTI Symptoms Assessment
UV	ultra violet
V	volts
v/v	volume to volume
w/v	weight for volume

# 1. Urinary Tract Defence

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The normally sterile urinary tract is constantly challenged by microbes through retrograde urethral spread. A number of defensive mechanisms have evolved in response to this threat comprising physical factors such as urine flow, pH and ionic urinary composition together with innate immune response characterized by the constitutive or inducible expression of host defence peptides with antimicrobial activity. In contrast, adaptive cellular and humoral immune elements are not thought to play a defensive role but remain crucial in combating established infection. Interest in endogenous molecular defence has been stimulated by increasing concern regarding the overuse of antibiotics with the expectation that enhancement of natural mechanisms will be an exciting and novel therapeutic avenue in the management of urinary tract infection (UTI).

## **1.1. The importance of cystitis and urinary tract infection**

Urinary tract infection (UTI) is one of the most common bacterial infections encountered in clinical practice. An estimated 150 million cases of UTI occur annually worldwide resulting in over £4 billion (€5 billion, \$8 billion) of healthcare expenditure (Kucheria *et al*, 2005). Women are most commonly affected with an estimated mean incidence of 0.5-0.7 episodes per year whilst other higher risk groups include children, the elderly, people with structural abnormalities of the urinary tract and those having urinary tract intervention such as catheter insertion (Foxman, 2002).

The incidence of UTI increases with age and the cumulative probability of a woman having had a UTI by the age of 50 is approximately 50% (Foxman *et al*, 2000). The normally sterile urinary tract is the site of an on-going, but complex interplay between

an evolving pathogen and a highly developed host immune defence system such that the pathogenesis of UTI generally requires either greater virulence in the pathogen or deficient host immune defences. Typically the process of infection begins with attachment of the uropathogen to the epithelial surface, it subsequently forms colonies which then disseminate and invade through the urothelial tissue. This dissemination is usually associated with ascent up the urinary tract which may manifest symptomatically as cystitis (in the bladder) or pyelonephritis (in the kidney). This bacterial process in turn evokes a powerful immune response and the interplay between pathogen and host continues, influencing the extent and level of invasion as well as the duration of infection and extent of tissue damage caused (Kaper *et al*, 2004).

The occurrence and severity of UTI can be related to genetic, hormonal and behavioural factors together with the virulence of the invading organism, chiefly uropathogenic *Escherichia coli* (UPEC), (Foxman, 2002). Although these infections rarely cause long term damage they are associated with a high burden of troublesome symptoms and healthcare costs, and also give a major contribution to the problem of nosocomial infection. Current treatment consisting of preventive behavioural advice and intermittent or long-term use of antibiotics, (Franco, 2005), gives limited symptom benefit and encourages emergence of resistant organisms - consequently there is a real need for alternative therapies (Stapleton, 1999).

## **1.2. Routes of Infection**

The ascending route is the commonest mode of infection of the urinary tract with most bacteria originating from the individual's own lower bowel and subsequently



colonising the periurethral tissue before ascending through the urethra and into the bladder (Handley *et al*, 2002). Colonisation of the periurethral mucosa with bowel flora is particularly problematic in females where the shorter urethra provides a convenient conduit for invading pathogens and rapid entry to the lower urinary tract. Even small variations in perineal anatomy in females can increase susceptibility; Hooton and colleagues for example demonstrated increased risk of female UTI where anal to urethral distance was less than 4.5 cm (Hooton *et al*, 1999). These anatomical risks can be further increased by the influence of external agents such as spermicides, faecal contamination of the perineum and the use of urethral catheters (Hooton *et al*, 1996; Foxman, 2002).

UTI is most frequently present only in the bladder (cystitis) but up to a half of infections may demonstrate some signs indicative of upper urinary tract involvement (Busch and Huland, 1984). Pyelonephritis is usually caused by ascent of bacteria from the bladder up the ureter and into the renal pelvis from where there is subsequent invasion of the renal parenchyma through the collecting ducts and disruption of the renal tubules. Certain pathogenic virulence factors including P-fimbriae and endotoxins can enhance the ability of bacteria to ascend the urinary tract as can host factors such as pregnancy and ureteral obstruction as these conditions inhibit ureteral peristalsis.

Haematogenous infection of the urinary tract is uncommon in normal individuals. However, patients with primary foci of infection elsewhere in the body involving *Staphylococcus aureus*, *Candida spp.*, *Salmonella spp.* and *Mycobacterium tuberculosis* can cause secondary infection in the kidney. The risk of such infection is enhanced when urine drainage from the kidney is obstructed (Smellie *et al*, 1975). Infection via the lymphatic route is rare, but can be caused by direct invasion of bacteria from

adjacent organs in conditions that result in retroperitoneal sepsis and suppuration. The lymphatic route is not thought to play a significant role in the majority of UTIs.

### **1.2.1. Urinary Pathogens**

The organisms that cause cystitis are surprisingly limited to a small number of bacterial species, which include *Escherichia coli* isolated in around 75-90% of cases (Ronald, 2002) and *Staphylococcus saprophyticus*, a skin commensal, isolated in around 5-10% of cases (Jordon *et al* 1980). Other enterobacteriaceae such as *Proteus sp.* and *Klebsiella sp* are also occasionally isolated as are gram-positive cocci such as *Enterococcus faecalis* (Naber *et al* 2008). Positive cultures in cystitis usually consist of a single organism, but occasionally two organisms may be isolated such as when a gram positive like bacteria *Enterococcus faecalis* is isolated with a gram-negative uropathogen. Any more than two strains would suggest external contamination and repeat culture is required.

### **1.3. Pathogen Virulence Factors**

As mentioned previously, at the heart of UTI, is the interplay between host and pathogen. UPEC strains are highly adaptable encoding a number of virulence factors that can be variably expressed, and which enable the bacteria to colonise the urinary tract and persist in the face of host defences. In recent years, great strides have been made in the understanding of these virulence factors. Prior to their migration, these bacteria will typically have come from a commensal site such as bowel where they do not generally have pathogenic effects. The role of virulence factors is therefore critical in the understanding of how commensals at one site act as pathogens at another.

UPEC strains exhibit a high degree of genetic diversity facilitated by the possession of specialised virulence genes. These are located on specific transferable genetic elements known as pathogenicity islands which may be as large as 170 kb and can increase the size of the pathogen genome by about 20% over a commensal strain (Wiles *et al*, 2008, Oelschlaeger 2002). Virulence factors of UPEC may be broadly divided into two groups according to whether they are involved in bacterial adhesion or not.

### **1.3.1. Adhesive Virulence Factors**

The presentation of cell surface adhesive organelles (adhesins) by UPEC is one of the most significant determinants of pathogenicity. UPEC may express several adhesins that allow it to attach to urinary tract tissues (Mulvey, 2002). UPEC adhesins can contribute to virulence in different ways which include: directly triggering host and bacterial cell signalling pathways, facilitating the delivery of other bacterial products to host tissues, and promoting bacterial invasion (Mulvey, 2002). The best characterised type of adhesin is the fimbriae.

#### ***Type 1 fimbriae***

Type 1 fimbriae are the most commonly expressed fimbriae on *E. coli* and are composed of a helical rod with repeating FimA subunits that are bound to a distal tip structure containing the FimH adhesin (Jones *et al*. 1995). Classically these fimbriae (also called type 1 pili) were shown to mediate hemagglutination of guinea pig erythrocytes (Duguid *et al*, 1979) and the reaction could be inhibited by the addition of mannose, hence the term mannose-sensitive hemagglutination (MSHA) (Svenson *et al*, 1983; Reid and Sobel, 1987).

However, while type 1 fimbriae have been shown to function as virulence factors in animal models of urinary tract infection where they facilitate bacterial colonisation, their function in human infection remains less clear (Hultgren *et al*, 1985; Connell *et al*, 1996; Mulvey *et al*, 2002; Emödy *et al*, 2003; Bergsten *et al*, 2007). The key difficulty with understanding their role lies in the observation that type 1 fimbriae are expressed in both pathogenic and commensal strains (Duguid *et al*, 1979; Hagberg *et al*, 1981), and furthermore there is no significant difference in Fim gene frequency between more or less virulent strains in the urinary tract (Plos *et al*, 1991). In the murine model, type 1 fimbriae bind to the urothelial mannosylated glycoproteins Uroplakin Ia (UPIa) and IIb (UPIb) via the adhesin subunit FimH, located at the fimbrial tip (UPIb) (Wu *et al*, 1996). Uroplakins are membrane proteins that are found on umbrella cells, which line the luminal surface of the urinary bladder. The interaction between FimH and the Uroplakins stimulates signalling pathways involved in invasion and apoptosis, and may also contribute to mucosal inflammation (Connell *et al*, 1996; Martinez *et al*, 2000; Schembri *et al*, 2001; Oelschlaeger *et al*, 2002; Thumbikat *et al*, 2009). In humans, the main evidence for the role of type 1 fimbriae has been obtained from the analysis of urinary bacterial isolates from patients with UTIs, which were found to express mannose-sensitive adhesins (Ljungh and Wadstrom, 1983).

Murine studies show that after binding to the urothelial surface, bacteria with FimH adhesins are quickly internalised as a result of localised actin rearrangement and engulfment of the bound bacterium by changes in the cell membrane around the bacteria (Martinez and Hultgren, 2002). Within the superficial urothelium, UPEC is able to establish a new niche that functions as a mechanism to avoid the host innate immune response (Anderson *et al*, 2004). Within the cell, UPEC proliferates in the

cytosol to form clusters known as intracellular bacterial communities (IBCs) (Anderson *et al.* 2004). After six to eight hours, the morphology of the bacteria changes to an engulfing 'biofilm' phenotype that further protects the uropathogen from host's immune response (Justice *et al.* 2004)

The switch to a biofilm phenotype is marked by a significant decrease in the rate of bacterial growth allowing the formation of a 'biofilm matrix'. This matrix is able to prevent the host's neutrophils from penetrating its surface and engulf the bacteria, and is also effective at preventing both host antimicrobials and external antibiotics from gaining access to the bacteria. Animal models also suggest that bacteria at the edge of IBCs are able to detach and become motile again to re-enter the urine and then re-adhere to the superficial urothelium, and reinvade cells to form further IBCs (Mulvey *et al.*, 2001). Ultimately, after a few days, possibly as a result of ongoing immune activity, the invasive bacteria enter quiescent state but persist in a dormant state in IBCs before re-emerging at a later to cause recurrent infection (Anderson *et al.*, 2004).

### ***P fimbriae***

The next most common UPEC virulence factor are the P fimbriae which mediate hemagglutination of human erythrocytes that is not altered by mannose and is thus termed mannose-resistant hemagglutination (MRHA). These fimbriae are believed to play a key role in ascending UTI and pyelonephritis (Leffer *et al.*, 1981, Vaisanen *et al.*, 1981). P fimbriae are heteropolymeric fibres made up of various peptides encoded by the papA-K gene (Hull *et al.*, 1981). The adhesin PapG, at the tip of these fimbriae recognise kidney glycosphingolipids carrying the Gal  $\alpha$  (Kaper *et al.*, 2004, Wiles *et al.*, 2008) Gal determinant on renal epithelia (Kaper *et al.*, 2004). The attachment of P

fimbriae, leads to the release of ceramide, which acts as an agonist of Toll-like receptor 4 (TLR4), a receptor involved in activation of the innate immune response including cytokines and antimicrobial peptides (Fischer *et al*, 2007). This then activates an inflammatory response and the associated pain of UTI (Bergsten *et al*, 2005).

P fimbriae may also work synergistically with type 1 fimbriae; with the former enhancing early colonisation of the tubular epithelium, while the latter mediates colonisation of the centre of the tubule by forming a biofilm. This colony then disrupts tubular filtration leading to obstruction of nephron and the symptoms of pyelonephritis (Melican *et al*, 2011). P fimbriae have also been implicated as one of the key virulence factors involved in acute kidney dysfunction within renal transplant patients (Rice *et al*, 2006).

### ***Other adhesins***

S fimbriae and F1C fimbriae have also been shown to play a role in UTI. S fimbriae bind to sialic acid residues via the SfaS adhesin, this facilitates bacterial dissemination within host tissues and is often associated with *E. coli* strains that cause sepsis, meningitis, and ascending UTIs (Mulvey, 2002). F1C fimbriae bind to glycosphingolipids in renal epithelial cells and induce an interleukin-8 inflammatory response (Backhed *et al*, 2002).

UPEC also expresses a group of afimbrial Afa adhesins, which have been clustered with the fimbrial Dr adhesin family due to similarity in structure plus their ability to bind to decay-accelerating factor (DAF) and type IV collagen in the kidney. Both fimbrial Dr and afimbrial Afa adhesins of *E. coli* are associated with UTIs, particularly recurrent infection and infection during pregnancy (Foxman *et al*, 1995; Garcia *et al*, 1996; Nowicki *et al*, 1990; Servin *et al*, 2005). Murine models have demonstrated that Dr

adhesins display a tropism to the basement membrane of the renal interstitium which is critical for the development of chronic pyelonephritis (Goluszko *et al*, 1997). Adhesins of the Afa family also demonstrate a unique renal tissue tropism; experimental findings suggest that UPEC strains with Afa adhesins are more likely to result in chronic or recurrent infection (Foxman *et al*, 1995, Bouguenec 2005).

### **1.3.2. Non-adhesive Virulence Factors**

While much attention is focussed on the adhesive virulence factors as many are relatively specific to UPEC, like other gram negative bacteria, UPEC has cell wall modifications, motility enhancements and secreted toxins that further enhance its pathogenicity.

#### ***Polysaccharides***

Both the bacterial capsule and the lipopolysaccharide (LPS) can also act as virulence factors. The capsule is a polysaccharide covering for the bacteria, which protects it from the host immune system in particular phagocytic engulfment and complement-mediated attack. Certain capsular types such as K1 and K5, mimic components of host tissue and can thus prevent an effective humoral response (Johnson *et al*, 1991).

LPS is a core component of the cell wall of Gram-negative bacteria; the LPS of UPEC is important in activation of a proinflammatory response in UTIs via the induction of nitric oxide as well as antimicrobial peptide and cytokine production (Bäckhed *et al*, 2001). However, the systemic immune response evoked by UPEC LPS may also have detrimental effects by causing acute kidney injury particularly in renal transplant patients with UTI (Wolfs *et al*, 2002, Samuelsson *et al*, 2004). Animal experiments

suggest that this systemic response and the consequential kidney injury are independent of the TLR4 LPS receptor mediated response (Cunningham *et al*, 2004).

### ***Flagellum***

The flagellum is a macromolecular organelle complex that protrudes from the exterior of the bacterial outer membrane up to 15 $\mu$ M long and is responsible for bacterial motility (Macnab *et al*, 2003). Its activity is linked to chemotactic receptors that sense environmental, chemical and physical information and stimulate motility when required for growth or survival (Berg *et al*, 2003). Structurally, it consists of a protein called flagellin and its shape is helical consisting of 3-parts: the motor, hook, and filament. The motor, also known as the body, is embedded in the cell envelope spanning both the inner and outer membranes. Its rotation is powered by the diffusion of protons from the periplasm into the cytoplasm (Sowa *et al*, 2008). The hook consists of a flexible curved helical structure 55 nm long made up of multiple copies of the FlgE flagellin protein which are formed into helical protofilaments (DePamphilis *et al*, 1971). The hook couples the motor to the filament which consists of a single flagellin protein, FliC, made into a rigid protofilaments, 10 to 15  $\mu$ m long that function as a propeller (Berg *et al*, 2003). An *E. coli* cell typically has between 4 and 10 flagella which are configured in a Peritrichous configuration i.e. they come together to form a bundle at the posterior pole when swimming. A change of direction in the bacterium can be achieved by one or more motors reversing direction and breaking from the bundle, causing the bacterium to re-orientate (Darnton *et al*, 2007).

Animal models of UPEC infection have indicated a significant role for flagellum in both lower and upper urinary tract infection. In upper tract infection flagella may allow bacteria to ascend from the bladder and cause pyelonephritis. These UPEC strains may



invade renal collecting duct cells through flagellin, with flagellin acting as an invasin in this process (Pichon *et al*, 2009). Other work in the murine model has suggested that mice deficient in TLR5 (the flagellin receptor) are more susceptible to UPEC infection in both the bladder and the kidney suggesting that flagellin may be involved in the original ascent into the bladder (Andersen-Nissen *et al*. 2007).

### 1.3.3. Secreted factors

Like other gram negative bacteria, toxins secreted by UPECs often contribute to or are responsible for inflammatory responses and symptoms. The most significant of these secreted toxins is a lipoprotein called  $\alpha$ -haemolysin (HlyA), which is frequently associated with pyelonephritis and renal scarring (Moblely *et al*, 1990). Alpha-haemolysin is a pore-forming toxin of the RTX (repeats in toxin) family that are common amongst gram-negative pathogens (Bhakdi *et al*, 1986; Eberspacher *et al*, 1989). At high concentrations it lyses erythrocytes and host cells, enabling UPECs to cross epithelial barriers, damage immune cells, and gain access to host iron stores (Cavalieri *et al*, 1984; Keane *et al*, 1987; Johnson *et al*, 1991). At low concentrations, it can induce apoptosis of host immune cells and promote the exfoliation of bladder epithelial cells (Russo *et al*, 2005; Chen *et al*, 2006; Smith *et al*, 2006) It can also affect intracellular calcium levels in renal epithelial cells, with consequent increases in IL-6 and IL-8 production (Uhlén *et al*, 2000).

Another secreted protein involved in ascending infections is cytotoxic necrotising factor 1 (CNF1), which is produced by around one-third of all pyelonephritis strains (Landraud *et al*, 2000, De Rycke *et al*, 1999). Experimental data suggests that CNF1 has effects on the epithelial cell membrane which enhance the ability UPEC to invade cells (Bower *et al*, 2005). In addition the protein has been shown to interfere with

polymorphonuclear phagocytosis and cause the death of bladder epithelial cells by apoptosis, which in turn increases bladder exfoliation and the exposure of underlying cells. (Mills *et al*, 2000; Fiorentini *et al*, 1997).

Other secreted proteins include secreted autotransporter toxin (SAT) and Toll/interleukin (IL-1) receptor (TIR) domain-containing protein (Tcp). *In vitro*, SAT has toxic activity against bladder and kidney cells suggesting a role in early pathogenesis of UTI (Guyer *et al*, 2002). Tcp is a more recently discovered type of virulence factors, which is able to subvert epithelial toll like receptor (TLR) signalling preventing early initiation of the innate immune response thereby facilitating bacterial survival and continuing infection (Cirl *et al*, 2008).

#### **1.4. Host Defences against UPEC Colonisation**

The normal urinary tract has a number of physiological and immunological defences geared towards preventing bacterial colonisation. First and foremost is the 'washing' effect of the flow of urine. Both the flow through the urinary tract and the 'bulk disposal' at the time of micturition helps rinse away loosely adherent or non-attached pathogens from the epithelial surface (Sobel *et al*, 1997). Adherence is further limited by the secretion of glucosamines by the urothelium which forms a mucous-type layer. In addition, the high concentrations of urea, salts and low pH due to organic acids make it difficult for poorly-adapted bacteria to survive.

Within the urine there are also a number of larger proteins which have been identified as important in the innate defence of the urinary tract. Of these, perhaps the most well-known is Tamm-Horsfall protein (THP), which is a glycoprotein secreted by epithelia lining the loop of Henlé and is present at high concentrations in the urine.

THP acts as an anti-adhesive urinary factor by binding to UPEC expressing type 1 fimbriae and forming the complex, which is then cleared by voiding (Serafini-Cessi, 2003). THP knockout mice have been shown to clear *E. coli* less rapidly than wild-type mice and go on to develop chronic bladder wall inflammation suggestive of persistent infection (Bates, 2004). The epithelial proteins lactoferrin and lipocalin also show antimicrobial activity through the sequestering of iron. Lipocalin knockout mice were more susceptible to systemic infection from *E. coli* (Berger *et al*, 2006). Lactoferrin is expressed in the distal collecting tubules and deposited on the luminal surface. It inhibits bacterial growth both by chelation of iron and membrane damage but does not appear to be induced by active infection (Abrink *et al*, 2000). Antimicrobial peptides are also secreted by urothelium in response to pathogens (Ali *et al*, 2009). These peptides work in a non-specific manner by attachment to the anionic phospholipids on the cell wall of pathogens and disruption of their cell membrane function, increasing cell permeability and causing cell death (Ganz, 2003). This non-specific action makes them a powerful first line defence against new pathogens and they have antimicrobial action against bacteria, fungi, and some encapsulated viruses (Ganz, 2003).

#### **1.4.1. Antimicrobial peptides**

##### ***Classification***

Antimicrobial peptides (AMPs) are a ubiquitous component of innate immune defence and are expressed by neutrophils or epithelial cells either constitutively or induced by exposure to pathogen. They consist of 15–45 amino acid residues and most have an overall positive charge. This cationic state is a key functional attribute common to most AMPs and results from numerous arginine and lysine residues. There are,

however, a small number of AMPs that are rich in glutamic and aspartic acids and consequently have a negative charge.

AMPs can be categorized into 4 groups according to amino acid composition, structure and size using nuclear magnetic resonance (NMR) with over 100 different entities described throughout the animal kingdom (Table 1.1).

**Table 1.1 Classes of antimicrobial peptides**

Group	Examples	Structure	Amino acid content	Human forms
Linear cationic $\alpha$ -helical	Cecropins Magainins Cathelicidin	Linear helical	No cysteine residues	LL37
Cationic peptides enriched for specific amino acids	Apidaecins Drosocins Indolicidin PR-39 Histatin	Linear extended helical	Rich in certain amino acids e.g. proline, arginine or typtophan but no cysteine residues	Histatin
Cationic peptides enriched for specific amino acids	Defensins Protegrins	$\beta$ -sheet structures which are stabilized by 2 or 3 intramolecular disulfide bonds	Cysteine residues	$\alpha$ and $\beta$ defensins
Anionic peptides	Maximin Dermicidin	small peptides with one intramolecular disulfide bond, characteristically near the C-terminus	Rich in glutamic and aspartic acids	Dermcidin

### ***Evidence for antimicrobial activity***

Both *in vitro* and *in vivo* models have provided compelling evidence that AMPs protect against a range of micro-organisms including bacteria, enveloped viruses, fungi and some protozoa (Zaiou, 2007). Much of the evidence relates to lower order animals which lack adaptive immunity and hence are totally reliant on innate mechanisms such as AMP expression for survival. Translation to human physiology can be difficult since

genome location, structure and activation from pro-peptides differ from other species, even within the mammalian kingdom such as rodents.

### ***Mechanisms of action***

Although the exact way in which AMPs kill microorganisms is not fully understood, it is known that their distinct structure containing both a charged, hydrophilic and a non-charged, lipophilic segment facilitates cell membrane disruption. Predilection for microbial rather than native cell membranes may relate to differing arrangements of charged and non-ionic lipid components of the membrane bi-layer (Tytler *et al*, 1995. Ludtke *et al*, 1996). Bacterial membranes tend to be rich in negatively charged lipids with phospholipid head groups such as phosphatidylglycerol on the outer surface, encouraging attachment of cationic peptides. Mammalian membranes in contrast have mainly neutral lipids such as phosphatidylcholine and also contain cholesterol, both tending to discourage cationic peptide attack (Shai, 1999). Many studies have demonstrated that the bacteriocidal activity of AMPs relates to their secondary structure, charge and hydrophobic nature which govern interaction with cell membranes (Mor, 2000; Wiepricht *et al*, 1997). A number of models of this interaction have been proposed. The barrel and stave model suggests that  $\alpha$ -helical peptides can form channels by boring through the bacterial cell membrane using the hydrophobic domain and then bind together like the vertical staves of a barrel with their hydrophilic domains orientated to the cell interior. Increasing polymerization enlarges the size and number of membrane pores resulting in cell lysis (Matsuzaki *et al*, 1998). The carpet model holds that the peptides coat the outer bacterial cell membrane in a carpet-like fashion and interaction between the hydrophobic domains and the lipid bilayer causes it to deform and disintegrate (Mor, 2000; Dagan *et al*, 2002). Another

possible mechanism of cell lysis is the toroidal pore model. Here AMPs encourage membrane permeabilisation by acting as bridges between the exterior and cytoplasmic membrane layers. Interaction between the membrane lipids and the peptide forms a channel through which ions can escape upsetting homeostasis and eventually leading to cell lysis (Matsuzaki *et al*, 1997). Other mechanisms for AMP activity under investigation include activation of autolysis as suggested in studies on bovine seminal plasmin, (Shai, 1999) together with non-lytic mechanisms such as inhibition of protein synthesis, degradation of proteins required for DNA replication and interference with the transport and energy metabolism of bacterial cells (Tossi *et al*, 2000; Sochacki *et al*, 2011).

It is perhaps not surprising that a number of resistance mechanisms have been developed by certain bacteria to protect against AMPs including altered cell surface charge, active efflux and peptide inactivation by trapping or protease digestion (Peschel, 2006). Consideration of these potential inactivating mechanisms will be an important part of harnessing the therapeutic potential of AMPs.

### ***Antimicrobial peptides in the urinary tract***

To date, four groups of AMPs have been identified within the genito-urinary tract. The evidence for these AMPs is discussed below.

#### **Alpha defensins**

Defensins, characterized by a 15-20 amino acid sequence including 6 cysteine residues, have received most attention in terms of human AMP research. The  $\alpha$ -defensins, HNP1 to HNP4 (Human Neutrophil Peptides) are primarily found in neutrophils where they provide non-oxidative antimicrobial activity. A clinical study found an 8-fold increase in urinary concentration of HNP-1 in patients with chronic pyelonephritis compared to

both normal controls and patients with glomerulonephritis. The rise in HNP-1 correlated with similar changes in urinary levels of the cytokine, interleukin-8 (IL-8) together with the leukocyte count (Tikhonov *et al*, 1997). No microbiological information was provided in this study however. The concentration of HNP1-3 has been shown to be elevated in vaginal washings from women during episodes of vaginitis/cervicitis compared to healthy controls (Bevins *et al*, 2006). In this study HNP content was two-fold higher for mixed or group B Streptococcal infections whilst levels in women with chlamydial infection did not differ from control.

The expression and function of epithelial human  $\alpha$ -defensins, HD5 and HD6, has been most characterized in the small intestine where they are secreted by Paneth cells into the intestinal crypts. Research findings indicate a key role in maintaining the numerical and species balance of intestinal flora together with a protective function in maintaining stem cell activity (Wiechula *et al*, 2007). HD5 has also been demonstrated in the female urogenital tract with highest concentrations identified in the vaginal epithelium and associated secretions. Here it is thought to form an intrinsic component of the innate immune defence system against infection by Chlamydia with its expression modulated by hormonal and pro-inflammatory factors (Quayle *et al*, 1998). More recently, Spencer *et al* (2012) demonstrated that HD5 is expressed in the urothelium of the lower urinary tract and the kidneys with evidence of induction in the kidney during pyelonephritis.

In addition to the antibacterial effects of HD5, interesting findings have also been made in relation to its antiviral action against BK virus, a polyoma virus that establishes a lifelong persistence in most humans and is a major problem in immunocompromised patients with kidney transplants. Dugan *et al* (2008) demonstrated that HD5 inhibited

BK virus by interacting with it directly and inducing aggregation of virions. The authors speculate that HD5 may also help clear BKV infection in urinary tract tissue during reactivation.

### **Beta defensins**

Beta-defensins are widely expressed throughout human epithelia. Whilst over 30 gene loci are believed to exist (Schutte *et al*, 2002), predominantly clustered on chromosome 8p23, only a few human  $\beta$ -defensins; HBD1 to HBD4, have been studied in depth. Expression of a variety of HBDs has been demonstrated throughout the urinary tract by protein and RNA molecular identification techniques but their functional roles remain uncertain. Apart from their characterized antimicrobial activity  $\beta$ -defensins have also been implicated in iron metabolism and tumour surveillance (Leong *et al*, 2004). In the kidney, HBD1 has been shown to be constitutively expressed by epithelial cells lining the loop of Henlé, distal tubule and collecting duct (Valore *et al*, 1998). Despite this, urine levels of HBD1 are insufficient to lyse invading bacteria. It is probable that there is a significantly higher concentration of HBD1 at the luminal surface of the tubular epithelium which, in conjunction with other antimicrobials, provides a fast-acting antimicrobial coating, destroying microbes that manage to gain attachment and thus preventing invasion. One could describe this layer as an 'antimicrobial paint' protecting the epithelial surface beneath. In contrast, expression of HBD2 is not found in normal kidney, but is induced by chronic renal infection suggesting that it is involved as a second line defence perhaps in co-operation with other induced mediators such as cytokines (Lehmann *et al*, 2002). The most convincing evidence that defensins play a direct role in the defence of the kidney against infection is provided by studies using knock-out mouse models. In one study, animals with



deletion of the murine HBD1 gene (*Defb1* -/-) showed a 30% increase in bacteriuria compared to wild-type mice (*Defb1* +/+) (Morrison *et al*, 2002).

The same group that demonstrated HBD1 secretion in the kidney also showed expression in the female urogenital tract (endocervix, ectocervix, vagina) and isolated peptides from vaginal lavage (Valore *et al*, 1998). More recently expression of HBD1-4 mRNA has been detected in endometrium and it has been shown that each defensin has a unique temporal expression profile (Fleming *et al*, 2003; King *et al*, 2003). HBD1 and 3 are expressed at highest levels during the secretory phase. This is similar to the pattern of expression described above for the alpha defensin, HD5 (Peschel, 2006). In contrast, HBD2 mRNA expression shows a dramatic peak during menstruation while HBD4 is expressed mainly in the proliferative phase.

It has been noted that within the HBD gene locus there are significant copy number polymorphisms (CNP) and that mRNA concentration correlates with copy number. This contention is supported by findings in patients with Crohn's disease affecting the colon where the gene copy number of HBD2 is lower than normal (Fellerman *et al*, 2006.) In addition to copy number, intragenic single nucleotide polymorphisms (SNP) have been reported. It is therefore possible that variations in amino acid content and structure of expressed peptide could affect host antimicrobial defence capability and may increase susceptibility to disease such as UTI

### **Cathelicidin**

The human cathelicidin, LL37 is an alpha-helical AMP expressed on all epithelial surfaces and by circulating neutrophils (Agerberth *et al*, 2000). Its transcription is determined by a single gene – *CAMP*, situated at 3p21 and its expression in most epithelial sites is induced by local injury or infection. There is limited but reasonably

convincing evidence that it has a role in human urinary defence against bacterial invasion. An investigation into the expression of cathelicidin in the human kidney suggested that it was induced to high levels when a microbial presence is detected in the urinary tract (Chromek *et al*, 2006). In support of this the normally low urinary levels of LL-37 were increased in children with UTI. The cathelicidin found in urine was thought to be of epithelial origin as levels did not correlate with urinary leucocyte count. This is supported by localization studies on renal biopsies which showed continuous synthesis of LL-37 by tubular epithelium with subsequent release into the lumen. The inducibility of LL-37 gene expression was demonstrated by exposure of renal tissue to UPEC. In these experiments cathelicidin mRNA rose to maximum levels within 5 minutes and continued at supra-normal levels for up to 2 hours, accompanied by a surge in peptide secretion. These findings suggest that the cathelicidin defence mechanism is designed to facilitate both an immediate and sustained response to bacterial threat.

The same study provided further evidence for the role of cathelicidin using a mouse model to investigate the activity of the murine cathelicidin homologue, CRAMP. In these experiments, a large inoculum of UPEC was used to overwhelm the defences of the lower urinary tract and induce severe pyelonephritis. The results showed that in early infection cathelicidin was secreted by renal tubular epithelium whilst in more advanced stages this was supplemented by release from leukocytes – suggesting a dual source for urinary cathelicidin in the mouse. Deletion of the CRAMP gene in knockout mice resulted in a greater and more rapid bladder colonisation and invasion together with higher rates of ascending infection. Although these results require confirmation

by others they do provide a powerful rationale for the possible therapeutic exploitation of LL-37 in the treatment or prophylaxis of UTI.

### 1.4.2. Toll like receptors

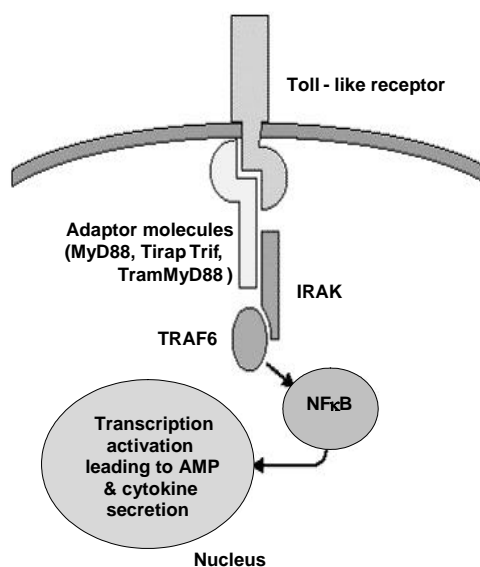
The ability of epithelium of the urogenital tract to mount a response to invading pathogens is highly dependent on the presence of immune surveillance molecules. In common with other epithelial surfaces, the lining of the urinary tract contains receptors capable of pathogen recognition via a set of specific pathogen associated molecular patterns (PAMPS), these receptors are known as the Toll-like receptor (TLR) family (Samuelsson *et al*, 2004; Adersen-Nissen *et al*, 2007). TLRs are type I trans-membrane proteins which structurally contain large, leucine rich, repeat domains in the extracellular region and a Toll/IL-1 receptor homology (TIR) domain in the cytoplasmic region. Each TLR shows responsiveness to a different microbial component and activation leads to production of innate immune effectors such as cytokines and AMPs. Other cellular responses are also induced, most particularly of chemotaxis of innate immune cells involved in regulating inflammation and adaptive immunity (Beutler *et al*, 2003). The primary receptors expressed on human urothelium are TLR 2, 4 and 5. TLR 2 is activated by peptidoglycan or lipotechoic acid, part of the cell wall of bacteria. TLR 4 and its co-receptors (CD14 and MD2) recognise bacterial LPS and TLR 5 is activated by flagellin.

#### ***Signalling***

Activation of TLRs by PAMPs triggers conformational changes in the receptor to allow recruitment adaptor molecules to the TIR Toll/interleukin-1 receptor (TIR)-domain of the TLR. Several adapter molecules are recognised but the most significant are MyD88 and TIR-domain-containing adaptor protein-inducing interferon (IFN)- $\beta$  (TRIF) (Akira *et*

*al*, 2006). The differential immune responses mediated by distinct TLR ligands are partly dependent on selective usage of these adaptor molecules in that MyD88 and TRIF activate distinct signalling pathways. The MyD88 pathway causes early activation of NF- $\kappa$ B (via several adaptor molecules) which then translocates into the nucleus and initiates expression of cytokine and AMPs (see Figure 1.1) whereas the MyD88-independent, TRIF-dependent pathway initiates expression of type I interferons and late activation of NF- $\kappa$ B (Akira *et al*, 2006).

**Figure 1.1 TLR simplified intracellular signalling pathway**



Activation of cell surface TLR recruits cytoplasmic adaptor molecules to propagate signal. These proteins, identified as MyD88, Tirap(Mal), Trif and TramMyD88, interact with TLR through Toll/IL-1receptor domain and in turn engage serine-threonine kinase(IRAK) through a death domain. Signal transduction factors such as TRAF6 carry signals through series of phosphorylations until NF $\kappa$ B is ultimately released to enter nucleus where it can activate transcription of appropriate immune response genes resulting in production of AMPs and cytokines.

### ***Toll-like receptor 4 (TLR4)***

TLR4 is the most studied of the TLRs and is well expressed on epithelial cells of the kidney and bladder (Samuelsson *et al*, 2004). TLR 4 and its co-receptors (CD14 and

MD2) recognise bacterial LPS. In the mouse urinary tract, its activation has been shown to promote a florid innate immune response against Gram negative bacteria but when the gene is knocked out, (TLR4<sup>-/-</sup>) mice develop an asymptomatic carrier state (Schilling *et al*, 2001). As well as the conventional cytokine and AMP responses, a more novel function of TLR4 has been recognised by Soman Abraham's group in North Carolina who demonstrated a cAMP regulated mechanism by which TLR4 is able to expel UPEC from infected bladder epithelial cells (Bishop *et al*, 2007).

The significance of TLR4 has been further highlighted by the effect of gene polymorphisms. In mice, polymorphisms of the TLR4 gene are associated with reduced sensitivity to LPS, absence of neutrophil recruitment, and delayed clearance of bacteria from the urinary tract (Haraoka *et al*, 1999). A role for polymorphisms in the TLR4 gene has also been suggested in human where a TLR4 polymorphism has been shown to increase susceptibility to septic shock and Gram-negative bacteraemia (Lorenz *et al*, 2002). Other studies have suggested that reduced TLR4 expression may play a role in promoting a clinically beneficial tolerance state in asymptomatic bacteriuria rather than a more harmful situation of severe disease (Ragnarsdottir *et al*, 2010). Nonetheless, there is still some uncertainty regarding the nature of expression of TLR4 in the human bladder as work by Jenny Southgate's group in York, UK demonstrated that finite cultured normal human urothelium NHU cells neither bound nor responded to LPS (Smith *et al*, 2011). Within the vagina also, there are also differing reports in relation to TLR4 expression. Fazeli *et al* (2005) investigated TLR expression in tissue samples from the uterine tubes, endometrium, endocervix, ectocervix and vagina of nine patients and found no expression of TLR4 in the vagina. In contrast, Pivarsci *et al* (2005) demonstrated the presence of TLR4 in both vaginal tissue and the PK vaginal

cell-line, and more recently, Dusio *et al* (2011) demonstrated the presence of TLR4 at protein and RNA level in the VK2 vaginal cell-line too.

### ***Toll-like receptor 5 (TLR5)***

TLR5 is the specific receptor for the bacterial flagellin (Hayashi *et al.* 2001). Its potential importance was first recognised in studies on TLR5 knockout mice (TLR5<sup>-/-</sup>). These mice, when challenged with the CFT073 UPEC strain, initially showed decreased inflammation compared to controls as an early response after challenge but subsequently demonstrated reduced bacterial clearance and a delayed inflammatory response (Andersen-Nissen *et al*, 2007). In the same year Lane *et al* (2007) demonstrated that while wild-type UPEC were able to establish infection in both the bladder and kidney by 6 hours post-inoculation, flagellin-mutant bacteria were only able to effectively colonise the bladder with limited presence and activity in the kidney at that early time point. Subsequently, by 48 hours post-inoculation, flagellin-mutant bacteria were attenuated in both the bladder and kidneys while wild type still demonstrated high numbers at both sites. The authors suggested that these data provided compelling evidence that flagellin was a key factor in the ability of UPEC to cause infection. The two studies together suggest a critical role in the innate immune response to UPEC.

TLR5 has also been of specific interest from genetic association studies in humans. In these studies, a common single nucleotide polymorphism (SNP) causing a premature stop codon (TLR5<sup>392STOP</sup>) has been identified which causes loss of the trans-membrane domain of TLR5 protein and the entire signalling cytoplasmic tail (Hawn *et al.* 2003). Population studies have demonstrated that this SNP is linked to susceptibility to Legionnaire's disease, a type of pneumonia caused by the flagellated bacterium

*Legionella pneumophila* (Hawn et al. 2003) and recurrent cystitis (Hawn et al. 2009). However, population studies have also shown that the SNP confers resistance to two autoimmune disorders: Crohn's disease (Gewirtz et al, 2006) and Systemic Lupus Erythematosus (SLE) (Hawn et al, 2005).

TLR5<sup>392STOP</sup> has been shown to cause a loss of function of TLR5 in an autosomal-dominant fashion and is found in geographically different populations at frequencies between 5% and 10% (Hawn et al. 2003, 2005; Gewirtz et al. 2006). This is a surprisingly high population frequency for a potentially detrimental SNP and indeed there is evidence that the SNP is actually being positively selected (Wlasiuk *et al*, 2009). One proposed explanation is that there may be some as yet unrecognised evolutionary advantage associated with defective TLR5-mediated signalling (Hawn et al, 2005). Irrespective of any potential advantage, the presence of this common SNP makes TLR5 particularly interesting from a UTI point of view.

### ***Toll-like receptor 2 (TLR2)***

TLR2 recognizes cell-wall components such as peptidoglycan, lipoteichoic acid and lipoprotein from Gram-positive bacteria. As well as TLR4, TLR2 expressed in tubular cells may also play a role in defending the upper urinary tract against UPEC. Stimulation of renal tubular cells *in vitro* by TLR2 ligands results in the secretion of inflammatory mediators and chemokines (Tsuboi *et al*, 2002). It has been suggested that TLR2 and TLR4 may work in a synergistic manner to produce a stronger response in the presence of ligands for both TLRs, particularly where the ligand for TLR2 is from a Gram positive bacteria and the ligand for TLR4 from a Gram negative (Amdekar *et al*, 2011). This synergy may provide a rationale for the use of probiotics (Amdekar *et al*, 2011) or other agonist agents that help enhance the TLR4 response. In the vagina,

Dusio *et al* (2011) demonstrated the use of low molecular weight hyaluronic acid (LMW-HA) as a non-bacterial agent to induce higher levels of antimicrobial secretion and enhanced wound repair through stimulation via TLR2 and TLR4.

### ***Toll-like receptor 11 (TLR11)***

TLR11 has been described in the urinary tract of mice where it seems to play a role in preventing UTI (Zhang *et al*, 2004). Mice lacking TLR11 are highly susceptible to pyelonephritis induced by UPEC indicating a potentially important role in preventing ascending infection. However, in humans, the gene for TLR11 contains a premature stop codon resulting in a non-functional peptide and therefore is unlikely to play a significant role.

### **1.4.3. Activated Responses**

If a microbe evades first line innate defences, contact with the urothelium results in the activation of further host immune defence mechanisms, which include the exfoliation of bladder cells and induction of an inflammatory response.

### ***Exfoliation of Infected Cells***

One of the most notable observations of the host response during a UTI is the disruption of the epithelial barrier by exfoliation of infected cells (Fukushi *et al*, 1979, Mysorekar *et al*, 2002). In the absence of infection the urothelium is relatively quiescent and the umbrella cell layer is renewed every few months. However, the normally repressed proliferation and differentiation cascades are rapidly activated by the FimH component of fimbriae that results in an exfoliation mechanism, which involves activation of caspases and cysteine proteases in a pathway that is similar to apoptosis (Mulvey *et al*, 1998, Klumpp *et al*, 2001). Following activation of this



pathway, there is potential for the umbrella cell layer to regenerate within 24 hours of the exfoliation process.

The significance of this response has been highlighted by experiments in which the exfoliation mechanism has been dampened using a pan-caspase inhibitor. The effect is to greatly reduce bacterial expulsion from the bladder in early infection, which in turn allows bacteria to escape from dying superficial cells and go onto infect other cells (Mulvey 2001). The net effect is to enhance dissemination of the bacteria but potentially also allows access to underlying cells where bacteria can persist in long term reservoir colonies. In mouse studies, a mild exfoliation process in response to UPEC was more likely to result in a biofilm-producing state allowing bacteria to migrate into deeper layers (Anderson *et al.* 2004b). Consequently, it is clear that exfoliation is a key mechanism in the eradication of both attached and internalised bacteria from urothelium.

### ***Inflammatory Response***

In addition to triggering exfoliation, bacterial adherence to urothelium or the detection of bacterial products also induces a variety of other innate immune responses. Such responses are characterised by the production of a number of proinflammatory mediators, including cytokines and chemokines (Agace *et al.*, 1993; Schilling *et al.*, 2001; Schilling *et al.*, 2003). Bladder and kidney epithelial cells appear to be a major source of interleukin-6 (IL-6) and interleukin-8 (IL-8) after infection with UPEC, which are important in the development of local tissue damage (Hedges *et al.*, 1991; Agace *et al.*, 1993; Otto *et al.*, 1999; Jantusch *et al.*, 2000; Gabay 2006). IL-6 possesses a variety of proinflammatory functions, including activation of signals involved in neutrophil recruitment and production of acute phase proteins (Gabay 2006). IL-8 is also a potent

neutrophil chemotactic molecule. In humans, the induction of IL-8 after infection with UPEC correlates with appearance of neutrophils in the urine (Agace *et al*, 1993). Neutrophil recruitment to the site of infection has been shown to be critical for bacterial clearance from both the bladder and kidney, and the presence of neutrophils in the urine is a hallmark of UTIs. Other immune cells such as macrophages, eosinophils and natural killer cells are also recruited and granulocytes synthesise nitric oxide which has a toxic effect on the invading bacteria (Poljakovic *et al*. 2001; Poljakovic and Persson 2003;).

Neutrophils play a key role in the early inflammatory response as they migrate towards the site of infection. This process is initiated by specific bacterial components which activate pathogen-associated molecular pattern receptors (PAMPs) such as the Toll-like receptors (Albiger *et al*, 2007; Song and Abraham, 2008). As discussed above, activation of these receptors triggers a signalling pathway which initiates inflammatory and antimicrobial response from the immune system.

### ***Antibody Response***

Due to the relatively short duration and adaptability of pathogens, the mainstay of the immune response to UTI is the innate response. However, in ascending infections of longer duration, the adaptive immune response is activated with the production of high affinity antibodies by B and T lymphocytes. In pyelonephritis there is immunoglobulin synthesis within the serum and kidney with antibodies targeting type 1 and P fimbriae detectable in serum as well as IgG and IgA antibodies in the urine (Rene *et al*. 1982). Local synthesis of these antibodies enhances opsonisation and reduces adherence of *E. coli* (de Ree and van den Bosch 1987). The presence of these antibody responses has led to attempts to create vaccines against fimbrial components

of the bacteria in an attempt to reduce colonisation and ascending infections in susceptible female patients (Uehling *et al*, 1994).

## 1.5. Implications for Recurrent UTI

The therapeutic potential of AMPs in preventing or treating infection has been clear since the discovery of their bactericidal properties, but development has been given added impetus by the realisation of increasing adverse effects and short-term usefulness of traditional exogenous antibiotics. One area where it is hoped that a better understanding of AMPs may provide new therapeutic options is in the troublesome area of recurrent urinary tract infection (rUTI). Recurrent UTI represents a common, debilitating and difficult-to-treat problem affecting 5% of women and increasing in prevalence with age (Wild *et al*, 2005). Current treatment consists of preventive behavioural advice and intermittent or long-term use of antibiotics (Franco, 2005). This gives limited symptom benefit and encourages emergence of resistant strains of the main pathogen, *E. coli* so there is consequently a real need for alternative therapies (Stapleton, 1999). It is possible that an increased understanding of the role and activity of AMPs in the urinary tract could result in therapeutic strategies to reinforce innate defences and condition the vaginal, urethral or bladder mucosa to hinder migration of bacteria and subsequent infection.

### **Note**

Some sections of this chapter have formed part of a published review article: **Maintaining a sterile urinary tract: the role of Antimicrobial Peptides.** AS Ali, CL Townes, J Hall and RS Pickard. *Journal of Urology* 2009; 182: 21-8.

Other sections have formed part of a textbook chapter written by Ased Ali for: **“The Oxford Textbook of Urological Surgery”**, edited by Professor Freddie Hamdy and Mr Ian Eardley, Oxford University Press, *for publication in 2014.*

## 2. Recurrent Urinary Tract Infection

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Lower urinary Tract Infection (UTI) or cystitis is generally regarded as having recurred if a second symptomatic infection follows clinical resolution of an earlier infection, although there is not a universally accepted definition. A practical working categorisation is the occurrence of at least two episodes of acute uncomplicated cystitis within a 6-month period, or at least three episodes in a 12-month period, or at least two episodes per year for 2-years (Franco, 2005; Schoof and Hill 2005; Hooton and Stamm 2006).

Recurrent cystitis is very common among young healthy women and is rarely associated with any anatomical or functional abnormality of the uro-genital tract. In the 1970s, Mabeck found that almost one-half of the women whose initial uncomplicated UTIs resolved spontaneously developed a recurrent UTI within the first year (Mabeck 1972). In a Finnish study of women aged 17–82 years who had *Escherichia coli* cystitis, there was an overall recurrence rate of 44% within 1 year with rates of 53% in women older than 55 years and 36% in younger women (Ikaheimo *et al* 1996). It should be noted that to date no large population based studies have been performed to determine what proportion of women with cystitis develop a pattern of repeated frequent recurrences over longer periods.

### 2.1. Aetiology

Recurrent cystitis occurs in one of two situations:

- Bacterial persistence i.e. infecting bacteria re-emerge from a reservoir within the uro-genital tract

- Re-infection i.e. new infection from bacteria outside the urinary tract (bowel or skin flora).

Bacterial persistence is typically characterised by infection with the same organism recurring at very short intervals by. In contrast, re-infections usually occur at more varied longer intervals which can be up to several months and may not necessarily be caused by the same organism. It is important to distinguish between these two categories since identification and removal of reservoirs of infection may resolve the problem. Re-infection, while more common in women than bacterial persistence, is a more complex situation where a single remediable abnormality is much less likely to be found. In men, recurrent UTI is less common and more likely to be associated with underlying abnormality such as bladder outlet obstruction.

## **2.2. Bacterial Persistence**

### **2.2.1. Diagnosis**

Once acute cystitis has resolved and there is no further microbiological evidence of bacteriuria, it is possible under certain circumstances for the organism to 'hide' within a part of the urinary tract or vagina which was exposed to less high concentrations of antimicrobial chemotherapy. The specific abnormalities that can result in this situation are outlined in Table 2.1.

**Table 2.1 Urinary Tract Abnormalities Causing Bacterial Persistence**

Anywhere in urinary tract	Stones
	Foreign bodies
Renal	Infected atrophic kidney
	Medullary sponge kidney
	Papillary necrosis
	Infected renal calyceal cyst
Ureter	Ureteral duplication
	Ectopic ureter
	Non-refluxing, infected ureteral stump
Bladder	Infected urachal cysts
	Perivesical / pelvic abscess
Prostate	Chronic bacterial prostatitis
Urethra	Urethral diverticula

Urinary culture is useful in the detection of *Proteus mirabilis*, the commonest organism linked with the presence of infection stones (although most patients with *P. mirabilis* cystitis do not go on to form infection stones). *P. mirabilis* can cause significant alkalinisation of the urine with precipitation of calcium, magnesium, ammonium, and phosphate salts and the subsequent formation of branched struvite (triple phosphate) renal stones. This has serious consequences as bacteria can persist inside struvite stones even when the urine shows no growth. Consequently, struvite infection stones are a possible cause of bacterial persistence in women.

Many of the other abnormalities can be identified by a combination of imaging and endoscopic evaluation of the urinary tract. Although conventional intravenous urography may still be used, CT KUB (non-contrast) and CT urography (late phase contrast-enhanced) and cystoscopy provide the most sensitive investigation

(particularly as Struvite stones are often relatively radiolucent), retrograde urography and ureteroscopy are also useful in some situations.

### **2.2.2. Treatment**

Where a reservoir for persistent infection is identified, the standard treatment is to remove the foreign body or correct the anatomical abnormality. Most of the abnormalities listed in Table 2.1 will require surgical intervention to facilitate removal and eradication of the source of bacterial persistence. For struvite infection stones, complete removal of the calculus either by percutaneous nephrolithotomy, ureteroscopic lithotripsy, extracorporeal shockwave lithotripsy, or rarely open removal, is needed for bacteriological cure and to prevent renal damage due to obstruction (Silverman and Stamey 1983). However, management with continuous antibiotic prophylaxis and acidification of the urine provides some symptomatic relief and may slow deterioration in renal function. Chronic bacterial prostatitis is usually treated initially with penetrative long course antibiotic therapies but transurethral resection may be helpful in some cases (Barnes *et al* 1982).

Where the reservoir of infection cannot be removed, long-term, low-dose antibiotic treatment may be the only option to suppress bacterial growth and prevent symptoms. Agents typically recommended for this use are nitrofurantoin and trimethoprim. Other drugs such as cefalexin, and the fluoroquinolones are effective but their use is avoided where possible due to higher risk of ecological bacterial change.

## **2.3. Re-infection**

### **2.3.1. Diagnosis**

Recurrent infections occurring at longer intervals or involving different species/type of bacteria are the hallmarks of re-infection and the diagnosis is therefore typically made on the basis of history, examination and urine culture. This pattern is more commonly seen in females of all ages and is usually secondary to infection by ascending bowel flora particularly coliforms. Though the ascending route is by far the most common route, fistulas (enterovesical or vesiovaginal) or other structural abnormalities are still important possibilities to consider particularly where other risk factors are present such as diverticulitis, and previous surgery or radiotherapy. In men, re-infection is more frequently associated with structural or functional abnormality impeding bladder emptying.

As in the case of bacterial persistence, it is important to identify and correct urinary tract structural and functional abnormalities where they exist. Any abnormality which either reduces the formation of urine or disrupts its orderly progress through the urinary tract can increase the risk of re-infection and limit the effectiveness of antibiotic therapy. Imaging typically by ultrasound is useful to demonstrate the anatomy of the urinary tract and indirectly indicate the emptying ability of kidneys, ureter and bladder. Cystoscopy should also be performed if symptoms are suggestive of persistent mucosal irritation (stones or cancer), obstruction (urethral stricture), bladder dysfunction (diverticula), and fistula.

### **2.3.2. Treatment**

Initial management should be directed towards identifying and where possible modifying any of the reversible risk factors shown in Table 2.2. Consequently, in



patients with diabetes, glycaemic control should be optimised. In women using spermicides, a diaphragm or depot hormonal contraception, alternative forms of birth control should be explored. Where an indwelling catheter or intermittent catheterisation is used, catheter management should be reviewed. In postmenopausal women, the local effects of reduced oestrogen should be examined and local replacement considered. In elderly patients, perineal hygiene, general hydration, and both faecal and urinary incontinence should be managed.

**Table 2.2 Risk Factors for Cystitis**

Sexual activity – increased inoculation
Urinary incontinence – increased inoculation
Faecal incontinence or constipation– increased inoculation
Spermicide – increased binding
Oestrogen depletion – increased binding
Antimicrobial agents – decreased commensal ‘healthy’ flora
Inadequate fluid uptake (dehydration) – decreased urine flow
Diabetes – enhanced medium for growth

General advice in terms of increased fluid intake, use of sanitary towels instead of tampons, post-coital micturition, and avoiding the use of soaps in the vaginal area is also often given, though evidence of benefit is weak (Remis *et al* 1987; Strom *et al* 1987; Foxman and Chi 1990).

When an infection occurs it should be treated with a full (three or seven-day) course of an appropriate antibiotic (as in the case of acute uncomplicated cystitis). After the resolution of an acute episode, there are several evidence-based antibiotic strategies which may be employed to prevent recurrences: long-term prophylaxis, post-coital

prophylaxis, and patient-initiated therapy as well as some alternate, non-antibiotic based strategies with weaker evidence of effectiveness.

## ***Antibiotic Strategies***

### **Low-Dose Continuous Prophylaxis**

Long term prophylaxis usually requires a single daily dose of antibiotic (typically taken at bedtime). The success of prophylaxis is dependent on the ability of an antimicrobial agent to eliminate or suppress proliferation of pathogenic bacteria from the urogenital tract prior to excitement of a host response and without causing the development of significant resistance or change to the ecology of bowel or vaginal commensal flora. Evidence of efficacy exists for the following antimicrobials: trimethoprim, trimethoprim / sulphamethoxazole, nitrofurantoin, cefalexin and fluorquinolones (Kodner and Gupton, 2010; Dason *et al*, 2011). It should be noted that the use of fluorquinolones for this purpose is discouraged by most guidelines due to their broad spectrum.

Long-term prophylaxis typically involves continuing the daily antibiotics for 6–12 months, although this can be extended to several years. It is possible that breaks of 1-2 months every 6 months or the use of alternating agents may reduce the risk of changing resistance patterns although good evidence of effectiveness is lacking. If symptomatic re-infection occurs during prophylactic treatment, then urine should be sent for culture and full therapeutic dosage of another antimicrobial should be used for treatment. Prophylaxis may then be resumed once the infection has resolved provided the culture results do not show the presence of resistance to the prophylactic agent. Long term prophylaxis is effective at preventing recurrences in 95% of patients

whilst they are on prophylaxis. Unfortunately, once prophylaxis is discontinued, around 50% of patients will have a further UTI within 3 months (Nicolle 2002).

As an alternative to these conventional antibiotics described earlier, weak evidence for the use of methanamine hippurate also exists. Methanamine decomposes at an acid pH to formaldehyde and ammonia, and the formaldehyde is bactericidal. Urinary acidity can be ensured by co-administering vitamin C (ascorbic acid) or ammonium chloride. Methanamine is particularly useful for long-term prophylaxis as bacteria do not develop resistance to formaldehyde. However, it should not be used in the presence of renal impairment.

### **Patient-Initiated Intermittent Therapy.**

Patient-initiated intermittent or self-start therapy is a useful alternative to long term prophylaxis. The key aim is to empower the patient to initiate a 3-day course of empirical antibiotic treatment at the first onset of their symptoms or at an 'early warning sign' which is consistent with development of previous episodes of cystitis (Schaeffer and Stuppy 1999; Gupta *et al* 2001). As an adjunct, the patient can be given reagent strips and instructed to also occasionally submit urine for culture when symptoms of infection occur with a subsequent culture a week after treatment to check for microbiological efficacy and changing resistance patterns.

As the antimicrobial agent selected for patient-initiated therapy will be largely used on an empirical basis, it should have a reasonably broad spectrum of activity and achieve high urinary levels to minimise development of resistance while having as little effect as possible on the bowel flora. Nitrofurantoin and trimethoprim are recommended in the UK whilst trimethoprim-sulphamethoxazole is also available in other countries. Agents such as tetracycline, amoxicillin, cefalexin and fluoroquinolones in full doses

should be avoided because they can give rise to resistant bacteria and ecological bacterial change (Wong *et al* 1985). Overall, there is much to commend patient-initiated therapy both from a bacterial point of view as the use of a short full dose course is less likely to result in resistant strains and from a patient point of view where it gives patients with less frequent infections the ability to play an active role in their diagnosis and management.

### **Post-intercourse Prophylaxis.**

In women where sexual intercourse has been identified as ‘triggering event’ in the history, post-intercourse prophylaxis is a useful option (Nicolle 2002). Taking a reduced dose of an antimicrobial agent such as nitrofurantoin 50 mg or trimethoprim 100 mg immediately after voiding post-intercourse can be very effective at reducing the rate of re-infection (Pfau *et al* 1983). Furthermore, studies have shown the efficacy to be comparable to daily prophylaxis with the advantage of fewer side effects as on average only a third of the total dosage of antibiotic is used (Melekos *et al* 1997).

### ***Estrogen Therapy***

Postmenopausal women are recognised as group more prone to frequent re-infections (Raz and Stamm, 1993; Raz *et al*, 2000). While in some cases, pelvic organ prolapse contributes to residual urine after voiding; in others, the lack of estrogen causes marked changes in the vaginal commensal microflora and vaginal pH which leads to reduced *Lactobacilli sp.* and increased colonization by *E. coli* (Melekos *et al* 1997). Estrogen replacement therapy is believed to partially restore the normal vaginal environment which allows re-colonisation with lactobacilli, reduces uropathogenic bacterial colonisation and hence occurrence of UTI (Raz *et al*, 1993). However, the mechanism of action is not typical of a classical endocrine effect. Local oestrogen

reduces UTI occurrence whilst systemic therapy does not (Perrottta *et al* 2008). The vaginal response is also rapid in onset but short-lived, lasting only for the duration of the therapy. The beneficial effects of local oestrogen therapy in recurrent UTI are also seen in younger women using the oral contraceptive without evidence of oestrogen deficiency (Pinggera *et al* 2005). Despite the unclear mechanism, the use of vaginal oestrogen in post-menopausal women is supported by evidence from a meta-analysis performed as part of a Cochrane systematic review and there may also be a role in pre-menopausal women (Perrotta *et al*, 2008).

### ***Other Strategies***

For women with a persistent post-void residual urine (> 100 mL) and evidence of reduced urethra calibre, a single dilation of the urethra under a general anaesthetic to improve bladder emptying may be appropriate. There is no evidence, however, that repeated urethral dilation is beneficial in the routine management of women with recurrent UTI.

Cranberry extract has been a popular preventive method for many years. It is believed to work partly by acidifying the urine, and partly by reducing bacterial adhesion and bacteriuria. Cranberry extract contains proanthocyanidins which has demonstrable *in-vitro* to both competitively inhibit the *E. coli* fimbrial subunit from binding to the uroepithelial cells, and prevent the expression of normal fimbrial subunits (Patel and Daniels 2000). From the clinical viewpoint, in 1994, Avorn *et al* studied 153 women and showed that 300 mL/day of cranberry juice reduces bacteriuria and pyuria by 42% and persistent bacteriuria and pyuria by 72% (Avorn *et al* 1994). However, the proportion of cranberry extract in commercial juices and tablets is highly variable therefore effects are unpredictable. Furthermore, subsequent randomised controlled trials of cranberry

products have not shown benefit and the latest Cochrane systematic review has shown no evidence that they are effective for management of UTI (Jepson *et al* 1998).

**Note**

Some sections of this chapter have formed part of a textbook chapter written by Ased Ali for: "**The Oxford Textbook of Clinical Nephrology (OTCN), Fourth Edition**", edited by Neil Turner, David Goldsmith, Christopher Winearls, Norbert Lameire, Jonathan Himmelfarb, Christoph Wanner, Guisepppe Remuzzi, Oxford University Press, *for publication in 2014*.

## 2.4. Aims of the Study

It is evident from review of the literature that while considerable strides have been made in the scientific understanding of UTI, treatment options for *recurrent* UTI still largely consist of preventive behavioural advice and intermittent or long-term use of antibiotics (Franco, 2005). These give limited symptomatic benefit and given the plasticity and adaptability of *E. coli*, encourage the emergence of resistant strains (Stapleton, 1999), potentially making the condition worse in the long term for both the individual patient and the wider community.

The host aspect of aetiology of rUTI is unclear with genetic, hormonal and behavioural factors all thought to play a part (Stapleton, 1999). The route of infection, however, does seem clear with increased colonisation of vaginal and periurethral mucosa by bowel and skin commensals, leading to migration up the urethra and attachment to the bladder mucosa (Handley *et al*, 2002). Despite this, much of the existing research outlined has focussed on either immune defence of the kidney from ascending infection (Zasloff, 2007), or pathogenic mechanisms of *E. coli* persistence within the bladder once an infection has been established (Andersson *et al*, 2004). There has been comparatively less research into which of the normal innate defence mechanisms have been breached to allow lower urinary tract infection to take hold in the first place. In particular, given the route of infection, it would seem appropriate that in addition to urothelium, the vaginal epithelium should also be considered as part of the first line of *bladder* defence. Studies in other tissues have suggested that epithelial production of cationic peptides such as cathelicidins and defensins are an important aspect of host anti-microbial defence (Boman, 2003) and an improved of

understanding of how these mechanisms protect and in rUTI, *fail to protect*, may give insights in how to prevent rUTI.

The overall aim of this doctoral research study was therefore to compare differences in antimicrobial peptide (AMP) expression and secretion between women with recurrent urinary tract infection (rUTI) and those without focussing on human beta defensin 2 (BD2). It was hypothesised that,

**“Women suffering from recurrent urinary tract infection (rUTI) have altered tolerance to infecting bacteria related to differences in expression of endogenous antimicrobial peptides (AMPs) and that identification of this deficiency represents a potentially useful opportunity for design of preventive or adjunctive treatment.”**

Within this overall aim, the study sought to:

- Determine the pattern of constitutive and inducible expression and activity of selected AMPs in bladder and vaginal epithelium using cultured cell lines. AMPs for analysis were selected on the basis of those already described in the literature and those found on analysis of human tissue samples in the present study
- Evaluate differences in expression and secretion of AMPs in urine and vaginal washings from 2 groups of women recruited to the study (rUTI/no UTI).
- Determine if any clinically identifiable factors influenced differences in AMP expression and secretion.



## 3. Laboratory Materials and Methods

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### 3.1. Tissue Culture

All tissue culture experiments were carried out in the sterile environment of a class II laminar flow cabinet (S@fflow 1.2, BIOAIR, Italy) using aseptic techniques to avoid contamination. A sterile environment was maintained by daily irradiation with UV light and regular spraying with 70% ethanol and TriGene (Starlab, UK). All medium was stored at 4°C and warmed to 37°C prior to use.

#### 3.1.1. RT4 Cells

The immortalised RT4 cell line was derived from a non-invasive papillary transitional cell carcinoma of the bladder at the Medical Research Council Biochemical Genetics Unit, London, UK (Master *et al.* 1986). The cell line was chosen as it is commercially available (ATCC HTB-2) and has been used by our research group previously as it has an antimicrobial peptide expression profile comparable to normal human urothelium (Townes *et al.* 2010).

The RT4 urothelial cell line was maintained without antibiotics in HEPES (4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid) modified RPMI (Roswell Park Memorial Institute) 1640 medium with 2 mM glutamine and 10% foetal calf serum (Sigma, Dorset, UK).

Cells were grown in 75cm<sup>2</sup> flasks (Corning, Tewksbury, Massachusetts, USA) at 37°C in 5% CO<sub>2</sub> in air until confluent at approximately four days (determined by light microscopy). After confluence, they were sub-cultured and re-seeded at a density of

$1 \times 10^6$  cells per flask. Cells from passage 10 to 30 were used for experiments beyond which a new culture was initiated from frozen stocks.

### **3.1.2. VK2 E6/E7 Cells**

The VK2 E6/E7 cell line was developed from vaginal mucosal tissue obtained from a 32-yr-old woman following anterior-posterior vaginal repair surgery and laboratory immortalised using HPV 16 E6/E7 transduction at the Harvard Medical School, Boston, Massachusetts, USA (Fichorova *et al*, 1997). This cell line was chosen as it is the only commercially available vaginal cell line (ATCC CRL-2616).

The VK2 vaginal epithelial cell line was maintained without antibiotics in Keratinocyte Serum-free medium (GIBCO, Paisley, UK) containing 0.4mM calcium with 0.1 ng/ml human recombinant Epidermal Growth Factor (EGF) and 0.05 mg/ml bovine pituitary extract supplements.

Cells were grown in 75cm<sup>2</sup> flasks (Corning, Tewksbury, Massachusetts, USA) at 37°C in 5% CO<sub>2</sub> in air until confluent at approximately seven days (determined by light microscopy). After confluence, they were sub-cultured and re-seeded at a density of  $5 \times 10^6$  cells per flask. Growth medium was replaced every three to four days. Cells from passage 5 to 25 were used for experiments beyond which a new culture was initiated from frozen stocks.

### **3.1.3. Primary tissue**

Primary tissue culture was carried out on my behalf by Claire Varley from Jenny Southgate's group at the University of York. The technique is described in detail in publications by Jenny Southgate's group (Southgate *et al*. 2002 and Smith *et al*. 2011). However, briefly, human ureter tissue removed during routine nephrectomy and

surplus to diagnostic requirements obtained was collected (with informed consent and ethical approval). Ureter used was from patients with no history of urothelial neoplasms and confirmed normal histology. Tissue was transferred from the hospital to the laboratory in transport medium consisted of HBSS (Hank's Buffered Saline Solution) without  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  (HCMF) with 0.01 M HEPES buffer pH 7.6 and 0.04 KIU/mL (Kallikrein Inhibitor Unit per mL) aprotinin.

In the laboratory, serosa, fat and debris were removed from the specimen, which was cut into 1-2  $\text{cm}^2$  pieces. Urothelium was isolated by leaving the pieces incubated overnight at 4°C with 15ml stripping solution that consisted of the transport medium supplemented with 0.1% EDTA (ethylene diaminetetraacetic acid, disodium salt). The tissue was transferred to a Petri dish and, using two pairs of sterile jeweler's forceps, the urothelium was gently separated from the underlying stroma. The urothelium detaches easily as intact sheets of cells. The pieces of stroma were picked out with a wide-tipped sterile plastic Pasteur pipette and the sheets of urothelium transferred into a 10ml centrifuge tube.

The urothelial cell sheets are collected by centrifugation at 250g for 4 min. The supernatant was aspirated and the tube flicked to resuspend the pellet in the residual volume. 2 ml collagenase (200 units of collagenase type 4 in HBSS with 10 mM sterile HEPES at pH 7.6) was added and the cells incubate at 37°C for 20 min. After incubation, 3ml of KSFMc [Keratinocyte Serum Free Medium (GIBCO, Paisley, UK) complete with 5ng/mL Epithelial Growth Factor (EGF) and 50ng/mL Bovine Pituitary Extract (BPE)] was added and the cell sheets disaggregated by gentle pipetting. The cells were once again pelleted by centrifugation and resuspended in a further 5 ml of KSFMc.

A hemocytometer was used to estimate the number of urothelial cells in the suspension, which contained a mixture of single cells and clumps of urothelial cells, erythrocytes, and leukocytes. Detached normal human urothelium (NHU) was cultured in flasks containing KSFMc growth medium at a minimum seeding density of  $4 \times 10^4$  urothelial cells per  $\text{cm}^2$  for primary cell culture and maintained at  $37^\circ\text{C}$  in a humidified atmosphere of 5% (v/v)  $\text{CO}_2$  in air. Medium was replaced after 24-hours and subsequently on alternate days.

Cells were sub-cultured by removing the medium from the flask and incubating the cell monolayer in  $0.2 \text{ ml/cm}^2$  of PBS with 0.1% EDTA solution for 5 minutes at  $37^\circ\text{C}$  until the cells separated. The solution was then aspirated and sufficient Trypsin-EDTA (HCMF with 0.025% Trypsin and 0.02% EDTA) to cover the monolayer and incubated again at  $37^\circ\text{C}$  for around 2-minutes for the cells to detach. 5ml of KSFMc with 2.5mg of trypsin inhibitor is added to the cells which were then centrifuged and resuspended in KSFMc re-seeded at around  $5 \times 10^5$  cells in a T25 culture flask. Cell were used for experimentation between passages 3 and 5. Cytodifferentiation was induced using 5% adult bovine serum and 2mM calcium chloride and verified by qualitative RT-PCR for uroplakin-2 expression.

## **3.2. Bacterial Culture**

### **3.2.1. Strains**

To model the induction of AMPs in a state of urinary tract infection, both RT4 and VK2 cells were initially challenged with three strains of Uropathogenic *E. coli* for varying times up to 24-hours. The strains used were:

- NU14 – a laboratory strain of UPEC with multiple virulence factors derived from a clinical isolate of *E. coli* originally obtained from the urine of a patient with cystitis in 1986 (Hultgren *et al.* 1986).
- NU14-1 – a non-functioning fimH mutant of NU14.
- NCTC 10418 (ATCC 10536) – A long established National Collection of Type Cultures (NCTC) *E. coli* strain used for testing antimicrobial and disinfectant agents originally isolated in 1947 (Personal communication from HPA Cultures)
- K12 – a long established gut strain originally isolated in 1922 (Bachmann, 1972) and regarded as a standard strain for use in microbiology experiments but lacking many virulence characteristics.
- CFT073 – a laboratory strain of UPEC with multiple virulence factors derived from a clinical isolate of *E. coli* originally obtained from the urine of a patient with pyelonephritis in 1990.

### **3.2.2. Luria Bertani Broth and Agar Plates**

Luria Bertani broth (LB) was prepared with 10% (w/v) Bacto-tryptone (10g), 10% (w/v) sodium chloride (10g), 5% (w/v) Bacto-yeast (5g) and 1L of deionised water with pH adjusted to 7.4 using 5M NaOH. The LB broth was sterilized at 121°C for 20 minutes at a pressure of 15 pounds per square inch (PSI). 2% (w/v) agar plates were prepared using LB broth and agar, and re-sterilised in the same manner as the original broth. 20-25ml of autoclaved agar was poured into each petri dish, allowed to solidify and dried at 37°C.

### **3.2.3. Storage and culture of *E. coli* strains**

Glycerol stocks of bacterial strains were prepared by inoculating 10ml of LB broth with a loopful of bacteria and incubating aerobically at 37°C overnight culture

(approximately 16-hours). An aliquot of this culture (0.5ml) was placed into a 1.5ml cryovial containing 0.5ml 50% (v/v) glycerol and stored at -80°C.

When required for experimentation, bacteria were taken simply by dipping a sterile 10µl pipette tip into the glycerol stocks and then inoculating 10ml of LB broth for overnight culture.

### 3.2.4. Challenge Experiments

For challenge experiments, both RT4 and VK2 E6/E7 cells were seeded onto 30 mm, six well plates (Corning, Tewksbury, Massachusetts, USA) at  $10^4$  cells/well and cultured at 37°C and 5% CO<sub>2</sub> until confluent. Prior to challenge, cells were washed in PBS and incubated for 24-hours in 1ml of new medium.

For bacterial challenges, bacteria from overnight culture were used to inoculate 5ml of LB medium for 1-hour then 50µl of this solution was grown in a further 5ml of LB medium for 3-hours (synchronisation by stationary phase method). Growth characteristics of the four *E. coli* strains have been previously characterised by our research group using serial optical density measurement and confirmed to be in log-phase using this method. 20µl of the 3-hour culture was re-suspended in 980µl PBS to give a working solution. 10µl of the working solution was serially diluted, plated in quadrants on agar plates and incubated overnight to confirm that 20µl was approximately  $5 \times 10^5$  colony forming units (CFU). Dead bacteria were produced by incubating live bacterial stock solution for 30 minutes at 65°C and death confirmed by overnight agar plating. Each well of eukaryote cells was inoculated with either 20µl of live or dead bacteria for the time required.

For bacterial component challenges, each well of eukaryote cells was inoculated with 20µL of LPS (0.25 – 1mg/mL), *S. typhimurium* flagellin (100 – 250ng/mL), Peptidoglycan

(5 – 10µg/mL) (Invivogen, San Diego, California, USA) and *E. coli* Flagellin (250 ng/mL) (gift from Marcelo Lanz, Newcastle University, UK).

When the appropriate time point in a challenge was reached, the medium from the well was removed, stored at -20°C and the cells prepared for RNA extraction as described in section 3.3.1.

### **3.2.5. Time kill antimicrobial assay**

Time kill antimicrobial assays using the laboratory uropathogenic *E. coli* (UPEC) strain NU14 were adapted from the technique described previously by our research group (Townes *et al.* 2010). A 20µL aliquot a 3-hour NU14 bacterial culture (described in section 3.2.4) was diluted to 2 mL in PBS. 50µL samples of eukaryote cell culture medium taken from either challenge or control experiments or synthetic defensin peptide were added to 50µL of the diluted bacterial culture, vortexed, and incubated for one hour at 37°C. Suspensions (taken at 0 hr and 1 hr time-points) were sequentially diluted to 1:10<sup>4</sup> in PBS, and each dilution was plated onto LB agar. All plates were incubated overnight at 37°C and the colonies counted.

### **3.2.6. Motility Assay**

The *E. coli* motility assay was carried out by Dr Chris Birchall and Dr Phil Aldridge on my behalf. Swarm plates were prepared one day prior to use with 25 ml of LB medium fortified with 0.3% Bacto agar, 1% tryptone and 0.5% Sodium chloride. To minimize water on the agar surface, plates were dried 20 min prior to inoculation. For qualitative swarm assays, plates were centrally inoculated with cells from a freshly grown overnight colony using a sterile stick. Plates were incubated at 37°C for 8-hours and pictures taken.

### **3.3. RNA Extraction**

#### **3.3.1. TRIzol® RNA Isolation**

Tissue samples were initially homogenized at room temperature in 1ml of TRIzol® reagent (Invitrogen, Paisley, UK) with a rotor-stator style homogenizer (TissueRuptor, Qiagen, Crawley, UK) ensuring that the homogenizer was cleaned thoroughly between samples to prevent cross-contamination.

For cells cultured during challenge experiments in 30 mm, six well plates, the culture medium was removed and the cells washed in 2ml of PBS. 1ml of TRIzol® reagent was then added to the well and left to lyse the cells for five minutes with the cell lysate passed several times through a pipette to facilitate the process.

Once the homogenised and lysed cells had been incubated at room temperature for five minutes, 200µl of chloroform was added to the lysate, gently mixed and left for a further three minute incubation period at room temperature. The samples were then centrifuged for 15 minutes at 12000g and 4°C, the clear supernatant removed, mixed with 700µL of Isopropanol and the solution re-centrifuged. The clear pellet was washed with 1ml of 70% ethanol, and after centrifugation the RNA pellet resuspended in 20µL of molecular grade water. To aid preservation of RNA, 1µl of RNAsin™ RNase inhibitor (Promega, Southampton, UK), was added to all samples. Samples were stored at -80°C.

#### **3.3.2. Analyses of RNA concentration, purity and integrity**

The concentrations of RNA extracted from the tissue samples and cells were determined spectrophotometrically. Essentially, RNA samples were diluted 1:10 or 1:100 in nuclease-free water and the absorption of RNA measured at 260nm. The



conversion factor 40µg/ml per 1 unit OD260nm was used to calculate RNA concentration. For initial calibration of the spectrophotometer nuclease-free water was used. Absorbance was also measured at 280nm and 230nm to check for contamination of the sample with protein (A260/A280) and guanidine isothiocyanate (A260/A230). In both cases ratios of 1.8-2.2 indicated that the sample was within an acceptable purity range and was free from protein and /or chemical contamination.

Random samples of extracted RNA were checked using a Bioanalyzer 2100 (Agilent Technologies, Berkshire, UK) to ensure quality of the nucleic acid. A minimum RIN-number of 8.0 was maintained for quantitative real time PCR (RT-qPCR) analyses of the *in vitro* samples and a minimum of 7.0 for biopsy samples.

## **3.4. Reverse Transcription**

### **3.4.1. DNase treatment of RNA**

Following quantification of RNA, all samples were treated with DNase (DNase kit, Promega, Southampton, UK) to remove any genomic DNA contamination. In a microfuge tube approximately 4.5µg of RNA was mixed with 2µl of DNase buffer (400mM Tris-HCL, pH 8.0, 100mM MgSO<sub>4</sub>, 10mM CaCl<sub>2</sub>) and 4.5µl of DNase. To this molecular grade water was added to give a total reaction volume of 20µl. Where samples had a poor RNA yield, water was omitted from the sample and instead a larger volume of RNA was added. The tube was briefly vortexed, incubated at 37°C for 30 min and the reaction terminated by the addition of 4µl of DNA stop solution (20mM EDTA, pH8). The reaction was heat treated at 65°C for a further 10 min. Samples were used for reverse transcription or stored at -20°C.

### **3.4.2. Complementary DNA Preparation**

Total RNA was reverse transcribed into complementary DNA (cDNA) for use in end-point and real-time qPCR analyses. For these reactions, 5µl of DNase treated RNA was set up in a microfuge tube reaction with an Applied Biosystems PCR set consisting of 2µl 10xPCR buffer, 1.6µl Magnesium Chloride, 8µl of 10mM dNTPs (dATP, dGTP, dCTP, dTTP) and 0.5 µl of muLV reverse transcriptase (Life Sciences, Paisley, UK). To this, 1µl of 15mM oligoDTs, 0.5µl of 40u/µl Human Placental RNase inhibitor, and 1.4µl nuclease-free water were added (Promega, Southampton, UK). These were mixed and the solution placed in a thermal cycler (Hybaid PCR express, Sprint™, Hybaid, Middlesex, UK) and treated for 1 hour at 42°C, 95°C for 5 min and 5°C for 5 min. All cDNA was stored at 4°C prior to PCR amplification. In addition to samples, 'no template' and 'no reverse transcriptase enzyme' controls were also used.

## **3.5. End-point polymerase chain reaction**

### **3.5.1. Primers**

Primers for cDNA amplification were designed using NCBI Blast sequences of targeted genes and designed to amplify across at least two exon containing regions. The primer sets have been extensively used in our research group with validation and optimisation outlined by Stephanie Bell in her PhD thesis (Bell, 2008) and by Marcelo Lanz in his MRes (Lanz 2010). Primers were produced by Invitrogen (Life Sciences, Paisley, UK), supplied laboratory ready as a stock solution of 100µM. Working solutions of 10µM in RNase and DNase free deionised water were prepared from the stock solutions. Ambion 18S primer pair was used as a positive control (Life Sciences, Paisley, UK). All

primers were used at an annealing temperature of 58°C, the sequences and their size are outlined in Table 3.1.

**Table 3.1 Primer sequences for End-point RT-PCR**

<b>Gene</b>	<b>Forward Primer</b>	<b>Reverse Primer</b>	<b>Product size (bp)</b>
<b>HBD1</b>	CCATGAGAACTTCTACCTTC	GTCACCTCCAGCTCACTTG	221
<b>HBD2</b>	GTGAAGCTCCAGCCATCAG	GATTGCGTATCTTTGGACACC	325
<b>HD5</b>	GCCATCCTTGCTGCCATTC	GATTTACACACCCCGGAGA	240
<b>LL37</b>	CATGAAGACCCAAAGGGATG	CACACTAGGACTCTGTC	518
<b>TLR4</b>	TGCAATGGATCAAGGACCAGAGGC	GTGCTGGGACACCACAACAATCACC	449
<b>TLR5</b>	CCTCATGACCATCCTCACAGTCAC	GGCTTCAAGGCACCAGCCATCTC	355
<b>Er<math>\alpha</math></b>	GGATACGAAAAGACCGAAGAG	GTCTGGTAGGATCATACTCGG	246
<b>Er<math>\beta</math></b>	TAGTGGTCCATGCCAGTTATCAC	GCACTTCTGTCTCCGCACAA	439
<b>18S</b>	Ambion commercial primer	Ambion commercial primer	344

### 3.5.2. Amplification

Polymerase chain reaction (PCR) was used to amplify cDNA exponentially using a hot-start Taq (*Thermus aquaticus*) polymerase. PCR reactions were set up using Biolinekit reagents consisting of 0.8 $\mu$ l Magnesium Chloride [25 mM], 4 $\mu$ l of 10 x PCR Buffer and 0.5 $\mu$ l Taq Polymerase (Bioline Reagents, London, UK) to which was added 4 $\mu$ l of Primer pair (2 $\mu$ l of forward [10 $\mu$ M] and 2 $\mu$ l reverse primer [10 $\mu$ M]) and 10 $\mu$ l of cDNA from the respective reverse transcription reaction. The reaction volume was made up to 20 $\mu$ l with deionised water.

The mixed solution was placed in a thermal cycler (Hybaid PCR express, Sprint <sup>™</sup>, Hybaid, Middlesex, UK) for amplification consisting of a 5-minute denaturation step at 95°C; then 35 cycles of the following sequence: 30 seconds at 95°C, 30 seconds at 58°C

(annealing temperature) and 30 seconds at 72°C, and a final extension step at 72°C for 12 min. A final 5-minute cooling step lowered the reaction temperature to 4°C.

### **3.5.3. Agarose gel electrophoresis**

PCR products were electrophoresed on 1.5% w/v TBE-agarose gels. Molecular grade agarose was dissolved in 1 X TBE buffer (0.089M Tris-borate, 2mM EDTA, pH8.3) with 5µg/ml ethidium bromide added before pouring into an electrophoresis tank. The solidified gel was submerged in TBE buffer and samples added. To 8µl of PCR product, 2µl of gel loading dye was added (50mM Tris, pH8.5, 5mM EDTA, 50% glycerol and 0.1% bromophenol blue). Each gel was also loaded with 4µl of Hyperladder IV DNA ladder (Bioline Reagents, London, UK). Electrophoresis was performed at a voltage of 70V for 45 to 60 minutes. Gels were visualized and photographed under ultraviolet illumination.

## **3.6. Real-Time Polymerase Chain Reaction**

### **3.6.1. Primers**

Primers for qPCR were designed in a similar manner as described for end-point PCR (section 3.5.1) and had also been previously validated and optimised by our research group for defensins (Bell, 2008) and Toll like receptors (Lanz, 2010). This validation included coefficient of variation (CV) calculations consistent with MIQE guidelines for each assay to ensure reproducibility using the first and second clone dilutions from standard curves.

Two types of primer were used: Probes Master, an oligonucleotide probe based detection system or the SYBR Green I Master intercalating DNA-dye based system (Roche Products, Welwyn Garden City, UK). Probes Master primers are produced by

Roche whereas SYBR Green primers were produced by Invitrogen (Life Sciences, Paisley, UK). Pre-designed primer pairs for ‘house-keeping’ reference genes were produced by Primer Design (Southampton, UK)

The primer type, sequence and annealing temperature (T<sub>m</sub>) are shown in Table 3.2.

**Table 3.2: Primer sequences for RT-qPCR**

<b>Gene</b>	<b>Primer Sequence</b>	<b>Primer Type</b>	<b>T<sub>m</sub> (°C)</b>
BD1	Reverse: <u>cgcc</u> GGTAGGAAGTTCTCATGGcG Forward: GTCAGCTCAGCCTCCAAAGGA	Probes Master	60
BD2	Forward: GTGAAGCTCCCAGCCATCAG Reverse: GATTGCGTATCTTTGGACACC	SYBR Green I Master	58
HD5	Forward: GCCATCCTTGCTGCCATTC Reverse: GATTCACACACCCCGGAGA	SYBR Green I Master	58
LL37	Forward: <u>cgct</u> GACGGGCTGGTGAAGcG Reverse: CCCAGCAGGGCAAATCTCTT	Probes Master	55
TLR4	Forward: GAATTTAGAAGAAGGGGTGCC Reverse: CCTTCATGGATGATGTTGGC	SYBR Green I Master	58
TLR5	Forward: CAGAGACTGGTGTTC AAGGAC Reverse: GTGTCCAGGTGTTTGAGCA	SYBR Green I Master	54
GAPDH	Primer Design commercial primer (House-keeping reference gene)	SYBR Green I Master	60
ATP5B	Primer Design commercial primer (House-keeping reference gene)	SYBR Green I Master	60

### 3.6.2. Amplification

Polymerase Quantitative PCR (qPCR) was carried out on a Roche LightCycler 480 using LightCycler 480 SYBR-green Master Mix (Roche, UK).

For amplification of genes of interest the protocol was as follows: 95 °C for 10 minutes, 45 cycles of 95°C (10 seconds), T<sub>m</sub>°C (20 seconds) and 72°C (10 seconds), followed by

melt curve analysis (cooling to 65°C followed by heating to 97 °C during which a continuous fluorescent reading is recorded) and a cooling step.

For house-keeping reference genes, the manufacturer's protocol was followed: 95°C for 10 minutes, 45 cycles of 95°C (15 seconds), 60 °C (30 seconds) and 72°C (10 seconds), followed by melt curve analysis and a cooling step.

All data was normalised to a geometric mean derived from the two appropriate reference genes.

### **3.6.3. GENORM Analysis and Choice of Reference Genes**

Utilising cDNA samples taken from several passages of each cell line (RT4 urothelial and VK2 vaginal) in challenged and unchallenged states, twelve house-keeping reference genes for use in data normalisation were analysed. Analyses utilised a Genorm kit with pre-designed primer mixes and software (PrimerDesign, Southampton, UK), which quantifies the stability of each potential gene relative to the others. Of the twelve, GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and ATP5B (ATP synthase) were selected as the two most suitable genes.

### **3.6.4. Relative quantification and Data Analysis**

For qPCR analysis, two types of quantification, absolute and relative, are commonly used. For absolute quantification a standard with a known copy number is required and assays must have highly reproducible amplification efficiencies between plates to allow extrapolation of an exact copy number value for a given sample. Relative quantification instead assigns relative values to individual samples allowing comparison between them. These values typically consist of either crossing point (CP) value (the number of cycles required for fluorescence to reach a pre-determined

level that is significantly increased from background) or by using a 'standard curve' to extrapolate relative expression in arbitrary units (Fleige *et al.* 2006, Larionov *et al.* 2005). For exploratory work such as that carried out in this study, relative quantification provides sufficient information to allow comparison of gene expression between samples without the need for the more rigorous control of interpolate variation required for absolute quantification. Relative quantification was therefore chosen as the quantification method in this study. Standard curves were formed using serial dilutions of plasmids previously created during validation of the qPCR assays (Bell 2008, Lanz 2010).

### **3.7. Sandwich ELISA**

BD1 and BD2 peptides were measured in clinical and cell culture samples using Human BD2 and Human BD1 ELISA Development Kits (Leinco Technologies, St Louis, Missouri, USA) as indicated by the manufacturer. Briefly, 100 $\mu$ L of 0.25  $\mu$ g/mL defensin capture antibody (purified rabbit anti- human BD1 or purified goat anti- human BD2) was added to each ELISA Nunc Maxisorp microplate well (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The plate was sealed and incubated overnight at room temperature to allow coating. After incubation, capture antibody was removed from each well and the plate washed four times with 300 $\mu$ L of wash buffer (0.05% Tween-20 in PBS) per well then blotted on paper towels. Block buffer (1% BSA in PBS) was applied for a minimum of two-hours at room temperature. The plate was again washed four times with wash buffer.

Standards, 500 pg/ml to 2 pg/mL, were prepared in diluent (0.05% Tween-20, 0.1% BSA in PBS). Samples and standards were added in duplicate to the coated

microplates, 100µl to each well, and incubated for two hours at room temperature. After incubation, a further four washes were carried out, 100µl of 0.5 µg/mL detection antibody (purified biotinylated rabbit anti-human BD1 or purified biotinylated goat anti human BD2) was added to each well and left to incubate for two-hours at room temperature. The plates were again washed four times, 100µl of Avidin HRP conjugate concentrate (diluted in diluent to a 1:200) was added, and left to incubate for 30 minutes at room temperature. After washing a further 4 times, TMB substrate was added to start the colour reaction, and the plates were incubated in a dark room for five to ten minutes. The colour reaction was stopped by adding 50µl of 1M Sulphuric acid as a stop solution. The plates were read at 450nm using a micro plate reader with wavelength correction set at 650 nm.

### **3.8. Immunohistochemistry**

Tissue specimens were stored embedded in paraffin. Cut sections (4 µm) were mounted on SuperFrost Plus microscope slides (VWR International, Lutterworth, UK) and allowed to dry at 56°C. The sections were dewaxed in xylene, rehydrated, and incubated for 5 minutes in 3% hydrogen peroxide. Blocking was carried out for 5 minutes with goat serum diluted 1:10 with Tris-buffered saline (TBS). This was followed by overnight incubation with anti-defensin antibody at a dilution of 1:100 at 4°C (Abcam, Cambridge, UK). Primary antibody binding was detected using Vectastrain Elite biotinylated goat anti-mouse antibody (Vectorlabs, Peterborough, UK) for 30-minutes at room temperature then visualised with NOVA red peroxidase agent incubated for 10-minutes at room temperature. Finally counterstaining was performed with haematoxylin for 30-second. Slides were washed three times with TBS at pH 7.6 slides between each step.



## 3.9. Single Nucleotide Polymorphism (SNP) Analysis

### 3.9.1. DNA extraction from blood

The GeneCatcher™ gDNA 3–10 mL Blood Kit (Invitrogen, Paisley, UK) was used for the extraction of gDNA (genomic DNA) from frozen whole blood. Purification was achieved using a magnetic bead based purification procedure in which cells were lysed using the GeneCatcher lysis Buffer and the lysate mixed with GeneCatcher Magnetic Beads for subsequent DNA binding to the beads. The DNA-bound magnetic beads were separated from the lysate using a magnetic separation rack. Any residual proteins were digested with the Protease (25 mg/mL in 50 mM Tris-HCl, pH 8.5, 5 mM CaCl<sub>2</sub>, 50% glycerol). The beads were thoroughly washed with the GeneCatcher buffers to remove contaminants. The genomic DNA was recovered in elution Buffer (10 mM Tris-HCl, pH 8.5).

### 3.9.2. PCR Amplification of SNP Region

PCR amplification of the TLR5<sup>392stop</sup> containing region of the TLR5 gene was carried out in a similar manner to the end point PCR described in section 3.5.2 but with some important differences. Instead of Taq polymerase, a high fidelity, proof reading Kod (*Thermococcus kodakaraensis*) DNA polymerase was used – Novagen Kod Host start DNA Polymerase (Merck Millipore, Darmstadt, Germany). This enzyme has a very low incidence of mispriming events which is important in SNP analysis.

PCR reactions were set up using the kit supplied with the enzyme: 2µl Magnesium sulphate [25 mM], 5µl of 10 x PCR Buffer, 5µl of dNTPs, 1µl Kod Polymerase to which was then added 3µl of Primer pair (1.5µl of forward primer, GGTAGCCTACATTGATTTGC [10µM] and 1.5µl reverse primer, GAGAATCTGGAGATGAGGTACCCG [10µM]) and 100ng of gDNA. The reaction volume was brought up to 50µl with deionised water.

The mixed solution was placed in a thermal cycler (Hybaid PCR express, Sprint <sup>™</sup>, Hybaid, Middlesex, UK) for amplification consisting of a 2-minute denaturation step at 95°C; 30 cycles of the following sequence: 20 seconds at 95°C, 10 seconds at 60.3°C (annealing temperature) and 10 seconds at 70°C, and a final extension step at 70°C for 10 min. A final 5-minute cooling step returned the reaction temperature to 4°C. A 10µL aliquot of the 461 bp product was used for sequencing.

### **3.9.3. Sequencing**

Prior to sequencing, all samples had their OD (optical density) values checked with a spectrophotometer at 260 nm and 280 nm to ensure that 260/280 ratio was between 1.7 and 1.9. DNA quality check, clean-up and DNA sequencing were performed by Genevision (Newcastle upon Tyne, UK) the latter employing an Applied Biosystems 3730xl DNA Analyzer.

### **3.9.4. Software Analysis**

Sequencing data was provided in AB1 data format and data analysis was carried out on the freeware FinchTV trace viewing software (Geospiza, Seattle, USA) – <http://www.geospiza.com/Products/finchtv.shtml>.

## **3.10. NF-κB Reporter Gene Transfection**

### **3.10.1. Stable Transfection**

The stably transfected RT4 NFκB reporter cell line was produced by Marcelo Lanz – the methodology and validation of this engineered cell line is described in his MRes dissertation (Lanz, 2010). Briefly, RT4 cells were seeded onto 30mm diameter, six well plates at a density of 10<sup>5</sup> cells/well and cultured in 2ml growth medium at 37°C and 5% CO<sub>2</sub> until 90% confluent. Plasmids for pBlue-NFκB and negative control pBlue-TATA

promoter were diluted in Optimem medium (GIBCO, Paisley, UK). The cells were washed twice in PBS, incubated for four hours with the appropriate plasmid and lipofectamine 2000 transfection mixture (Life Sciences, Paisley, UK) and then with normal growth medium. After 24 hours, the media was changed to include G418 Geneticin aminoglycoside antibiotic (Sigma, Dorset, UK) at 0.5mg/ml for positive selection. The selection medium was changed every 72-hours and after six weeks, stably transfected colonies were trypsinised, pooled and passaged in flasks as for non-transfected RT4 cells.

### **3.10.2. Transient Transfection**

A procedure for developing a transient VK2 E6/E7 NFκB reporter cell line was achieved using the Attractene fast-forward transfection protocol (Qiagen, Hilden, Germany) by Bea Suarez M-Falero during her summer studentship working with our research group. Briefly, in this procedure  $5 \times 10^4$  VK2 cells were seeded in 100µl of medium in a Costar 96-well plate (Corning, Tewksbury, Massachusetts, USA). To this medium, 50µl of TE buffer containing 0.2 µl of either NFκB-GFP, CMV promoter or negative control TATA promoter plasmid (Qiagen, Hilden, Germany) and 0.75µl of Attractene transfection reagent were added. Cells were cultured for 24-hours, washed with PBS and medium changed to normal medium prior to challenge experiments. To account for transfection efficiency, GFP fluorescence results were calculated relative to fluorescence by the CMV promoter.

### **3.11. Statistical Analyses**

All statistical analyses were carried out using the Prism 5 Software package (GraphPad Software Inc, La Jolla, California, USA)

Two tailed Student's t-test was used for all *in vitro* experiments to compare a given challenge against the control. Unless otherwise indicated, analysis was always carried out at the time point where maximum difference between challenge and control was apparent. Significance level was set at the 5% level.

## 4. Clinical Study Material and Methods

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The aim of the clinical study was to compare the types and amounts of antimicrobial peptides (AMPs) expressed by urogenital epithelium in women with recurrent cystitis against those found in age-matched women without cystitis (controls) and determine whether any patient or clinical characteristic influenced AMP response. It was anticipated that the results would indicate the importance of AMP innate immune mechanisms in protecting against cystitis and suggest how this might be enhanced therapeutically in the future.

### 4.1. Study Design

At the inception of the study it was decided that a group of women with recurrent cystitis and a group of age-matched women without a history of symptomatic urinary infection would be identified and recruited. Each participant would be requested to provide demographic and clinical data together with specimens of blood, urine, vaginal washings, bladder epithelium and vaginal epithelium. These samples would then be analysed in a blinded manner in the laboratory for mRNA expression and where appropriate peptide secretion of relevant AMPs (as directed by *in vitro* findings). The results would be analysed and compared statistically to determine whether there were any significant differences between the affected and control groups of subjects.

### **4.1.1. Funding**

The study including salaries, materials and other direct costs were all met by a Clinical Research Training Fellowship awarded to the author by the Wellcome Trust (WT085040MA) on the 15<sup>th</sup> of July 2008 (letter in appendix B ).

### **4.1.2. Ethical & NHS approval**

The County Durham & Tees Valley 1 Research Ethics Committee reviewed and approved the study (09/H0905/15) on the 26<sup>th</sup> February 2009 (letter in appendix B).

Newcastle upon Tyne Hospitals NHS trust gave research and development (ID 4841) approval on the 15<sup>th</sup> May 2009 (letter in appendix B).

The National Institute for Health Research (NIHR) adopted the study (ID 7018) into the Clinical Research Network (CRN) Portfolio on the 19<sup>th</sup> June 2009 (letter in appendix B).

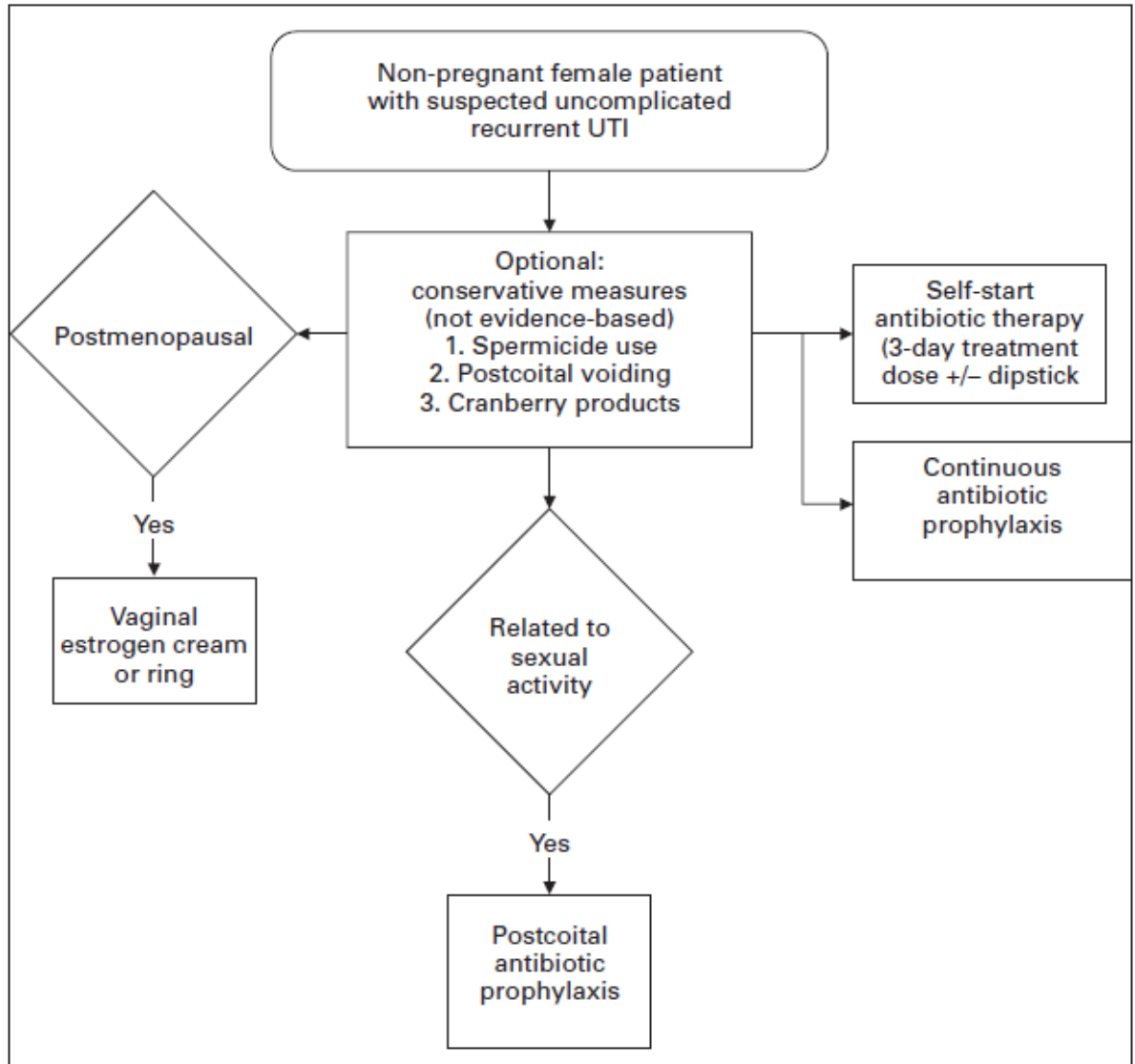
### **4.1.3. Development of 'One-Stop' Clinic for Women with Recurrent UTI**

To enhance the delivery of care for women with recurrent urinary tract infection (rUTI) and facilitate the recruitment of patients into this research study and future research studies, a new 'one-stop' rUTI clinic was developed. The concept was based on a similar idea to the prevalent 'one-stop' haematuria diagnostic clinic with the purpose to ensure that:

- All patients had their clinical history and symptomatology recorded in a uniform and consistent manner. Previously, patients had attended a variety of general urology out-patient clinics with varying degrees of details of their clinical history recorded.

- All patients had the appropriate investigations performed on the same day as their clinical consultation. These investigations included haematological (full blood count) and biochemical (urea and electrolytes) blood tests, a renal tract ultrasound scan and a flexible cystoscopy to inspect the urethra and bladder epithelial surface. Previously patients would attend an out-patient consultation then subsequently attend on a minimum of three further occasions – one visit for an ultrasound scan, one for a flexible cystoscopy and a further consultation with all the results. The number of visits was therefore reduced from up to four to one.
- All patients were managed according to an agreed treatment protocol based on guidelines from the European Association of Urology (current version of guidelines: Grabe *et al*, 2013) and a simple treatment algorithm adapted from the Canadian Urology Association guidelines (current version of guidelines: Dason *et al*, 2011) – Figure 4.1 .

Figure 4.1 UTI Treatment Flowchart



Choice of antibiotic was determined by past microbial sensitivity, potential drug interaction and patient tolerance and/or preference. Typically however, this would be (in order of preference):

1. Trimethoprim
2. Nitrofurantoin
3. Cefalexin ( only if 1 and 2 poorly tolerated or resistance previously encountered)



All patients were booked for routine out-patient follow-up in 6-months to assess benefit.

#### **4.1.4. Patient Recruitment**

Control and affected women with structurally normal urinary tracts willing to provide biopsies, samples of urine and vaginal washings were recruited from the Urology and Urogynaecology departments of Newcastle upon Tyne Hospitals NHS Foundation Trust from the 14<sup>th</sup> July 2009 to February 24<sup>th</sup> 2011. The cohort of women with recurrent cystitis (rUTI) and the control group of unaffected women were stratified according to menopausal status to create 4-groups:

- Pre-menopausal controls
- Pre-menopausal cases (rUTI sufferers)
- Post-menopausal controls
- Post-menopausal cases (rUTI sufferers)

Prior to the start of the study, sample size calculations based on urinary LL-37 urinary concentration suggested that 18 women in each group will give an 80% chance of detecting a difference at the 5% significance level. A recruitment target of 80 was set with an aim to recruit 20 in each group but a minimum target of 18.

#### ***Inclusion and exclusion criteria***

The main inclusion criterion was women who have been referred for assessment of recurrent UTI and who had suffered at least 2 episodes per year for 2 years or 3 episodes in the last year. Controls were recruited from women scheduled to attend the 'one-stop' clinic for investigation of haematuria or other non-infection related urogynaecological assessment. Women with a history of symptomatic urinary infection

or who had previous microbiological evidence of urinary infection in the past 2-years were not recruited as controls.

Other inclusion criteria were:

- Age > 18 years
- Pre-menopausal or at least 6-months post-menopause.
- Good general health [American Society of Anaesthesiologists (ASA) grade < 3]
- Both cases and controls could not have had antibiotic therapy within 4 weeks of their recruitment.

Exclusion criteria were:

- Structurally abnormal urinary tract.
- Inability to give informed consent.
- Severe impairment of renal function [estimated glomerular filtration rate (eGFR) < 30 ml/min] because this could lead to reduced renal secretion of AMPs and confound results.
- Inability to complete trial tasks.

## **4.2. Clinic protocol**

Patients were informed about the study via an information sheet (see appendix A) sent out in the post with their clinic appointment. This sheet described the study and explained the need for urine and tissue samples. On the day of the patient's clinic appointment, the research nurse confirmed that the patient had received the information pack and discussed the study further, answering any questions.

Agreement to take part was then confirmed by written informed consent taken by a research nurse before the clinic.

At the out-patient appointment itself, in the consultation room, relevant demographic and clinical data were collected on a pro-forma, and a blood sample was taken (at the same time as routine blood tests) by a nurse practitioner. The patient was then requested to give a mid-stream sample of urine (MSSU) for culture. An ultrasound scan (USS) of the urinary tract was also performed by a sonographer.

The UTI Symptoms Assessment (UTISA) questionnaire and the EuroQol 5-domain (EQ-5D) general health status questionnaire were filled in by the patient herself in the waiting room before entering the endoscopy room.

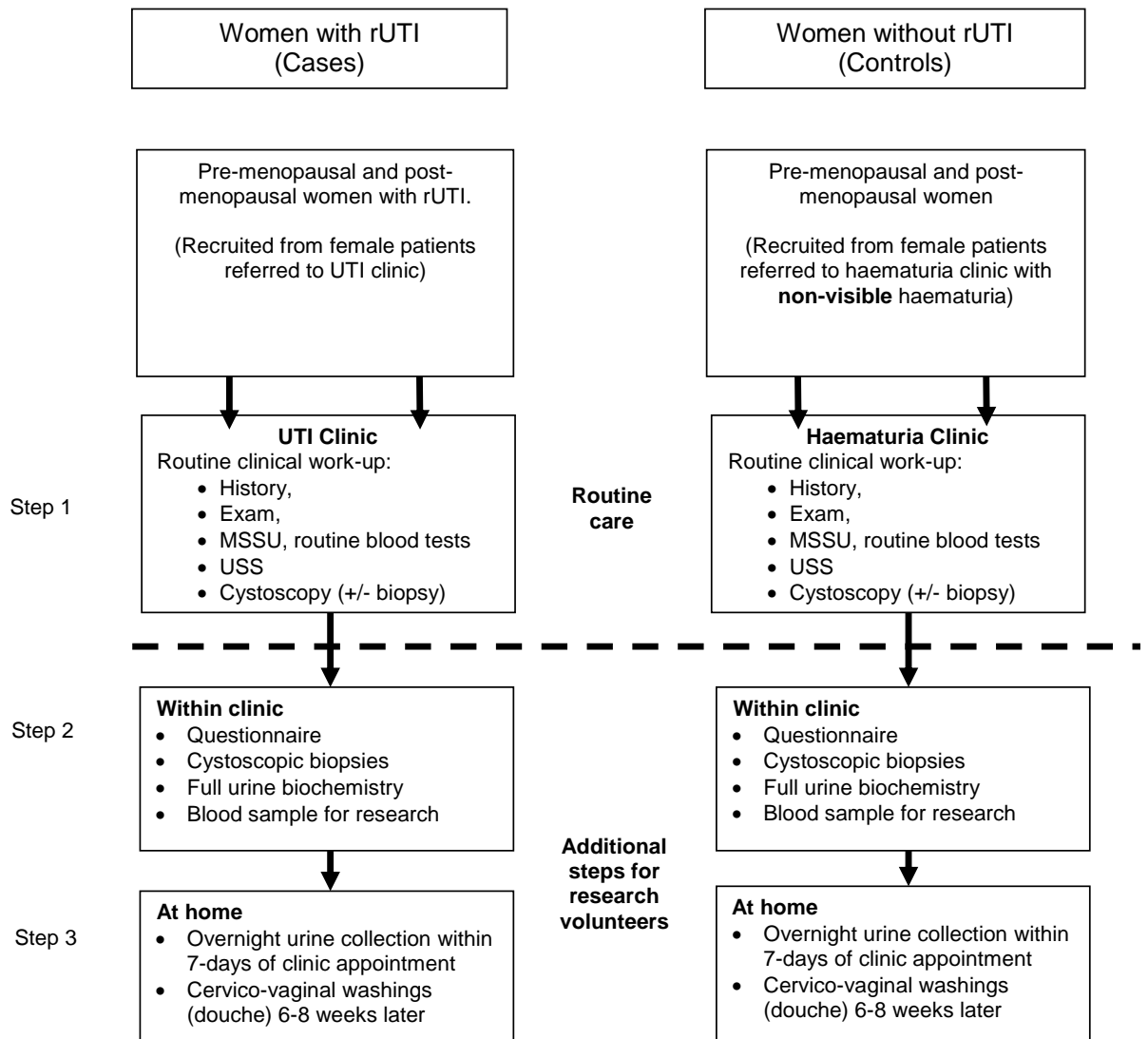
In the endoscopy room, the clinical history was briefly reviewed by the author and a clinical examination plus cystoscopy performed. Biopsies from the posterior bladder wall were taken during cystoscopy. Following cystoscopy, the first vaginal douche was collected. Then a vaginal biopsy from the first 2-3 cm of the vagina was obtained using the flexible cystoscope to carry out a vaginoscopy during the vaginal examination.

After the consultation, each subject was given the necessary instructions and containers to collect an overnight urine specimen (approximately 600-800 ml) and a further vaginal douche 6-8 weeks later. They were given instructions to collect the douche (at approximately the mid-point of their menstrual cycle for pre-menopausal women) and bring the specimens to a designated collection point at Freeman Hospital or Newcastle University on the morning of the next day.

Figure 4.2 shows a summary of the steps involved in the protocol.

Figure 4.2 Clinical study summary flowchart

## Summary Flow Chart



### Abbreviations

<b>MSSU</b>	Mid-stream sample of urine for microbiological culture
<b>rUTI</b>	Recurrent urinary tract infection
<b>USS</b>	Ultrasound scan of urinary tract to look for abnormalities
<b>UTI</b>	Urinary tract infection

## 4.3. Clinical Details

### 4.3.1. Medical history

The following details were recorded for each rUTI subject:

- Presenting complaint and urinary symptoms
- Predominant type of UTI symptoms
- Length of history of proven rUTI
- How many treatment courses of antibiotic for UTI had been taken in the last year?
- Relationship of UTI to sexual activity

The following details were recorded for every patient (rUTI and control)

- Menopausal status (pre-, peri- or post- menopausal)
- Presence of vaginal symptoms
- Use of vaginal estrogen (currently or previously)
- Use of vitamin tablets
- Use of cranberry products
- Use of oral contraceptive
- Use of systemic hormone replacement therapy (HRT)
- Previous sexually transmitted infection (STI)
- Previous gynaecological or urological history
- Number of pregnancies
- Other medical conditions and treatment
- Smoking history

A clinical examination including vaginal examination was performed for all patients. Ultrasound and cystoscopy results were also recorded.

### **4.3.2. Symptom and Quality of life score**

All recruits were requested to fill in the UTISA symptoms questionnaire (see appendix A). This questionnaire rates severity and bother of the following eight symptoms:

1. Frequency of urination (going to the toilet very often)
2. Urge of urination (a strong & uncontrollable urge to pass urine)
3. Pain or burning when passing urine
4. Not being able to empty bladder completely/passing only small amounts of urine
5. Feeling feverish or shivers
6. Pain or uncomfortable pressure in the lower abdomen/pelvic area caused by your urinary tract infection
7. Low back pain caused by your urinary tract infection
8. Blood in your urine

For each of the eight symptoms, recruits are asked to give a rating from 0-3 relating to the severity of the symptom and the degree of bother it causes thus:

- Please indicate whether you have had the following symptoms during a urine infection by circling one number for each symptom.

0. Did not have
1. Mild
2. Moderate
3. Severe

- If you experienced these symptoms during a urine infection please indicate how much they troubled you by circling one number for each symptom.

0. Not at all
1. A little
2. Moderately
3. A lot

The recruits were also requested to fill in a general health status questionnaire, the EQ-5D (see appendix A). This asked participants to rate the degree of problems they have on a 3-point scale (0 – No problems, 1 – Some problems, and 2 – Unable to carry out) in relation to the following five domains:

1. Mobility
2. Self-care
3. Usual activities (e.g. work, study, housework, family or leisure activities)
4. Pain / discomfort
5. Anxiety / depression

## **4.4. Clinical samples**

### **4.4.1. Blood**

Blood for DNA analysis was collected in a 4 ml container with EDTA bottle using the Vacutainer System (BD Diagnostics, Oxford, UK). Samples were stored temporarily in a -20°C freezer in the hospital prior to long-term storage in the University.

#### **4.4.2. Urine**

A conventional mid-stream sample of urine (MSSU) was collected in a 30 ml universal container (Sterilin, Newport, UK) in the clinic and sent for culture and sensitivity to the hospital microbiology lab. Overnight urine collection was taken in a 1-litre 24-hour collection container (Sterilin, Newport, UK) and stored in a -20°C freezer in the hospital prior to transfer for long-term storage in the University.

#### **4.4.3. Vaginal douche**

Vaginal douches were carried out using the 'Summer's Eve Cleansing Douche' (Fleet Laboratories, Lynchburg, Virginia, USA) according to the manufacturer's instructions and collected in a 100ml container (BD Diagnostics, Oxford, UK).

#### **4.4.4. Biopsies**

Biopsies were taken during cystoscopy using an Olympus rigid or flexible cystoscope with a standard 1.8mm flexible biopsy forceps.

Biopsy samples for RNA extraction were immediately immersed in 1.5ml of RNAlater solution (Life Technologies, Paisley, UK) in a 1.7ml centrifuge tube (Eppendorf AG, Hamburg, Germany). Tubes were then stored overnight at -4°C to allow the RNA later to penetrate through the tissue and then put into the -20°C freezer in the hospital prior to transfer for long-term storage in the University at -80°C in a secure freezer.

Samples for immunohistochemistry were fixed in formalin overnight in an appropriate container before being transferred to 10ml Falcon tube (BD Diagnostics, Oxford, UK) containing 2-3 ml of 70% ethanol for long term storage.



## **4.5. Sample Anonymisation and Data Handling**

Demographic, clinical and questionnaire data were transferred from paper collection sheets (kept in the participants' medical record) onto an electronic spread sheet stored on the hospital network drive. An anonymised version was created for transfer to the university network drive for analysis. Caldicott and Data Protection approval (reference 676) was granted on the 9<sup>th</sup> February 2009 (see appendix B).

All samples had patient identification labels removed and were pseudo-anonymised using a standard coding system with patient identification held at the hospital. Samples were transferred on ice to the laboratory for storage and further processing.

## **4.6. Statistical Analysis**

All statistical analyses were carried out using the Prism 5 Software package (GraphPad Software Inc, La Jolla, California, USA) with significance level set at 5%.

### **4.6.1. Demographics and Clinical History**

For demographic details, the age of the control and recurrent UTI groups were compared according to menopausal status to make sure that there was no significant difference in the age distribution of cases and controls. Given the expected continuous parametric distribution, a Student's t-test carried out between control and rUTI subjects.

For all other comparisons on clinical history criterion between control and recurrent UTI groups, Fisher's exact test was used.

#### 4.6.2. SNP Analysis

The relative prevalence of TLR5<sup>392Stop</sup> SNP was compared between the control and rUTI groups using Fisher's exact test.

#### 4.6.3. Symptom and quality of life scores

Symptom and health status scores between control and rUTI groups were rated on an interval ordinal scale and were not necessarily normally distributed therefore comparisons were carried out using the non-parametric, Mann-Whitney U test.

When comparing matched scores relating to severity and bother, a Wilcoxon matched paired test was used.

The EQ-5D scores were analysed according to the descriptive system looking at each category separately which is regarded as the most appropriate method for clinical observational studies (Krabbe and Weijnen, 2003) with a Student's t-test carried out between control and rUTI subjects.

#### 4.6.4. Biopsy, urine and douche analyses

For biopsies, urine and douche analyses, two-tailed Student's t-test (with Welch's correction to correct for unequal variance) was used to compare control and rUTI subjects. For analyses of the 12-patients that had infection (which involved comparison with the TLR5<sup>392Stop</sup> subjects), a one-tailed t-test was used as this was a smaller subgroup and from the *in vitro* data there was a reasonable expectation that differences in expression or secretion would be in the negative direction potentially making a two-tail test arguably unnecessary. However, for the sake of completion, data is included for both types of analyses.

For comparison of the first and second douche, a paired two-tail test was used as each measurement was taken from the same individual.

Data for gene mRNA expression is presented on a log scale in line with the nature of PCR based amplification. Peptide data is presented on a linear scale as the ELISA technique is not based on an amplification protocol.

The significance level for all these analyses was set at 5%. However, the p-value has been also been indicated on each graph to allow the reader to appreciate the exact probability that any given result is due to chance. It is my opinion that unlike the *in vitro* data, since clinical data contains great variability, confounding factors plus many sub-groups and can therefore never be repeated exactly in the same way, then it is beneficial for the reader to have the opportunity to make their own judgement also on the significance of any given result.

# 5. In-Vitro Model of Cystitis

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## 5.1 Introduction

Innate immunological mechanisms are important in protection of the lower urinary tract against bacterial colonisation. These mechanisms are remarkably effective in maintaining a bacteria free bladder. When infection does occur it begins with increased colonisation of vaginal and periurethral mucosa by bowel and skin commensals with subsequent migration up the urethra and attachment to the bladder mucosa. A key component of the innate defence is the synthesis of cationic antimicrobial peptides (AMP) following activation of epithelial cell surface Toll-like receptors (TLR) by bacterial components. Existing research suggests that epithelial production of cationic antimicrobial peptides (AMPs) such as cathelicidins and defensins are an important aspect of host anti-microbial defence. Several AMPs have been previously identified in the urogenital tract: beta-defensin 1 (BD1), beta-defensin 2 (BD2), alpha-defensin 5 (HD5) and cathelicidin (LL37). However, the roles and nature of these peptides in the lower urogenital tract has not been fully delineated (Zasloff 2007).

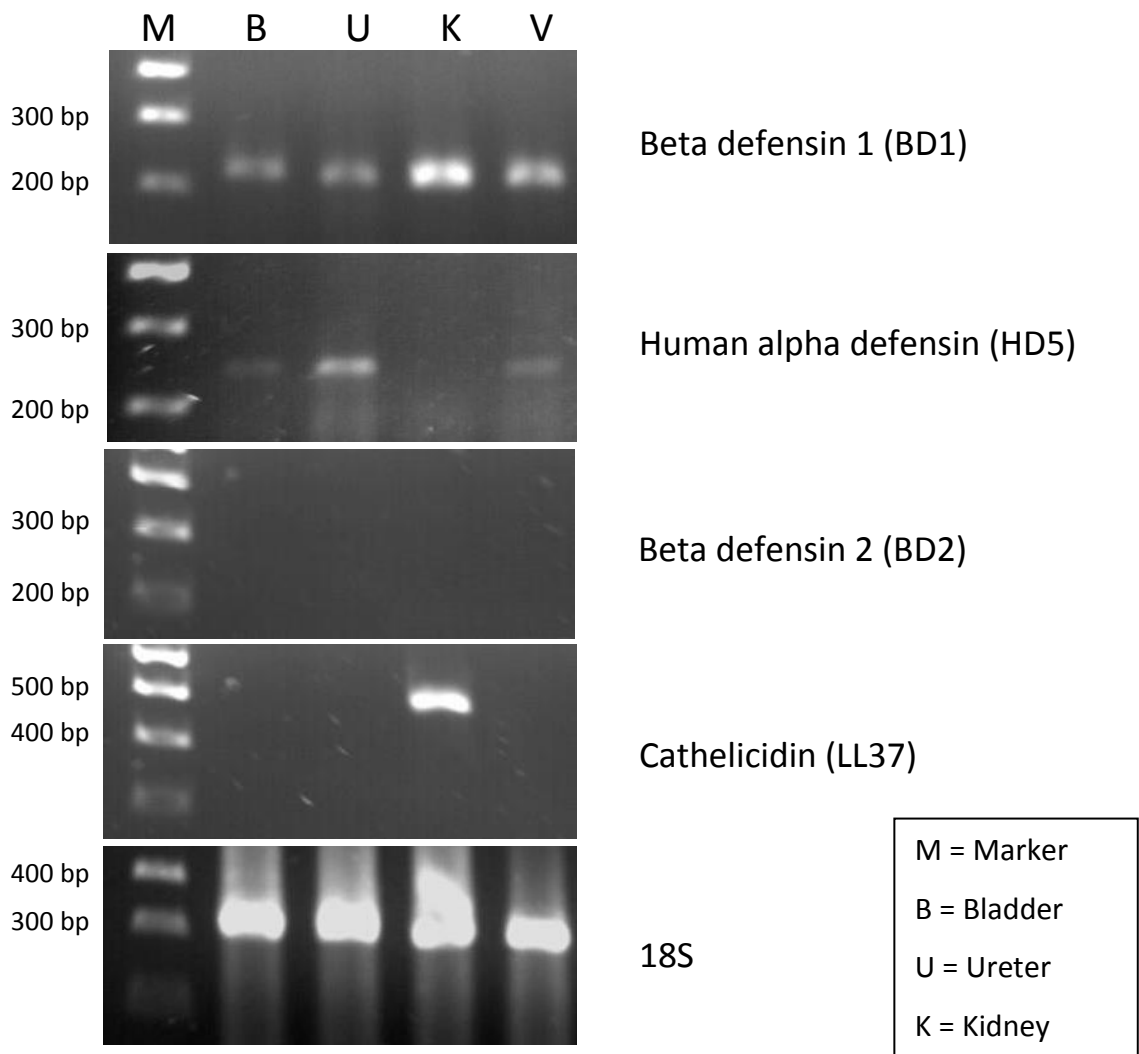
The aims of the *in vitro* experiments outlined in this chapter were to:

- Delineate which AMPs were involved in the normal innate immune response of the bladder and vagina against *E. coli* infection.
- Determine the nature of the AMP response induced by *E. coli* in terms of contrasting the effects of laboratory *E. coli* strains and their cell wall components alone.
- Determine what effect if any estrogen had on the AMP response.

## 5.2 Basal expression of AMPs

To examine the basal expression of AMPs in human urogenital tissues, five samples each of kidney, ureter, bladder, and vagina were analysed. The end-point PCR gel pictures in Figure 5.1 gives a representative example of each tissue type (Bladder, Ureter, Kidney and Vagina) tested. BD1 was expressed in all tissues with level of expression highest in the kidney. HD5 show highest levels of expression in the ureter with lower levels in the bladder and vagina and only a very faint band for the kidney. No BD2 expression was seen. LL37 expression was only seen in the kidney tissue. 18S expression was qualitatively similar for all tissues.

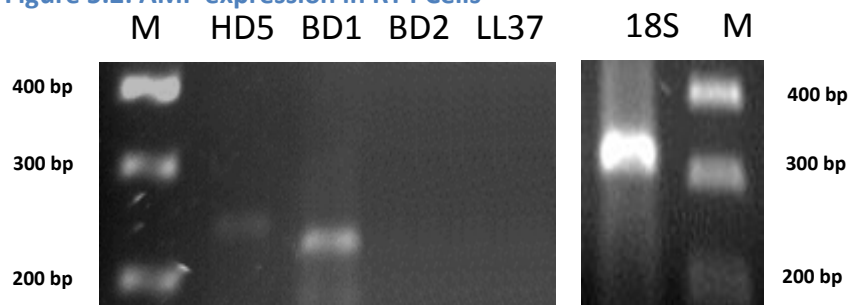
**Figure 5.1: End-point PCR of One Representative Sample of each Tissue**



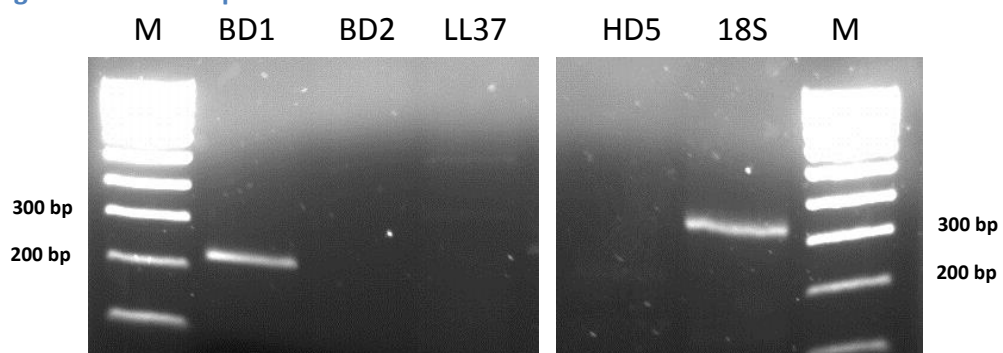
The RT4 Urothelial cell line was chosen as a suitable *in vitro* immortalised cell line modelling AMP response in the bladder as this had been previously used by both our own group and others for examining AMP expression (Townes *et al.* 2010). Other possibilities considered included the alternative immortalised commercially available cell lines UROtsa (Rossi *et al.*, 2001) and J82 (Xu *et al.*, 1994) but basal AMP expression in RT4 was more consistent with that of normal bladder urothelium. Figure 5.2 shows basal expression of AMPs in RT4 cells.

For the selection of the vaginal cell line, there were fewer options available, with only two immortalised cell lines described in the literature, VK2 E6/E7 (Fichorova *et al.*, 1997) and PK2 E6/E7 (Rajan *et al.*, 2000). Of these, the VK2 cell line was more widely used in the literature and this was also the only commercially available vaginal cell line (from ATCC), although the PK2 cell line is available from the Antony Schaeffer and David Klumpp's group at North West University, Chicago. Figure 5.3 shows basal expression of AMPs in the VK2 cell line.

**Figure 5.2: AMP expression in RT4 Cells**



**Figure 5.3: AMP Expression in VK2 Cells**



**Legend for Figure 5.2 and Figure 5.3**

**BD1 = Beta defensin 1**

**HD5 = Human alpha defensin 5**

**BD2 = Beta defensin 2**

**LL37 = Cathelicidin**

**18S = PCR Positive Control**

**M = Base pair size marker**

## **5.3 Urothelial Cells**

In this series of experiments show the RT4 Urothelial cell line is used as *in vitro* model for analysis of AMP expression following challenge by bacteria and bacterial components.

### **5.3.1 Bacterial challenge**

#### **5.3.1.1 Live bacteria**

Initial challenges were attempted with 3 strains of uropathogenic *E.coli* for varying duration of exposure:: 15 minutes, 60 minutes 120 minutes. Challenge exposure to live bacteria continued beyond this time resulted in visibly significant cell death with up to 50% of cells having lost adhesion by 4 hours.

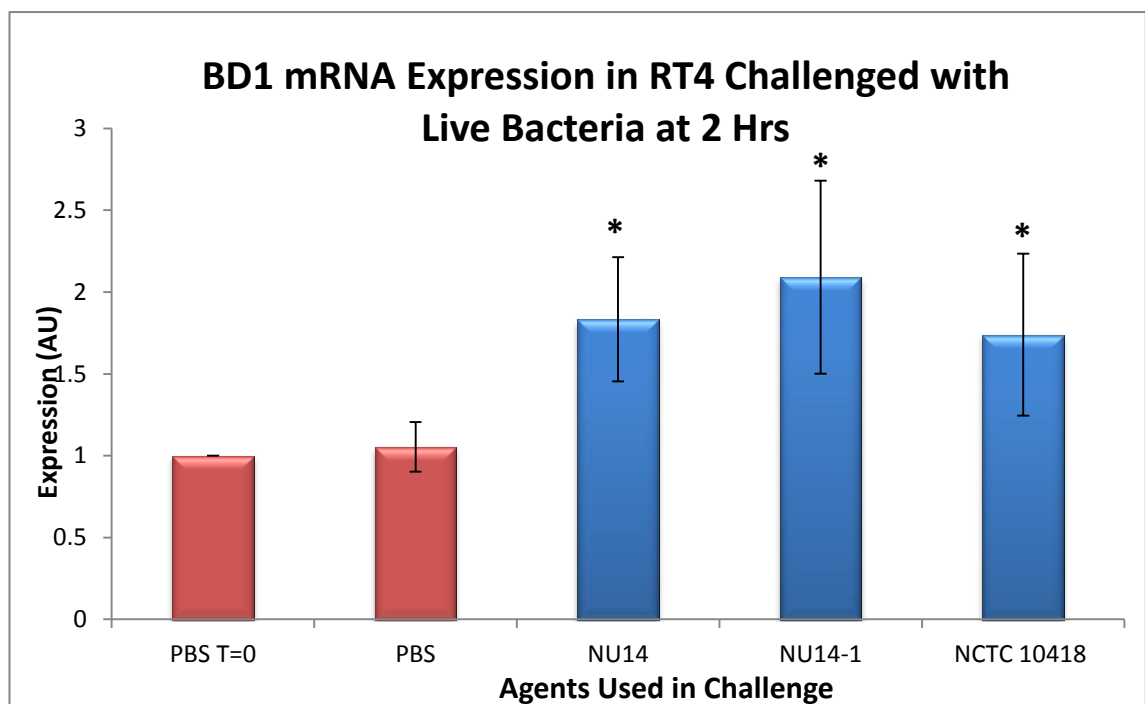
The three *E. coli* strains used were:

- NU14 – a laboratory strain of UPEC with multiple virulence factors derived from a clinical isolate of *E. coli* originally obtained from the urine of a patient with cystitis.
- NU14-1 – This is the corresponding non-functioning *fimH* mutant.
- NCTC 10418 (ATCC® 10536) – A long established National Collection of Type Cultures (NCTC) *E.coli* strain used for testing antimicrobial and disinfectant agents.

For each time point a phosphate buffered saline (PBS) control was also used.

RT-qPCR analyses were carried out for the four AMPs: BD1, BD2, HD5 and LL37. With live bacterial challenges, no significant change in expression was seen at any of the time points for HD5 and LL37. LL37 showed no expression and HD5 was only detectable at very low levels, and not affected by infection. However, significant, reproducible differences were seen at 2-hours for BD1 as shown in Figure 5.4.

**Figure 5.4: BD1 mRNA Expression in RT4 cells challenged with live *E. coli* for 2-hours**



\*Significant difference from PBS control ( $p < 0.05$  Student's t-test)

Measurements made by qRT-PCR with GAPDH and ATP5B house-keeping gene and shown relative to PBS control at time 0.

AU = Arbitrary Units

N = 9 (3 experiments with 3 replicates)

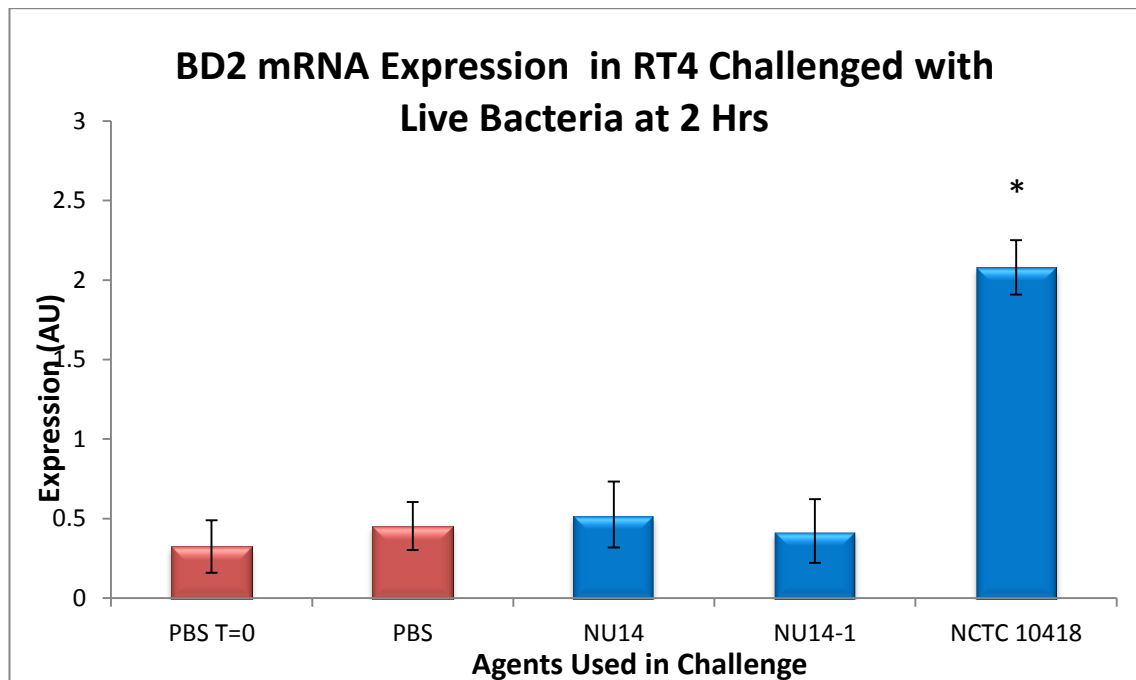
Error bar shows standard error of the mean



Figure 5.4 shows a modest but statistically significant increase in the expression of BD1.

The pattern for BD2 expression was however quite different. There was almost no constitutive expression and no significant difference was seen following challenge with the NU14 strain. However exposure to NCTC 10418, resulted in a significant increase in BD2 expression, which was observed at 2-hours after challenge and shown in Figure 5.5.

**Figure 5.5: BD2 mRNA Expression in RT4 cells challenged with live *E. coli* for 2-hours**



\*Significant difference from PBS control ( $p < 0.05$  Student's t-test)

Measurements made by qRT-PCR with GAPDH and ATP5B house-keeping gene and shown relative to PBS control at time 0.

AU = Arbitrary Units

N = 9 (3 experiments with 3 replicates)

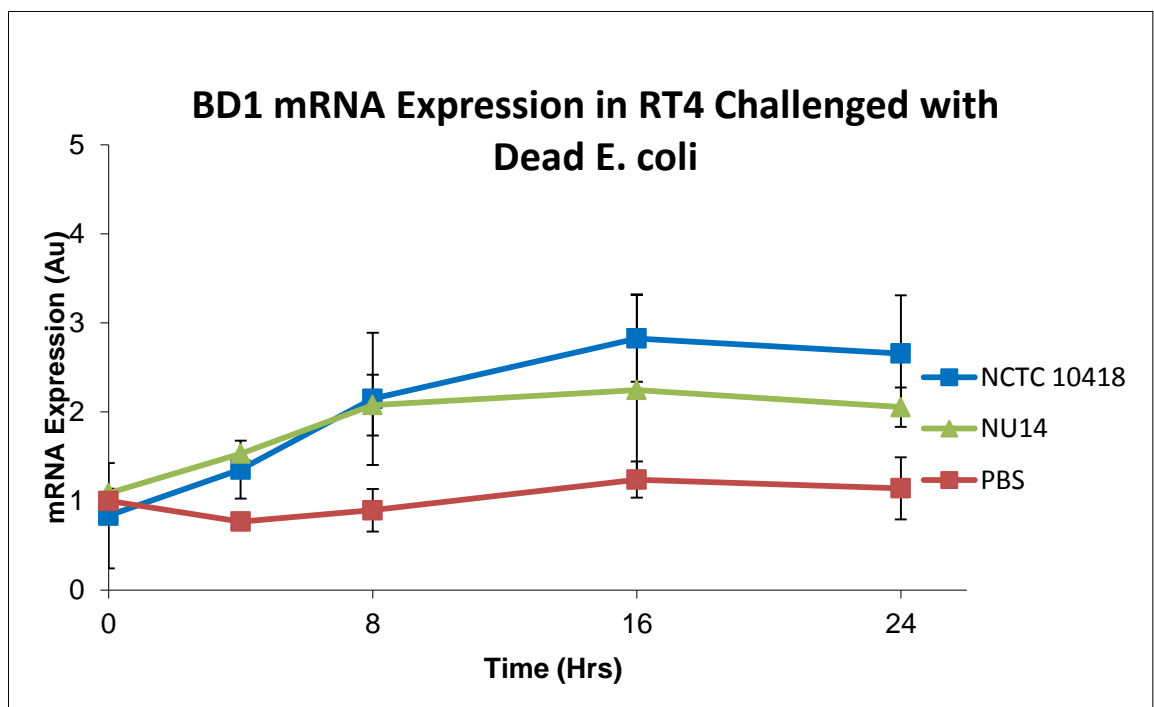
Error bar shows standard error of the mean

### 5.3.1.2 Dead Bacteria

Rapid epithelial cell death following cellular damage during challenge with live bacteria made it difficult to carry out experiments with reliable and reproducible results beyond 2-hours. While this gave a very early snap-shot of the AMP response during bacteria challenge, it was too early to fully understand how the response develops over an extended time period and when peptide secretion begins. Consequently, to model over a longer period, a decision was made to use dead bacteria. The rationale for this being that cell surface ligands known to be involved in TLR-mediated AMP induction (such as LPS and flagellin) would still be present after bacterial killing.

Bacteria were killed as described in the methods section and challenges carried out for 24-hours. Although there was the suggestion of an increase in BD1 expression when the cells were challenged with both *E. coli* strains this was not statistically significant (see Figure 5.6). In contrast a rapid and significant rise ( $p < 0.05$ ) was detected in the expression of BD2 in NCTC 10418 challenged cells as can be seen in Figure 5.7.

Figure 5.6: BD1 mRNA Expression in RT4 cells challenged with dead *E. coli* for 24-hours



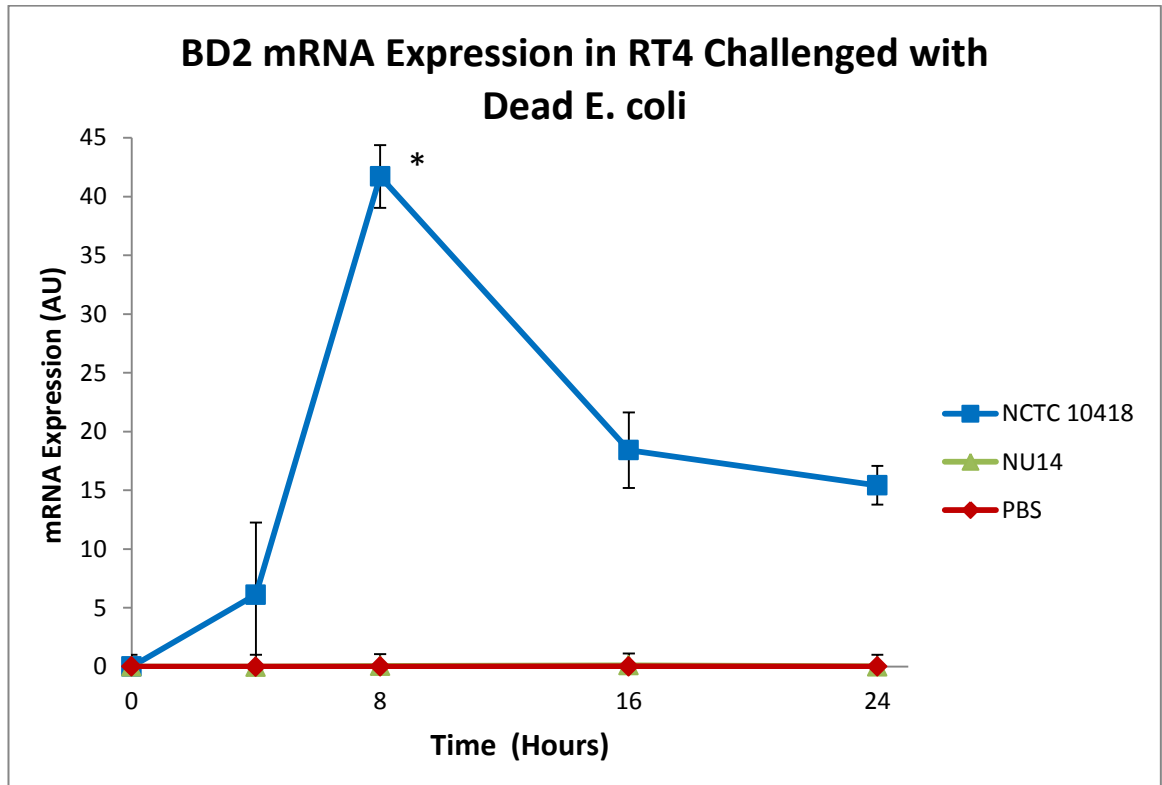
Measurements made by qRT-PCR with GAPDH and ATP5B house-keeping gene and shown relative to PBS control at time 0.

AU = Arbitrary Units

N =9 (3 experiments with 3 replicates)

Error bar shows standard error of the mean

Figure 5.7: BD2 mRNA Expression in RT4 cells challenged with dead *E. coli* for 24-hours



\*Significant difference from PBS control ( $p < 0.05$  Student's t-test)

Measurements made by qRT-PCR with GAPDH and ATP5B house-keeping gene and shown relative to PBS control at time 0.

AU = Arbitrary Units

N =9 (3 experiments with 3 replicates)

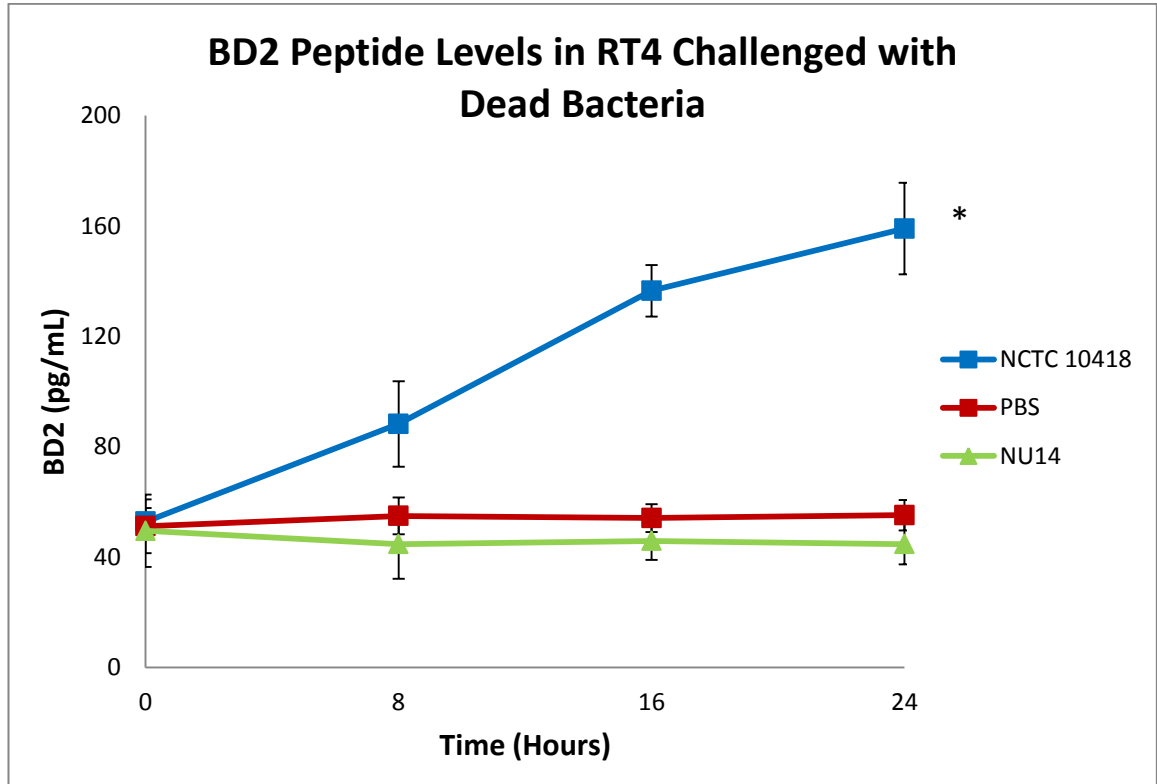
Error bar shows standard error of the mean

The BD2 mRNA expression in RT4 cells challenged by NCTC 10418 rapidly increased to a peak at 8 hours, was decreasing at 16 hours and started to level out at 24 hours but still remained at a higher level of expression than baseline. In contrast, there was no

such response to NU14 challenge with levels of BD2 expression comparable to the PBS control.

To confirm that these findings were not purely at a transcription level, further experiments were carried to check for peptide synthesis using the BD2 sandwich ELISA described in Methods. In Figure 5.8, it can be seen that peptide levels increased from baseline following challenge with NCTC 10418. The maximal rate of increase was detected between 8 and 16 hours which corresponded to the period following the maximal mRNA expression seen at 8 hours. By 24-hours, peptide levels had increased from a baseline 52 pg/mL to 158 pg/mL, while the control showed no change. In contrast, RT4 challenged with NU14 showed no peptide response and comparable to the basal level.

Figure 5.8: BD2 Peptide Secretion in RT4 cells challenged with dead *E. coli* for 24-hours

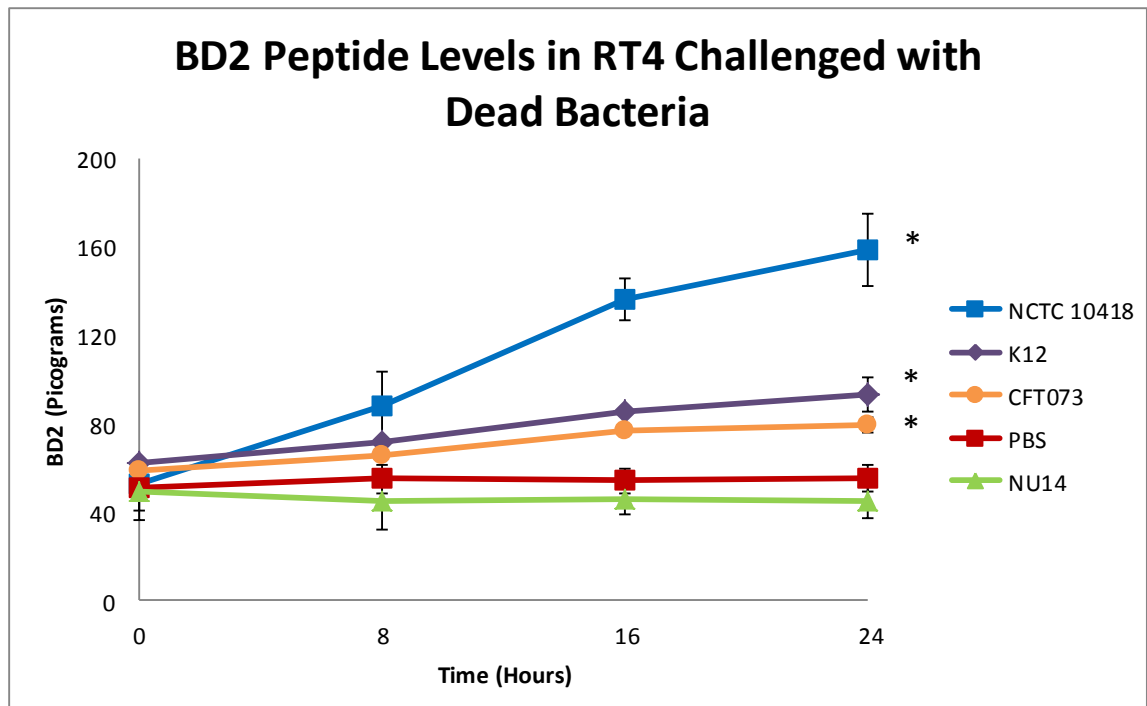


\*Significant difference from PBS control ( $p < 0.05$  Student's t-test)

**N = 6 (3 experiments with 2 replicates)**  
Error bar shows standard error of the mean

To further explore the BD2 epithelial response to *E. coli*, BD2 peptide secretion was analysed following challenge by two other *E. coli* strains: K12, a commensal (asymptomatic bacteriuria) type strain and CFT073, a UPEC strain associated with pyelonephritis. The results are shown in Figure 5.9.

**Figure 5.9: BD2 Peptide Secretion in RT4 cells challenged with four different strains of dead *E. coli* for 24-hours**



\*Significant difference from PBS control ( $p < 0.05$  Student's t-test)

**N = 6 (3 experiments with 2 replicates)**  
Error bar shows standard error of the mean

A clear range of magnitude in BD2 responses evoked in RT4 by the different strains of *E. coli* was demonstrated with highest response induced by NCTC 10418 and the lowest response by NU14.

Concurrently with these experiments, my research colleague, Marcelo Lanz carried out bacterial challenge experiments in RT4 using the same protocol as my experiments in his MRes but using a luciferase based NF $\kappa$ B reporter gene as the outcome measure. Where the previously described experiments had examined the link between cell surface receptor activation and expression/secretion of immune effector, his experiments examined the intermediary signalling molecule NF $\kappa$ B response using luciferase luminescence measurements as illustrated in Figure 5.10.

**Figure 5.10: Induction of NF- $\kappa$ B then AMPs by PAMPs acting through TLRs**

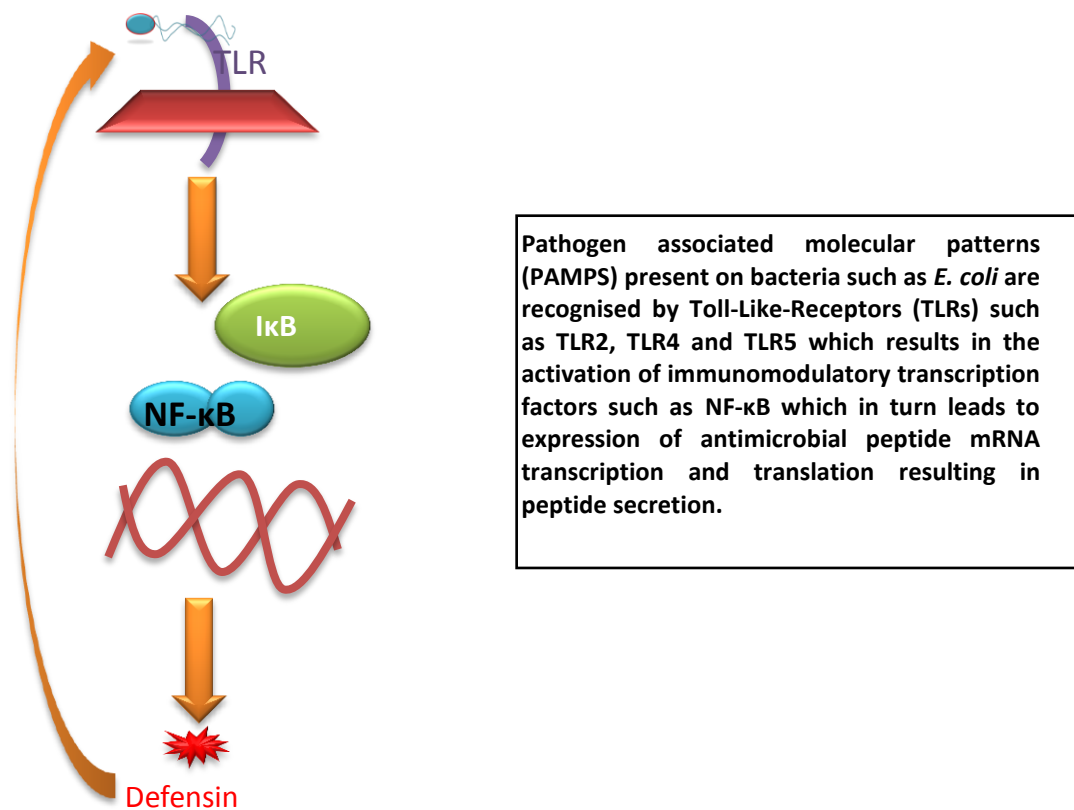
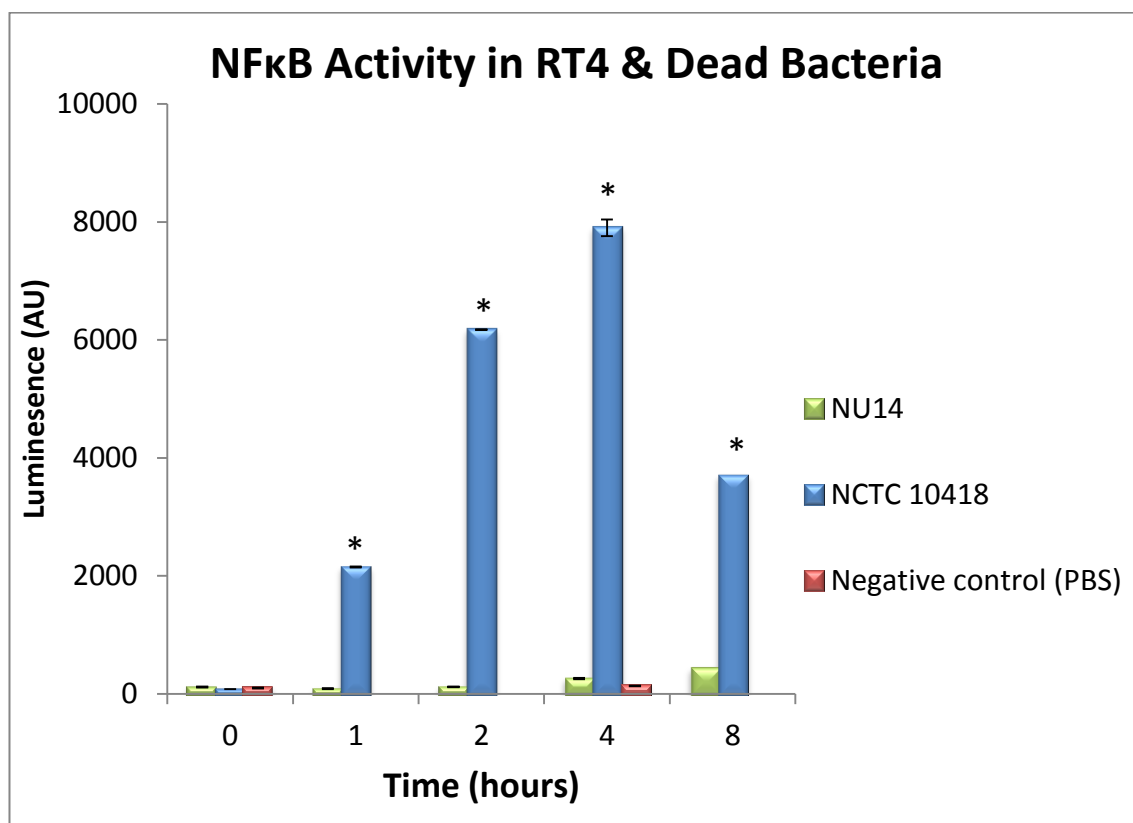


Figure 5.11 (reproduced with permission) demonstrates the NF $\kappa$ B response in RT4 cells challenged with dead *E. coli* strains over 8 hours using the NF- $\kappa$ B reporter assay.

Figure 5.11: NFκB response by luciferase luminescence in RT4 cells challenged with dead *E. coli* for 8-hours



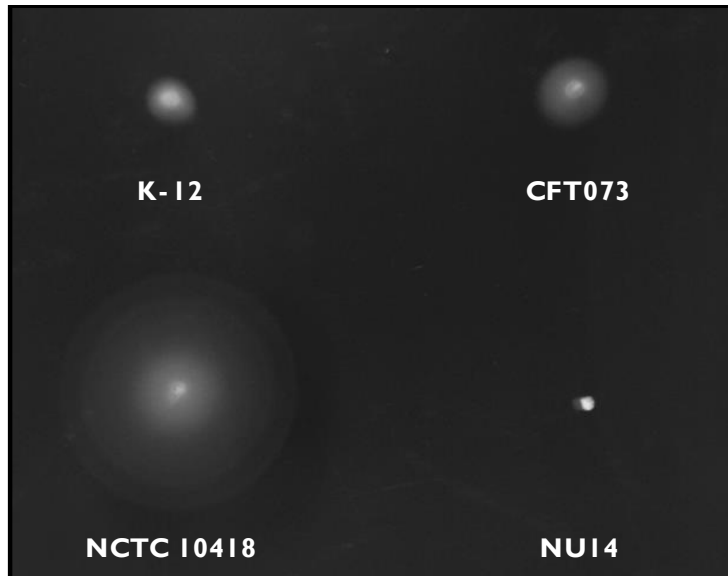
\*Significant difference from PBS control and NU14 ( $p < 0.05$  Student's t-test)

N = 6 (3 experiments with 2 replicates)

Error bar shows standard error of the mean

These data emphasised that the differences in immune response elicited by NU14 and NCTC 10418, were manifest from an early point in the signalling cascade. A key phenotypic difference between strains that was observed in our laboratory was motility. The NU14 was the least motile and NCTC the most motile. The results of a motility assay carried out by Drs Chris Birchall & Phil Aldridge are demonstrated in Figure 5.12.

Figure 5.12: *E. coli* Motility assay over 8-hours (data from Chris Birchall)



*E. coli* swarming motility on LB media. Colonized agar appears white and uncolonized agar appears black. Swarming cells colonize a larger surface area than non-swarming cells.

This motility assay demonstrated bacterial movement using time capture over 8-hours. The differences in strain motility reflected by the size of each halo led us to frame the hypothesis that BD2 secretion may be activated by the presence of the *E. coli* cell wall component, flagellin. Consequently to investigate this further experiment were carried out using specific components of *E. coli*.

### 5.3.2 Bacterial component challenge

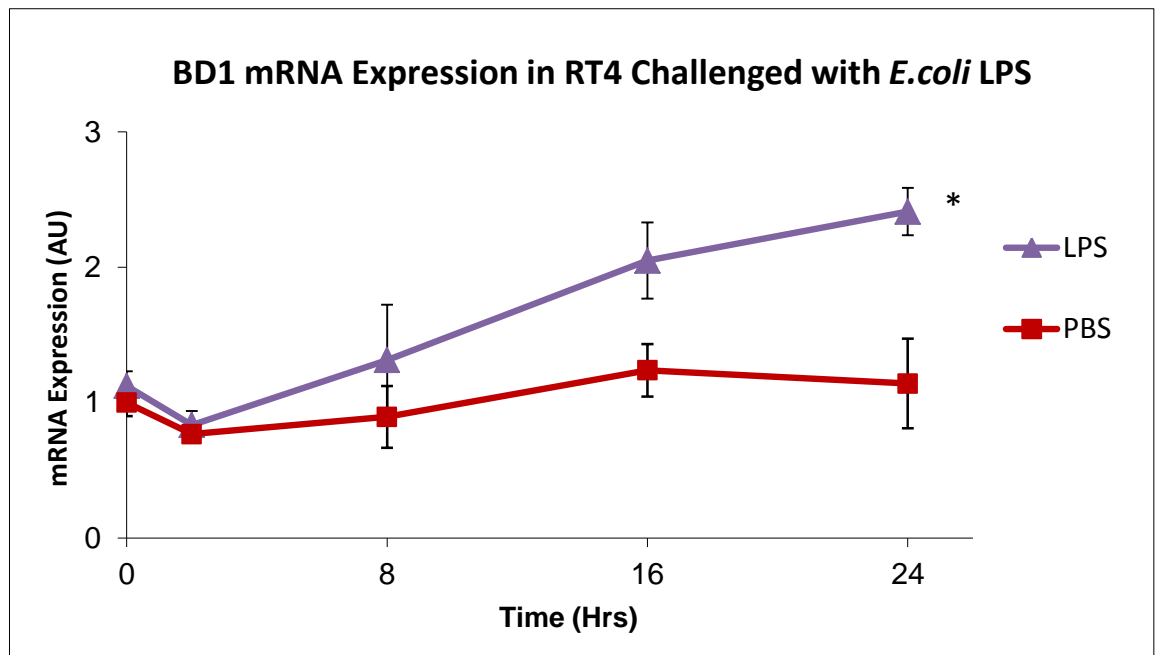
#### 5.3.2.1 LPS

RT4 cells were challenged with commercially sourced *E. coli* LPS from both *E. coli* 0111:B4 and K12 (Invivogen, California, USA) at varying concentrations up to a maximum of 1 mg/ml based on manufacturer's guidelines and published literature (Song *et al*, 2007). LPS is a recognised TLR4 agonist. A small but significant increase was seen in BD1 expression, which increased slowly over the 24-hour challenge period. The effect was best demonstrated with the 0.5 mg/mL LPS challenge and shown for *E. coli*



0111:B4 in Figure 5.13. The pattern seen broadly conformed with that seen with the dead bacterial challenge with a slow rise in BD1 expression in challenged cells, levelling out by 24-hours to approximately 2.4 AU vs 1.1 AU for unchallenged cells.

**Figure 5.13: BD1 mRNA Expression in RT4 cells challenged with *E. coli* 0111:B4 LPS for 24-hours**



\*Significant difference from PBS control ( $p < 0.05$  Student's t-test)

Measurements made by qRT-PCR with GAPDH and ATP5B house-keeping gene and shown relative to PBS control at time 0.

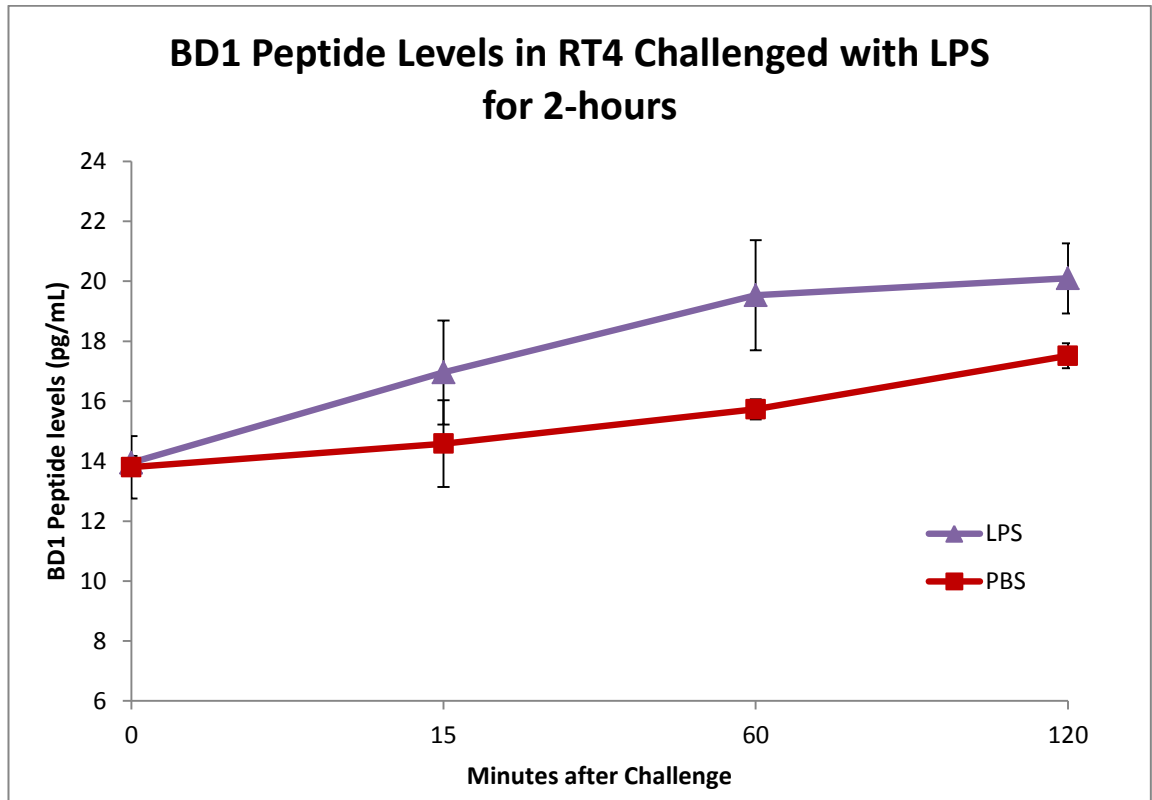
AU = Arbitrary Units

N = 9 (3 experiments with 3 replicates)

Error bar shows standard error of the mean

To examine if this difference could also be detected in BD1 peptide synthesis, sandwich ELISA was carried out on the culture medium as shown in Figure 5.14 and Figure 5.15.

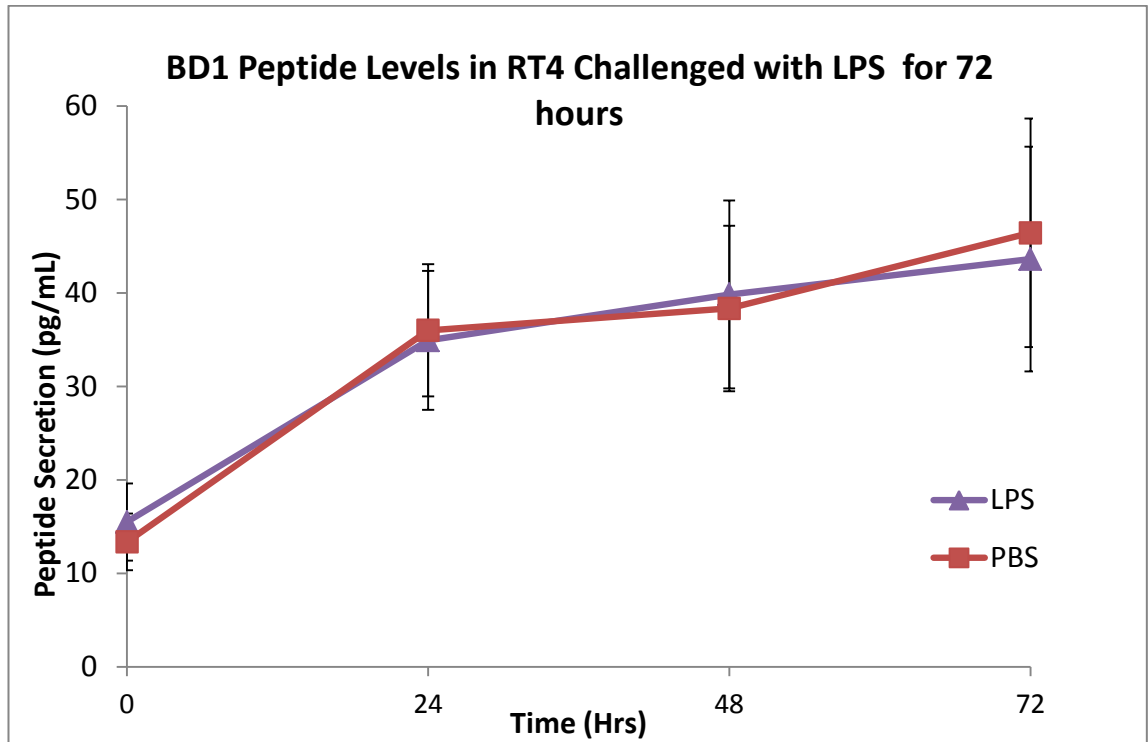
Figure 5.14: BD1 Peptide Secretion in RT4 cells challenged with *E. coli* LPS for 2-hours



N = 6 (3 experiments with 2 replicates)  
Error bar shows standard error of the mean

Figure 5.14 shows that while there was a consistent numerically small difference in BD1 levels peptide levels by ELISA within the first hour, it was not significant and became undetectable as BD1 levels increased due to peptide accumulation in both control and challenged cells over a 72 hour period (Figure 5.15). These findings are consistent with existing studies demonstrating that BD1 secretion is largely constitutive with only a small capacity for induction beyond basal levels.

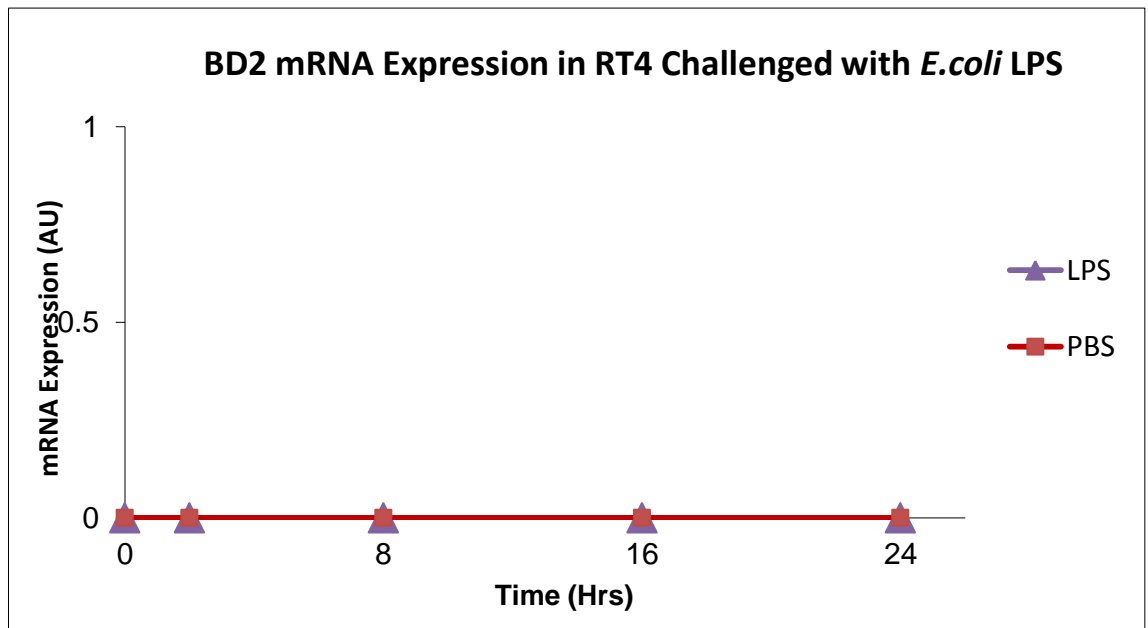
Figure 5.15: BD1 Peptide Secretion in RT4 cells challenged with *E. coli* LPS for 72-hours



N = 6 (3 experiments with 2 replicates)  
Error bar shows standard error of the mean

In contrast to BD1, no induction of BD2 mRNA was seen, as demonstrated in Figure 5.16. These data suggested that LPS is not involved in the induction of BD2 in RT4 cells.

Figure 5.16: BD2 mRNA Expression in RT4 cells challenged with *E. coli* LPS for 24-hours



Measurements made by qRT-PCR with GAPDH and ATP5B house-keeping gene and shown relative to PBS control at time 0.

AU = Arbitrary Units

N =9 (3 experiments with 3 replicates)

Error bar shows standard error of the mean

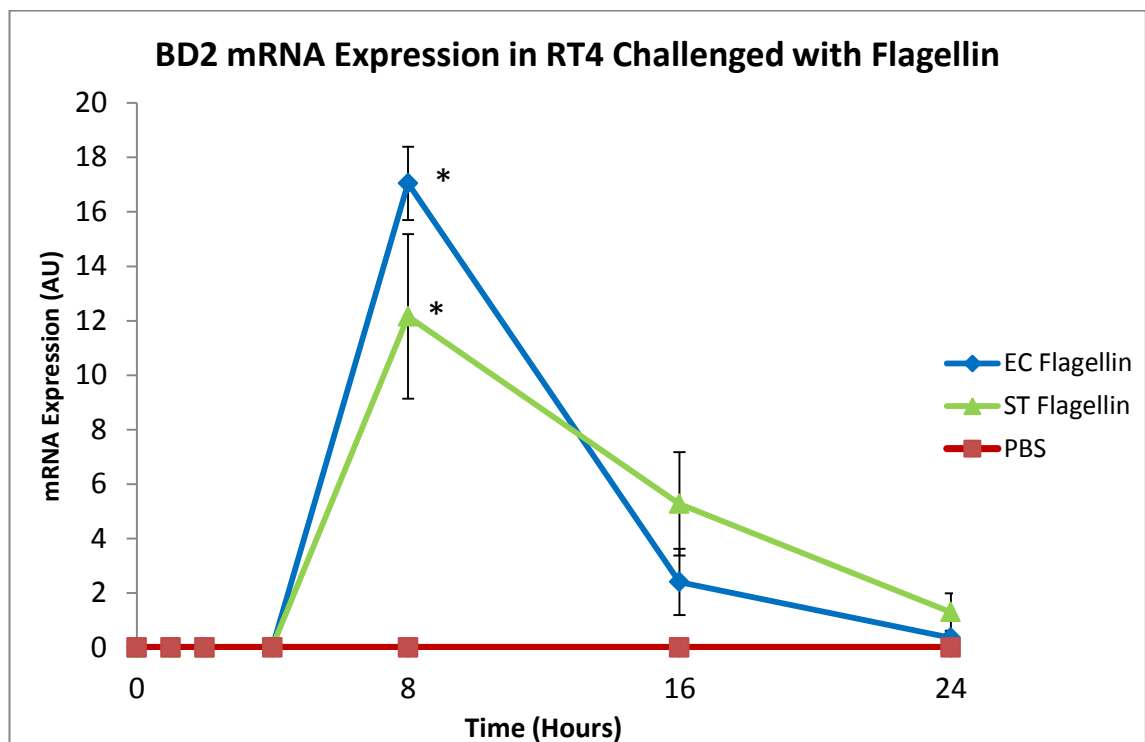
### ***5.3.2.2 Peptidoglycan***

RT4 was also challenged with commercially sourced peptidoglycan, a TLR2 agonist, from both *E. coli* 0111:B4 and K12 (Invivogen, California, USA). No significant change in BD1 or BD2 was seen between challenged RT4 cells and controls at a range of concentrations up to 48 hours – data not shown.

### ***5.3.2.3 Flagellin***

For challenges using flagellin, commercially sourced *S. typhimurium* flagellin (Invivogen, California, USA) and *E. coli* NCTC 10418 flagellin produced in our own laboratory (by Marcelo Lanz) were used. The results for 250 ng challenge are shown in Figure 5.17.

Figure 5.17: BD2 mRNA Expression in RT4 cells challenged with *E. coli* & *S. typhimurium* flagellin for 24-hours



\*Significant difference from PBS control ( $p < 0.05$  Student's t-test)

Measurements made by qRT-PCR with GAPDH and ATP5B house-keeping gene and shown relative to PBS control at time 0.

EC = *E. coli*

ST = *S. typhimurium*

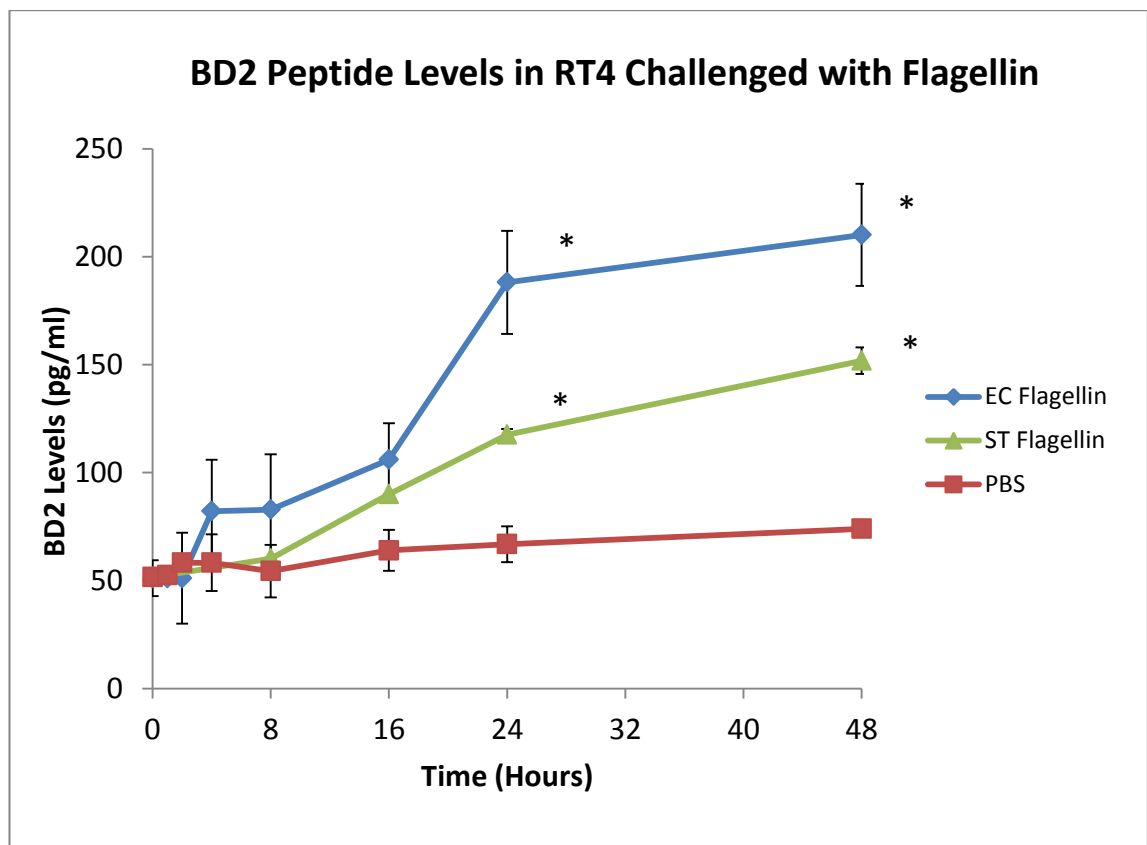
AU = Arbitrary Units

N = 9 (3 experiments with 3 replicates)

Exposure of RT4 cells to flagellin evoked a strong response in term of increasing BD2 mRNA expression. This response was seen for both types of flagellin but that seen for *E. coli* flagellin (17 AU) was higher than with an equivalent concentraion of *Salmonella typhimuirum* (12 AU). Comparable to the response seen when RT4 was challenged with dead NCTC 10418 *E. coli*, the first detectable rise in BD2 expression was at 4 hours with a peak response at 8 hours and then a decline to 24 hours. No BD2 expression was seen in the unchallenged cells. However, unlike with the dead bacterial challenge, the decline in expression was more rapid, which was most likely due to the lower level of maximal induction compared to that seen with dead bacteria. Nonetheless, the fact

that two different flagellin preparations evoked a BD2 response, coupled with the observations on motility provides strong evidence that TLR-5 is the PAMP linked to the induction of BD2. To delineate the response further, sandwich ELISA was used to examine BD2 peptide concentrations within the medium of the RT4 cells and the results are shown in Figure 5.18.

**Figure 5.18: BD2 Peptide Secretion in RT4 cells challenged with *E. coli* Flagellin for 48-hours**



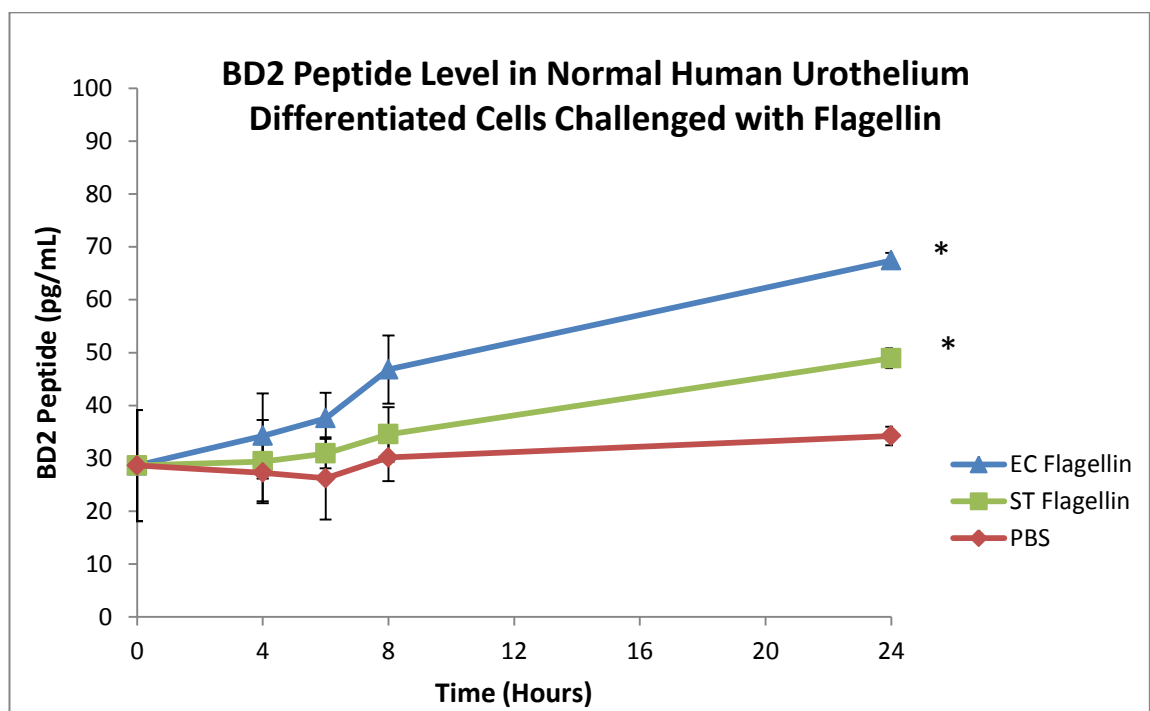
\*Significant difference from PBS control ( $p < 0.05$  Student's t-test)  
**N = 6 (3 experiments with 2 replicates)**  
**Error bar shows standard error of the mean**

These peptide data confirmed that there was significantly more BD2 detected in the media of cells challenged with *E. coli* flagellin compared to unchallenged cells. The increase was from 50 pg/mL to 190 pg/mL representing a greater than 3-fold increase by 24-hours, after which concentrations stabilised at 200 pg/mL by 48-hours. A similar

pattern but at lower BD2 concentrations was observed using *Salmonella typhimurium* flagellin, where BD2 values of approximately 120 pg/mL were measured at 48 hours.

To validate these data further, additional experiments were carried out using differentiated normal human urothelium (NHU) cells grown from primary tissue taken during urological surgery. These experiments were carried out in conjunction with Dr Claire Varley and Professor Jenny Southgate, York University. The NHU cells were established in York as described in methods, challenged with flagellin and the peptide levels in the medium assayed using the same ELISA technique as for the RT4 medium. The results from these experiments are shown in Figure 5.19.

**Figure 5.19: BD2 Peptide Secretion in NHU cells challenged with *E. coli* & *S. typhimurium* Flagellin for 24-hours**



\*Significant difference from PBS control ( $p < 0.05$  Student's t-test)  
N = 6 (3 experiments with 2 replicates)  
Error bar shows standard error of the mean

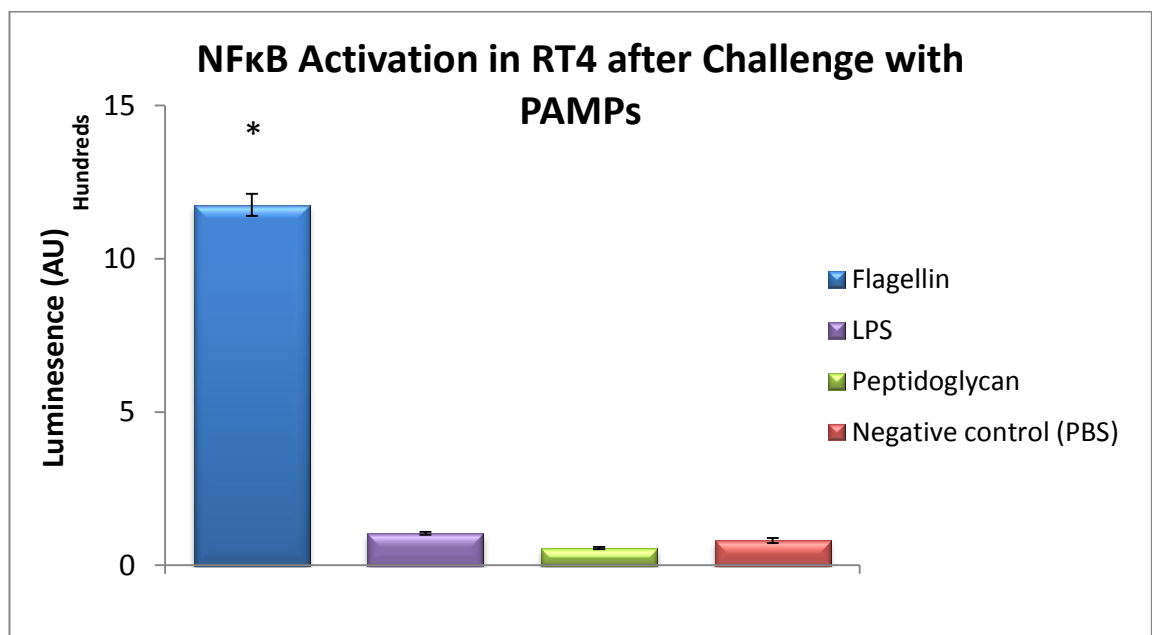
The observed pattern in BD2 production was similar to that seen with the RT4 cells with significant increases ( $p < 0.05$ ) in BD2 peptide concentrations in response to both

*E. coli* and *Salmonella typhimurium* flagellin. Although the absolute concentrations measured were different reflecting the different nature of the two cell models, the trend and relative changes were similar.

#### 5.3.2.4 Reporter Assay Response to Flagellin

In parallel to the experiments measuring AMP effector output following PAMP treatments Bea Suarez-Falero (an undergraduate project student working under my supervision) examined the NFκB response to the same PAMPs i.e. LPS, Peptidoglycan and Flagellin using an NF-κB luciferase reporter luminescence assay. The data from these experiments is shown in Figure 5.20:

Figure 5.20: NFκB response by luciferase luminescence in RT4 cells challenged with *E. coli* PAMPs for 4-hours



\*Significant difference from PBS control ( $p < 0.05$  Student's t-test)

AU = Arbitrary Units

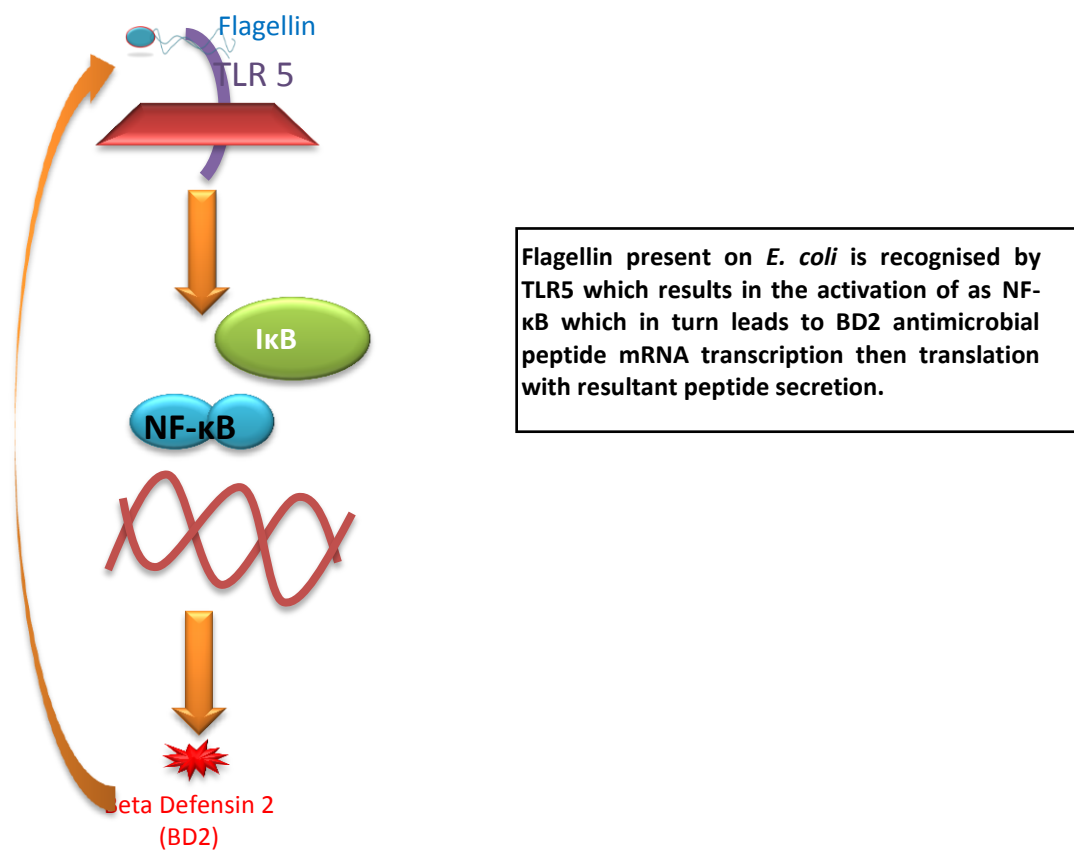
N = 6 (3 experiments with 2 replicates)

Error bar shows standard error of the mean



The data from the NFκB luciferase reporter assay supported the findings of increased BD2 expression and secretion following challenge by flagellin. Increased NFκB activation was seen following challenge with *E.coli* flagellin while no significant increase was detected using either TLR-4 or TLR-2 activators. Coupling these data, with the mRNA expression and peptide secretion data discussed earlier, and the knowledge that flagellin works through TLR5, a signalling pathway similar to that presented in Figure 5.21 can be suggested.

**Figure 5.21: Flagellin Induced Secretion of Beta Defensin 2 (BD2)**

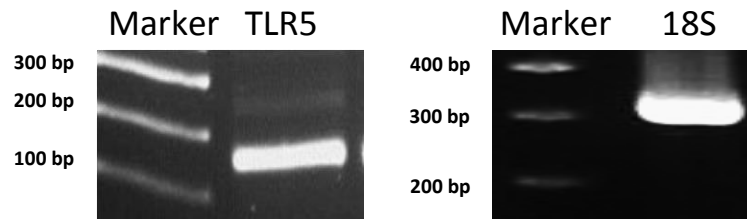


### ***5.3.2.5 Validation of Flagellin Induced BD2 Secretion with Anti-TLR 5 Antibody***

Having found strong evidence for the induction of BD2 secretion by flagellin, it was expected, referring to the schematic diagram shown in Figure 5.21, that this rise would

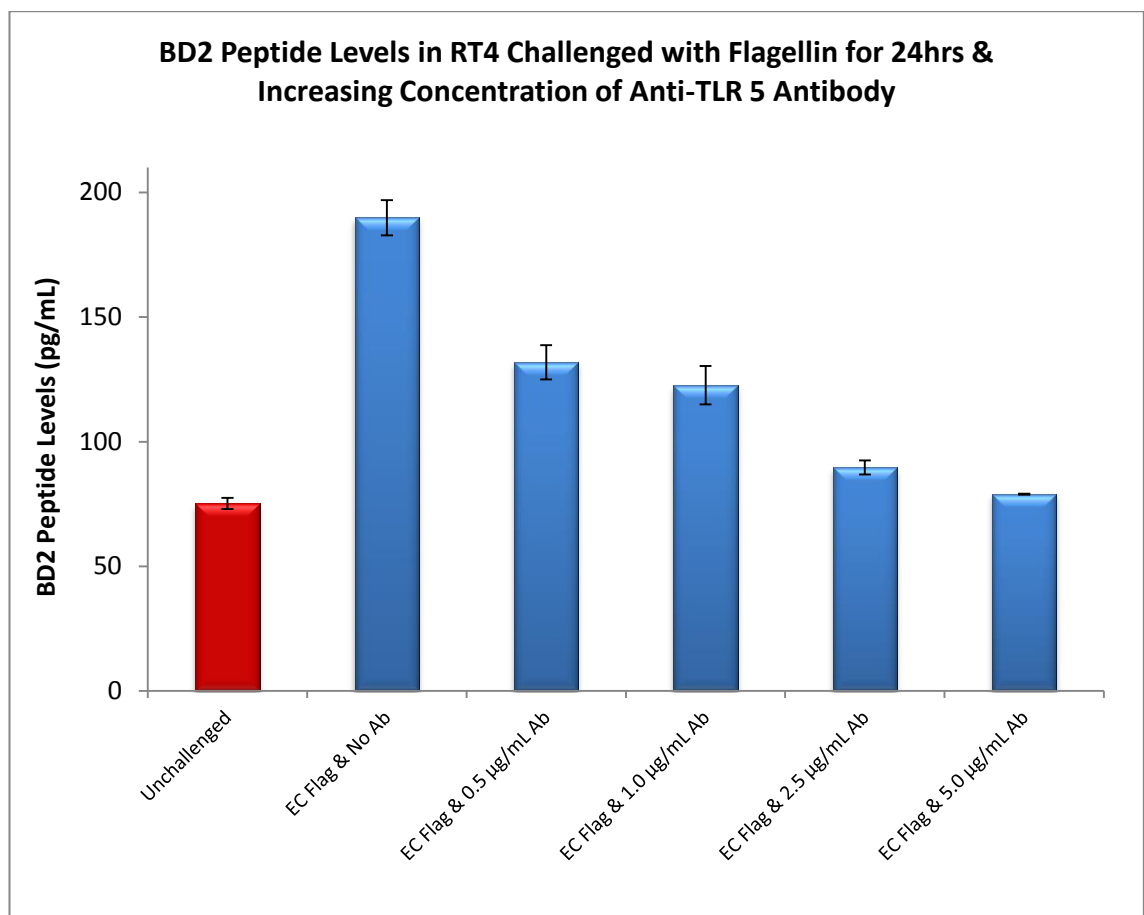
be inhibited by blocking of the TLR5 receptor.. To investigate this further, the expression of the TLR5 receptor was demonstrated in RT4 cells using endpoint PCR (see Figure 5.22) and DNA sequencing.

**Figure 5.22: PCR Confirmation of TLR5 Expression in RT4 cells**



Having confirmed expression, TLR5 was then blocked in the RT4 cell model with an anti-TLR5 antibody (Invivogen, California, USA) using varying dilutions to establish a concentration-dependent response. The results are shown in Figure 5.23.

**Figure 5.23: Anti-TLR5 Antibody inhibition of BD2 peptide secretion in RT4 challenged with flagellin**



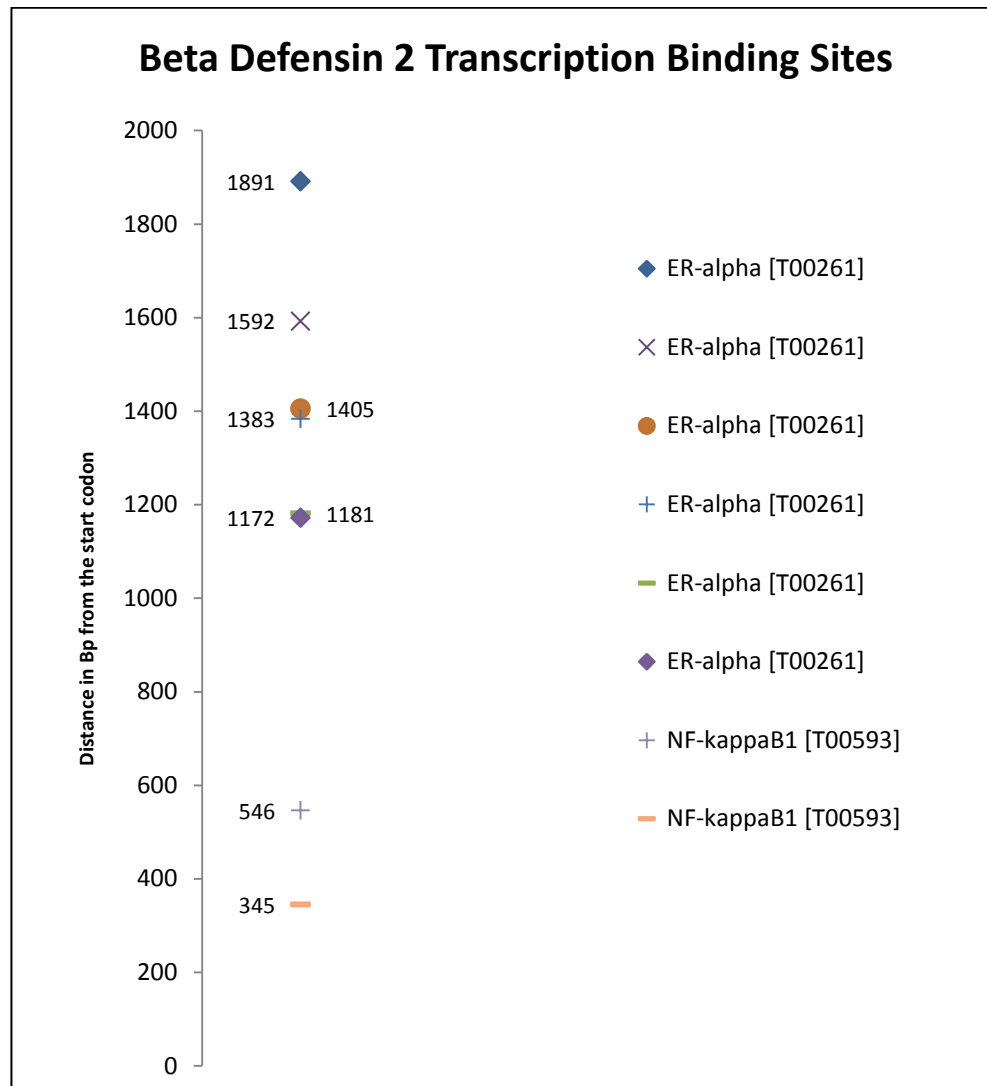
**N =6 (3 experiments with 2 replicates)**  
**Error bar shows standard error of the mean**

These data showed that with increasing concentrations of antibody, there was a progressive reduction in the assayed concentration of BD2 peptide with control BD2 levels measured at an antibody concentration of 5.0 µg/mL. This provided further evidence to suggest that flagellin, acting through TLR5 was inducing secretion of BD2 peptide. To confirm that a similar effect was seen in the signalling pathway, the same concentration of anti-TLR5 antibody was used in the luciferase NFκB reporter assay by Bea Suarez Falero and similar results found (data not shown).

#### ***5.3.2.6 Effect of Estrogen on RT4 Cells***

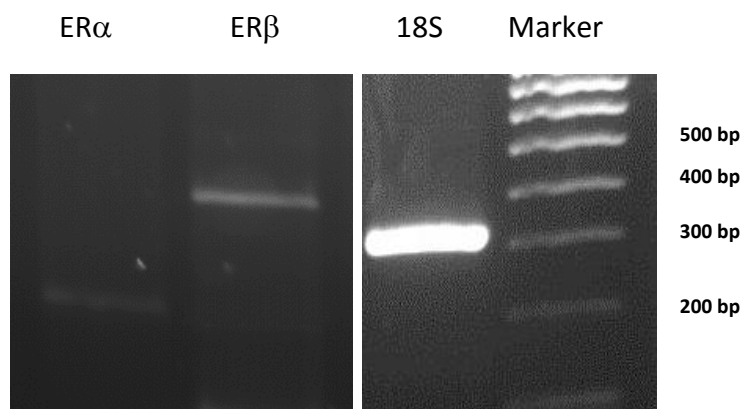
As discussed previously, the risk of UTI is higher for women with relative estrogen deficiency and the epithelium of at least some parts of the bladder are thought to express estrogen receptors (Wolf *et al*, 1991). Furthermore, from bioinformatics analysis using PROMO (Messeguer *et al*, 2002), the BD2 gene is predicted to have several alpha estrogen (ER-alpha) binding sites within the promoter region as shown in Figure 5.24.

Figure 5.24: Beta Defensin 2 Gene Promoter Region Transcription Binding Sites



To investigate this further, the expression of the estrogen receptors, ER $\alpha$  and ER $\beta$  was assessed in RT4 cells employing end-point PCR and using primer pairs gifted by Fatema Al-Atawi and Professor Diane Ford, and described in Methods. The results are shown in Figure 5.25.

**Figure 5.25: Estrogen receptor expression in RT4 cells**



It can be seen that both receptors are expressed in RT4, however, qualitatively ER $\alpha$  band seems fainter than ER $\beta$ .

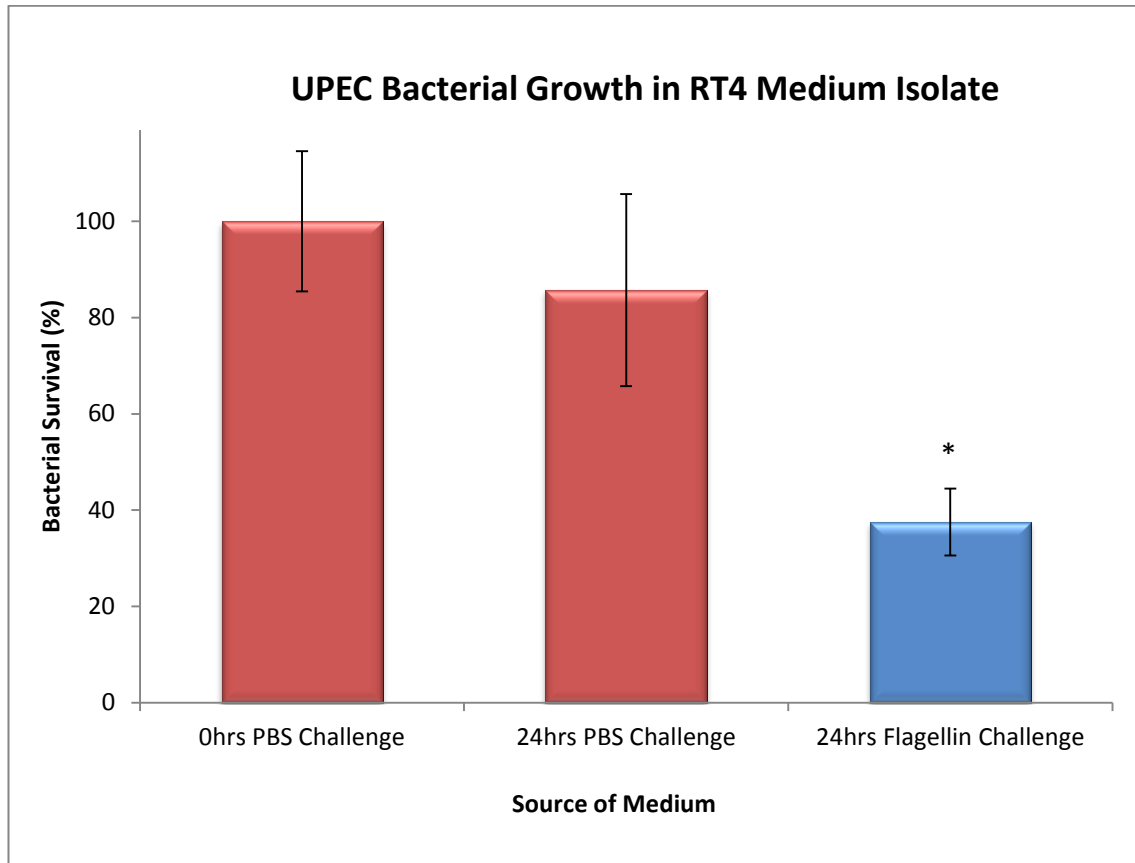
Despite the presence of both estrogen receptors, when RT4 cells were grown in varying concentrations of estrogen ranging from 2nM to 16nM, no significant difference was seen in either basal BD2 AMP gene expression or basal expression of any of the other AMPs studied (BD1, HD5 and LL37) – data not shown.

### **5.3.3 Antimicrobial Effects**

Having established that flagellin from *E. coli* elicits a significant AMP response in RT4 cells through TLR-5 activation and NF $\kappa$ B activation resulting in BD2 mRNA expression and peptide secretion, the potency of BD2 as an antimicrobial against *E. coli* was investigated. The importance of BD2 antimicrobial activity against gram negative bacteria on skin is well recognised (Schröder and Harder, 1999), however, the activity in urothelium has not been described previously.

The antimicrobial activity was investigated in two ways. Firstly, as shown in Figure 5.26, medium from RT4 challenged with PBS and flagellin was used in a 1-hour *E. coli* NU14 time-kill antimicrobial assay.

Figure 5.26: Antimicrobial activity of RT4 medium following challenge with flagellin



\*Significant difference from PBS control ( $p < 0.05$  Student's t-test)

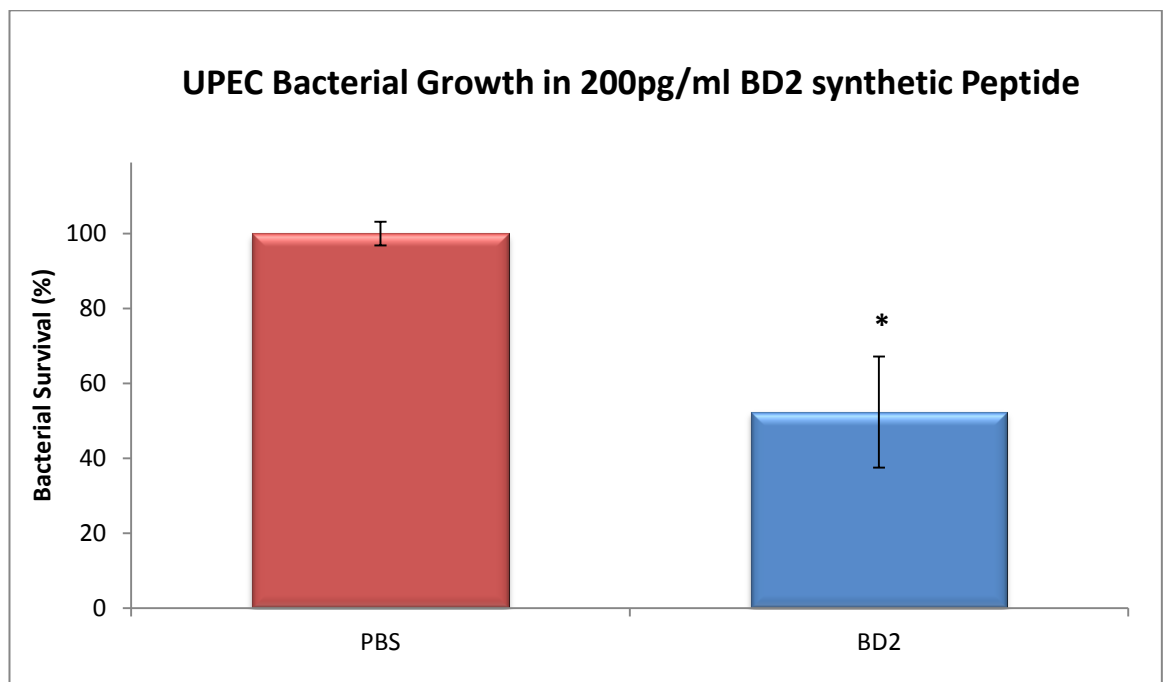
**1-hour Antimicrobial Time Kill assay against *E. coli* NU14**  
**N =9 (3 experiments with 3 replicates)**  
**Error bar shows standard error of the mean**

The results from this experiment showed that the antimicrobial activity of the medium bathing the flagellin challenged cells was increased. Indeed the medium bathing the unchallenged cells showed only a small degree of antibacterial activity with NU14 survival at approximately 86%; in contrast there was considerably enhanced killing activity in the medium from the flagellin challenged cells with bacterial survival reduced to 38%. It is likely that this antimicrobial activity was due to a number of factors in the medium in addition to BD2, and probably explains why the medium of the unchallenged cells showed antimicrobial activity. It is also worth noting that this

difference could only be demonstrated in a short time-kill assay as in a longer assay, any antimicrobial activity was depleted as there is no mechanism to replenish the antimicrobial agents.

To determine what contribution BD2 was making to the total antimicrobial activity, a further time-kill assay was performed using fresh media with synthetic BD2 added at a concentration similar to that measured previously via ELISA in the challenge assays (see Figure 5.18) i.e. 200 pg/mL. The results of this time-kill assay, shown in Figure 5.27 demonstrated reduced bacterial survival in BD2 supplemented media to a mean  $\pm$  SEM of  $52 \pm 15\%$  compared to  $100 \pm 3\%$  in the non-supplemented control ( $P < 0.05$ ).

**Figure 5.27: Antimicrobial activity of synthetic BD2 at concentration comparable to challenge with flagellin**



\*Significant difference from PBS control ( $p < 0.05$  Student's t-test)

**1-hour Antimicrobial Time Kill assay against *E. coli* NU14**

**N =9 (3 experiments with 3 replicates)**

**Error bar shows standard error of the mean**

### 5.3.4 Summary of RT4 Data

To summarise, a number of important positive and negative findings have been made in the RT4 experiments outlined:

- Of the AMPs examined, the main ones expressed *in vitro* were BD1 and BD2. BD1 was mainly expressed constitutively with a small inducible component in response to *E. coli* LPS challenge. BD2 was not expressed constitutively but RT4 cells showed a rapid and significant rise in BD2 expression when challenged with dead samples of motile *E. coli* and flagellin. LL37 and HD5 showed very low levels of expression and no induction on bacterial challenge.
- Of the PAMPs examined, the response to flagellin was the most marked stimulator of the AMP effector response and the increase in BD2 expression seen at the mRNA level was also found at peptide level by ELISA for both motile *E. coli* and flagellin. The BD2 response to flagellin was inhibited by blocking TLR5, the archetypal receptor for flagellin.
- Challenge with flagellin also resulted in induction of NFκB, which could be inhibited by anti-TLR5 antibody suggesting that the intracellular pathway from TLR5 to BD2 secretion is NFκB mediated.
- Estrogen had no effect on AMP responses in RT4 cells despite the presence of estrogen receptors.
- The increased levels of BD2 secreted during flagellin a challenge considerably enhanced antimicrobial activity with bacterial survival reduced to a mean of 52% compared to control.



## 5.4 Vaginal Cells

The vagina provides a primary site of defence against bacterial ascent into the female urinary tract. As described in the introductory chapters, preliminary vaginal colonisation by potential *E. coli* pathogens has been hypothesised to occur prior to symptomatic UTI.

### 5.4.1 Bacterial challenge

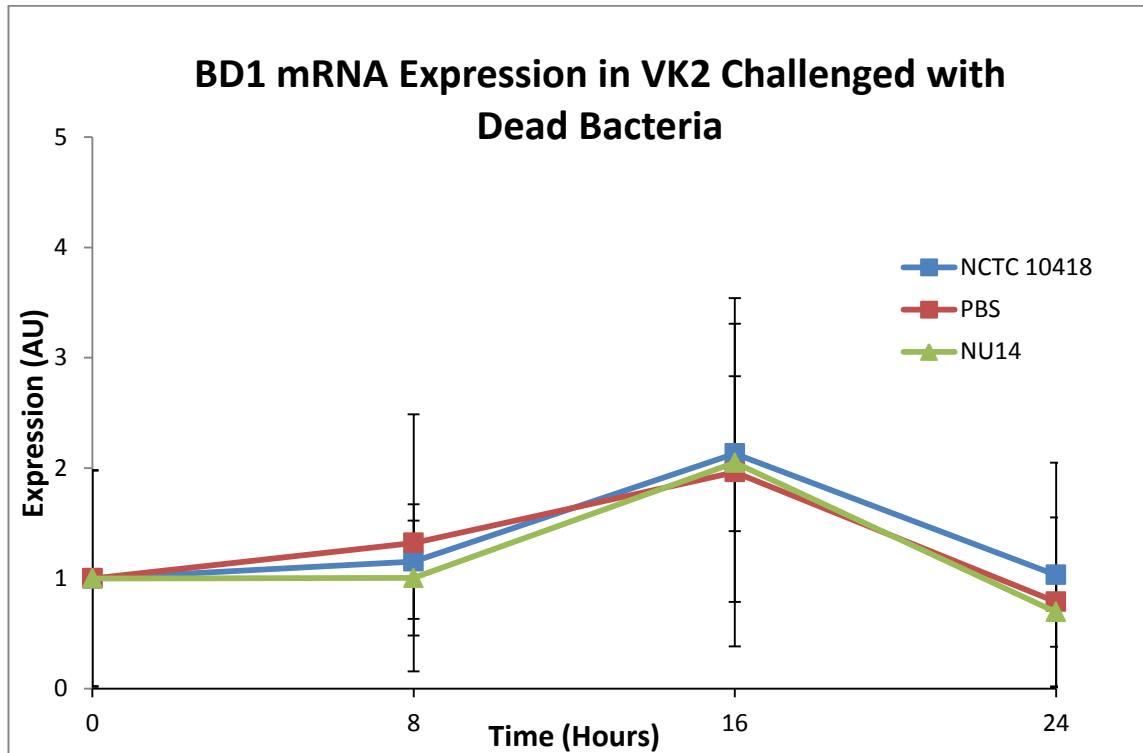
#### 5.4.1.1 Live bacteria

Live bacterial challenges to vaginal cells showed no significant change in AMP expression by 2 hours. At later time points more than 50% of cells lost their adhesion to the well indicating significant cell damage. As a consequence, a decision was made to concentrate analyses on the responses elicited by dead bacteria similar to RT4 urothelial experiments.

#### 5.4.1.2 Dead Bacteria

Bacteria were killed as described in methods and challenges were carried out for up to 24 hours. Very low basal levels of expression were seen for both HD5 and LL37 with no induction on challenging with dead bacteria. Higher basal levels of expression were seen with BD1 but no change was seen on challenge (Figure 5.28)

Figure 5.28: BD1 mRNA Expression in RT4 cells challenged with dead E. coli for 24-hours



Measurements made by qRT-PCR with GAPDH and ATP5B house-keeping gene and shown relative to PBS control at time 0.

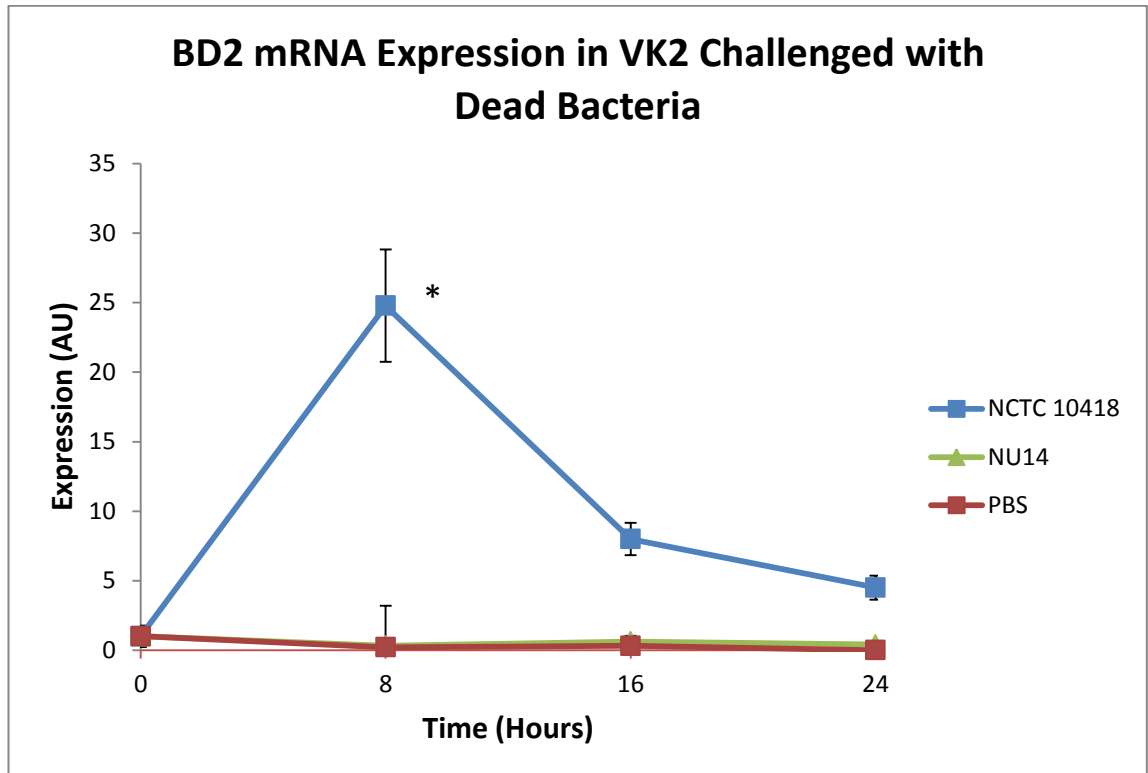
AU = Arbitrary Units

N = 9 (3 experiments with 3 replicates)

Error bar shows standard error of the mean

In contrast, a significant and rapid rise was seen in the expression of BD2 following challenge by NCTC 10418 (Figure 5.29). BD2 was expressed at very low levels although detectable and bacterial challenge leads to a rapid rise. Of note, the maximum rise in expression occurred at a similar time, 8-hours, to that seen in RT4, but the gradient of increase was lower (see Figure 5.7) although the increase remained significantly higher ( $P < 0.05$ ) than control. Furthermore, BD2 expression returned to baseline more rapidly in VK2 cells and was equivalent to baseline at 24 hours. This suggests a more 'measured' response to NCTC 10418 in the vaginal cells with a less elevated response and more rapid reduction than was seen in the urothelium.

Figure 5.29: BD2 mRNA Expression in VK2 cells challenged with dead *E. coli* for 24-hours



\*Significant difference from PBS control ( $p < 0.05$  Student's t-test)

Measurements made by qRT-PCR with GAPDH and ATP5B house-keeping gene and shown relative to PBS control at time 0.

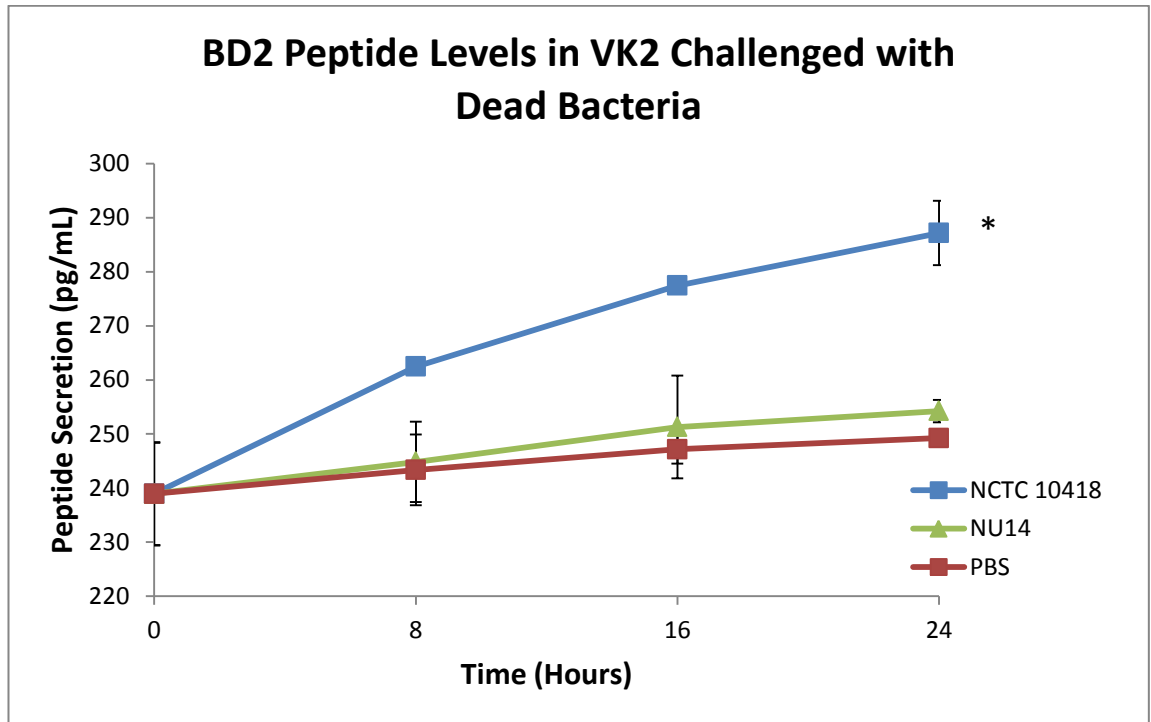
AU = Arbitrary Units

N = 9 (3 experiments with 3 replicates)

Error bar shows standard error of the mean

The pattern seen in peptide secretion confirmed that the rise in BD2 expression was translated to protein with BD2 significantly higher in the 24 hour challenged cells than the PBS control (Figure 5.30 below). Interestingly, despite little basal BD2 mRNA expression, high levels of BD2 peptide were detected in unchallenged cells indicating considerably higher basal secretion. On challenge, a gradual rise was observed with an absolute increase of 48 pg/mL (239 pg/mL to 287 pg/mL) compared to the rise of 106 pg/mL that was seen in RT4 (Figure 5.8).

Figure 5.30: BD2 Peptide Secretion in VK2 cells challenged with dead *E. coli* for 24-hours



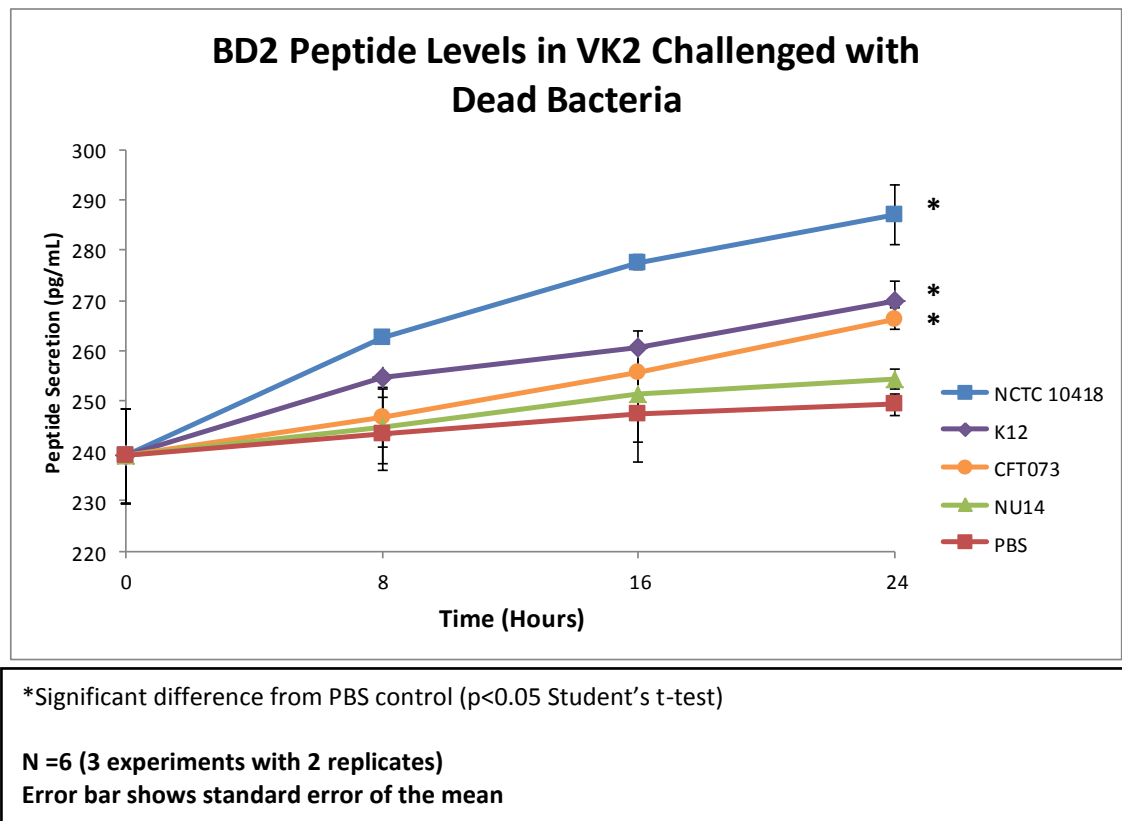
\*Significant difference from PBS control ( $p < 0.05$  Student's t-test)

N = 6 (3 experiments with 2 replicates)

Error bar shows standard error of the mean

As with RT4, it was possible to compare the BD2 peptide response seen with other uropathogenic *E. coli* strains of varying motility (Figure 5.31). As with RT4, there appeared to be a relationship with motility i.e. NCTC 10418, the most motile, eliciting the highest response and the non-flagellated and least motile NU14 causing little if any BD2 secretory response.

Figure 5.31: BD2 Peptide Secretion in RT4 cells challenged with four different strains of dead *E. coli* for 24-hours



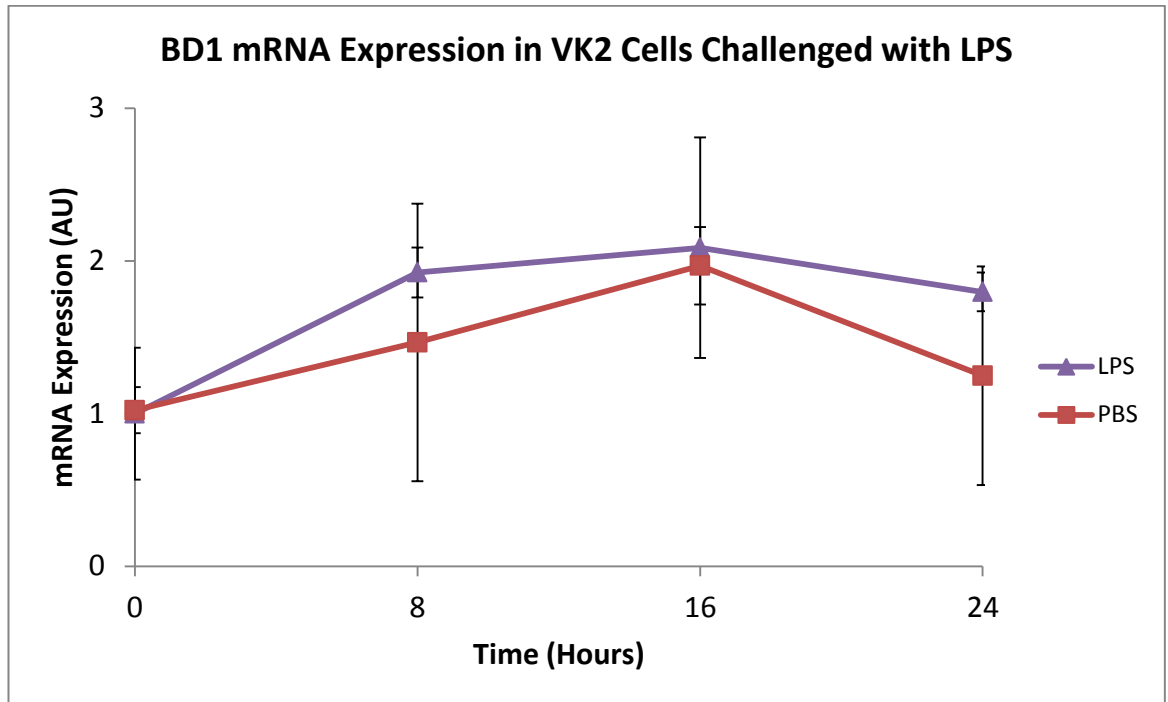
Having examined the effect of dead bacteria and seen similar trends to that of RT4, it was decided to examine the role of the individual bacterial components and this is outlined in the next section.

## 5.4.2 Bacterial component challenge

### 5.4.2.1 LPS

Similar to RT4, VK2 cells were challenged with commercially sourced *E. coli* LPS from both *E. coli* 0111:B4 and K12 (Invivogen, California, USA) at varying concentrations up to a maximum of 1mg/ml. No AMP expression response was seen with any of the challenges and representative graphs for BD1 and BD2 are shown Figure 5.32 and Figure 5.33 respectively.

Figure 5.32: BD1 mRNA Expression in VK2 cells challenged with *E. coli* LPS for 24-hours



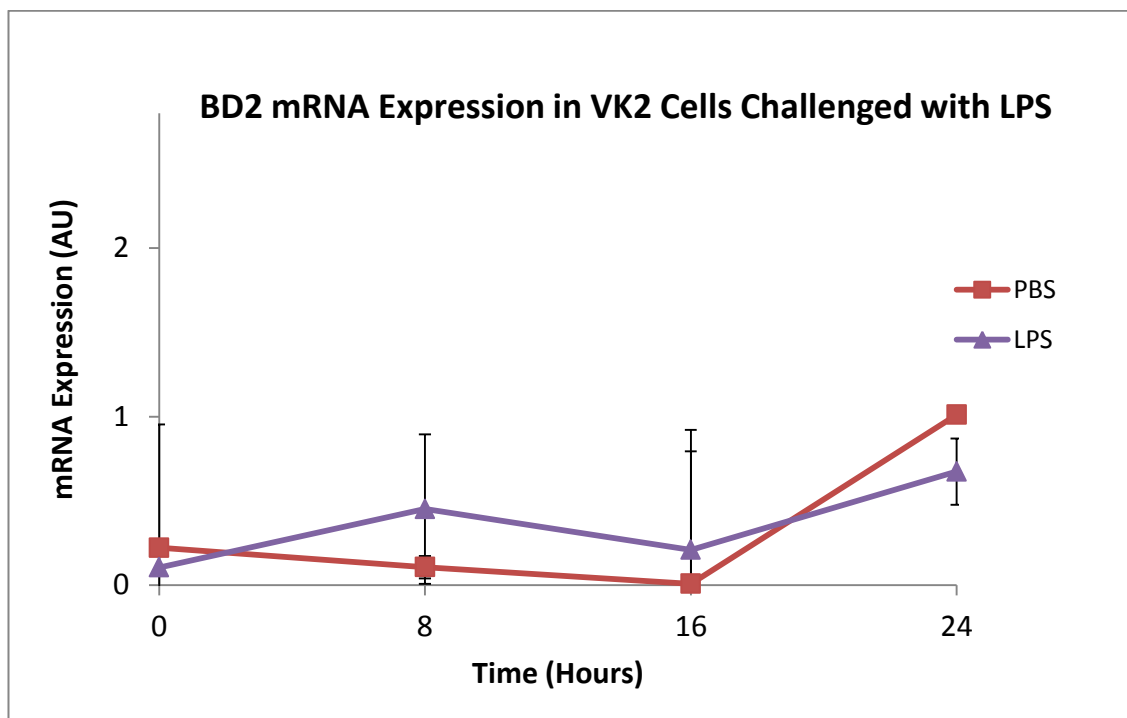
Measurements made by qRT-PCR with GAPDH and ATP5B house-keeping gene and shown relative to PBS control at time 0.

AU = Arbitrary Units

N = 9 (3 experiments with 3 replicates)

Error bar shows standard error of the mean

Figure 5.33: BD2 mRNA Expression in VK2 cells challenged with *E. coli* LPS for 24-hours



Measurements made by qRT-PCR with GAPDH and ATP5B house-keeping gene and shown relative to PBS control at time 0.

AU = Arbitrary Units

N =9 (3 experiments with 3 replicates)

Error bar shows standard error of the mean

It can be seen that while both BD1 and BD2 were constitutively expressed (BD2 at only very low levels), there was no significant change following LPS challenge.

#### 5.4.2.2 Peptidoglycan

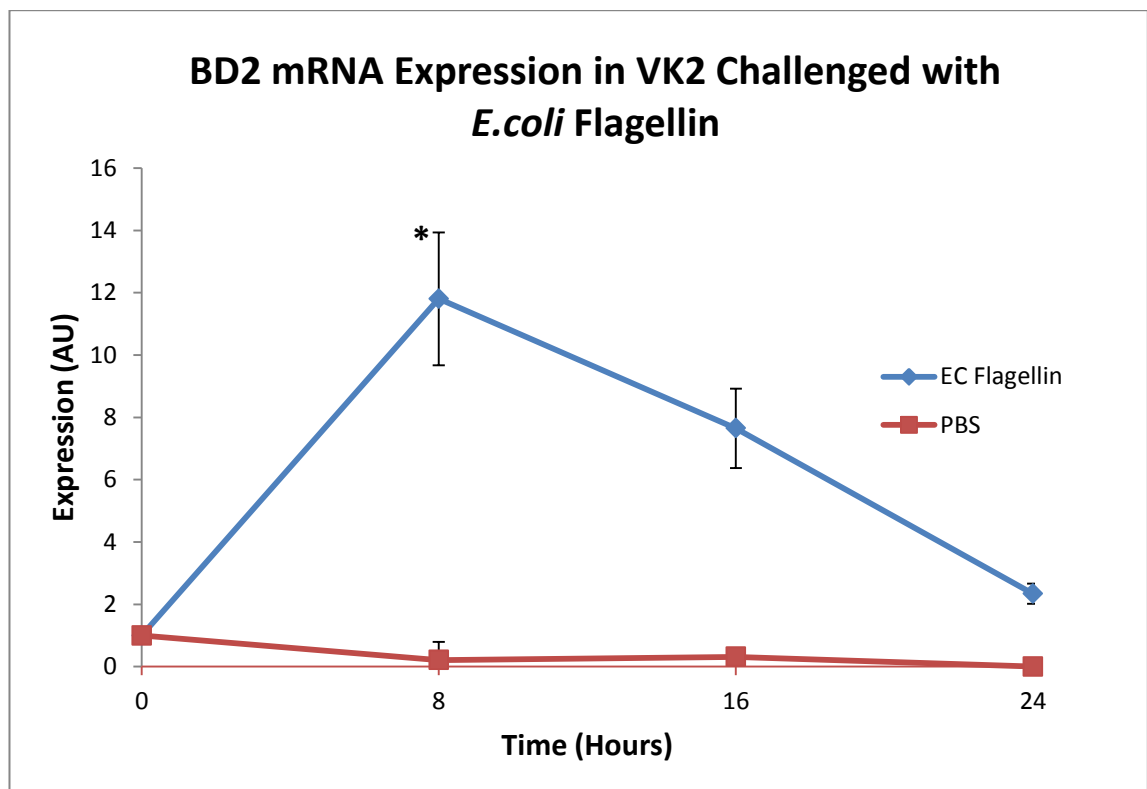
Challenges using peptidoglycan were not specifically carried out during the work for this thesis. However, in parallel to this work, my colleague Dr Claire Townes did carry out challenges using peptidoglycan did not identify any significant changes amongst the four AMPs (data not shown).

#### 5.4.2.3 Flagellin

Given the apparent relationship between motility of the *E. coli* strains and BD2 expression, and secretion in VK2 (and previously in RT4), the effect of *E. coli* flagellin

was also investigated. As reported with RT4 cells, flagellin resulted in increased BD2 expression in VK2 cells with significantly higher levels by 8 hours (Figure 5.34). However, as was observed with the *E. coli* challenges, the absolute values for increased expression were not as high as with RT4 urothelial cells and the decline was again much faster reaching baseline at 24 hours.

**Figure 5.34: BD2 mRNA Expression in VK2 cells challenged with *E. coli* flagellin for 24-hours**



\*Significant difference from PBS control ( $p < 0.05$  Student's t-test)

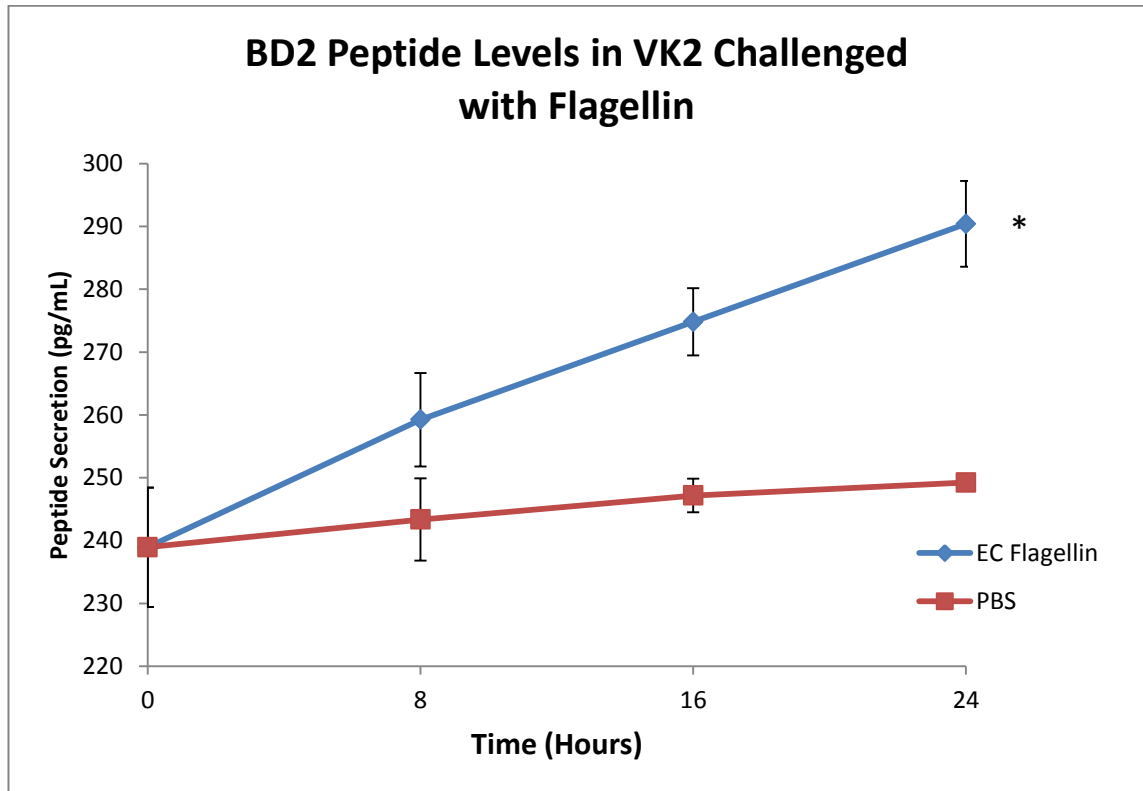
Measurements made by qRT-PCR with GAPDH and ATP5B house-keeping gene and shown relative to PBS control at time 0.

EC = *E. coli*  
 AU = Arbitrary Units  
 N = 9 (3 experiments with 3 replicates)  
 Error bar shows standard error of the mean

Peptide analysis via ELISA confirmed a significant rise in BD2 secretion (Figure 5.35). The rise was similar to that seen with dead NCTC 10418 (the most motile of the *E. coli* strains).



Figure 5.35: BD2 Peptide Secretion in VK2 cells challenged with *E. coli* Flagellin for 24-hours



\*Significant difference from PBS control ( $p < 0.05$  Student's t-test)

EC = *E. coli*

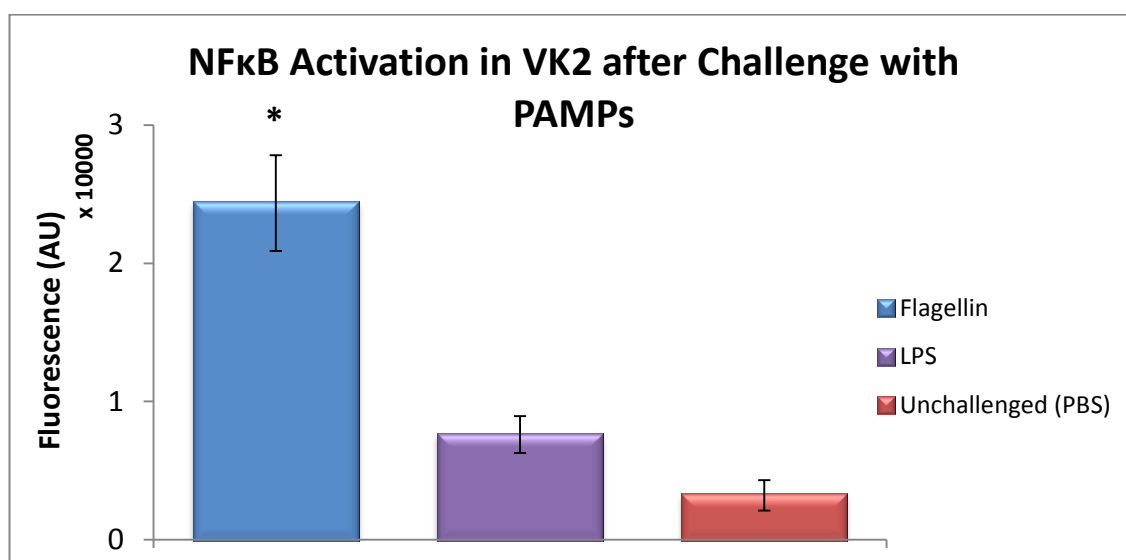
N = 6 (3 experiments with 2 replicates)

Error bar shows standard error of the mean

#### 5.4.2.4 Reporter Assay Response to Flagellin

In parallel to the measurement of downstream effector molecules, Bea Suarez-Falero (the undergraduate student) also examined the response of VK2 cells to *E. coli* flagellin and LPS using a NF $\kappa$ B GFP fluorescence assay (Figure 5.36). Similar to the luciferase reporter assay used for RT4 cells, this again demonstrated a rise in NF $\kappa$ B in response to the flagellin challenge, again suggesting that the BD2 response seen with flagellin was likely to be functioning through the NF $\kappa$ B pathway.

Figure 5.36: NFκB response by GFP fluorescence in VK2 cells challenged with *E. coli* PAMPs for 24-hours



\*Significant difference from PBS control ( $p < 0.05$  Student's t-test)

AU = Arbitrary Units

N = 3

Error bar shows standard error of the mean

(Experiment done by Bea Suarez-Falero)

#### 5.4.2.5 Validation of Flagellin Induced BD2 Secretion with Anti-TLR 5 Antibody in VK2 E6/E7 Cells

Having found strong evidence that flagellin also induces BD2 expression and secretion in VK2 cells, and given that flagellin works through the TLR5 receptor, it was expected this response would be inhibited by the use of TLR5 blocking antibody, as was seen with RT4. To provide further validation, expression of TLR5 was checked using endpoint PCR (Figure 5.37), and increasing concentrations of TLR5 antibody were used to inhibit BD2 secretion in response to flagellin (Figure 5.38).

Figure 5.37: PCR Confirmation of TLR5 Expression in VK2 cells

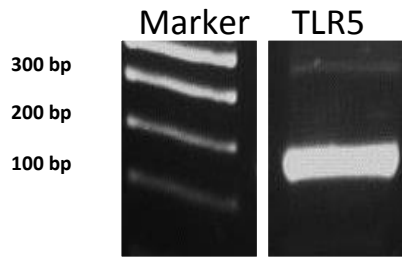


Figure 5.38: Anti-TLR5 Antibody inhibition of BD2 peptide secretion in VK2 challenged with Flagellin

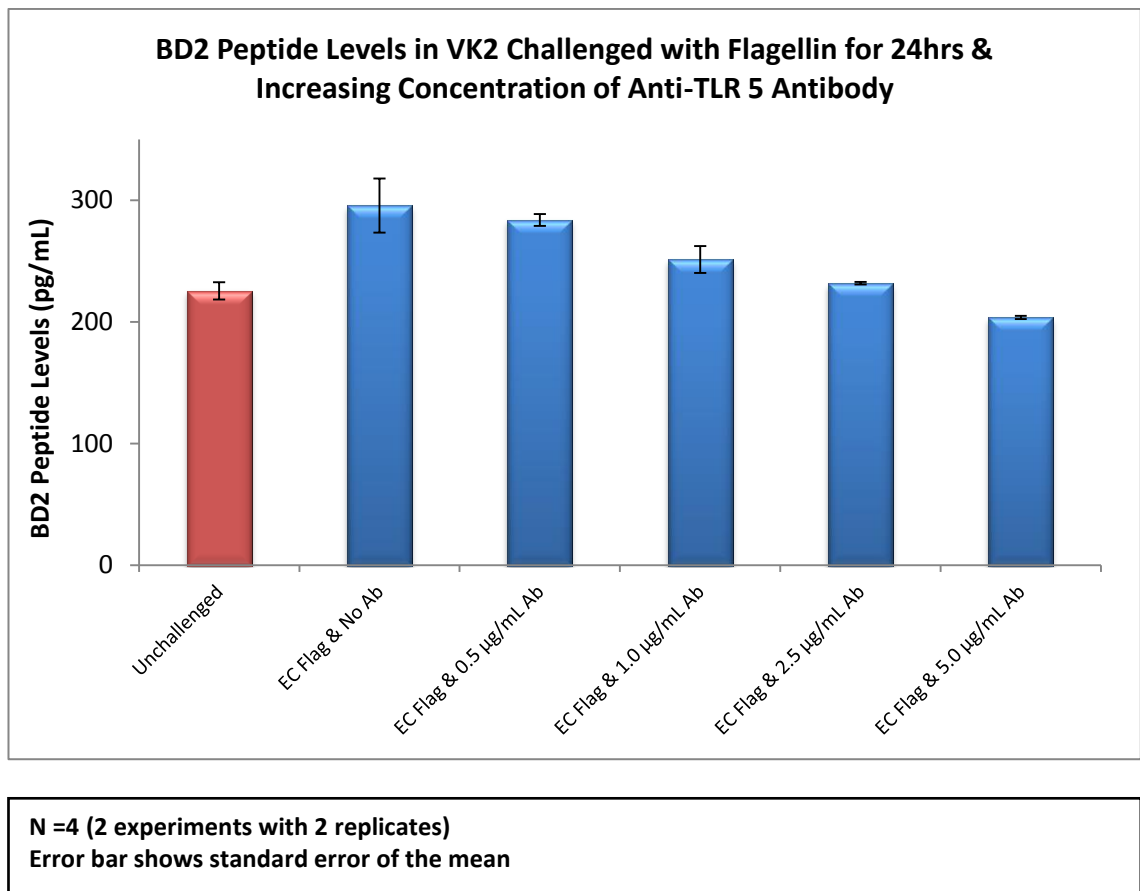
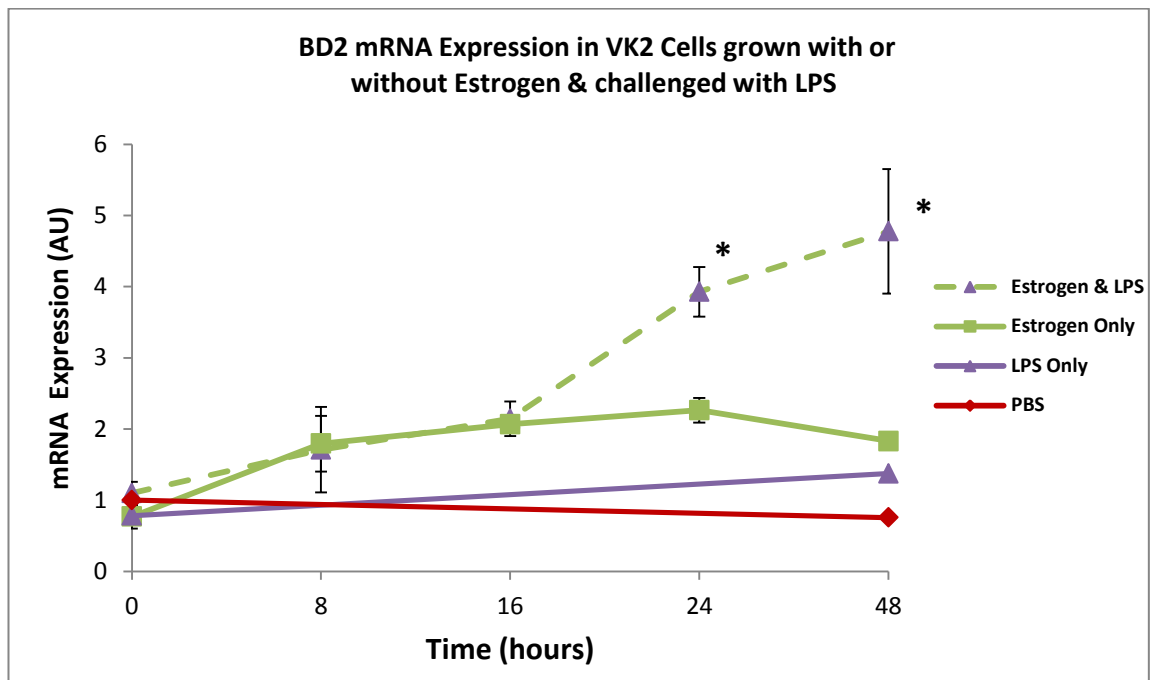


Figure 5.38 demonstrated that, comparable to what was seen RT4, increasing concentration of anti-TLR5 antibody inhibited the BD2 response induced by flagellin. Although with VK2, the effect was seen even with more dilute antibody. A similar inhibitory effect with the use of 5µg of anti-TLR5 antibody was seen by my colleague Marcelo Lanz using a NFκB GFP fluorescence assay (data not shown).

#### ***5.4.2.6 Effect of Estrogen on VK2 E6/E7 cells***

It is well recognised that tissues of the genital tract are responsive to estrogen. As discussed in the introduction, there are clearly differences in vaginal tissue estrogen levels dependent on the pre- or post-menopausal status of women, and application of topical vaginal estrogen is recognised as a preventative treatment option for recurrent urinary tract infection in women. It has also been reported by Charles Wira's group (Pioli *et al*, 2006; Fahey *et al*, 2008) in New Hampshire, USA that estrogen up-regulated BD2 expression in uterine cells in culture. Although experiments in RT4 with estrogen (section 5.3.2.6) did not yield positive results, given that both the uterus and the vagina have a shared embryological origin from the Müllerian duct, it was considered reasonable to consider that that estrogen may have a similar effect on vaginal cells. To explore this further, VK2 cells were cultured in varying concentrations of estrogen as a pre-treatment and then challenged with LPS. Although, as described earlier (section 5.4.2.1) LPS challenge alone did not result in any increased BD2 expression, the adjuvant addition of estrogen changed the response substantially. Figure 5.39 illustrates the effect of 4 nM estrogen on the response of VK2 to *E. coli* LPS and Figure 5.40 demonstrates the effect of varying concentrations of estrogen in a 24-hour challenge.

Figure 5.39: BD2 mRNA Expression in VK2 cells grown with or without 4nM Estrogen and challenged with *E. coli* LPS



\*Significant difference from PBS control ( $p < 0.05$  Student's t-test)

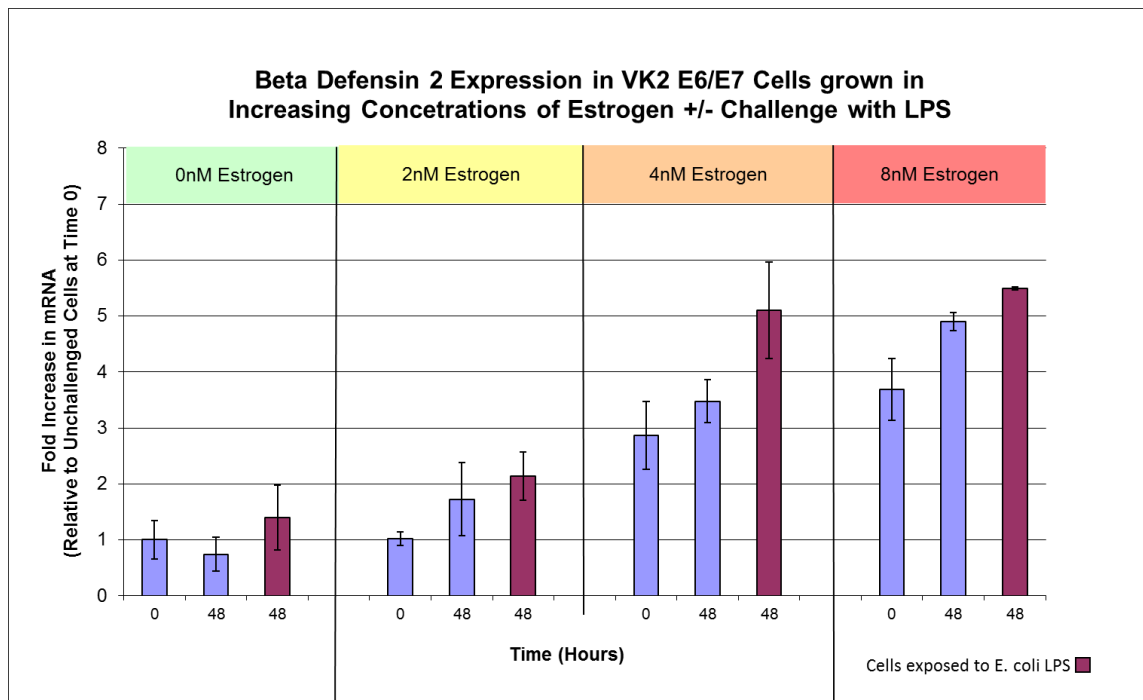
Measurements made by qRT-PCR with GAPDH and ATP5B house-keeping gene and shown relative to PBS control at time 0.

AU = Arbitrary Units

N = 9 (3 experiments with 3 replicates)

Error bar shows standard error of the mean

**Figure 5.40: BD2 mRNA Expression in VK2 cells grown in varying concentrations of Estrogen and challenged with *E. coli* LPS**



Measurements made by qRT-PCR with GAPDH house-keeping gene and shown relative to PBS control at time 0.

AU = Arbitrary Units

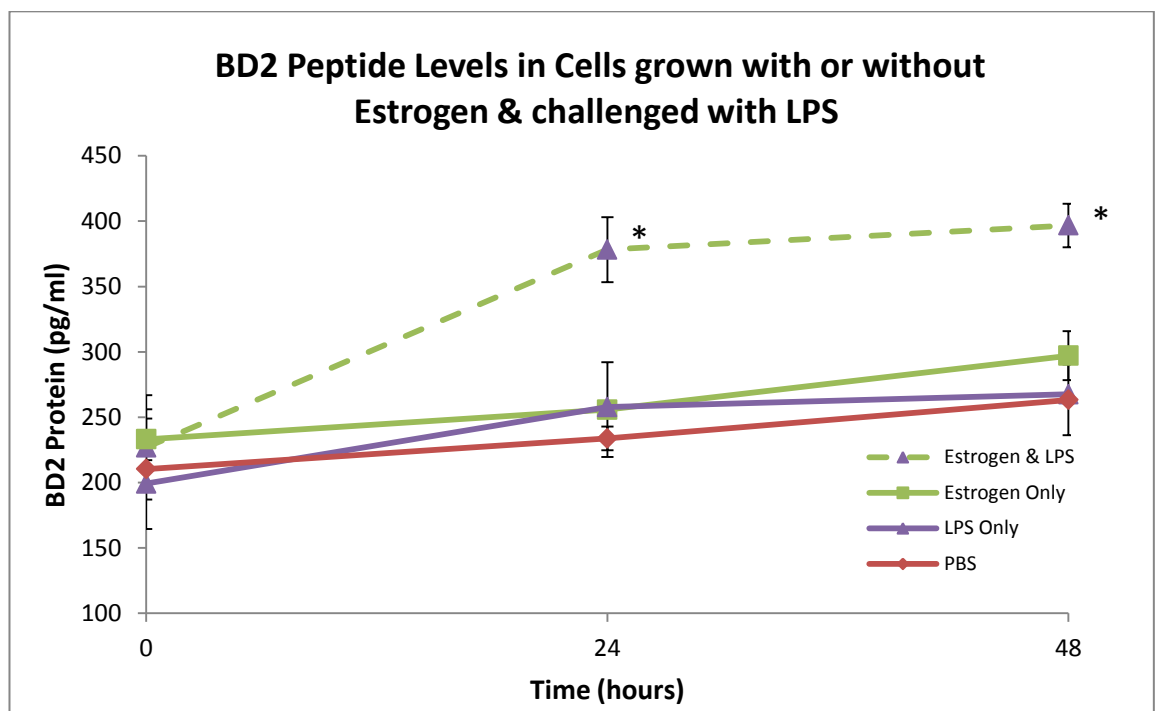
N = 3

Error bar shows standard error of the mean

It can be seen from Figure 5.39 that a physiological concentration of estrogen has a significant potentiating effect on BD2 expression. VK2 cells, which are otherwise largely unresponsive to LPS stimulation showed increased BD2 expression when grown in estrogen-supplemented medium and then challenged by LPS. By 24 hours the increase in BD2 response to LPS by VK2 in the presence of supplementary estrogen was significant ( $p < 0.05$ ). Furthermore, this effect of estrogen appeared to be dose-dependent reaching maximal effect at 4 nM (Figure 5.40). It is important to note that there did appear to be some positive effect on BD2 expression with estrogen alone i.e. without any immune stimulation, but this was not statistically significant. No effect was seen for BD1, HD5 or LL37 expression.

Examining induced peptide secretion following estrogen supplementation, a similar overall pattern was observed when assaying for BD2 via ELISA as shown in Figure 5.41. There was a significant increase in peptide secretion by 24-hours when compared to the control with no increase in peptide secretion detected in either unchallenged cells grown in estrogen, or those challenged with LPS but without estrogen supplementation. There was a slight upward trend under all the experimental conditions most likely due to the accumulation of basally secreted peptide over time.

**Figure 5.41: BD2 Peptide Secretion in VK2 cells grown with or without 4nM Estrogen and challenged with *E. coli* LPS**

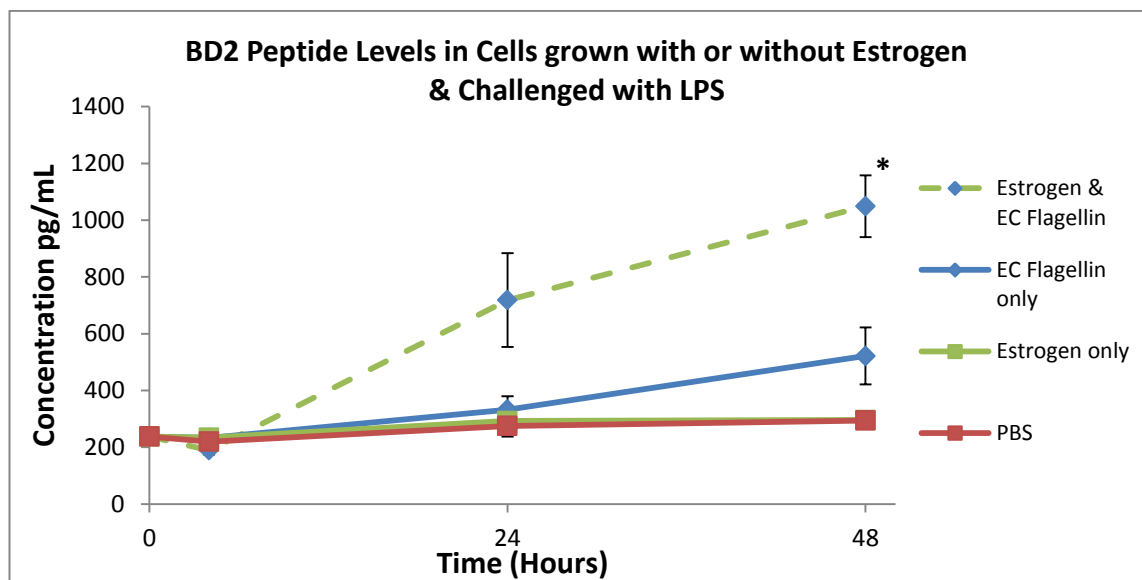


\*Significant difference from PBS control ( $p < 0.05$  Student's t-test)  
**N = 6 (3 experiments with 2 replicates)**  
**Error bar shows standard error of the mean**

Having examined the effects in relation to LPS, the effect on peptidoglycan was also examined but no effect was observed (data not shown). In contrast, with flagellin an enhanced BD2 response was again seen (this challenge was carried out by Dr Claire Townes using my methodology). This response was particularly marked with BD2

responses reaching approximately 700 pg/mL by 24 hours when VK2 cells were grown in estrogen and challenged with flagellin (Figure 5.42). It also demonstrated that even where a response is initiated by the PAMP itself as is the case with flagellin, there is still potential for further amplification of the response when estrogen is present i.e. in VK2 cells flagellin alone does not evoke the maximal BD2 epithelial secretory response.

**Figure 5.42: BD2 Peptide Secretion in VK2 cells grown with or without 4nM Estrogen and challenged with *E. coli* Flagellin**



\*Significant difference from PBS control ( $p < 0.05$  Student's t-test)  
 N = 6 (3 experiments with 2 replicates)  
 Error bar shows standard error of the mean  
 (Figure produced from data provided by Claire Towne)

### 5.4.3 Summary of VK2 E6/E7 Data

To summarise, the key finding from the VK2 E6/E7 experiments are as follows:

- Similar to RT4, the main detectable AMPs in VK2 are BD1 and BD2. BD1 mRNA is constitutively expressed and no significant up-regulation was seen during the challenges using bacteria or bacterial components. BD2 mRNA is only very



lowly expressed at baseline but is highly up-regulated in the presence of heat-killed motile strains of *E.coli* and flagellin. HD5 and LL37 were not expressed.

- Challenge with *E. coli* flagellin evokes a strong BD2 response which is detectable at peptide level via ELISA. However, even in the absence of challenge, VK2 cells secrete significant amounts of BD2 peptide. The rise seen during challenge by flagellin can be inhibited by treatment with TLR5 antibody.
- Similar to RT4, the response seen with flagellin appears to be transduced through the intermediary molecule, NFκB as is demonstrated by the NFκB GFP fluorescence assay which shows up-regulation in the presence of flagellin.
- Unlike RT4, VK2 shows a significantly enhanced innate immune response after estrogen treatment. This response is particularly profound as it works by potentiating the response to PAMPs. Estrogen alone does not cause any significant increase in BD2 expression or secretion, however, when cells are challenged with LPS, a response is seen despite the fact that LPS alone also results in no response. Stimulation with flagellin in the presence of physiological estrogen concentration results in a higher level of BD2 expression and secretion.

## 6. Clinical Sample Analysis

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### 6.1 Introduction

A key element of this research study was the analysis of samples from patients with the target condition; recurrent UTI, and from age-matched female controls. As discussed in chapter 4, the aim of the clinical study was to compare the AMP profiles of women with recurrent UTI with those without. Given the evidence that AMPs are influenced by hormonal changes and that recurrent cystitis tends to present most commonly in two periods of a women's life; during the third and fourth decade, or post-menopausal, controls and cases were also categorised into sub-groups according to menopausal status. To allow for single nucleotide polymorphism (SNP) analysis, blood samples were also collected.

### 6.2 Clinical data

For all subjects a standard clinical history was taken and information recorded on the clinical data sheet found in appendix A. All subjects were also asked to fill in symptom severity and quality of life questionnaires (sample questionnaires available in appendix A). The key data is outlined below.

#### 6.2.1 Demographics and Clinical History

Table 6.1 shows the key demographic and clinical history for both the control and rUTI subjects.

**Table 6.1 Summary table of demographics and clinical history**

	Control		rUTI	
	Pre-menopause	Post-menopause	Pre-menopause	Post-menopause
<b>Number (infection at time of biopsy)</b>	19 (0)	19 (0)	31 (5)	29 (7)
<b>Median Age (Range)</b>	35 <sup>a</sup> (18-46)	58 <sup>b</sup> (42-76)	31 <sup>a</sup> (18-41)	60 <sup>b</sup> (40-75)
<b>Taking vaginal estrogen</b>	1 (5%)	2 (11%)	0 (0%)	0 (0%)
<b>Taking vitamins</b>	4 (21%)	3 (16%)	4 (13%)	4 (14%)
<b>Taking cranberry-juice</b>	5 (26%)	5 (26%)	16 (52%)	15 (52%)
<b>On oral contraception</b>	6 (32%)	0 (0%)	4 (13%)	0 (0%)
<b>Had HRT</b>	0 (0%)	4 (21%)	0 (0%)	9 (31%)
<b>Previous STI</b>	1 (5%)	0 (0%)	3 (10%)	0 (0%)
<b>Previous urological or gynaecological surgery</b>	10 (53%)	10 (53%)	16 (52%)	17 (59%)
<b>Median no of pregnancies (Range)</b>	1 (0-6)	2 (0-5)	1 (0-3)	2 (0-6)
<b>Smoker</b>	5 (26%)	5 (26%)	9 (29%)	16 (55%)

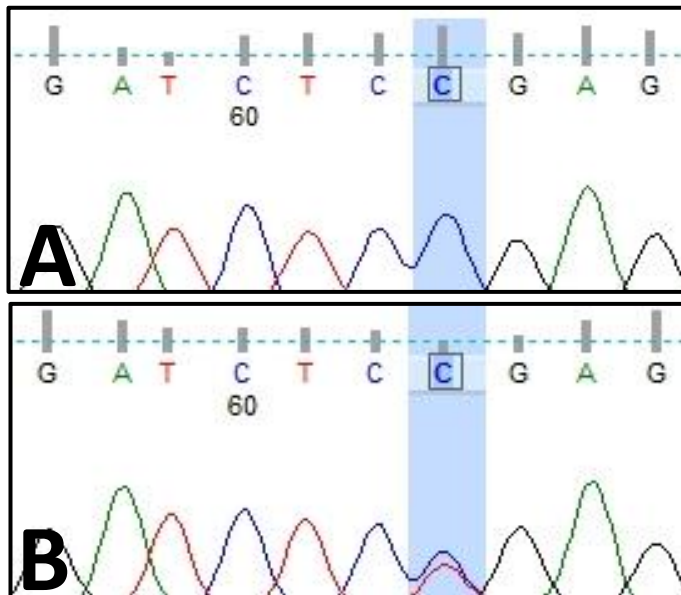
<sup>a</sup> No significant difference between control and rUTI pre-menopausal subjects (t-test)

<sup>b</sup> No significant difference between control and rUTI post-menopausal subjects (t-test)

### 6.2.2 TLR5<sup>392Stop</sup> SNP Status

As described in methods, blood samples collected from recruits to the study had a specific sequence (461bp) of the TLR5 gene amplified by PCR and sequenced by Sanger sequencing at Genevision, Newcastle upon Tyne, UK. The sequence was analysed for the TLR5<sup>392Stop</sup> Single Nucleotide Polymorphism (SNP) using the Finch TV software (Geospiza, Seattle, USA). Figure 6.1 illustrates the sequence found in a normal subject and that from one of the subjects found to have the heterozygous TLR5<sup>392Stop</sup> SNP. In the SNP sequence the two different base pairs can be seen with the normal blue cytosine (C) base at half height and a thymine base in the other allele resulting in amino acid-coding triplet 392 changing from CGA (Arginine) to TGA (stop codon).

Figure 6.1: TLR5 Sequence (A = Normal, B = TLR5<sup>392Stop</sup> SNP)



In total nine out of the 98 subjects recruited were found to have the TLR5<sup>392Stop</sup> SNP giving a frequency of 9.2%. However, all nine subjects carrying the SNP were from the recurrent UTI group (60-patients) giving a prevalence within that group of 15% versus 0% in the control group.

Of the nine subjects with the TLR5<sup>392Stop</sup> SNP, four were pre-menopausal and five post-menopausal. Three had infection at the time of biopsy but six did not. Five were treated with estrogen, three with long term antibiotic prophylaxis; one was satisfied with general UTI avoidance advice following clinic attendance.

### 6.2.3 Symptoms score

All recruits were requested to fill in the UTISA questionnaire to gauge their symptoms at the time of enrolment into the study (see appendix A). The UTISA questionnaire tests seven domains; a shortened clinician description of each domain is indicated in [brackets]:

- Frequency of urination (going to the toilet very often) [*Frequency*]

- Urgency of urination (a strong & uncontrollable urge to pass urine) [*Urgency*]
- Pain or burning when passing urine [*Dysuria*]
- Not being able to empty your bladder completely/passing only small amounts of urine [*Poor Emptying*]
- A high temperature or shivers [*High temp*]
- Pain or uncomfortable pressure in the lower abdomen/pelvic area caused by your urinary tract infection [*Lower abdo pain*]
- Low back pain caused by your urinary tract infection [*Lower back pain*]
- Blood in your urine [*Blood in urine*]

Each symptom was scored on a scale of 0-3 for the severity of the symptom i.e. 0 = Did not have, 1 = Mild, 2 = Moderate, 3 = Severe. Each symptom was also scored on a scale of 0-3 for the amount of bother it caused i.e. 0 = Not at all, 1 = A little, 2 = Moderate, 3 = A lot.

Figure 6.2 and Figure 6.3 show the mean score comparison data for severity and bother of symptoms in the pre-menopausal group:

Figure 6.2: UTISA – Severity of Symptoms in Pre-menopausal control and rUTI subjects

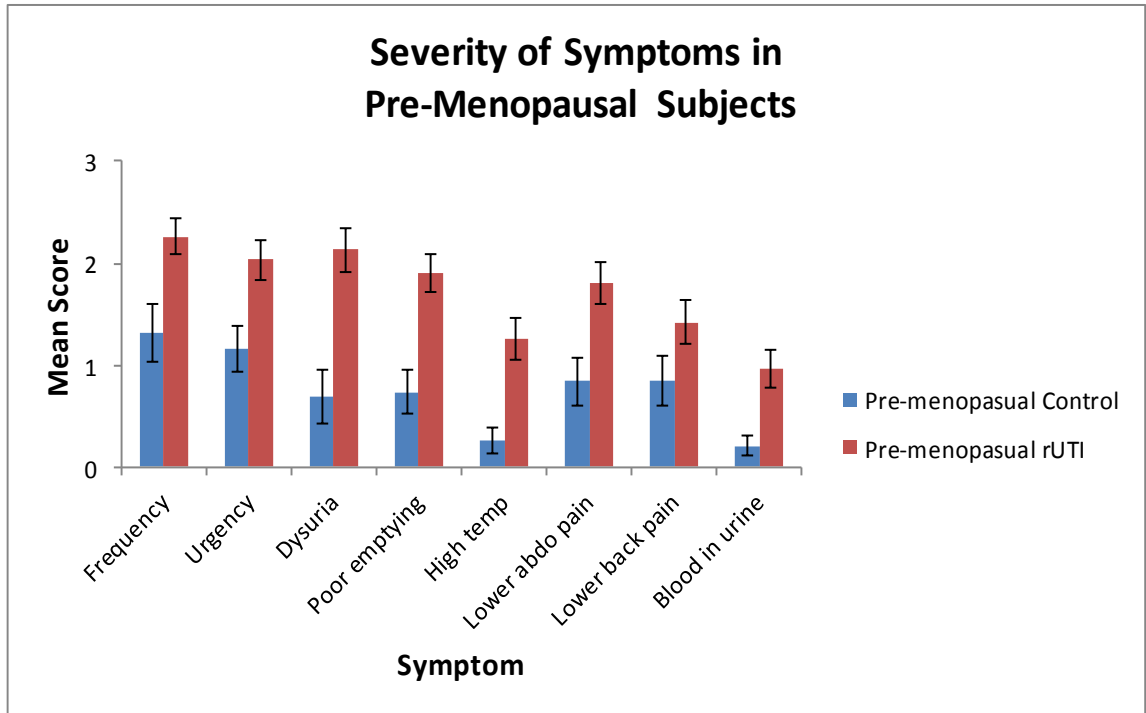
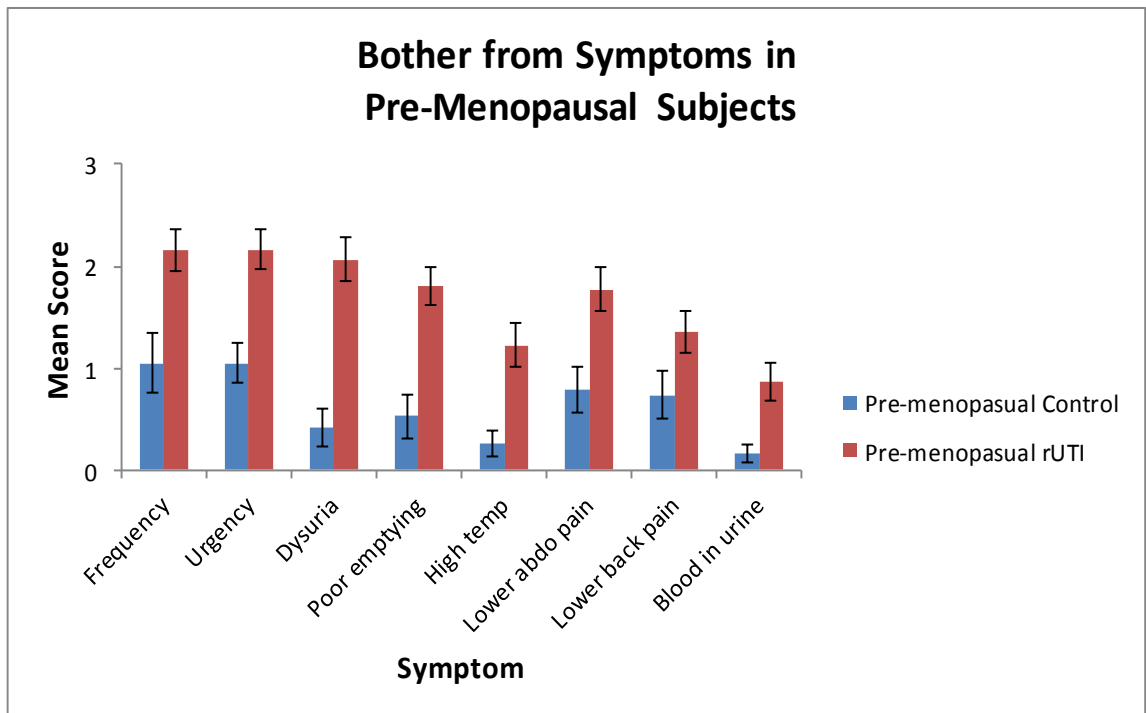


Figure 6.3: UTISA – Bother from Symptoms in Pre-menopausal control and rUTI subjects



There is a marked, statistically significant difference in both severity of symptoms and bother between the control and rUTI groups ( $p < 0.05$ , Mann Whitney non-parametric test) on all domains except lower back pain severity and bother.

A similar pattern was observed in relation to the post-menopausal patients (Figure 6.4 and Figure 6.5):

**Figure 6.4: UTISA – Severity of Symptoms in Post-menopausal control and rUTI subjects**

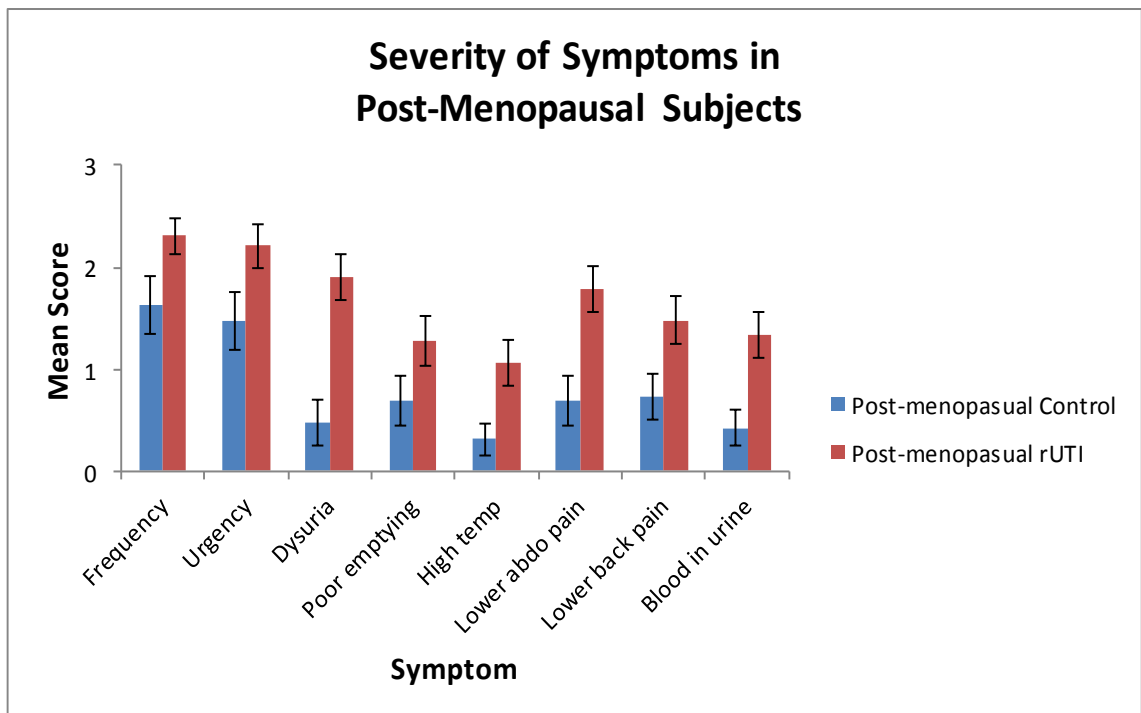
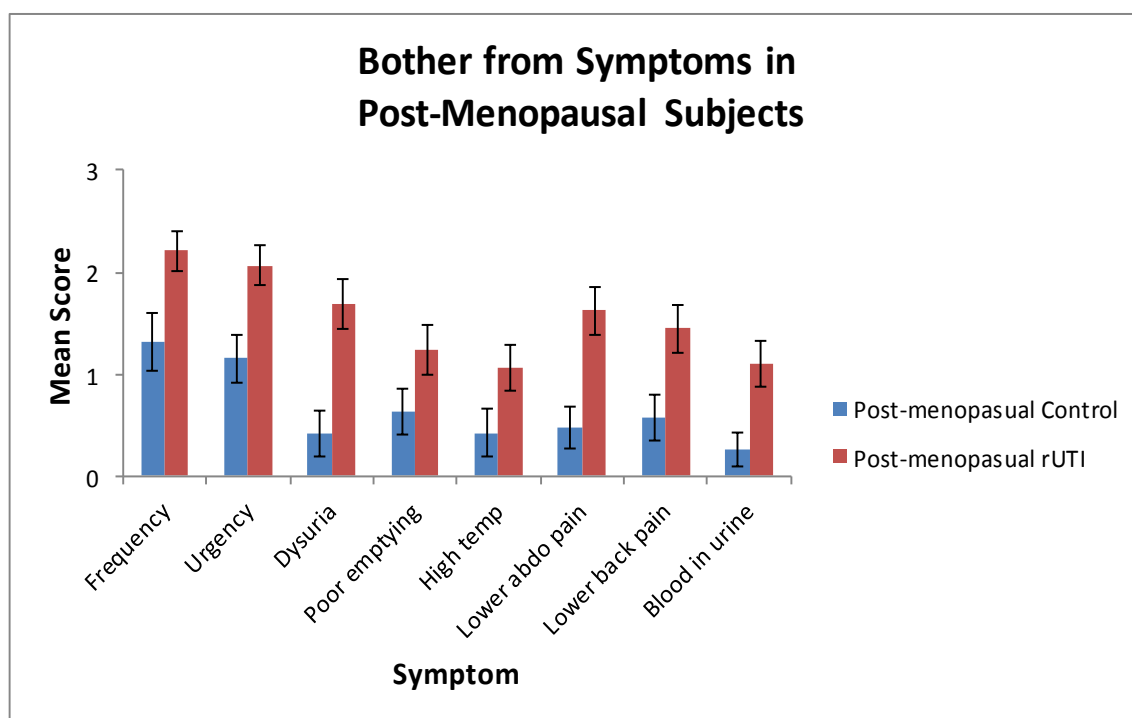


Figure 6.5: UTISA – Bother from Symptoms in Post-menopausal control and rUTI subjects



Again a marked difference in severity of symptoms and bother was observed. However, for the post-menopausal group, the difference reached statistical significance ( $p < 0.05$ , Mann Whitney non-parametric test) on all domains except the severity and bother of poor bladder emptying.

When comparing the seeming discrepancy between severity of symptoms vs bother, the only domains in which there was a significant difference in the scoring was dysuria for pre-menopausal subjects and urgency plus blood in urine for post-menopausal subjects ( $p < 0.05$ , Wilcoxon matched paired non-parametric test). For dysuria in pre-menopausal patients, the mean bother score was higher than the severity score. For urgency and blood in urine in post-menopausal subjects, the mean bother score was lower than the severity score.



## 6.2.4 Health State scores

The EQ-5D quality of life questionnaire (see appendix A) is a well-validated generic health status questionnaire that compares health state across five domains:

- Mobility
- Self-Care
- Usual Activities (e.g. work, study, housework, family or leisure activities)
- Pain/Discomfort
- Anxiety/Depression

Each domain has three categories scored from 1-3 depending on the current state of health. Figure 6.6 and Figure 6.7 show the average scores in each domain for the pre-menopausal and post-menopausal subjects respectively.

Figure 6.6: EQ-5D score in Pre-menopausal control and rUTI subjects

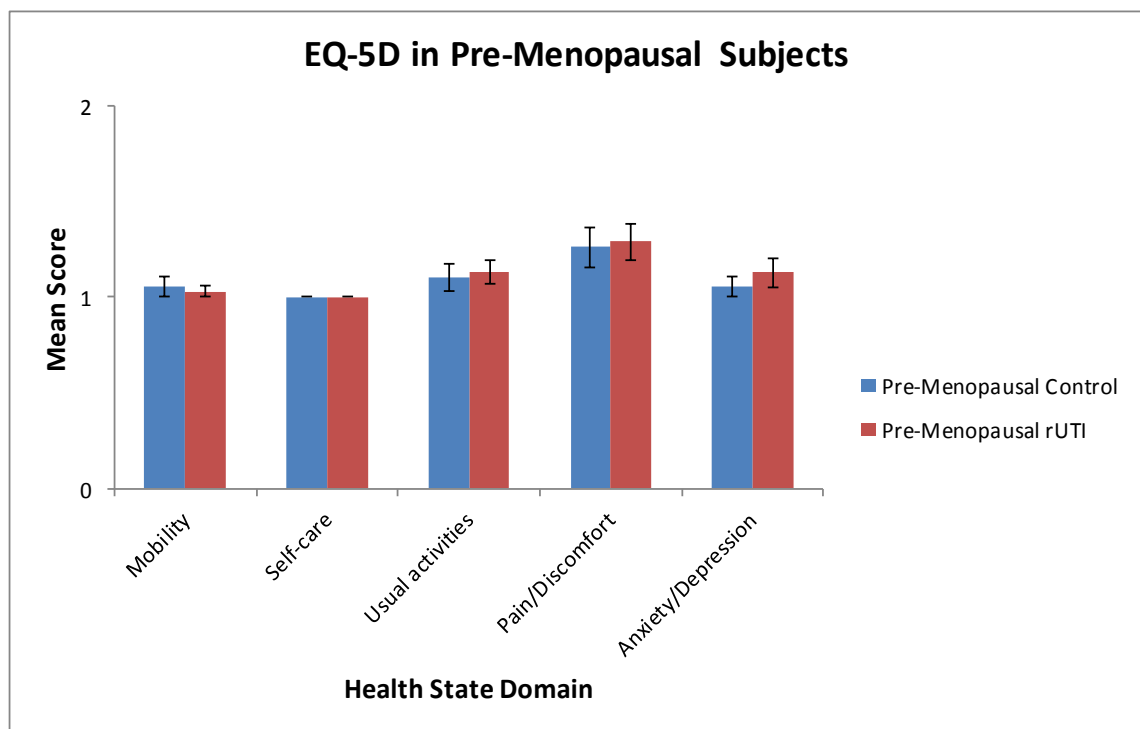
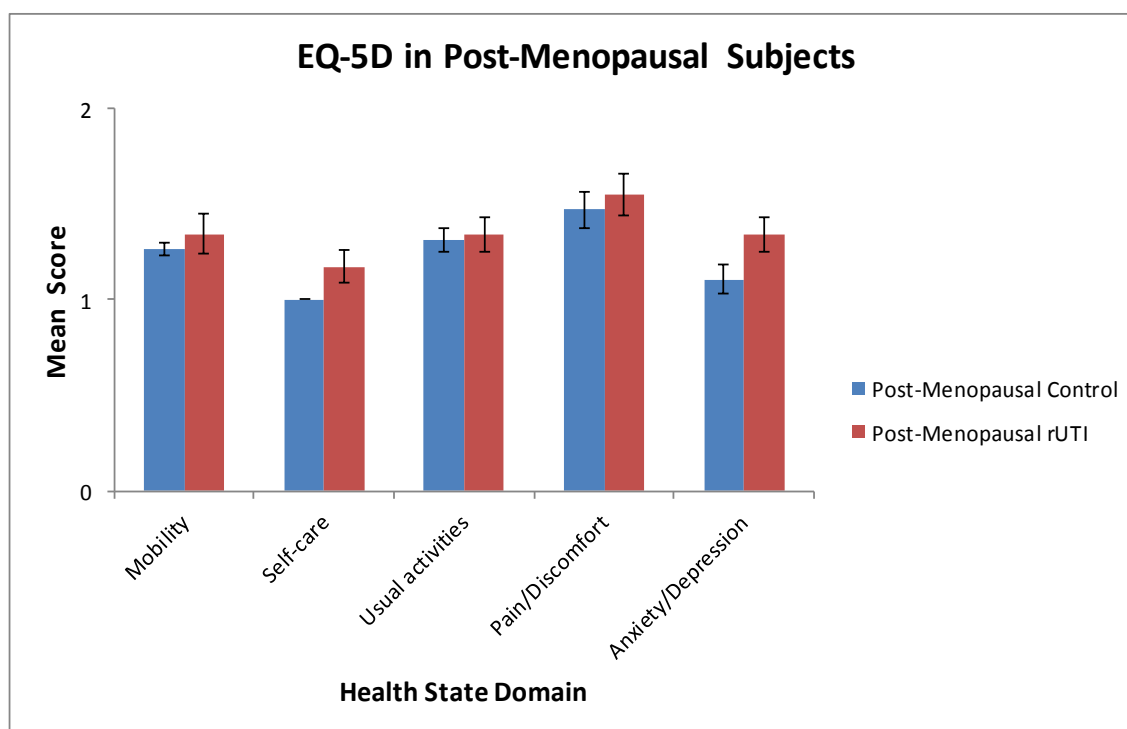


Figure 6.7: EQ-5D score in Post-menopausal control and rUTI subjects



There was no significant difference between controls and rUTI in either the pre- or post-menopausal cohorts in any of the health domains tested by EQ-5D.

### 6.2.5 Summary of Clinical data

In summary, the following findings were noted in relation to the clinical data collected:

- A total of 98 subjects were recruited into the study of which 12 had infection at the time of their biopsy and therefore were noted as requiring separate subgroup analysis. Pre-investigation power calculations had suggested that a minimum of 18-patients would be required in each cohort, this was therefore exceeded.
- There was no significant difference in age between the control and rUTI subjects recruited to the study.
- Nine patients were found to have TLR5<sup>392Stop</sup> SNP, all had recurrent UTI (four were pre-menopausal and five post-menopausal).

- Pre-menopausal rUTI patients were found to have significantly greater severity and bother symptoms compared to controls on the UTISA questionnaire in terms of frequency, urgency, dysuria, poor bladder emptying, raised temperature, lower abdominal pain and blood in the urine. Only lower back pain showed no significant difference. The bother score for dysuria was significantly greater than the severity in this sub-group.
- Post-menopausal rUTI patients were found to have significantly greater severity and bother symptoms compared to controls on the UTISA questionnaire in terms of frequency, urgency, dysuria, poor bladder emptying, raised temperature, lower abdominal pain, lower back pain and blood in the urine. Only poor bladder emptying showed no significant difference. The bother score for urgency and blood in urine was significantly less than the severity in this sub-group.
- No significant difference was found between control and rUTI subject in any of the EQ-5D health state domains.

### 6.3 Bladder Samples

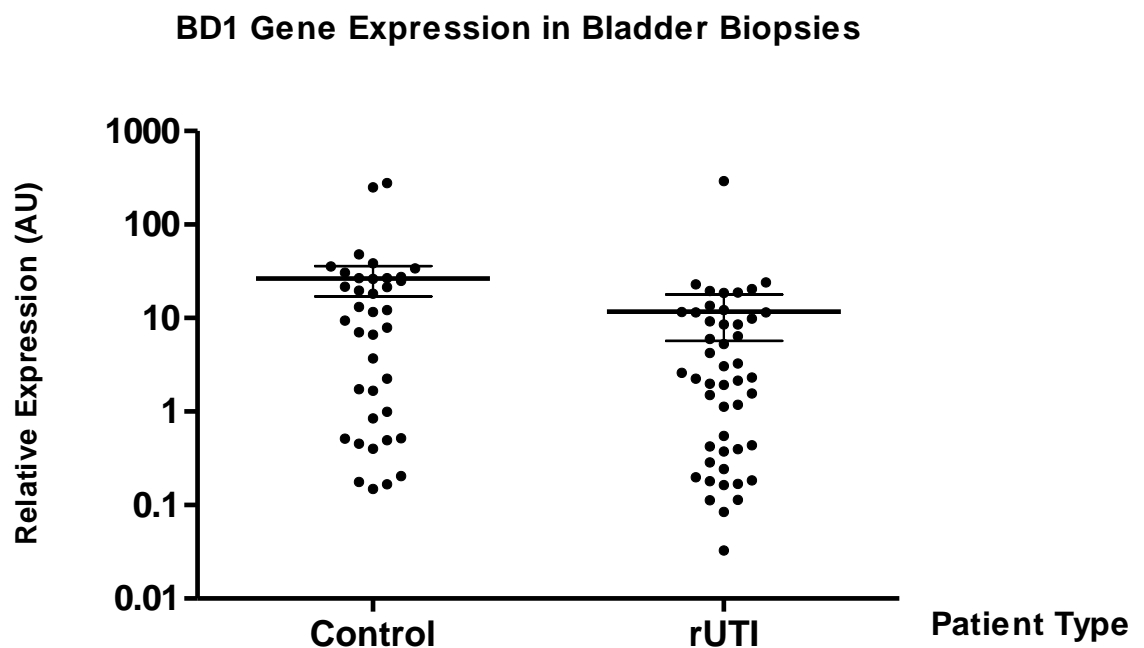
A crucial part of the clinical study protocol was that patients with recurrent UTI were preferably recruited at a time when they didn't have infection. Although some subjects were ultimately recruited at the time of infection (as it wasn't known that they had infection at the time of recruitment), these patients are analysed separately later and have serendipitously given interesting sub-group data. *In vitro* studies described in the previous chapter showed that in the absence of infection, only BD1 is expressed constitutively; BD2 is primarily expressed in response to infection. No urothelial

expression of LL37 was found in bladder urothelium and HD5 could only be detected at very low expression levels. So to examine differences in basal expression in the clinical samples, the analysis was focussed initially on BD1.

### 6.3.1 Recurrent UTI vs Controls

To investigate whether there was any 'basal' difference in BD1 AMP expression between control and rUTI subjects, bladder epithelial biopsies were assayed using qRT-PCR, and the results are shown in Figure 6.8.

**Figure 6.8: BD1 Gene mRNA expression in bladder biopsies from control and rUTI patients with no infection at the time of biopsy**



Measurements made by qRT-PCR with GAPDH and ATP5B house-keeping gene.  
AU = Arbitrary Units, note vertical access is on logarithmic scale  
N = 38 (Control) and N = 48 (rUTI)  
Central bar shows mean with standard error of the mean above and below

The data presented in Figure 8 shows that there is no statistically significant difference between the two groups in terms of BD1 expression with a comparable mean and spread of expression between the two populations. From the outset, the study design

had sought to separate pre- and post-menopausal women into similar-sized sub-groups due to the potential differences in the nature of rUTI within these two groups. It is therefore possible to look at these groups separately as shown in Figure 6.9.

**Figure 6.9: BD1 Gene mRNA expression in bladder biopsies from pre-Menopausal & post-Menopausal control and rUTI patients with no infection at the time of biopsy**



Measurements made by qRT-PCR with GAPDH and ATP5B house-keeping gene.

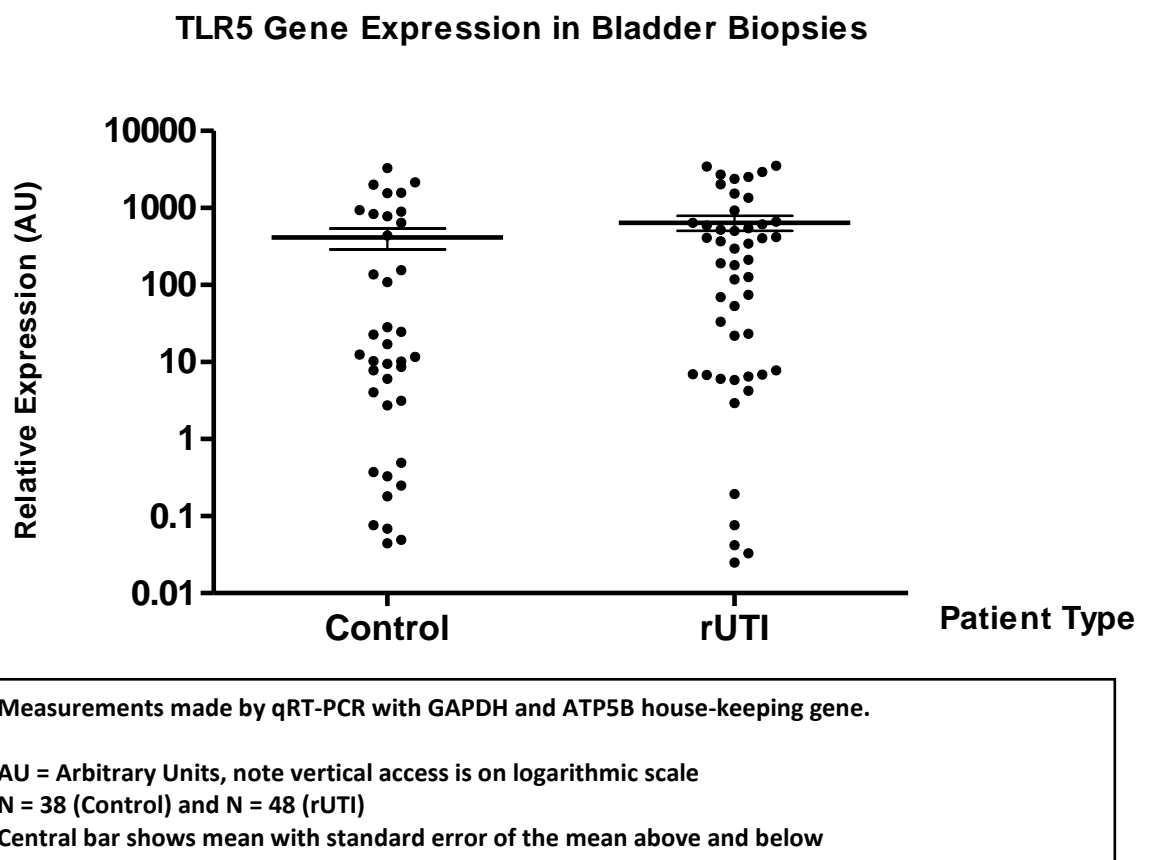
AU = Arbitrary Units, note vertical axis is on logarithmic scale  
 N=19 (Pre-menopausal control), N=26 (Pre-menopausal rUTI),  
 N = 19 (Post-menopausal control), and N=22 (Post-menopausal rUTI)  
 Central bar shows mean with standard error of the mean above and below

Even when the different sub-groups were examined separately, there was no difference in BD1 expression with similar levels detected across all four sub-groups.

From the *in vitro* work, it was apparent that BD2 expression in response to flagellin was a particularly important component of the early AMP response. However, as an

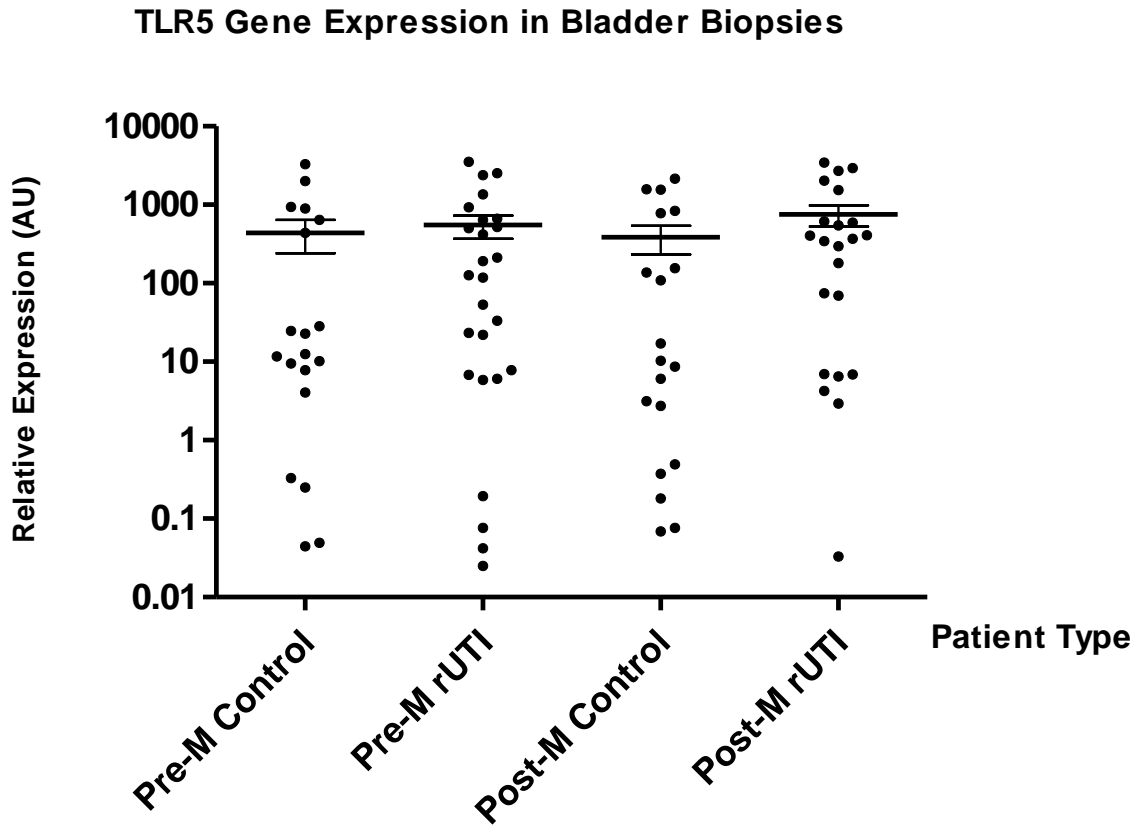
inducible AMP rather than a constitutively expressed AMP like BD1, **BD2 was essentially undetectable in biopsy samples in the absence of infection.** Nonetheless, the receptor for flagellin, TLR 5, is constitutively expressed and it's possible that differences in expression of TLR5 could cause variable AMP response in subjects with rUTI. TLR 5 expression in the bladder biopsies was therefore analysed (Figure 6.10):

**Figure 6.10: TLR5 Gene mRNA expression in bladder biopsies from control and rUTI patients with no infection at the time of biopsy**



Again, the data indicated that there was no difference in TLR5 gene expression between the two groups and even if further sub-categorised into pre- and post-menopausal categories (Figure 6.11), the same pattern was maintained:

Figure 6.11: TLR5 Gene mRNA expression in bladder biopsies from pre-Menopausal & post-Menopausal control and rUTI patients with no infection at the time of biopsy

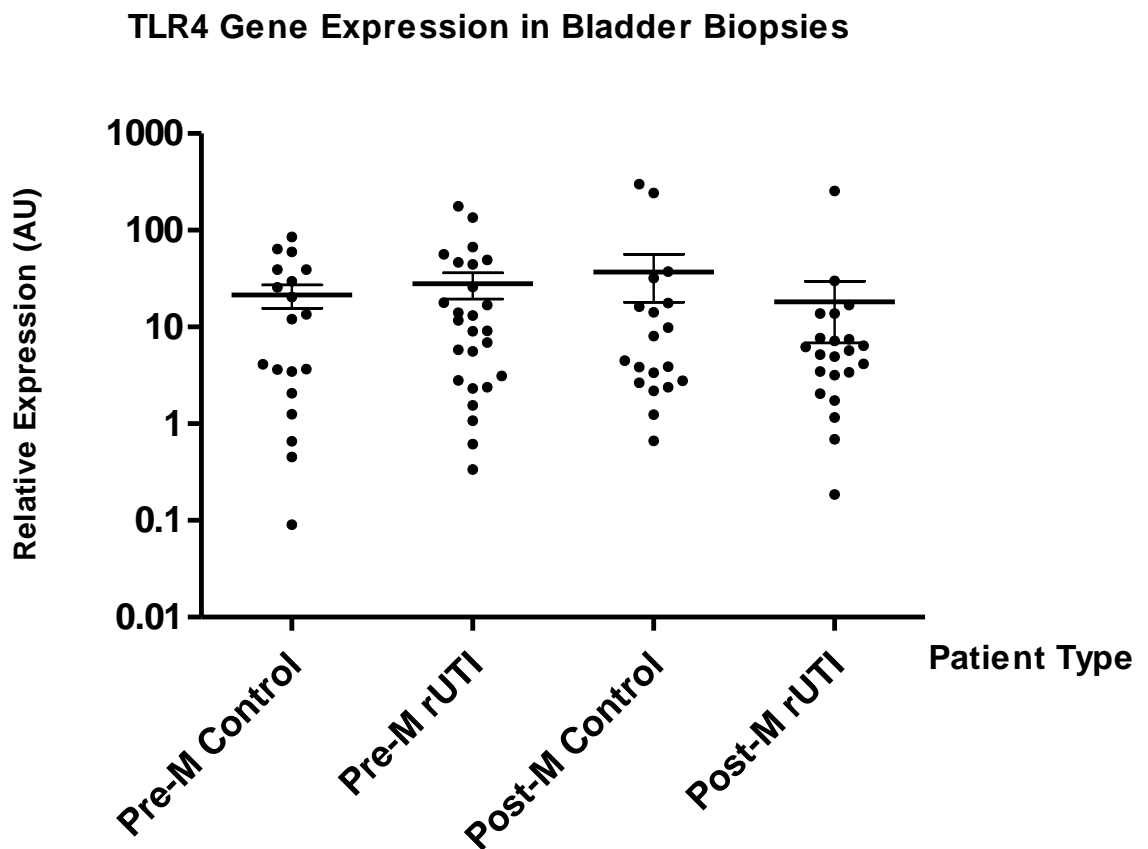


Measurements made by qRT-PCR with GAPDH and ATP5B house-keeping gene.

AU = Arbitrary Units, note vertical axis is on logarithmic scale  
 N=19 (Pre-menopausal control), N=26 (Pre-menopausal rUTI),  
 N = 19 (Post-menopausal control), and N=22 (Post-menopausal rUTI)  
 Central bar shows mean with standard error of the mean above and below

The sub-groups showed similar levels of TLR5 expression and if this was compared with TLR4 expression in the same samples (as a comparator, independent of TLR5), the pattern was comparable, although there was perhaps somewhat less spread in the data (Figure 6.12):

Figure 6.12: TLR4 Gene mRNA expression in bladder biopsies from pre-Menopausal & post-Menopausal control and rUTI patients with no infection at the time of biopsy



Measurements made by qRT-PCR with GAPDH and ATP5B house-keeping gene.

AU = Arbitrary Units, note vertical axis is on logarithmic scale  
 N=19 (Pre-menopausal control), N=26 (Pre-menopausal rUTI),  
 N = 19 (Post-menopausal control), and N=22 (Post-menopausal rUTI)  
 Central bar shows mean with standard error of the mean above and below

Overall, there was no evidence supporting a difference in basal AMP expression (outside periods of infection) between women in the recurrent UTI and control groups; the absence of differences in the activating Toll like receptors demonstrated the lack of difference in the afferent arm of the pathway too. The next section therefore examines the samples taken from 12 women at the time of infection.

### 6.3.2 Non-SNP vs SNP Patients

The *in vitro* work outlined in the previous results chapter demonstrated that during *E. coli* urinary infection, a key role for flagellin in induction of BD2 could be demonstrated



mediated by the TLR5 receptor and which could be blocked by the use of TLR5 inhibiting antibody. As described in section 6.2.2, within the total recruited population of 98-patients, nine (9.2%) were found to have the TLR5<sup>392Stop</sup> SNP in heterozygous form. All nine were found in the 60 patient rUTI sub-group (15%). Given that the TLR5<sup>392Stop</sup> SNP results in a truncated protein with absent signalling then an interesting comparison is possible between those with TLR5<sup>392Stop</sup> against those without. Unfortunately, in the case of the urothelium, as BD2 is only expressed during infection, such an analysis is only possible on the twelve patients who happened to be recruited at a time when they had infection. Interestingly these patients had negative urine dipsticks as otherwise for patient safety reason, patients with active infection would not normally have a flexible cystoscopy. It is possible that either human error on the part of the clinical staff interpreting the dipstick, or problems with the dipstick itself may have allowed this to happen. Nonetheless, it was serendipitous in terms of being able to explore this hypothesis, that of those twelve patients, three also had TLR5<sup>392Stop</sup>. Figure 6.13 shows localisation of BD2 in infected bladder epithelium using immunohistochemistry (carried out on my behalf by Liz Shiels and Dr Alison Tyson-Capper). The results for BD2 mRNA analysis are shown in Figure 6.14.

Figure 6.13: Immunohistochemistry staining of BD2 peptide secretion in normal infected bladder (Inset shows negative control)

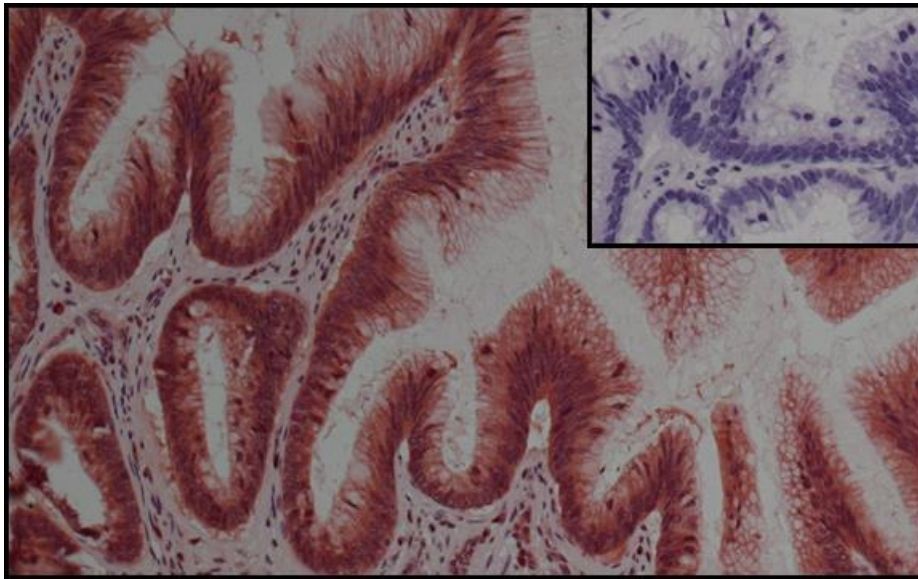
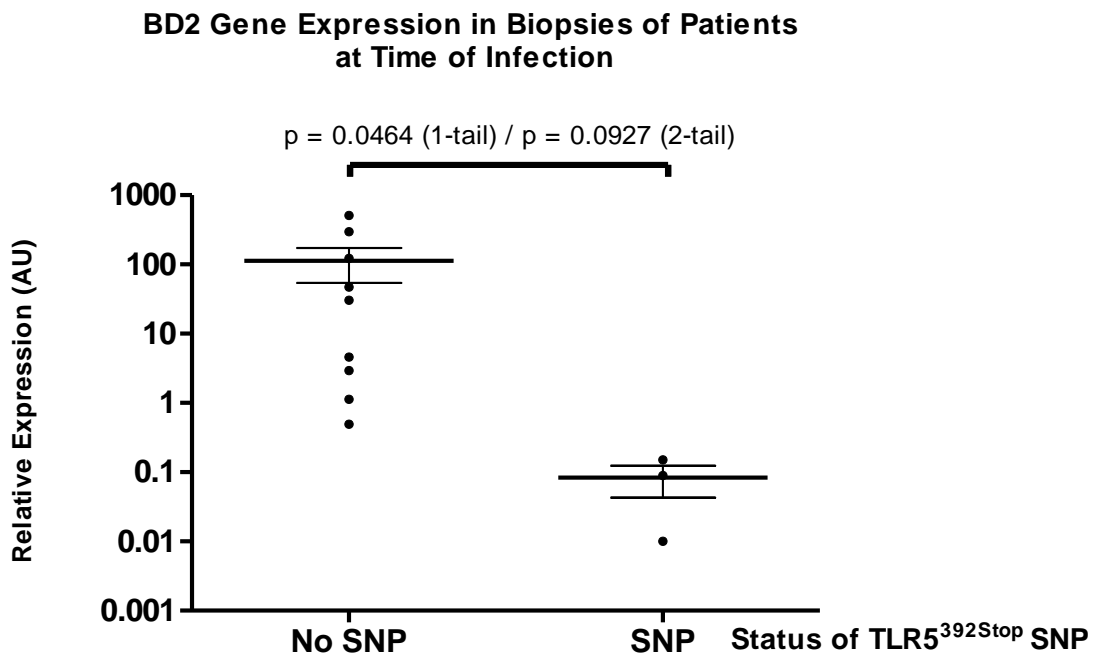


Figure 6.14: BD2 Gene mRNA expression in bladder biopsies from non-SNP and SNP rUTI patients with infection at the time of biopsy



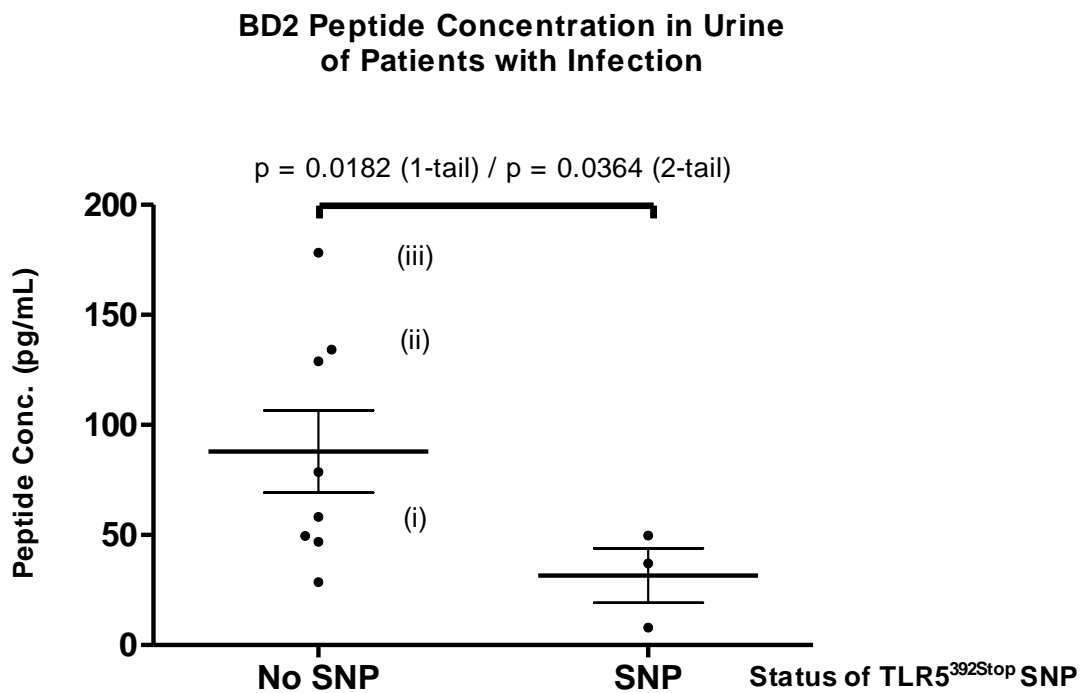
Measurements made by qRT-PCR with GAPDH and ATP5B house-keeping gene.

AU = Arbitrary Units, note vertical axis is on logarithmic scale  
 N = 9 (No SNP) and N = 3 (SNP)  
 Central bar shows mean with standard error of the mean above and below  
 Statistical analysis by one-tail and two-tail Student's t-test with Welch's correction (for unequal variance)

The BD 2 expression data shows that clinically, subjects with TLR5<sup>392Stop</sup> SNP and active infection do have statistically significant lower BD2 expression than non-SNP subjects with active infection with a mean of 112.9±59.1 AU for the non-SNP rUTI patients compared with 0.08±0.04 AU for the SNP patients. Given that the expectation was that BD2 would be lower i.e. the direction of the change was as expected from the *in vitro* data, it was felt reasonable with these relatively small numbers to carry out a one tail t-test (with Welch's correction for unequal variance) and this gives a p-value of 0.046 which is significant at the 5% level. However, with a two tail test, the p-value would be 0.0927 which would not be significant.

Peptide analysis of BD2 levels in infected urine samples demonstrates mean levels 87.4±21.0 pg/mL for the non-SNP rUTI subjects vs 35.6±16.1 pg/mL for the SNP subjects (p=0.02) (Figure 6.15):

**Figure 6.15: BD2 Peptide levels in urine from non-SNP and SNP rUTI patients with infection at the time**



Each ELISA assay was done with three replicates & compared against a standard curve of synthetic defensin.

N=8 (No SNP) and N=3 (SNP) – note one non-SNP patient with infection did not provide an overnight urine collection

Central bar shows mean with standard error of the mean above and below

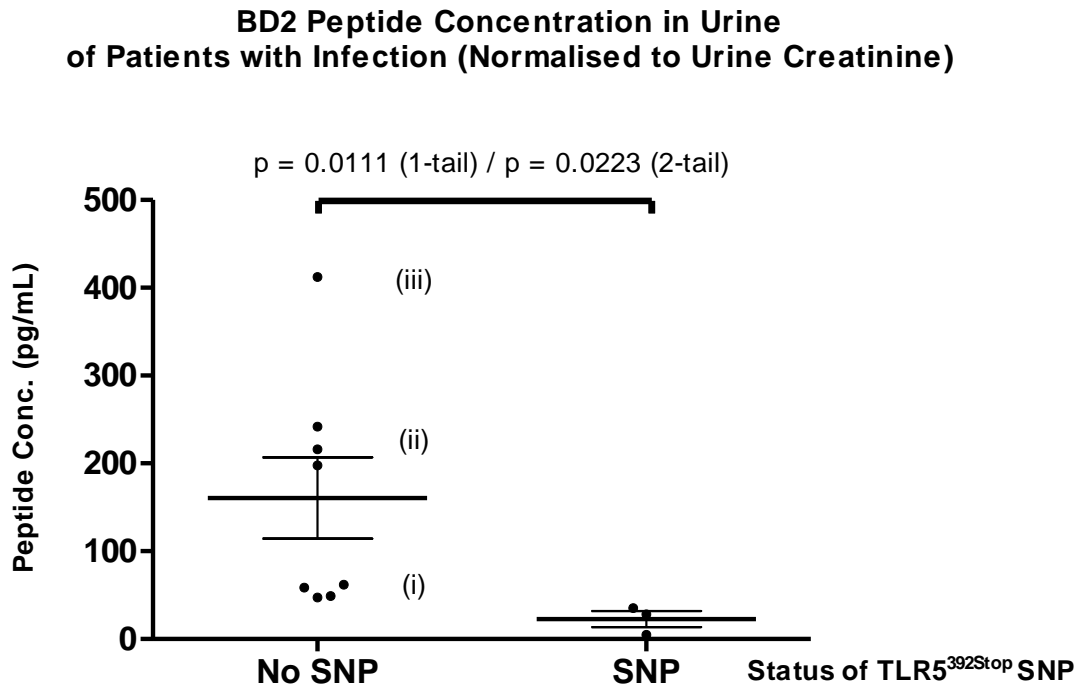
Statistical analysis by one and two tail Student's t-test with Welch's correction (for unequal variance)

Again, there is a statistically significant difference between the two groups at the 0.5% significance level in this case with both a 1-tail or 2-tail analysis with lower levels in the SNP group as expected. Of course, it is worth bearing in mind that the sample size is small and qualitative consideration of the range in peptide measurement for the group with no SNP, suggest that there may be 3-groups of patients:

- (i) a group with relatively low level of BD2 around 25 to 75 pg/mL
- (ii) a pair with intermediate levels between 100 and 150 pg/mL
- (iii) a single subject with higher levels at around 180 pg/mL

In contrast, the SNP group seem to have consistent low levels. The spread in the non-SNP group may reflect differences in the stage of infection, those with more recent infection not yet having fully mounted a BD2 response which may be consistent with the narrower range seen in expression of mRNA level. Given that urine can vary considerably in concentration between individuals and different times of day within the same individual, this could have been a factor that potentially accounted for the variability. However, when the results are normalised to urine creatinine, the three groups are just as apparent.

Figure 6.16 BD2 Peptide levels in urine from non-SNP and SNP rUTI patients with infection at the time (normalised to urine creatinine)



Each ELISA assay was done with three replicates & compared against a standard curve of synthetic defensin.

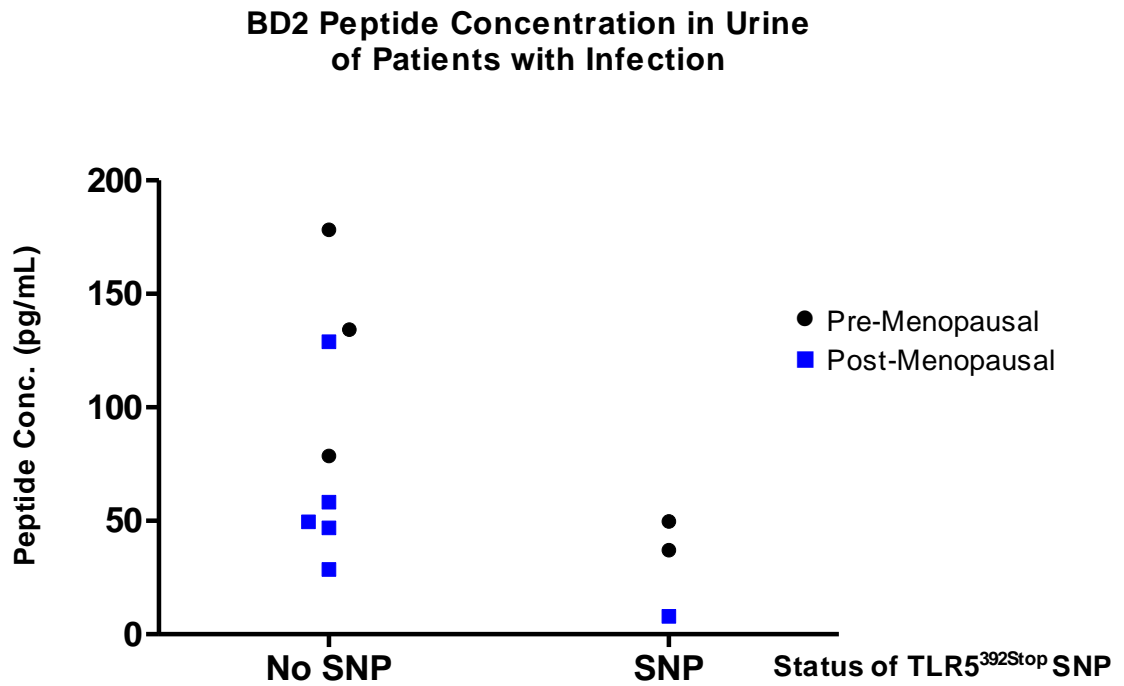
N=8 (No SNP) and N=3 (SNP) – note one non-SNP patient with infection did not provide an overnight urine collection

Central bar shows mean with standard error of the mean above and below

Statistical analysis by one and two tail Student's t-test with Welch's correction (for unequal variance)

Another variable which could have accounted for some of the spread was the menopausal status of the women and indeed if the original peptide data presented in Figure 6.15 is re-examined, taking this into account then it can be seen that two of the SNP subjects were pre-menopausal and three of the non-SNP patients were pre-menopausal. These sub-categories are shown in Figure 6.17:

Figure 6.17: BD2 Peptide levels in urine from non-SNP and SNP rUTI patients with infection at the time categorised by menopausal status



Each ELISA assay was done with three replicates & compared against a standard curve of synthetic defensin.

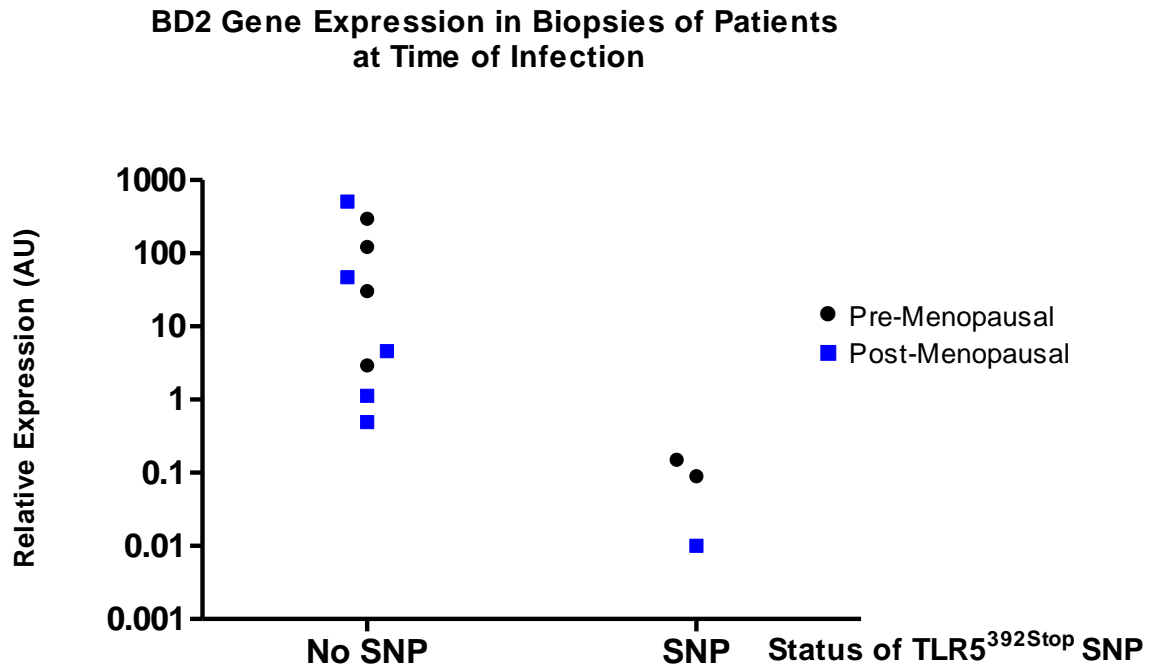
N=3 (Pre-menopausal, no SNP), N=5 (Post-menopausal, no SNP), N=2 (Pre-menopausal, SNP) and N=1 (Post-menopausal, SNP)

Central bar shows mean with standard error of the mean above and below

Given the small very numbers in each sub-group, it's difficult to come to any firm conclusion but the figure serves to illustrate how other factors may be affecting peptide concentrations and may merit further research. If the BD 2 peptide concentrations in the two pre-menopausal women with TLR5<sup>392Stop</sup> are compared statistically with the three without then there is still a significant difference but the validity of this comparison in such small numbers is questionable. Such a comparison is not even possible with the post-menopausal group as there is only one post-menopausal subject with TLR5<sup>392Stop</sup>. Nonetheless, on ELISA, the post-menopausal women would appear to demonstrate a trend towards lower BD2 peptide levels than

pre-Menopausal women although this is not statistically significant and mRNA expression of BD2 seems to show even less support for such a trend (Figure 6.18):

**Figure 6.18: BD2 Gene mRNA expression in bladder biopsies from non-SNP and SNP rUTI patients with infection at the time of biopsy categorised by menopausal status**



Measurements made by qRT-PCR with GAPDH and ATP5B house-keeping gene.

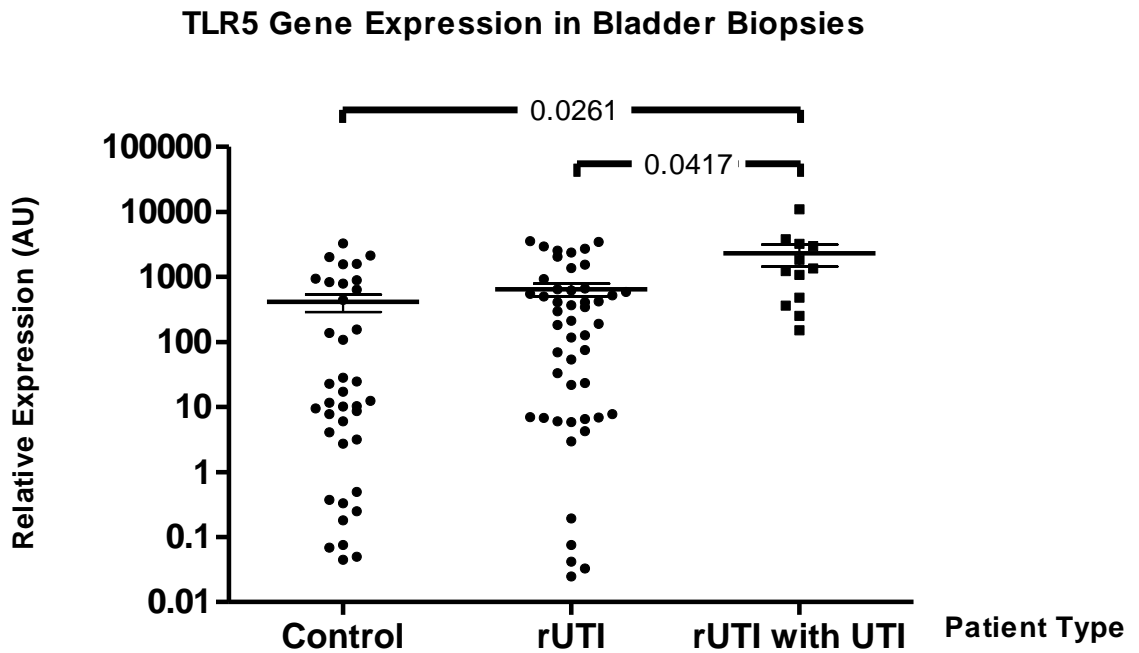
AU = Arbitrary Units, note vertical access is on logarithmic scale

N=3 (Pre-menopausal, no SNP), N=6 (Post-menopausal, no SNP), N=2 (Pre-menopausal, SNP) and N=1 (Post-menopausal, SNP)

Central bar shows mean with standard error of the mean above and below

Given that *in vitro* data indicated that TLR5 activation is key a component of mounting a BD2 response, another consideration to explore in the clinical material is the expression of TLR5 during infection. Previously, in Figure 6.11, it was demonstrated that there was no significant difference in TLR5 expression between rUTI patients and controls. However, from Figure 6.19, which shows a comparison of TLR5 expression in those patients with rUTI who had an infection at the time of biopsy against rUTI patients without active infection and control patients, it is evident that there was an increase in TLR5 expression during infection.

Figure 6.19: TLR5 Gene mRNA expression in bladder biopsies from uninfected control and rUTI patients compared with rUTI patients with infection at the time of biopsy



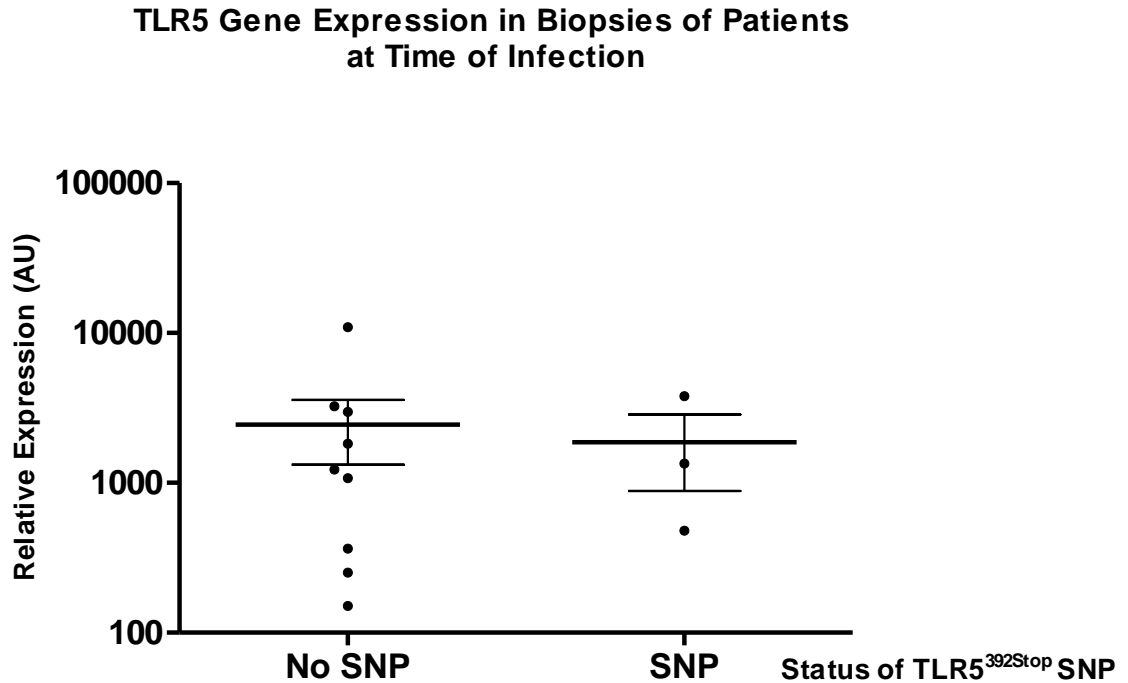
Measurements made by qRT-PCR with GAPDH and ATP5B house-keeping gene.

AU = Arbitrary Units, note vertical access is on logarithmic scale  
 N = 38 (Control), 48 (rUTI without active infection), 12 (rUTI with active infection)  
 Central bar shows mean with standard error of the mean above and below  
 Statistical analysis by one tail Student's t-test with Welch's correction (for unequal variance)

However, Figure 6.20, indicates that there was no difference in expression of TLR5 between those with TLR5<sup>392Stop</sup> and those without during infection with both showing higher levels than subjects without infection. This lends support to the notion that the differences in downstream response are due to signalling from TLR5 rather than expression of the receptor itself.



Figure 6.20: TLR5 Gene mRNA expression in bladder biopsies from non-SNP and SNP rUTI patients with infection at the time of biopsy



Measurements made by qRT-PCR with GAPDH and ATP5B house-keeping gene.

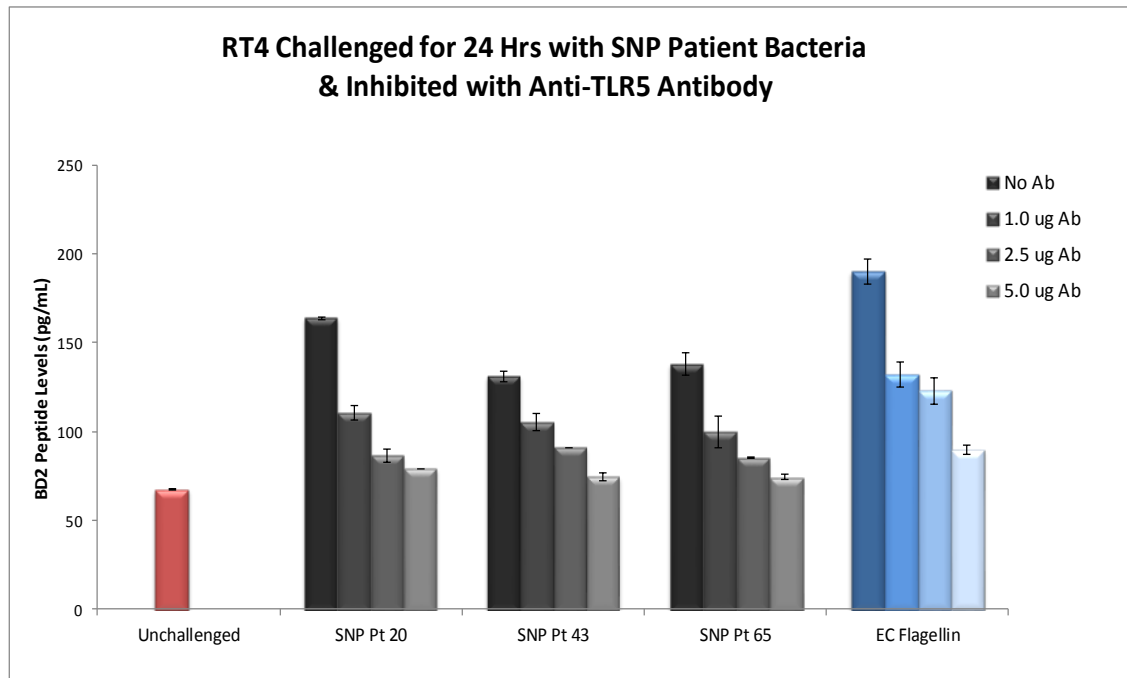
AU = Arbitrary Units, note vertical access is on logarithmic scale

N = 9 (No SNP) and N = 3 (SNP)

Central bar shows mean with standard error of the mean above and below

Having identified that TLR5 expression itself was not deficient. The bacteria isolated from the patients' infected urine were used in an *ex vivo* challenge in RT4 cells to check that a BD2 peptide response could be induced and if this response could be reduced by inhibiting TLR5 signalling using antibody. The latter experiment was done to attempt to mimic the effects of the TLR5<sup>392Stop</sup> *ex vivo*. The results are shown in Figure 6.21, the response to flagellin is also shown as a positive control.

**Figure 6.21: RT4 cells challenged for 24 hours with bacteria isolated from SNP patients with infection & inhibited with TLR5 antibody**



Each ELISA assay was done with three replicates & compared against a standard curve of synthetic defensin.

N =3 (1 experiment with 3 replicates)

Error bar shows standard error of the mean

From this experiment it is clear that *ex vivo* in urothelial cells with intact TLR5, the bacteria isolated from the patient with SNP who did not appear to mount a BD2 response *in vivo* can elicit a BD2 response in the *ex-vivo* model system. However, this response can be progressively inhibited with the use of anti-TLR5 antibody, hence mimicking what may be happening in patients with the TLR5<sup>392Stop</sup> SNP. A similar response was also seen at NFκB level (data not shown) when examined by my colleague Marcelo Lanz as part of other work in our research group.

### 6.3.3 Summary of Bladder Sample Analyses

The key findings from the clinical patient bladder and urine sample analyses were as follows:

- In the absence of infection, differences in the basal expression and secretion of BD1 or BD2 were found. Also no difference was found in expression the relevant Toll like receptors for LPS and flagellin, TLR4 and TLR5 respectively.
- Examination of the smaller cohort of women with active infection at the time of sampling demonstrated significantly lower BD2 mRNA expression and peptide levels in patients with the TLR5<sup>392Stop</sup> SNP. Hormonal influence may also play part but the 'infection' cohort is too small to analyse this further
- Expression of TLR5 was higher in subjects with infection when compared with both normal control and rUTI sufferers without infection.
- Overall expression of TLR 5 was no different in TLR5<sup>392Stop</sup> SNP patients compared to other rUTI patients without the SNP.
- Bacteria isolated from the infected urine of TLR5<sup>392Stop</sup> SNP patients with UTI, do elicit a BD2 response *ex vivo* but this can be inhibited by blocking TLR5. This adds weight to possibility the lack of response in SNP patients is due to host deficiency of functional TLR-5.

## 6.4 Vaginal Samples

The *in vitro* work has already demonstrated that the nature of AMP expression in vaginal epithelium is different to that of urothelium. In particular, expression of LL37 and HD5 was very low, and while BD1 was expressed constitutively, there was no significant response seen during infection. However, unlike urothelium, a degree of basal secretion of BD2 was noted even in the absence of infection which was then further elevated during infection. The nature of the vaginal epithelium is such that only much smaller biopsies were possible during the study mainly because the epithelium is more taut than urothelium (which folds with changes in bladder size). This prevents the forceps gaining as much purchase on the epithelium, furthermore the process was further confounded by the presence of mucus. Consequently one limitation of the vaginal samples is that the volume of vaginal epithelium material obtained from the biopsy was much lower than for urothelium, consequently the focus of assays on the vaginal samples was on BD2. However, one great advantage of the vaginal samples was that the douches (for peptide analysis) were taken at the same time as the biopsy potentially allowing greater validation of the mRNA expression data.

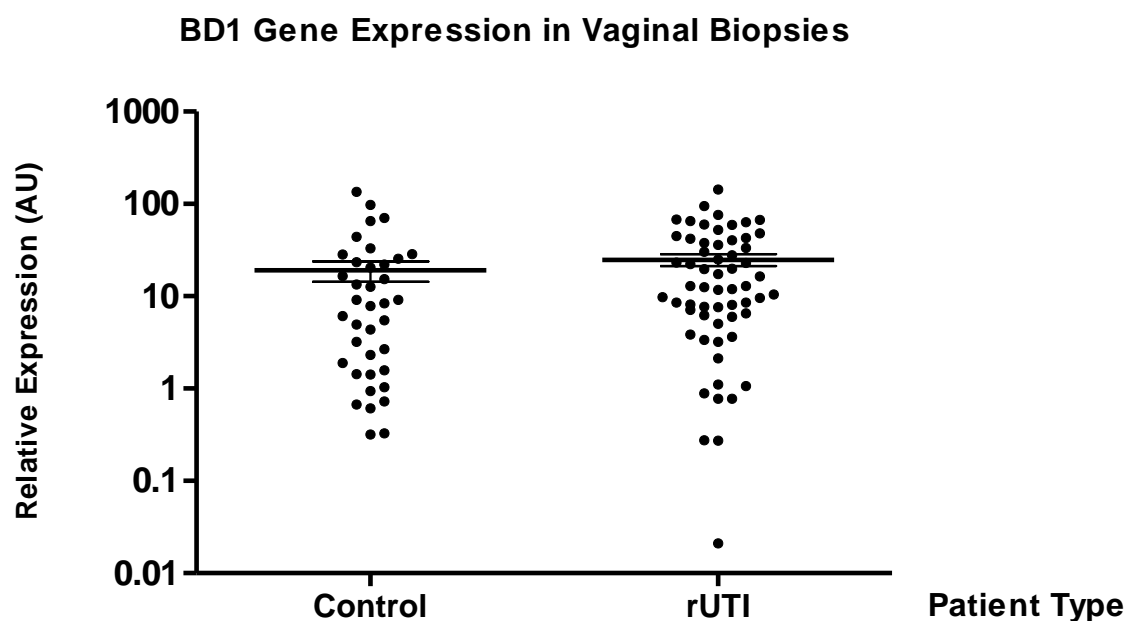
### 6.4.1 Controls vs Recurrent UTI

One of the key questions for the clinical arm of this study was whether there was any significant difference in the AMP expression between women with and without recurrent urinary tract infection. From the *in vitro* studies, it was clear that of the four examined AMPs, BD2 seems to be both the most responsive to *E. coli* PAMPs within the vaginal epithelium and the most active against the bacteria itself. However, as BD2 in the urothelium is only expressed during infection, an effective comparison cannot be made outside of infection. However, in the vaginal epithelium, due to on-going

basal secretion of BD2 even in the absence of infection, there was opportunity for comparison in both states. Similar to urothelium, BD1 is also present constitutively in the vaginal epithelium, consequently this was AMP was also assayed.

First a broad comparative analysis was made comparing mRNA expression of BD1 and BD2 between the two study groups. This data is shown in Figure 6.22 and Figure 6.23

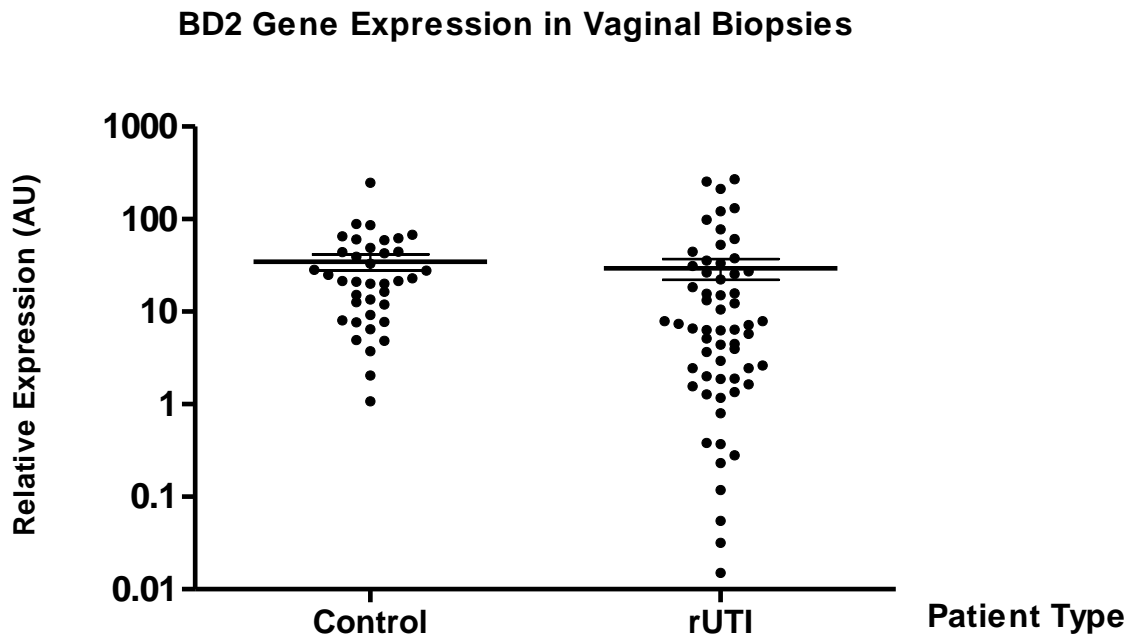
**Figure 6.22: BD1 Gene mRNA expression in vaginal biopsies from all control and rUTI patients**



Measurements made by qRT-PCR with GAPDH and ATP5B house-keeping gene.

AU = Arbitrary Units, note vertical access is on logarithmic scale  
N=38 (Control) and N=60 (rUTI)  
Central bar shows mean with standard error of the mean above and below

Figure 6.23: BD2 Gene mRNA expression in vaginal biopsies from all control and rUTI patients



Measurements made by qRT-PCR with GAPDH and ATP5B house-keeping gene.

AU = Arbitrary Units, note vertical axis is on logarithmic scale

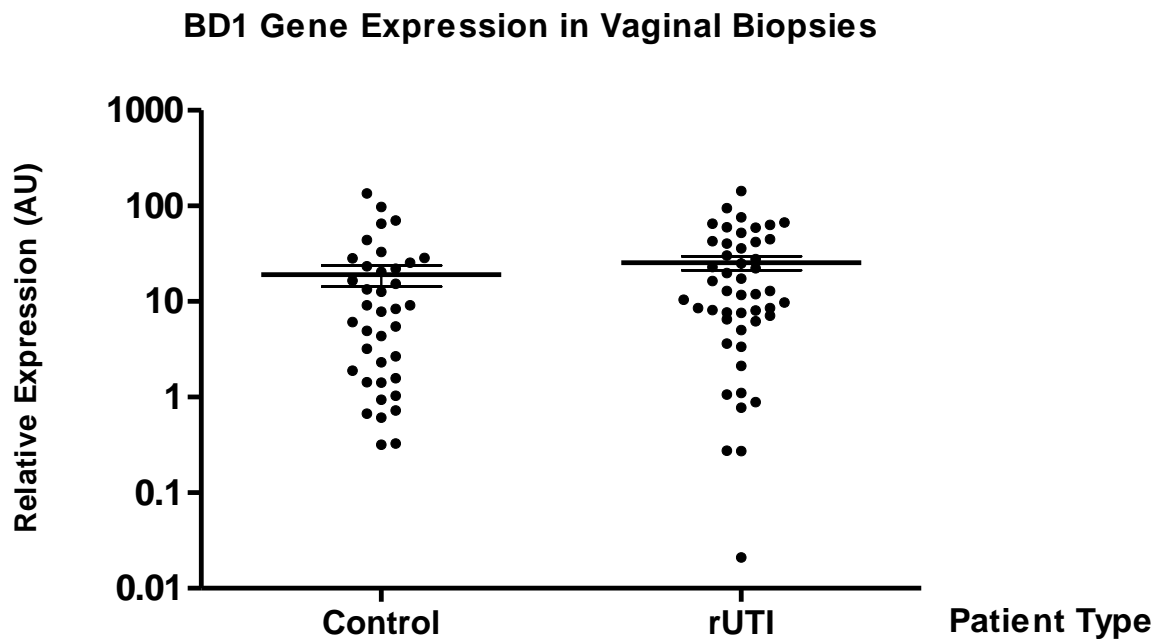
N=38 (Control) and N=60 (rUTI)

Central bar shows mean with standard error of the mean above and below

From this initial broad comparison, it is quite apparent from the graphs that there was no significant difference in gene expression between the two groups and this was confirmed statistically with a t-test. However, from the clinical details, it is also clear that some subjects had infection even at the time of biopsy and *in vitro* data has already demonstrated that infection potentially leads to a rise in defensin expression. Since, the state of infection only affects those in the rUTI group, it could act as a skewing factor. Indeed, just looking at BD2 expression the rUTI group in Figure 6.23, there was a wider range of expression with considerably reduced levels of expression at the lower end of the spectrum – a more prominent ‘tail’.

To better focus the analysis and take out this major confounding factor, the twelve patients with infection at the time of the biopsy, all of whom were in the rUTI group, were separated from this 'basal' analysis. The data for BD1 then looks as follows:

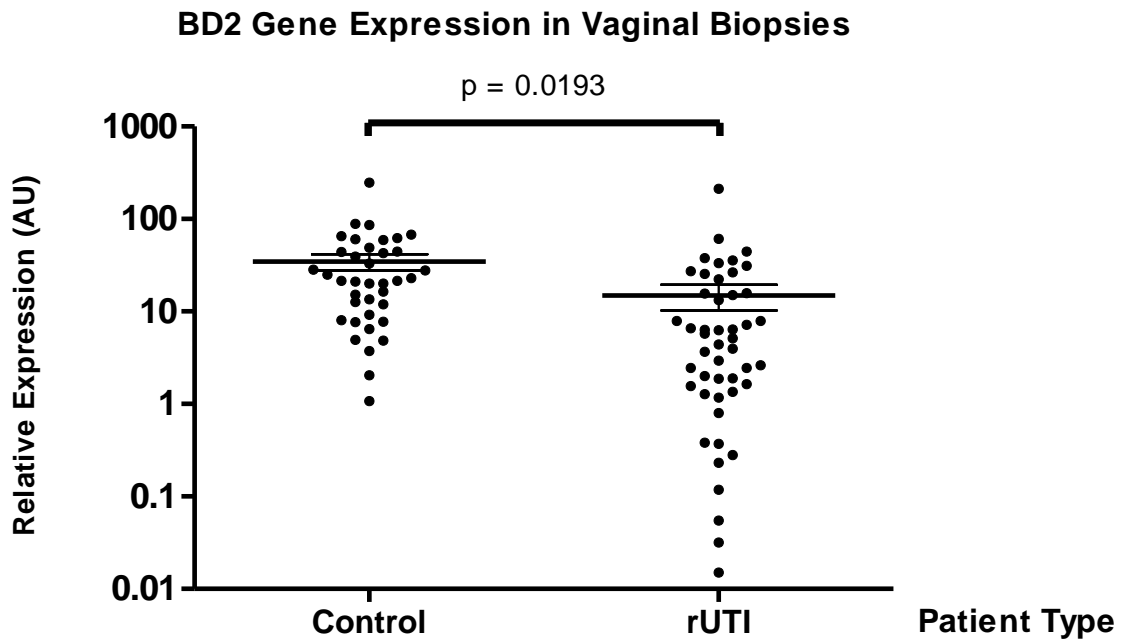
Figure 6.24: BD1 Gene mRNA expression in vaginal biopsies from control and rUTI patients with no infection at the time of biopsy



Measurements made by qRT-PCR with GAPDH and ATP5B house-keeping gene.  
AU = Arbitrary Units, note vertical axis is on logarithmic scale  
N=38 (Control) and N=48 (rUTI)  
Central bar shows mean with standard error of the mean above and below

Analysis of the samples with 'no infection' at the time of biopsy indicated that there was no significant difference in BD1 expression between the control and rUTI group. However, for BD2 expression, the difference between the two groups emerges, with the basal BD2 mRNA expression in the rUTI group being lower (Figure 6.25).

Figure 6.25: BD2 Gene mRNA expression in vaginal biopsies from control and rUTI patients with no infection at the time of biopsy



Measurements made by qRT-PCR with GAPDH and ATP5B house-keeping gene.

AU = Arbitrary Units, note vertical axis is on logarithmic scale

N=38 (Control) and N=48 (rUTI)

Central bar shows mean with standard error of the mean above and below

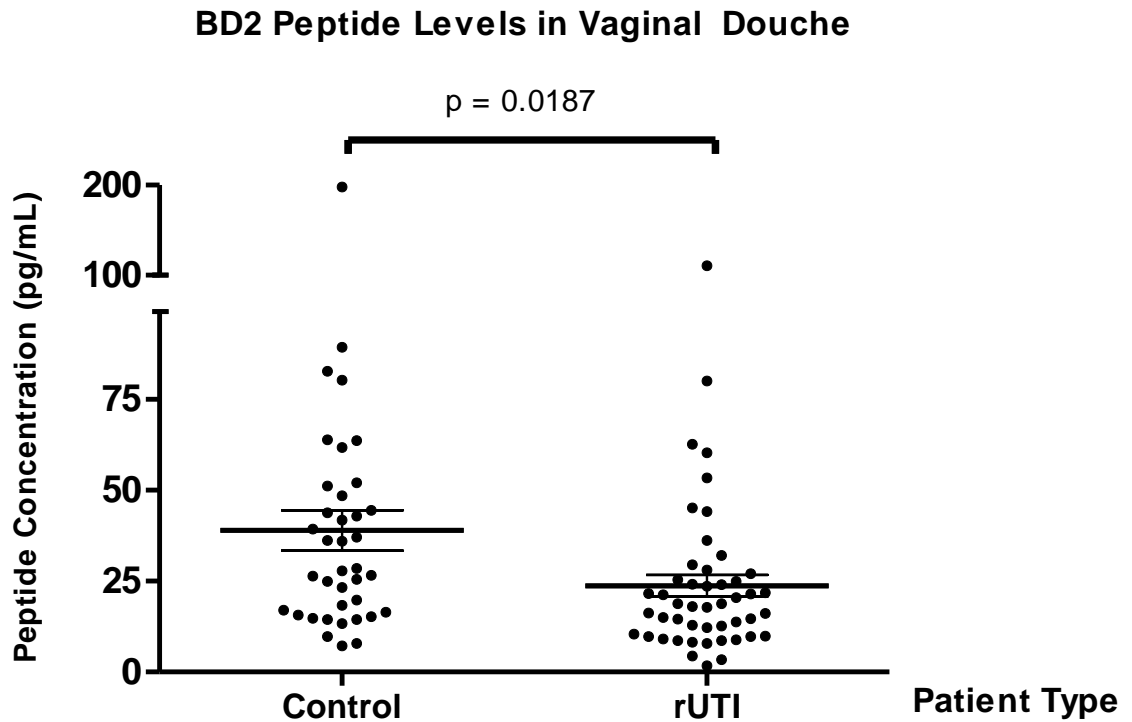
Statistical analysis by Student's t-test with Welch's correction (for unequal variance)

In Figure 6.25, it is possible to see that the difference between the two groups is become clearer with the basal BD2 mRNA expression in the rUTI group being lower. It is worth noting these data are presented on a logarithmic scale and that the mean and SEM for the control and rUTI groups were  $34.8 \pm 6.9$  and  $14.9 \pm 4.6$  respectively. A two tailed unpaired t-test with Welch's correction (to allow for different underlying variance) demonstrated a significant difference between the two groups at the 5% significance level ( $p = 0.0193$ ).

This difference was further validated by the peptide concentrations (Figure 6.26).



Figure 6.26: BD2 Peptide levels in vaginal douches from control and rUTI patients with no infection at the time of the douche



Each ELISA assay was done with two replicates & compared against a standard curve of synthetic defensin.

N=38 (Control) and N=48 (rUTI)

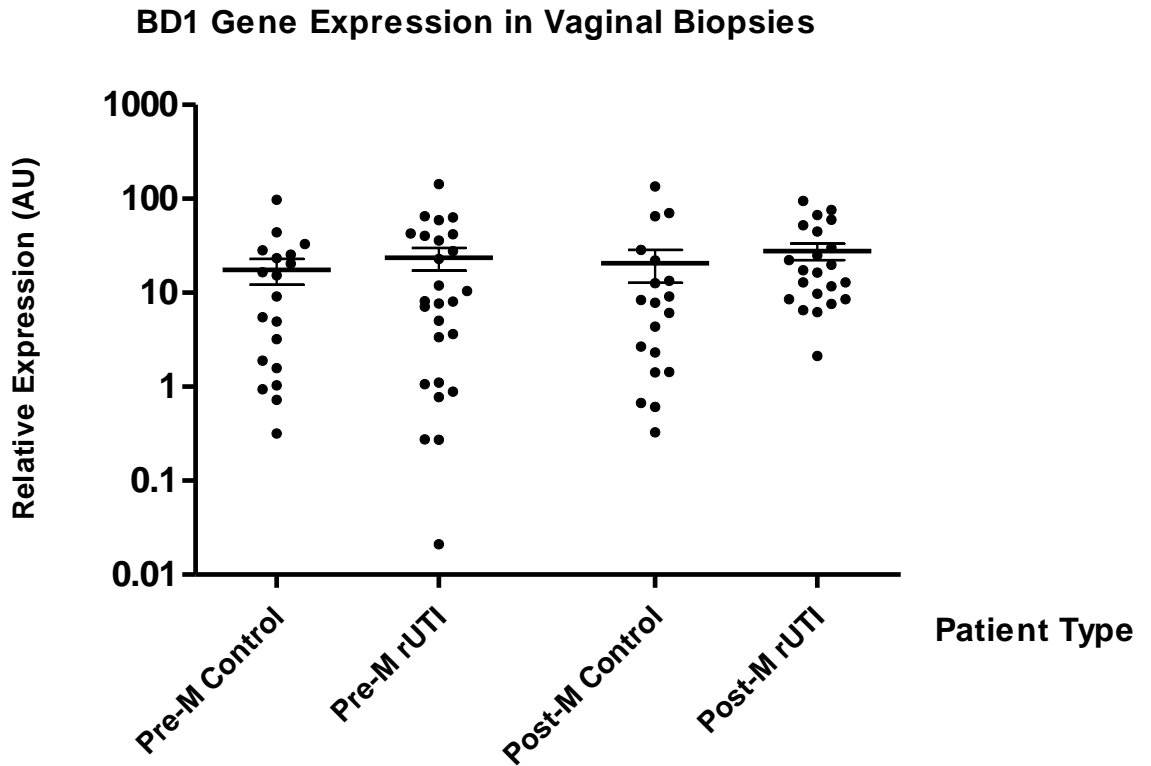
Central bar shows mean with standard error of the mean above and below

Statistical analysis by Student's t-test with Welch's correction (for unequal variance)

Figure 6.26 demonstrates that the levels for BD2 were significantly lower in the rUTI group with a mean and SEM of  $23.7 \pm 3.0$  vs  $38.9 \pm 5.5$  for the control group, resulting in a significant difference at the 5% level ( $p = 0.0187$ ).

From the outset the study was powered to examine pre-menopausal and post-menopausal subjects. Published data has previously shown that AMPs can be influenced by estrogen and the *in vitro* data has demonstrated that BD2 in particular is influenced by estrogen. Examining the data for pre-menopausal and post-menopausal subjects yielded the following for BD1 (Figure 6.27).

Figure 6.27: BD1 Gene mRNA expression in vaginal biopsies from pre-menopausal & post-menopausal control and rUTI patients with no infection at the time of biopsy

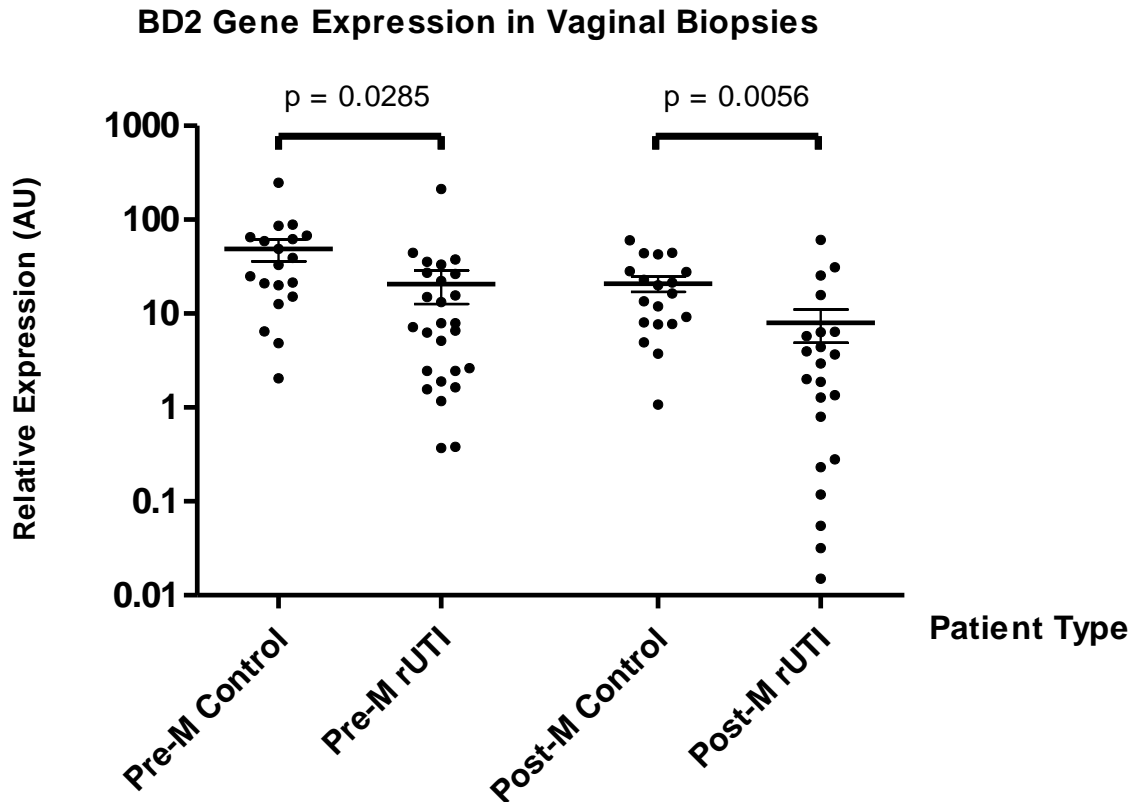


Measurements made by qRT-PCR with GAPDH and ATP5B house-keeping gene.

AU = Arbitrary Units, note vertical access is on logarithmic scale  
 N=19 (Pre-menopausal control), N=26 (Pre-menopausal rUTI),  
 N = 19 (Post-menopausal control), and N=22 (Post-menopausal rUTI)  
 Central bar shows mean with standard error of the mean above and below  
 Statistical analysis by Student's t-test with Welch's correction (for unequal variance)

Thus, sub-categorising by menopausal status for BD1 analysis provided results that were not different to that of the overall population with similar BD1 levels expressed by all four sub-groups. However, again for BD2 mRNA expression, a very different pattern emerged (Figure 6.28):

Figure 6.28: BD2 Gene mRNA expression in vaginal biopsies from pre-menopausal & post-menopausal control and rUTI patients with no infection at the time of biopsy

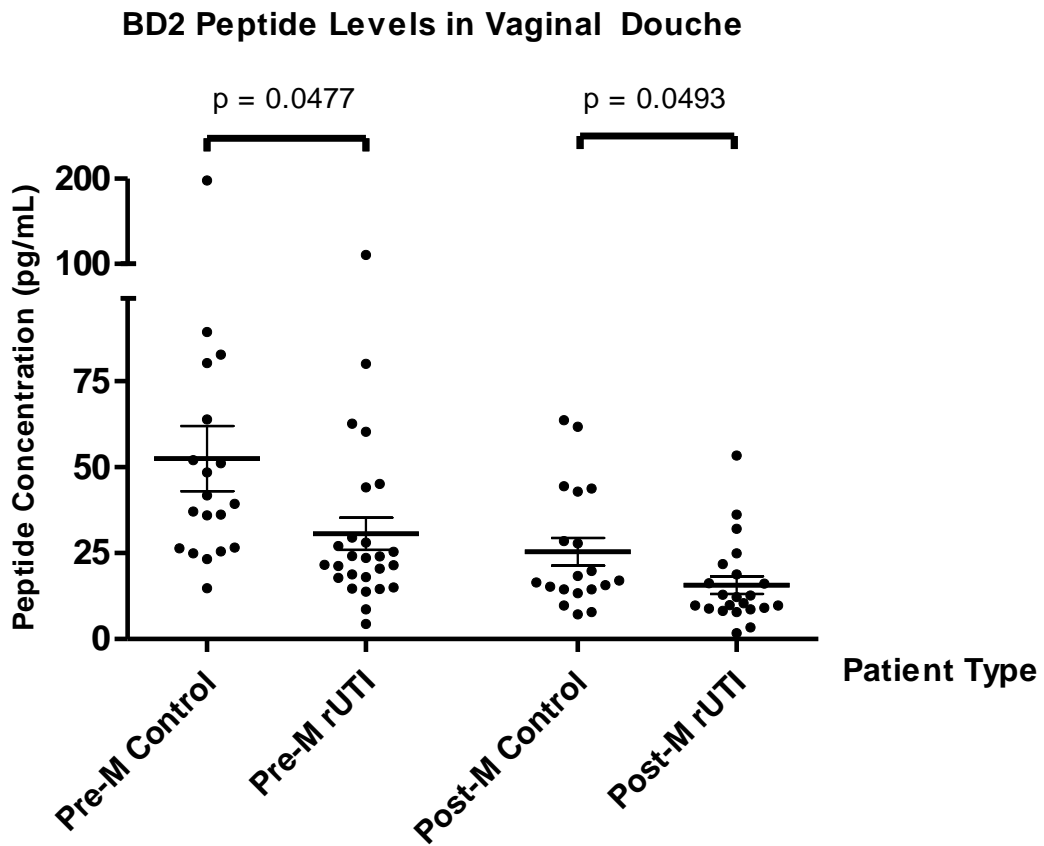


Measurements made by qRT-PCR with GAPDH and ATP5B house-keeping gene.

AU = Arbitrary Units, note vertical access is on logarithmic scale  
 N=19 (Pre-menopausal control), N=26 (Pre-menopausal rUTI),  
 N = 19 (Post-menopausal control), and N=22 (Post-menopausal rUTI)  
 Central bar shows mean with standard error of the mean above and below  
 Statistical analysis by Student's t-test with Welch's correction (for unequal variance)

Here it was observed that while subjects with rUTI had lower basal BD2 mRNA expression than those without, there was also a difference between the pre-menopausal and post-menopausal cohorts, with the post-menopausal group showing lower BD2 expression. Looking closely, the levels expressed by post-menopausal controls were equivalent to those amongst the pre-menopausal group with rUTI. A similar pattern was observed at peptide level from the douches (Figure 6.29):

Figure 6.29: BD2 Peptide levels in vaginal douches from pre-Menopausal & post-Menopausal control and rUTI patients with no infection at the time of biopsy



Each ELISA assay was done with two replicates & compared against a standard curve of synthetic defensin.

N=19 (Pre-menopausal control), N=26 (Pre-menopausal rUTI),

N = 19 (Post-menopausal control), and N=22 (Post-menopausal rUTI)

Central bar shows mean with standard error of the mean above and below

Statistical analysis by Student's t-test with Welch's correction (for unequal variance)

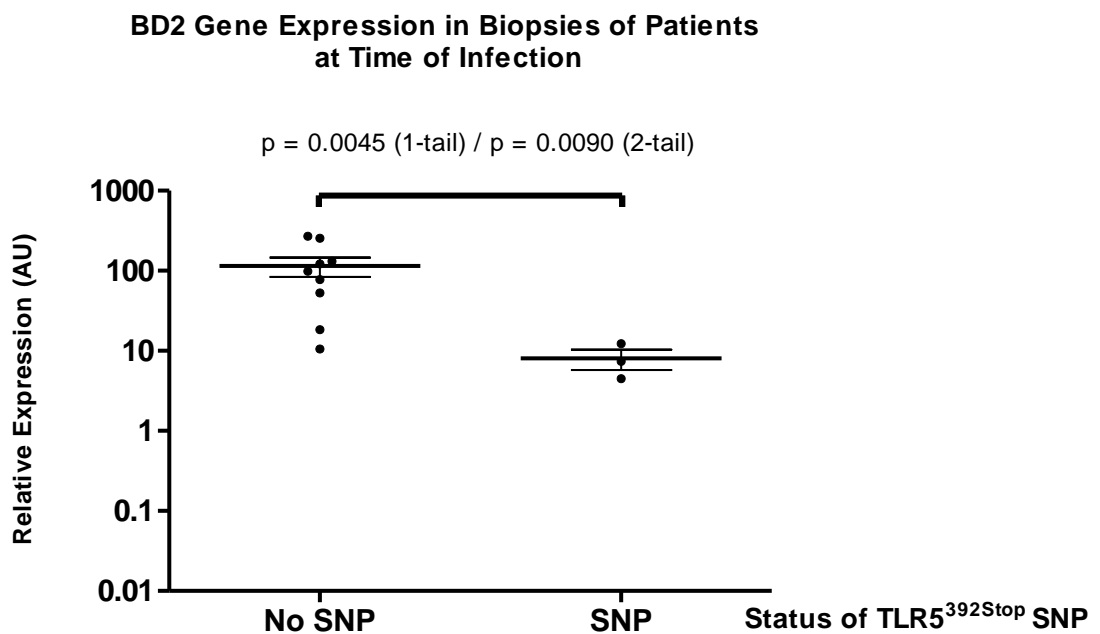
The premenopausal rUTI subjects have a mean level of detectable BD2 21.9 pg/mL (42%) lower than control (30.6±4.7 versus 52.5±9.5 pg/mL). In the postmenopausal group, those with recurrent UTI have a mean level of detectable BD2 around 9.7 pg/mL (37%) lower than control subjects (15.7±2.6 pg/mL versus 25.4±4.0). The effects of hormonal status will be analysed more closely in section 6.4.3.

## 6.4.2 Non-SNP vs SNP Patients

As with the bladder samples, in section 6.3.2, given the nature of BD2 induction via flagellin acting through TLR5, BD2 expression and secretion in subjects with the TLR5<sup>392Stop</sup> SNP were compared with subjects without the SNP. However with vaginal epithelium, as mentioned previously, there appears to be greater basal secretion even in the absence of bacterial challenge. Furthermore, in the clinical situation, the vagina is always colonised, this therefore affords an opportunity to compare BD2 levels in the TLR5<sup>392Stop</sup> SNP subjects with others in both an infection and non-infection state.

Looking first in the infection state, Figure 6.30 shows BD2 expression in rUTI subjects who had infection at the time of their biopsy categorised by whether or not they had the TLR5<sup>392Stop</sup> SNP. These results show that those with the SNP had significantly lower BD2 expression with a mean of  $8.0 \pm 2.3$  AU vs  $115.0 \pm 31.1$  AU for those without ( $p < 0.05$ ).

**Figure 6.30: BD2 Gene expression in vaginal biopsies from non-SNP and SNP rUTI patients with infection at the time of biopsy**



Measurements made by qRT-PCR with GAPDH and ATP5B house-keeping gene.

AU = Arbitrary Units, note vertical access is on logarithmic scale

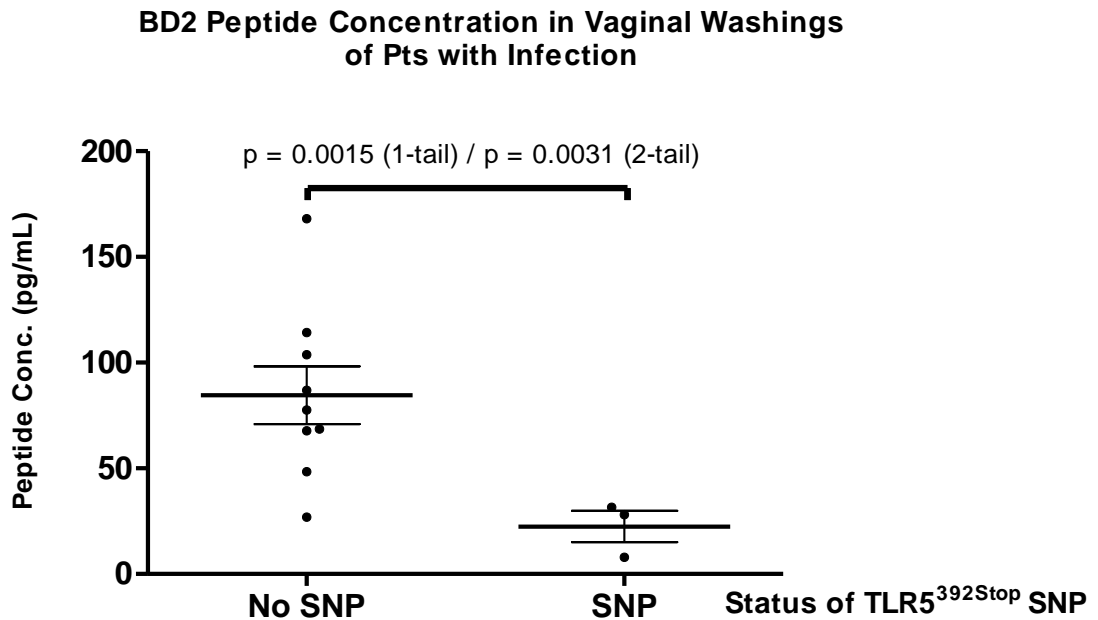
N = 9 (No SNP) and N = 3 (SNP)

Central bar shows mean with standard error of the mean above and below

Statistical analysis by one and two tail Student's t-test with Welch's correction (for unequal variance)

A similar pattern was detected following peptide analysis of the douches for BD2 levels with  $22.5 \pm 7.4$  pg/mL for the SNP subjects vs  $84.7 \pm 13.7$  pg/mL for the non-SNP rUTI subjects (Figure 6.31):

Figure 6.31: BD2 Peptide levels in vaginal douches from non-SNP and SNP rUTI patients with infection at the time



Each ELISA assay was done with two replicates & compared against a standard curve of synthetic defensin.

N=9 (No SNP) and N=3 (SNP)

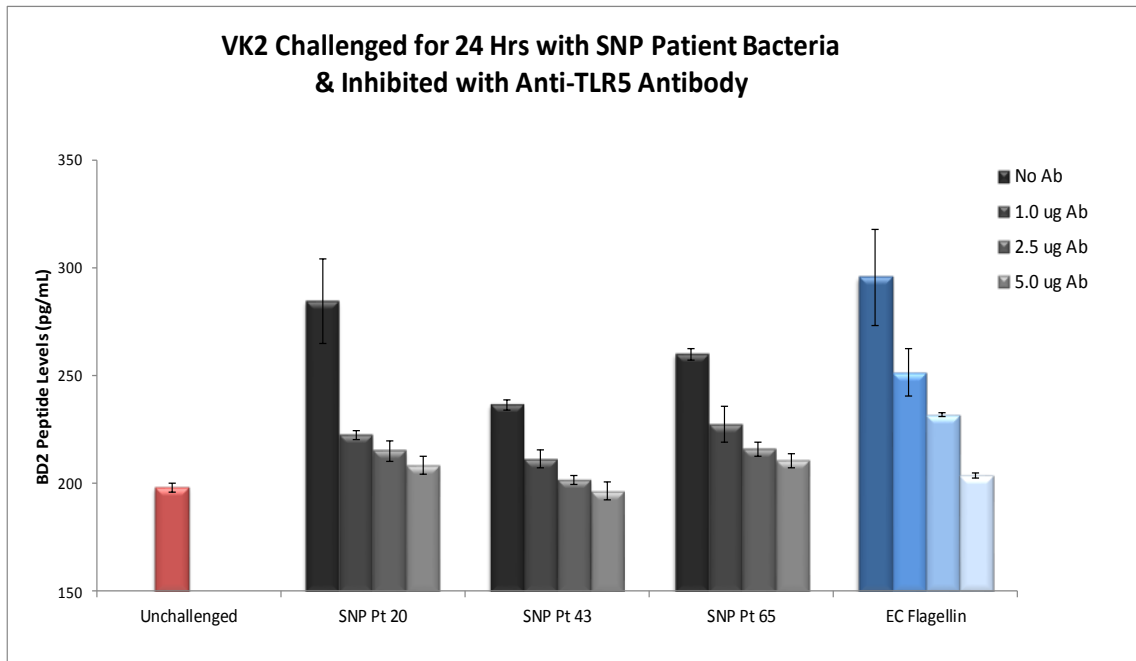
Central bar shows mean with standard error of the mean above and below

Statistical analysis by one and two tail Student's t-test with Welch's correction (for unequal variance)

As was done with RT4 in section 6.3.2, the bacteria isolated from the patients infected urine were also used in an *ex vivo* challenge in VK2 cells to check for BD2 peptide

response in vaginal cells and to investigate if this response could be reduced by inhibiting TLR5 signalling using antibody. The results are shown in Figure 6.32, the response to flagellin is also shown as a positive control.

**Figure 6.32: VK2 E6/E7 Cells Challenged for 24 hours with bacteria isolated from SNP patients with infection & inhibited with TLR5 antibody**



Each ELISA assay was done with three replicates & compared against a standard curve of synthetic defensin.

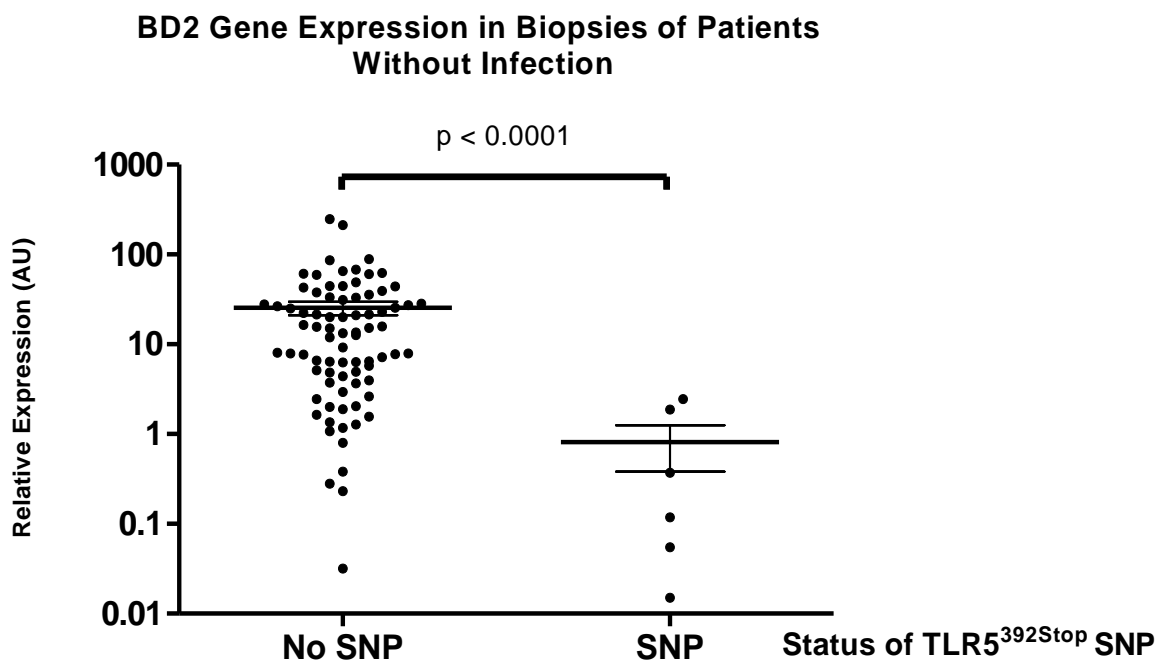
N =3  
Error bar shows standard error of the mean

As was seen with RT4 urothelial cells, the bacteria isolated from the infected urine can elicit a BD2 response in VK2 cells *ex vivo* but this response again can be inhibited with anti-TLR5 antibody mimicking the situation that may be occurring clinically in patients with the SNP. A similar response was also seen at NFκB level by my colleague, Marcelo Lanz (data not shown).

From the clinical vaginal samples, , as was seen with the bladder biopsy and urine analyses, it is clear that in the presence of infection there is greater expression and secretion of BD2 from the vaginal tissue of patients with the SNP than those without.

This is similar to the pattern seen with bladder biopsies and urine analyses. However, what is more interesting in the vaginal epithelium is that a similar situation can be seen even in the absence of infection. In Figure 6.33, the BD2 mRNA expression of all subjects without infection is shown but categorised according to the presence or absence of the TLR5<sup>392Stop</sup> SNP.

**Figure 6.33: BD2 Gene mRNA expression in vaginal biopsies from non-SNP and SNP patients without infection at the time of biopsy**



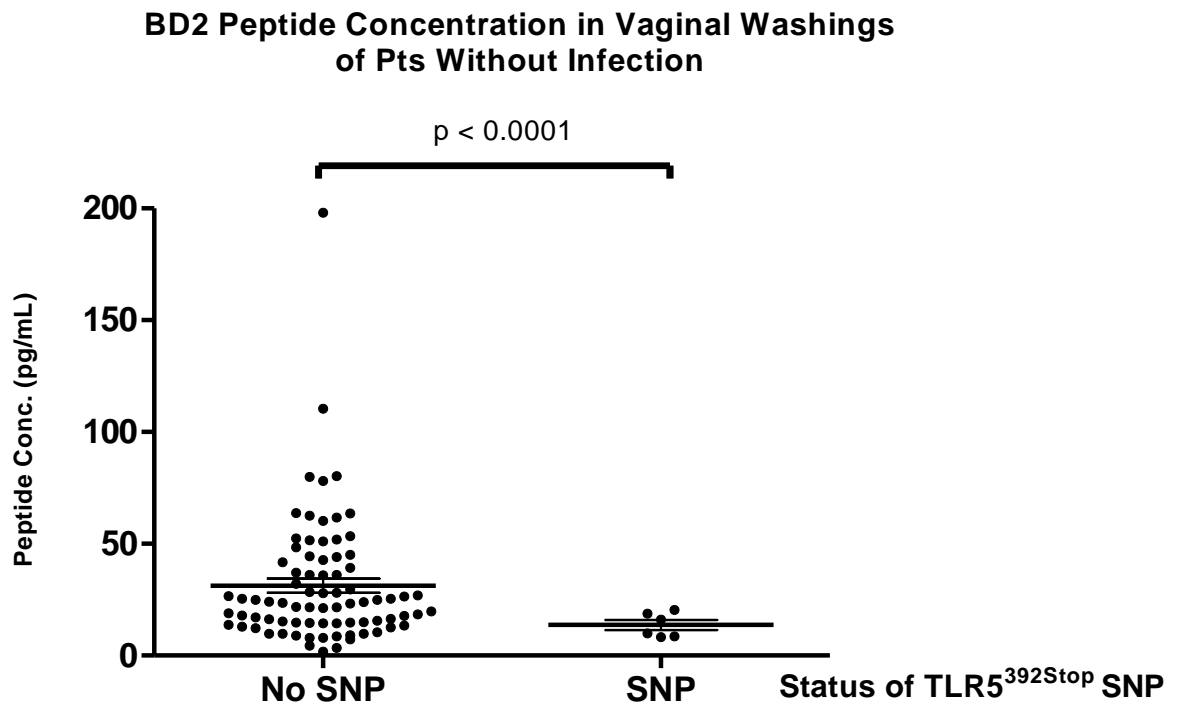
Measurements made by qRT-PCR with GAPDH and ATP5B house-keeping gene.

AU = Arbitrary Units, note vertical access is on logarithmic scale  
 N = 80 (No SNP) and N = 6 (SNP)  
 Central bar shows mean with standard error of the mean above and below  
 Statistical analysis by Student's t-test with Welch's correction (for unequal variance)

It is apparent that even in the absence of urinary tract infection, there is significantly lower BD2 expression in those subjects with the SNP vs those without ( $p < 0.0001$ ). A similar pattern is seen at peptide level in the douche analysis in Figure 6.34:



Figure 6.34: BD2 Peptide levels in vaginal douches from non-SNP and SNP patients without infection at the time



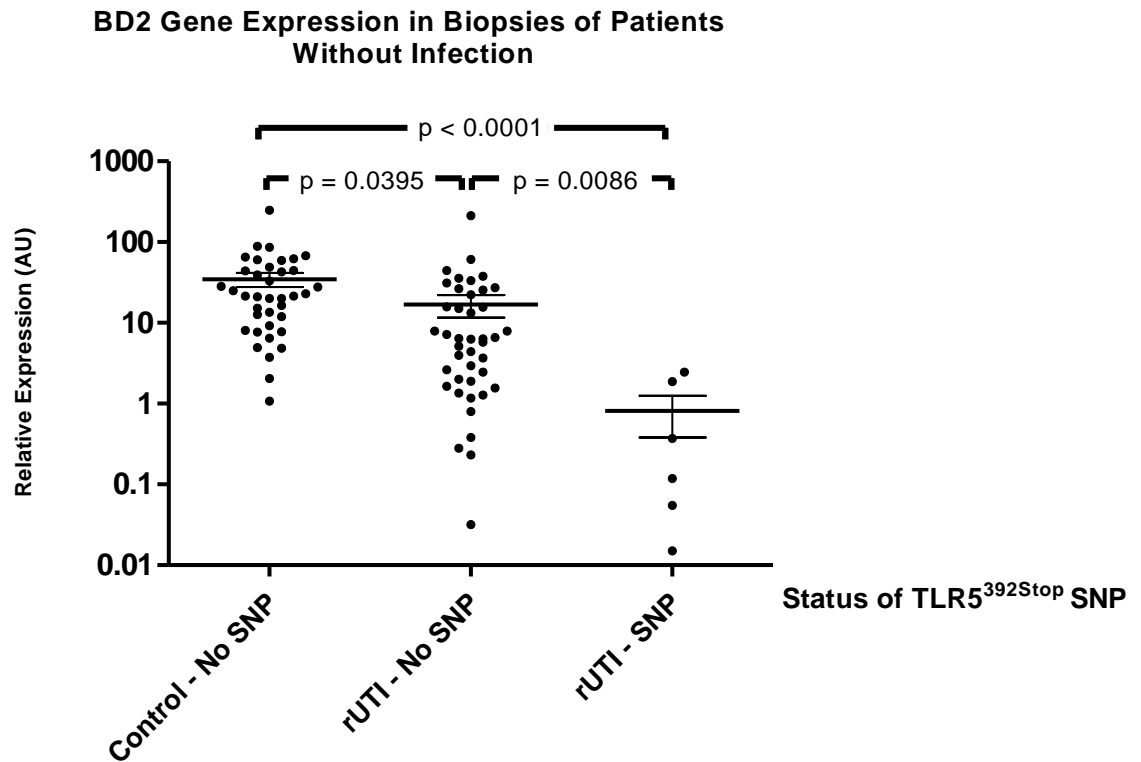
Each ELISA assay was done with two replicates & compared against a standard curve of synthetic defensin.

N=80 (No SNP) and N=6 (SNP)

Central bar shows mean with standard error of the mean above and below  
Statistical analysis by Student's t-test with Welch's correction (for unequal variance)

The mean BD2 peptide level in the no SNP group was  $31.3 \pm 3.2$  pg/mL vs  $13.7 \pm 2.2$  pg/mL in the TLR5<sup>392Stop</sup> SNP group. This indicates that even in the absence of infection, the mean level of basal BD2 secretion in the non-SNP group is 2.3 fold higher than that of the SNP group. It is possible to subdivide these data further and categorise the non-SNP group into those who had rUTI and those that did not, as shown in Figure 6.35.

Figure 6.35: BD2 Gene mRNA expression in vaginal biopsies from non-SNP and SNP patients without infection at the time of biopsy further categorised by whether or not they have recurrent UTI



Measurements made by qRT-PCR with GAPDH and ATP5B house-keeping gene.

AU = Arbitrary Units, note vertical axis is on logarithmic scale  
N = 38 (Control – no SNP); N = 42 (rUTI – no SNP) and N = 6 (rUTI – SNP)

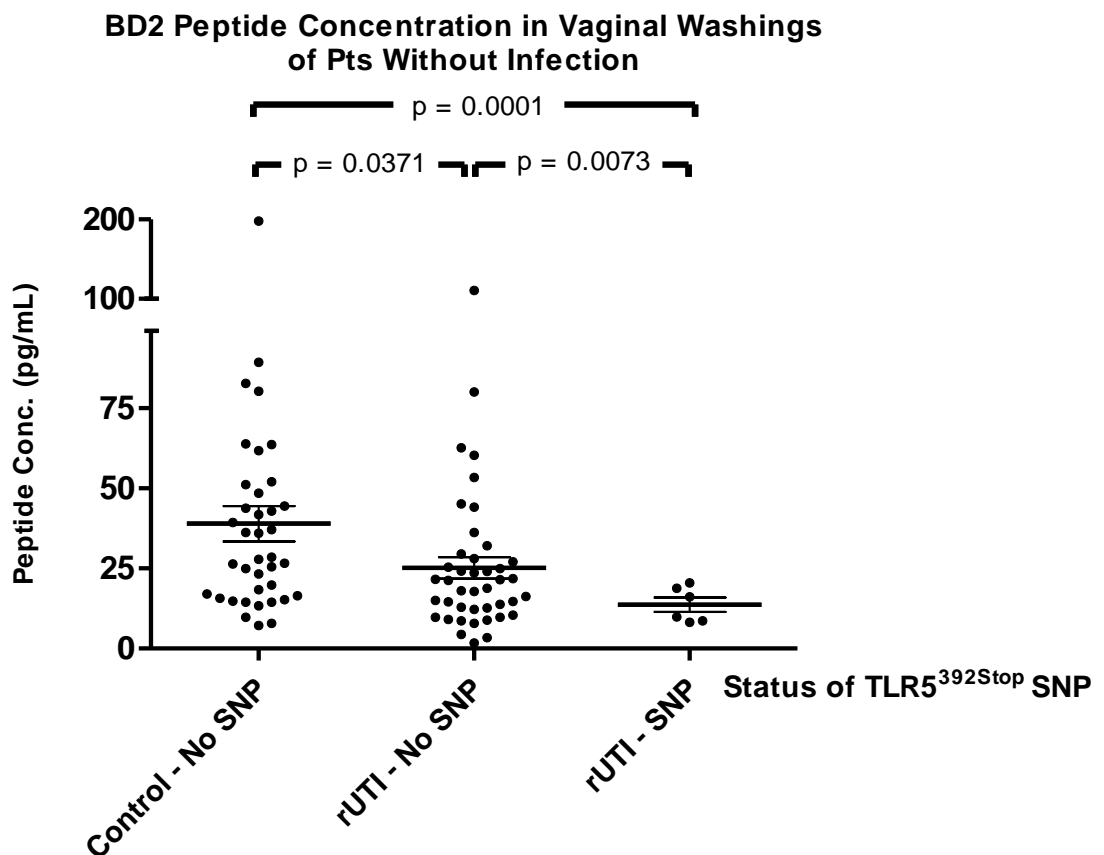
Central bar shows mean with standard error of the mean above and below  
Statistical analysis by Student's t-test with Welch's correction (for unequal variance)

Figure 6.35 throws further light on the relationship between BD2 expression and recurrent UTI. It is evident that those patients with the SNP had lower levels of BD2 expression ( $0.8 \pm 0.4$  AU) than both those with rUTI and no-SNP ( $16.9 \pm 5.2$  AU) and the normal controls ( $34.8 \pm 6.9$  AU). Interestingly, however, subjects with rUTI that did not have the SNP also had mean BD2 expression lower than controls (51% reduction). This is in line with findings discussed in section 6.4.1 and in relation to Figure 6.25 and Figure 6.26 i.e. that those with rUTI do seem to have lower vaginal BD2 expression and secretion. It is clear from data presented in Figure 6.35 that the lower BD2 expression

in the rUTI group is *not just* due to the presence of TLR5<sup>392Stop</sup> SNP subjects within that group, but rather that the group as a whole seems to have lower expression with the SNP subjects being a particularly affected sub-group.

Figure 6.36 shows a similar pattern in peptide secretion. Again, mean peptide secretion in the SNP group was lowest (13.7±2.2 pg/mL), but the rUTI group without the SNP was still lower (25.2±3.3 pg/mL) than the control group (38.9±5.5 pg/mL). It is perhaps worth noting that in the rUTI group without the SNP, there were some subjects with very low BD2 peptide levels, even lower than those with the SNP. There may be various reasons for this including other factors influencing BD2 secretion (e.g. hormonal or differences in commensal colonisation) as well as potentially other genes encoding signalling pathways.

**Figure 6.36: BD2 Peptide levels in vaginal douches from non-SNP and SNP patients without infection at the time further categorised by whether or not they have recurrent UTI**



Each ELISA assay was done with two replicates & compared against a standard curve of synthetic defensin.

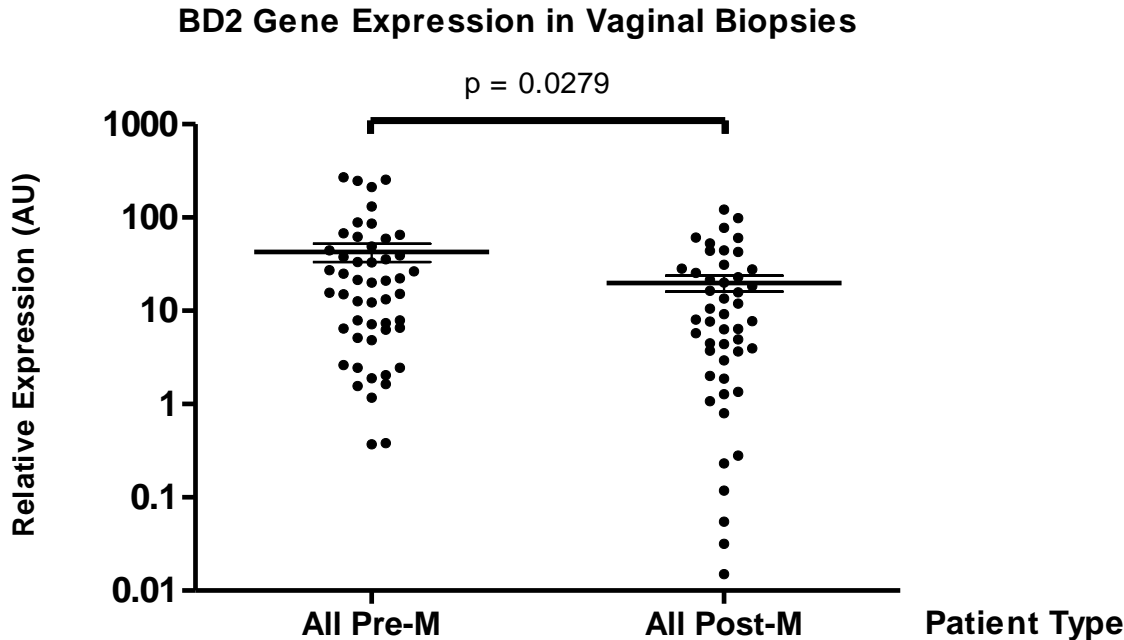
N = 38 (Control – no SNP); N = 42 (rUTI – no SNP) and N = 6 (rUTI – SNP)

Central bar shows mean with standard error of the mean above and below

### 6.4.3 Estrogen Effects

From both the *in vitro* data and the analyses of clinical samples from the control and rUTI groups in Figure 6.28 and Figure 6.29, it seems evident that the presence or possible deficiency of estrogen has a significant impact on BD2 expression and secretion in response to bacterial challenge. If one first compares all the pre-menopausal with all the post-menopausal subjects, it is evident that those who are post-menopausal have lower BD2 expression.

Figure 6.37: BD2 Gene mRNA expression in vaginal biopsies from all pre-menopausal and post-menopausal recruits



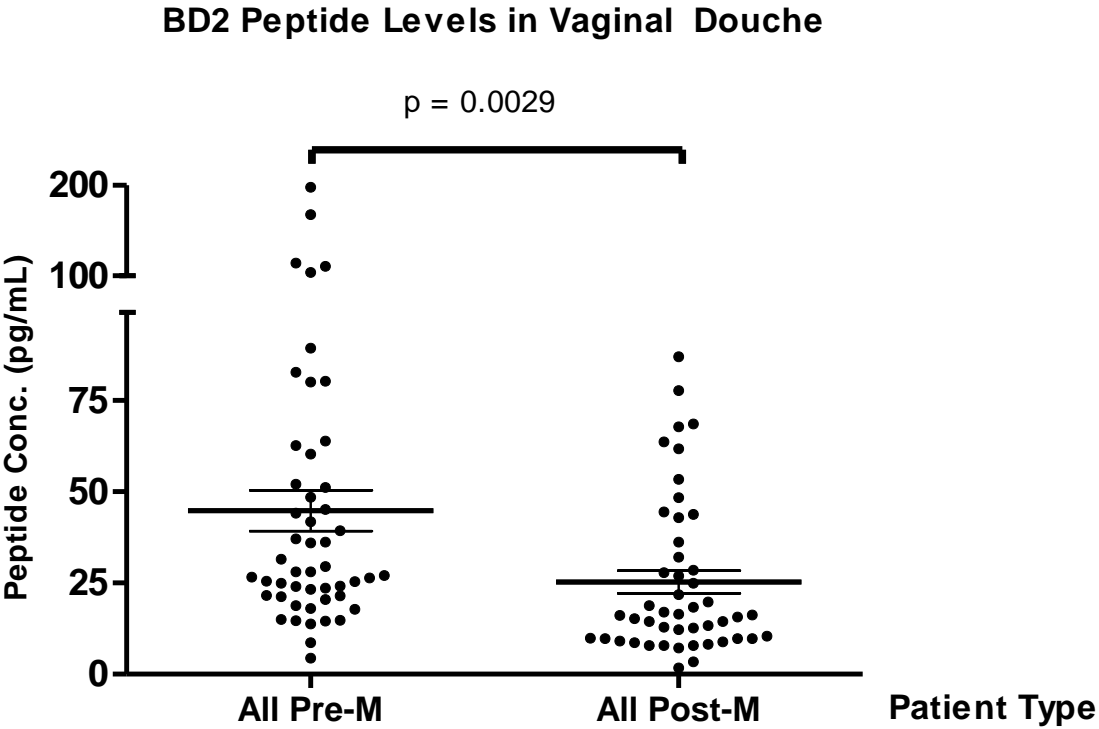
Measurements made by qRT-PCR with GAPDH and ATP5B house-keeping gene.

AU = Arbitrary Units, note vertical access is on logarithmic scale  
 N = 50 (Pre-menopausal) and N = 48 (Post-menopausal)

Central bar shows mean with standard error of the mean above and below  
 Statistical analysis by Student's t-test with Welch's correction (for unequal variance)

This pattern remains significant at the peptide level with mean BD2 levels in the premenopausal group being  $44.7 \pm 5.5$  pg/mL vs  $25.2 \pm 3.1$  pg/mL in the post-menopausal group which gives a difference between the two means of 19.5 pg/mL (44% reduction) . This is shown in Figure 6.38 which illustrates the significant difference between the two groups ( $p=0.003$ ).

Figure 6.38: BD2 Peptide levels in vaginal douches from pre-menopausal and post-menopausal patients



Each ELISA assay was done with two replicates & compared against a standard curve of synthetic defensin.

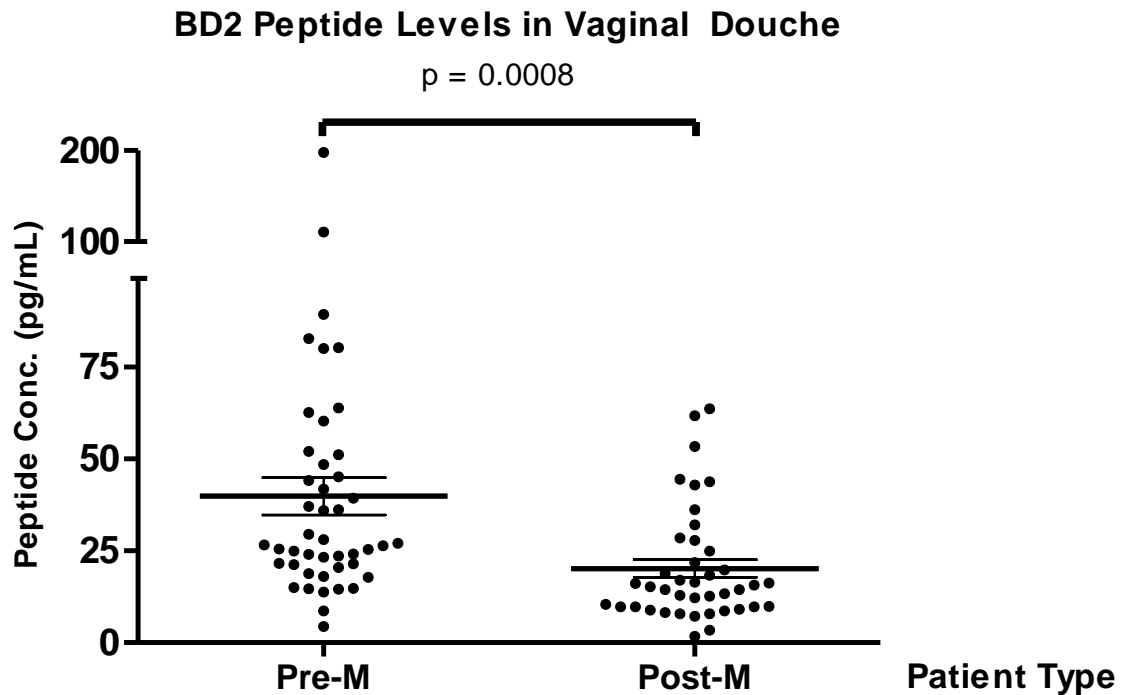
N = 50 (Pre-menopausal) and N = 48 (Post-menopausal)

Central bar shows mean with standard error of the mean above and below

Statistical analysis by Student's t-test with Welch's correction (for unequal variance)

As noted in section 6.4.1, when analysing differences between control and rUTI groups, it is possible that infection may cause artificial skewing of the data and confound the analysis (as it is known that BD2 levels rise with infection). Therefore, Figure 6.39 compares the two groups with exclusion of subjects who had UTI at the time of sampling. The effect is to reduce the mean BD2 level in both groups to  $39.8 \pm 5.0$  in the pre-menopausal group and  $20.2 \pm 2.4$  pg/mL in the post-menopausal group but there is still a difference between the mean secretion of the two groups of 19.6 pg/mL.

Figure 6.39: BD2 Peptide levels in vaginal douches from pre-menopausal and post-menopausal patients without infection at the time



Each ELISA assay was done with two replicates & compared against a standard curve of synthetic defensin.

N = 45 (Pre-menopausal) and N = 41 (Post-menopausal)

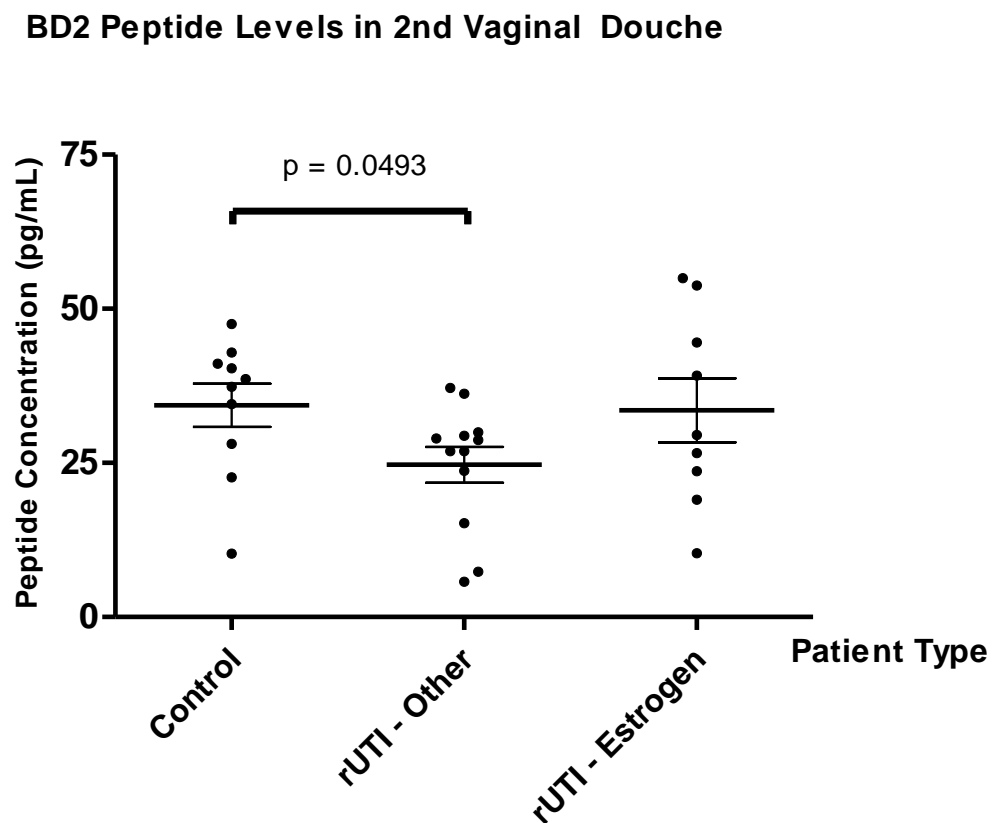
Central bar shows mean with standard error of the mean above and below  
Statistical analysis by Student's t-test with Welch's correction (for unequal variance)

In addition to the original douche taken at the time of the biopsy, recruits were asked to return a second douche approximately 6-8 weeks after the original. In total 38 of the recruits returned a second douche of which 31 were part of a pair in which neither douche was taken at the time of active infection which forms the dataset for analysis.

By comparing levels for the paired douches and considering treatment prescribed following the first clinical visit, it is potentially possible to examine the effect that administration of vaginal estrogen has on BD2 levels. Of the 31 subjects that gave a second douche, 9 had been rUTI sufferers and were prescribed vaginal estrogen supplementation, 12 had been rUTI sufferers given some other treatment to manage

their rUTI symptoms (antibiotic prophylaxis, self-initiated antibiotics, advice, etc.) and 10 were normal controls. Figure 6.40 shows the result of the analyses of the 3-groups. It can be seen that the second douche shows a broadly similar pattern to the data discussed in section 6.4.1. Subjects with rUTI treated with agents other than estrogen, continue to show significantly lower levels of BD2 than controls ( $p = 0.049$ ), while those treated with estrogen show levels of BD2 comparable with controls and there is no statistically significant difference between them (Figure 6.40).

**Figure 6.40: BD2 Peptide levels in second vaginal douche from controls and rUTI subjects treated with estrogen or other therapies**



Each ELISA assay was done with two replicates & compared against a standard curve of synthetic defensin.

N = 10 (Control); N = 9 (rUTI treated with estrogen) and N = 12 (rUTI treated with other therapy)

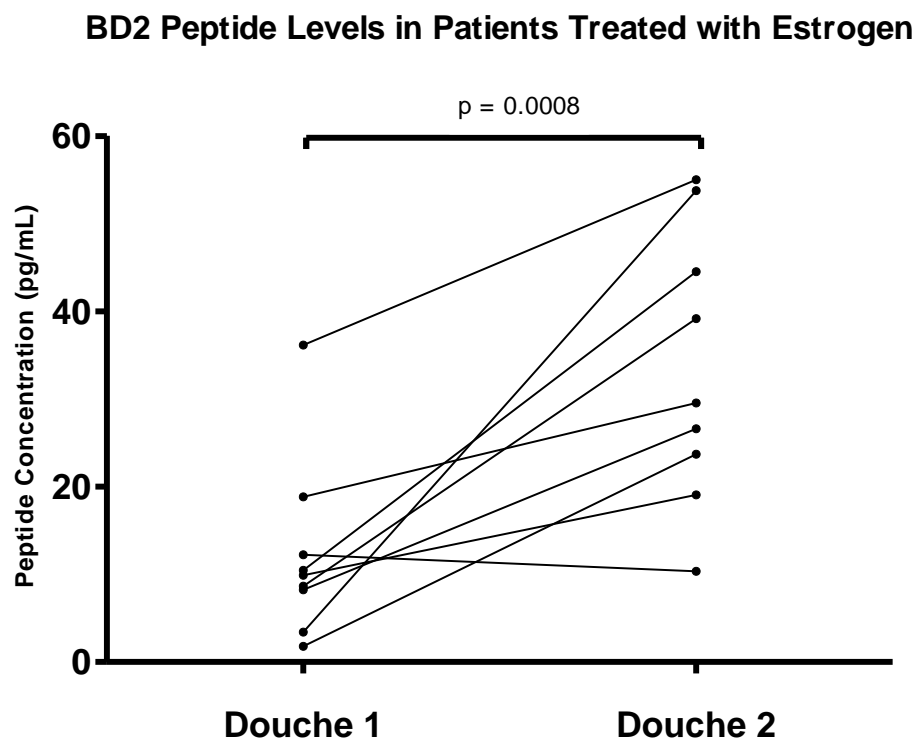
Central bar shows mean with standard error of the mean above and below

Statistical analysis by Student's t-test with Welch's correction (for unequal variance)



Comparison of the first and second douche data for the three groups above demonstrates the rise in BD2 levels seen in those patients treated with estrogen. Individually plotted data shown in Figure 6.41, confirms that there is overall a rise in the BD2 levels detected in the second douche of patients treated with estrogen ( $p=0.0008$ ).

**Figure 6.41: BD2 Peptide levels in first and second vaginal douches from rUTI subjects treated with estrogen**

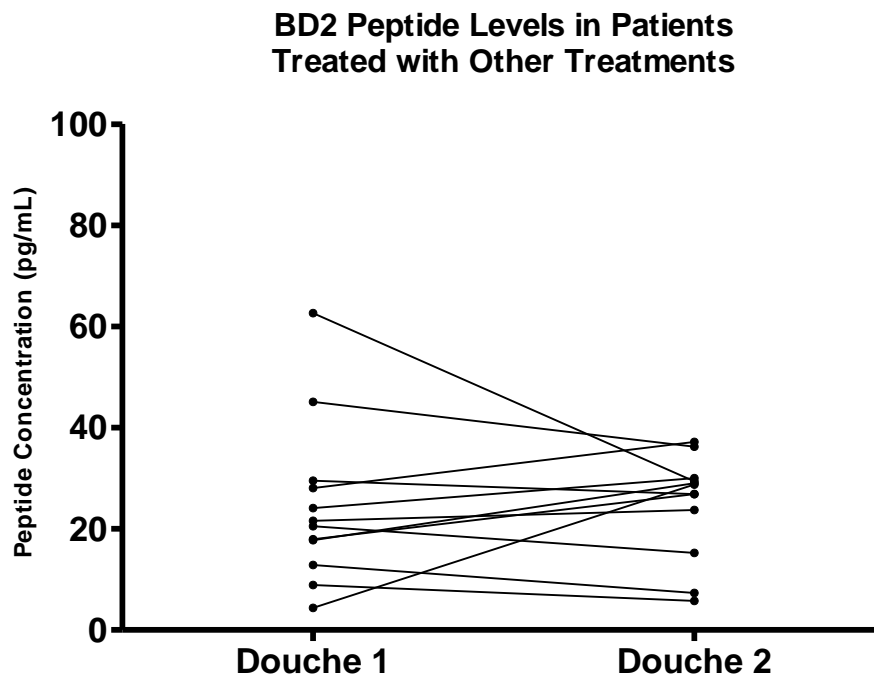
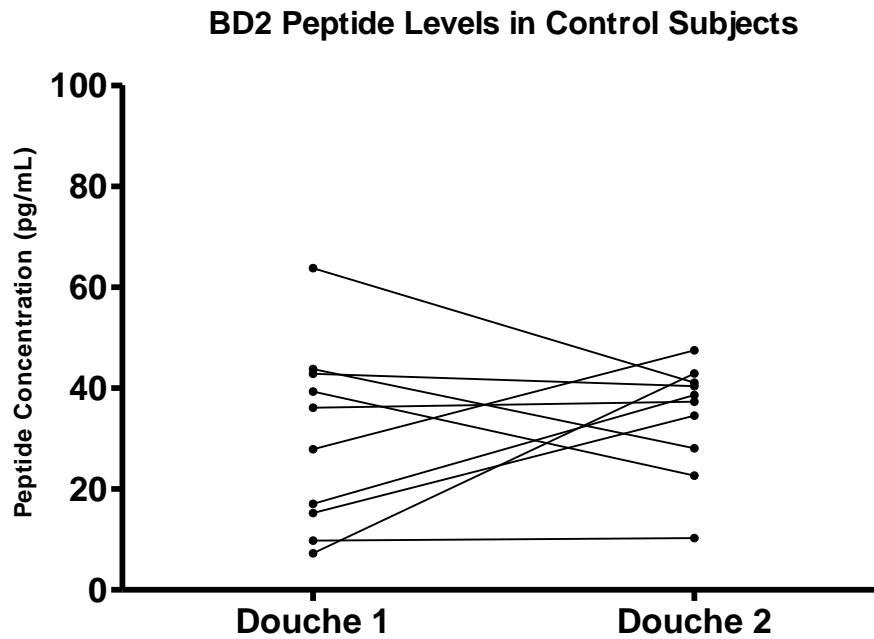


Each ELISA assay was done with two replicates & compared against a standard curve of synthetic defensin.

N = 9 pairs  
Statistical analysis by paired t-test

In contrast, the second douches from both the control and rUTI subjects treated with other therapies showed no significant change between the first and second douche (Figure 6.42).

Figure 6.42 BD2 Peptide levels in first and second vaginal douches from controls and rUTI subjects treated other therapies



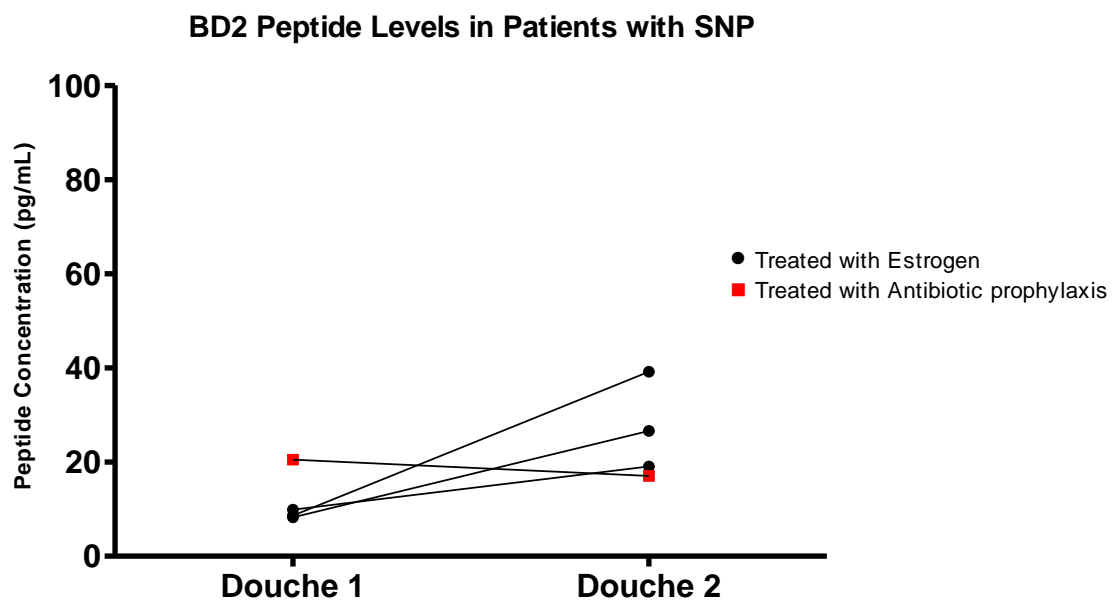
Each ELISA assay was done with two replicates & compared against a standard curve of synthetic defensin.

N = 10 pairs (Control) and N = 12 pairs (rUTI treated with other therapy)

Four of the nine patients that have the TLR5<sup>392Stop</sup> SNP also had two douches both sampled at times when they didn't have an infection. Three had been prescribed

vaginal estrogen as part of their management and one was on long term antibiotic prophylaxis. The result of this sub-group's douche BD2 analyses is shown in Figure 6.43. Although, there are only a small number of patients, it is evident that those carrying the TLR5 SNP conform to the trend seen with the other patients treated with estrogen (Figure 6.41). Furthermore, if data analysis is confined to *only* the three estrogen treated SNP patients then using a 1-tail paired t-test the second douches are significantly higher than the first ( $p = 0.0444$ ). Again, it is reasonable in this situation to carry out a 1-tail test as our *in vitro* data gives us a reasonable expectation that estrogen cause an increase in BD2, however, it is still worth noting that a two-tail taste would not show significant with such small numbers.

Figure 6.43: BD2 Peptide levels in first and second vaginal douches from treated TLR5<sup>392Stop</sup> SNP rUTI subjects



Each ELISA assay was done with two replicates & compared against a standard curve of synthetic defensin.

#### 6.4.4 Summary of Vaginal Sample Analyses

Key findings in the vaginal sample analyses were as follows:

- Basal vaginal BD2 mRNA expression and douche peptide levels in the absence of infection were significantly lower in the rUTI group compared to control. This was seen both when comparing the groups as a whole and when subcategorising them according to menopausal status. No difference was seen in BD1 expression.
- As was seen in the bladder, during active urinary tract infection, vaginal BD2 mRNA expression and douche peptide levels were significantly lower in TLR5<sup>392stop</sup> SNP patients compared to other infected rUTI patients without the SNP. This was also the case when comparing basal vaginal mRNA and peptide levels between TLR5<sup>392stop</sup> SNP subjects and non-SNP patients (control and rUTI).
- The bacteria isolated from the urine of SNP patients with UTI does elicit a BD2 response from VK2 E6/E7 cells *ex vivo*, reinforcing the notion that the lack of response is due to host deficiency rather than bacterial virulence.
- Women with rUTI and without the SNP also showed significantly lower basal BD2 mRNA expression and peptide levels than normal control subjects but not as low as those with the SNP.
- Pre-menopausal subjects had significantly higher basal BD2 mRNA expression and peptide levels than post-menopausal subjects. This suggests that relative local estrogen deficiency may reduce BD2 expression and secretion.
- rUTI subjects treated with vaginal estrogen showed significantly higher BD2 peptide levels in their second douche compared to those treated with other

agents/measures. A significant increase in BD2 was also observed in the second douche samples of the three SNP patients treated with estrogen with a 1-tail paired t-test.

## 7. Discussion

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The lower urinary tract is continuously exposed to a range of bacteria migrating up the urethra, many of which are potentially pathogenic. The ability of the host to detect such microbes and mount an appropriate response is critical to preventing on-going infection and systemic sepsis. Any delay or deficiency in this response could potentially result in increased frequency of infection or increased severity with greater local and systemic injury. Recurrent urinary tract infection (rUTI) is a common condition where, seemingly, the immune response is insufficient to prevent frequent re-infection with an organism (predominantly *Escherichia coli*) that also persists/exists in the host as a commensal. Whether this is due to a primary deficiency in the immune response, subversion by bacterial pathogenic mechanisms, host environmental changes favouring bacterial invasion, or a mixture of these factors will vary in each individual.

The focus of this thesis has been on the antimicrobial peptide (AMP) component of the innate immune response. The innate immune system provides the first line of defence against pathogens and is an important part of the immune system in preventing initial infection. It plays a critical role in the detection and elimination of invading pathogens as well instigating secondary and adaptive immune responses. The detection of pathogens occurs via a range of receptors on eukaryotic cells, an important set of which are the Toll like receptors (TLRs), which can recognise conserved pathogen-associated molecular patterns (PAMPs) shared by different classes of microbes. Activation of the Toll pathway results in the activation of transcription factors such as NF- $\kappa$ B and AP-1 that initiate expression of various host immune responses including the production of cytokines and AMPs (Akira *et al*, 2006).

AMPs are small, cationic, amphipathic, molecules that function as a form of ‘natural antibiotic’ displaying broad spectrum antimicrobial activity against many microorganisms including both gram-positive and gram-negative bacteria. The beta defensin class is particularly prevalent within human epithelia (Pazgier *et al*, 2006). Their cationic charge and hydrophobic domains gives selectivity for the anionic bacterial surface and allows integration within membranes the membrane bilayer causing disruption and rapid killing (Mor, 2000). This bactericidal action makes them potentially more potent than many therapeutic antibiotics which are bacteriostatic. Furthermore, as well as their direct killing role, AMPs are fully integrated into the immune system with key roles in mediating inflammation and tissue repair (Takeda and Akira, 2005). For this reason, AMPs made a logical target for this investigation in relation to the condition of rUTI.

## 7.1. Key Findings from *In vitro* Model

The first stage of this study was an *in vitro* experimentation approach using cell culture models that helped guide the analyses performed on the clinical samples. This section will discuss the key findings from those experiments.

### 7.1.1. Type of AMP

For urothelial cells, the *in vitro* cell culture studies demonstrated that of the four commonly detected AMPs within urine: (beta defensin 1 [BD1], beta defensin 2 [BD2], human alpha defensin 5 [HD5] and cathelicidin [LL37]), only beta defensins 1 and 2 appear to play a key role in **urothelial** defence (as opposed to renal defence). The former being constitutively expressed without challenge (with some further induction

on challenge) and the latter induced only on challenge. A similar pattern was seen for vaginal cells, with beta defensin 1 constitutively expressed, beta defensin 2 induced following challenge, no detectable expression of LL37 and only low levels of HD5 expression.

These findings are broadly supported by work from other authors. Human BD1 is recognised as a constitutive AMP in a number of epithelia including the urinary tract, genital tract, skin, and respiratory tract (Zhao *et al*, 1996; Pazgier *et al*, 2006), and this is consistent with findings from Tom Ganz's group in their original description of BD1 in human urinary tissues (Valore *et al*, 1998). Interestingly, however, Ganz's group focussed their effort on localising BD1 only in the kidney and female reproductive tissues. So while they also found BD1 expression in the vagina, no details were published in relation to urothelium. The porcine orthologue of human BD1 was reported as expressed in bladder urothelium by Zhang *et al* in 1998, but the first formal report of BD1 in human urothelium was not until 2001, when a Korean group carried out end-point RT-PCR for BD1 on a variety of male urogenital tissues including the bladder in which they demonstrated expression (Chang *et al*, 2001).

LL37 in the urinary tract was first described by Chromek *et al* in 2006. The authors reported that it was a key peptide in protecting the urinary tract. Their research described in detail the localisation of LL37 in human kidney from 12 individuals; however in relation to urothelium, they focussed on two immortalised cell lines and performed bacterial challenge experiments in mouse bladders. No data for LL37 expression in human samples was shown. Indeed, a later paper published by the same group (Hertting *et al*, 2010) showed very low expression of LL37 in the bladders of the control group, which would support the findings of this study. To date, while LL37 has



been found to be expressed under certain conditions within the cervical squamous epithelium (Nilsson *et al*, 1999) it has not been reported in vaginal epithelium. This may be because the pro-peptide precursor of LL37, hCAP-18 is found in semen and processed into a cathelicidin similar to LL37 by the prostate-derived protease gastricsin (also found in semen) when incubated at a pH corresponding to vaginal pH (Sørensen *et al*, 2003). At higher concentrations, LL37 has also been demonstrated to be cytotoxic against several types of eukaryotic cell (Johansson *et al*, 1998), which may indicate why its basal expression and induction is limited only to epithelia where it can be cleared rapidly.

There have been a limited number of studies investigating HD5 within the urinary tract with only two papers investigating its expression in urothelium. The first of these was from our own group (Townes *et al*, 2011), which showed very low levels of expression in normal ureter and a second study by Spencer *et al* in 2012, which found that while there was low levels of expression in the kidney and lower urinary tract, higher levels of expression were seen in the kidney during pyelonephritis. In the vagina, constitutive expression of HD5 was first reported by Quayle *et al*. in 1998, using end-point RT-PCR (prior to the availability of RT-qPCR); moreover the cDNA band densities relating to vaginal tissue HD5 expression were comparable to that seen in this study suggesting a low level of basal expression. Although, there are studies demonstrating induction of HD5 in response to bacterial vaginosis (Fan *et al*, 2008; Zhang *et al*, 2009), no published literature was found examining the expression of HD5 in response to challenge by *E. coli* or components of *E. coli*.

Expression of BD2 has not been previously described in the human bladder or urothelium. The Korean group who first described BD1 in the human bladder in 2001,

also carried out end-point BD2 RT-PCR on a variety of male urogenital tissues but only examined the bladder in a non-infected state and therefore found no expression (Chang *et al*, 2001). This was despite the fact that the authors themselves recognised that BD2 was an inducible peptide and demonstrated its expression in renal samples during episodes of pyelonephritis. A more detailed localisation of BD2 in the urinary tract by Lehmann *et al*, 2002 focussed on the distal tubules, loops of Henle and collecting ducts of the kidney with no investigation of the urothelium. Nitschke *et al*. also investigated BD2 in renal tubular cells in the same year and demonstrated induction with *E. coli* challenge but also did not look at urothelium. Consequently, BD2 expression is a novel finding from this study in relation to urothelium.

In contrast, BD2 expression and secretion in primary vaginal tissue has been described in 2010 by Han *et al*. who, in a contemporary study (carried out at the same time as this work), carried out challenge experiments using *E. coli* lipopolysaccharide (LPS). Results in VK2 cells have also been described recently by Christiano Rumio's group in Italy who induced expression following stimulation using low molecular weight hyaluronic acid rather than bacterial component challenge (Dusio *et al*, 2011).

### **7.1.2. Response to Flagellin**

BD2 expression and secretion was demonstrated in both RT4 urothelial and VK2 vaginal cells in response to challenge by flagellin and flagellated bacteria. The results for RT4 urothelial cells were also validated in finite cultures of primary cells. The BD2 response induced in both cell types by this specific bacterial component is a key finding of the *in vitro* studies and to my knowledge, the first time it has been described in the urinary or genital tracts. Furthermore, through inhibition experiments (done in conjunction with colleagues) it has been possible to delineate the main steps in this

process, namely detection via TLR5 and induction of the NF $\kappa$ B signal transduction pathway.

While not previously reported in the urogenital system, comparable responses have been reported in other epithelial systems. Flagellin was first reported as a potent activator of defensin expression in *Drosophila* (Samakovlis *et al*, 1992), and its role as a potent inducer of the inflammatory response seen in Gram negative sepsis is also well recognised (Eaves-Pyles *et al*, 2001). In 2001, the first report of a specific link with BD2 was described by Ogushi *et al* in the Caco-2 colorectal cell line, which when challenged with *Salmonella enteritidis* flagellin showed induction of BD2 via the NF $\kappa$ B pathway. In the same year the cell surface receptor for flagellin, TLR5 was reported (Hayashi *et al*, 2001). Induction of BD2 by *E. coli* was also first described in Caco-2 cells in response to the probiotic *E. coli* strain, Nissle 1917 (Schlee *et al*, 2007).

It is perhaps not surprising that the BD2 response to flagellin has not been described in the urothelium as previous studies into BD2 have primarily looked at the kidney. However, BD2 expression has been previously described and investigated in vaginal cells but *in vitro* studies have focussed only on responses to LPS (Han *et al*, 2010) or non-bacterial induction agents (Dusio *et al*, 2011). Herbst-Kralovetz *et al* reported their findings on the stimulation of a variety of TLRs in vaginal epithelial cells in 2008, (including TLR5), but they only assayed for the cytokine response, not the defensin response. Some clinical studies have described induction of BD2 in response to non-*Escherichia coli* related genitourinary infections (Wiechula *et al*, 2010; Jian *et al*, 2012), but these did not focus specifically on flagellin as the PAMP of interest.

The relative importance of flagellin in evoking an immune response in the urogenital epithelia is an area where understanding is still evolving. Much of the work focussing

on the urothelial innate immune response has been performed using immortalised urothelial cell-lines and mouse models (particularly by Scott Hultgen's group in Missouri), where experimental evidence has suggested a vital role for TLR4, the LPS receptor (Schilling *et al*, 2001; Schilling *et al*, 2003). It was also suggested by Soman Abraham's group in North Carolina that TLR4 has a role in bacterial expulsion (Bishop *et al*, 2007). In addition, TLR11 has been shown to have a specific role in detecting uropathogenic *E. coli* in mice (Zhang *et al*, 2004), but not in humans in whom the TLR11 gene contains a stop codon and therefore does not code for a full-length protein. However, in recent years, there has been increasing evidence supporting the importance of TLR5 in urothelial defence.

The importance of TLR5 was first noted by Thomas Hawn and the Seattle group in TLR5 knockout mice, which were more susceptible to urinary tract infection (Andersen-Nissen *et al*, 2007) and then subsequently in a population study which demonstrated that the TLR5<sup>392Stop</sup> polymorphism was associated with increased risk of rUTI (Hawn *et al*, 2009). More recently, Walter Hopkin's group from Wisconsin demonstrated in experiments employing offspring of genetic crosses of C3H/HeJ mice with infection resistant and susceptible inbred strains, that mice with a normal TLR4 on different genetic backgrounds were unable to clear *E. coli* bladder infections, while animals with a defective TLR4 could successfully resolve infections. This suggested the presence of a gene other than in TLR4 as an important genetic determinant of infection resistance/susceptibility (Suhs *et al*, 2011). Gene trees of TLR phylogeny have also suggested the possibility of gene duplication in a mouse or human progenitor such that human TLR5 may perform a similar function to mouse TLR11 (Lauw *et al*, 2005). This theory coupled with afore mentioned observations continue to add to the weight of

evidence regarding the potential role of TLR5 and its ligand, flagellin, in UTI while simultaneously questioning the traditional understanding of the prime importance of TLR4.

Jenny Southgate's group from York, UK (with whom I collaborated in this study) demonstrated that while immortalised T24 urothelial cells secreted interleukin 6 (IL6) and interleukin 8 (IL8) following challenge with LPS and flagellin, finite cultured normal human urothelium (NHU) was only responsive to flagellin (Smith *et al*, 2011). They also noted that only proliferative cells showed an IL6 and IL8 cytokine response while those that were induced to differentiate did not. This was despite the fact that they were able to demonstrate the presence a 97 kDa protein species on western blot with a TLR5 antibody in both proliferative and differentiated cell types. The 97 kDa moiety is the expected band for TLR 5 but they hypothesised that another 50 kDa moiety which was also detected in western blot by their antibody may be responsible for the interleukin response seen only in the proliferative cells. Although interestingly in experiments with THP monocytic cell lines, which they used as a positive control, the THP cell line demonstrated a dose dependent interleukin response to flagellin in both the differentiated and proliferative state despite not having the 50 kDa moiety.

In the work presented in this thesis, differentiated NHU cultured by Prof Southgate's group and challenged with *S. typhimurium* and *E. coli* flagellin demonstrated BD2 expression and secretion. One explanation for this may be that BD2 expression, unlike cytokine expression, is not dependent on the presence of the 50 kDa moiety. Indeed, the THP cells described in their study were able to secrete cytokines even in the absence of this moiety. An alternative explanation is that there were differences between the tissue used for primary culture in the BD2 experiments compared to

those used in their original reported experiments. It is well recognised primary culture that each sample having been taken from a separate subject can show different phenotypic attributes and indeed, in the group's paper, it is noted that there were differences between individual cultures, with some samples showing significant differences while other did not. Overall, however, it is my feeling that the NHU data in this thesis is in line with expectations from the immortalised cell data particularly since the RT4 cells used as a model in this study are regarded as demonstrating a phenotype more in keeping with differentiated cells due to their expression of human uroplakins, UPIa, UPIb, UPII, and UPIII – a finding itself reported by the Prof Southgate's group (Lobban *et al* 1998). Nonetheless, this is an area that would be worthy of further investigation and is undoubtedly at least partly compromised by the relative small number of samples on which the data is based.

### **7.1.3. Response to Estrogen**

BD2 expression and secretion in VK2 vaginal cells but not RT4 urothelial cells was influenced by estrogen concentration. A role for estrogen in the expression of BD2 was suggested by the presence of estrogen receptor binding sites in the promoter region of the BD2 (DEF4B) gene. Charles Wira's group in New Hampshire first demonstrated that estrogen could have an influence on BD2 expression in uterine epithelial cells in 2006 (Pioli *et al*, 2006; Fahey *et al*, 2008). Han *et al* then showed induction of BD2 expression and secretion in primary cultured vaginal cells in 2010, contemporary to the experiments carried out for the present study. There is therefore a good body of evidence to support the effects of estrogen on BD2 in the vaginal cells.

Although the RT4 urothelial cell-line does express both types of estrogen receptor (ER), this is likely to be due to the tumorous origin of the cell-line as ER expression

(particularly beta ER) is associated with oncological transformation (Shen *et al*, 2006). There is considerable uncertainty regarding the nature of ER expression in the normal bladder with the trigone and bladder neck being the only areas where the ER is commonly found (Wolf *et al*, 1991; Croft *et al*, 2005). Given this background, it is likely that much of the bladder epithelium will not be responsive to estrogen *in vivo* and the specific site of origin of the cell line may determine any effects. In the case of cell-lines originating from tumorous tissues, if expression of the ER was part of the transformation process, rather than endogenously expressed, then it may not function in the expected manner. It is difficult to draw any further conclusion in relation to this without further investigation and the use of primary cultures from different regions of the human bladder.

#### **7.1.4. Antimicrobial Activity**

It was demonstrated using antimicrobial assays that the medium from RT4 cells collected after challenge with flagellin reduced NU14 UPEC bacterial survival to only 38% (i.e. 62% killed), suggesting that flagellin evokes a strong soluble antimicrobial response. Synthetic BD2 at a similar concentration to endogenous BD2 found in the media reduced UPEC bacterial survival to 52% in 1-hour (i.e. 48% killed) suggesting that a large portion of the killing seen in response to flagellin is achieved by BD2 although undoubtedly other effector antimicrobials are also likely to be involved. This is consistent with evidence from other studies, which have shown potent action of BD2 against *E. coli* (Nitschke *et al*, 2002). Furthermore, BD2 action may be enhanced further in urine where the presence of lysozyme and lactoferrin has a synergistic effect (Bals *et al*, 1998). While minimum inhibitory concentrations (MIC) for BD2 against *E. coli* are reported (Bals *et al*, 1998), this is probably not the most appropriate measure

for an AMP. The reason for this is that AMPs are secreted continuously by epithelial cells replacing AMPs used up as microbes are killed. This is in contrast to the action of an antibiotic, which is given as a single burst at a fixed concentration. Consequently, the measure used in this study and others involving AMP (Nitschke *et al*, 2002, Townes *et al*, 2007), is based on time kill assays which analyse the amount of killing at a specific concentration over a fixed time period.

## **7.2. Key Findings from Clinical Study**

The second stage of this study utilised the information gathered from the *in vitro* models to analyse the clinical samples. This section will discuss the key findings.

### **7.2.1. Symptoms and Health State**

The difference between symptoms of rUTI patients and controls was as expected with significant differences in occurrence of most symptoms commonly associated with UTI. In pre-menopausal recruits, the only symptom not significantly different between rUTI patients and controls was the presence of back pain – this may possibly be because these symptoms are also commonly experienced in premenstrual syndrome (Dennerstein *et al*, 2011) therefore making it a less discriminating symptom in pre-menopausal women.

In post-menopausal recruits, the only symptom not significantly different between rUTI patients and controls was poor bladder emptying. This was probably because of the high prevalence of pelvic organ prolapse in post-menopausal women, which is commonly associated with feelings of incomplete emptying (Dain *et al*, 2010). Consequently, in post-menopausal women, this is like to be a less discriminating symptom.



In terms of health state, no significant difference was found between rUTI sufferers and controls. This was somewhat surprising as previous studies have demonstrated significantly lower health state scores in UTI sufferers (Ellis and Verma, 2000; Abrahamien *et al*, 2011). While both of these studies used the SF36 questionnaire, the scores can be converted to EQ-5D health state values as outlined in a 2012 systematic review by Bermingham and Ashe. It was feasible that the lack of a significant difference was due to some skewing of the data. This could be explained by either poorer than expected health state within the control group (as many were attending hospital urological investigation albeit not for UTI) or better than expected health state in the rUTI group; the latter possibly due to the fact that the questionnaire was taken at a time when they didn't have an infection or due to optimism regarding their attendance and ultimate treatment at the UTI clinic.

### 7.2.2. Clinical Samples

The TLR5<sup>392Stop</sup> single nucleotide polymorphism (SNP) analysis of the collected blood samples showed that out of the total population of 98 subjects recruited 9.2% were found to have the SNP, which was within expectations as previously described frequencies have ranged from 5 to 10% (Wlasiuk *et al*, 2009). However, all subjects carrying the SNP were from the recurrent UTI group giving a prevalence within that group of 15%, which was higher than the 12.2% quoted by Hawn *et al* in 2003. Conversely, the prevalence of 0% in the control group was considerably lower than the 6.8% found by Hawn *et al*. One explanation for this difference is that the exclusion criterion for control group (such as no history of UTI in the past two years and no abnormal urological investigations) is essentially selecting a cohort of 'super-controls' i.e. a group which is less likely to suffer from rUTI than the general female population.

For the clinical tissue samples, the RT-qPCR and ELISA analyses indicate that subjects with TLR5<sup>392Stop</sup> have lower bladder and vaginal BD2 expression, and secretion, than other subjects with rUTI during an infection. Furthermore, in the absence of infection vaginal BD2 expression and secretion is also lower than both control and other rUTI sufferers that do not have TLR5<sup>392Stop</sup>. This is potentially a key novel finding as it provides a potential mechanism by which subjects with TLR5<sup>392Stop</sup> may have compromised immunity against invading *E. coli*. While there are no comparable studies in human urothelium, TLR5<sup>392Stop</sup> has also been noted to increase susceptibility to Legionnaires' disease, a pneumonia caused by *Legionella pneumophila*, another flagellated bacterium (Hawn *et al*, 2003). Given that the first description of BD2 was in the lung (Bals *et al*, 1998) and that *L. pneumophila* has been shown to induce BD2 (Scharf *et al*, 2010), one might speculate that reduced BD2 secretion in the lung of subjects with TLR5<sup>392Stop</sup> may also be responsible for susceptibility to this bacterium. Also in the lung, it was noted by Singh *et al*. in 1998, that BD2 was detected in much higher concentrations in bronchoalveolar lavage (BAL) fluid of cystic fibrosis (CF) and inflammatory lung disease patients compared to normal controls. In a large cohort study of adults living with CF, subjects with TLR5<sup>392Stop</sup> had improved nutritional status compared to those without the SNP (Blohmke *et al*, 2010). Again one might speculate that in CF patients, where injury by inflammatory products is a significant part of the lung pathology, reduced BD2 secretion as a result of TLR5<sup>392Stop</sup> may be advantageous as local tissue damage may be mitigated.

As well as the very specific findings in relation to the TLR5<sup>392Stop</sup> SNP, analysis of the clinical samples also revealed BD2 expression and secretion was significantly lower in the vaginal epithelium of rUTI subjects even in the absence of infection. This is an

important finding as according to the ascending theory of pathogenesis of rUTI, the vagina is a key initial site of colonisation by *E. coli* (Pfau and Sacks, 1977; Fowler and Stamey 1977; Larsen and Galask, 1980). Unlike the bladder, the vagina is always colonised with commensal bacteria species and therefore there the epithelium is continuously under challenge by a mixed flora consisting primarily of diphtheroids, streptococci and micrococci while Gram-negative enteric bacteria are relatively rare (Pfau and Sacks, 1977). It is possible therefore that BD2 plays a key role in maintaining commensal flora but preventing gram negative colonisation, similar to its role in the skin (Schröder and Harder, 1999). Consequently, it is possible that in at least some of the rUTI patients, low BD2 is responsible for their susceptibility to recurrent infection. This could be particularly important in post-menopausal women as BD2 levels in this group were observed to be lower in vaginal biopsies and douches regardless of whether they had a history of rUTI or not. This, coupled with the *in vitro* data for estrogen, may explain why using topical estrogen as a treatment can help reduce the frequency of rUTI (Perrotta *et al*, 2008) in addition to the previously described beneficial effects on vaginal thickness, mucus secretion and pH (Suckling *et al*, 2006). The observation in relation to the second douches after estrogen treatment would suggest that estrogen is able to increase BD2 secretion clinically as well as *in vitro*. The direct effects of estrogen on epithelial expression and secretion of BD2 may also help explain why local estrogen can be beneficial when utilised by pre-menopausal women with rUTI (Pinggera *et al*, 2005).

### **7.3. Strengths and Weaknesses**

A key strength of this study was the planned synergy of *in vitro* model data and clinical samples from the relevant patient group and controls. Starting with *in vitro* cell culture

models of the host–pathogen interaction occurring in the bladder and vaginal epithelium, BD1 and BD2 were identified as the key AMPs involved in urogenital defence. The study sought to use a hypothesis driven methodology to investigate the roles of AMPs in the urothelium, and having established the importance of BD1 and BD2, patient samples were collected to investigate the clinical relevance of these AMPs with an appropriate power calculation that guided sampling numbers.

This study also demonstrated one of the first examples of a post-genomics approach to investigation of this condition. By taking note of the population-based studies that highlighted the TLR5<sup>392stop</sup> SNP as a risk factor for rUTI, it was possible to sequence patient DNA and identify this particular SNP, and the *in vitro* studies were focussed specifically on flagellin, the ligand for TLR5. The post-genomic translational approach that was adopted yielded dividends by producing novel findings from the *in vitro* models and the clinical samples, plus it also provided early indicators as to how the findings might be exploited therapeutically.

However, despite these strengths, there are also a number of weaknesses in the study. Perhaps the most significant drawback was in the recruitment of subjects when they did not have firm evidence of active infection. There was, however, a clear justification for this in that at the start of clinical recruitment, which occurred in parallel to the *in vitro* challenge experiments, it was not known which AMPs would be investigated and that BD2 would need to be examined during infection. Furthermore, it would have been clinically and ethically more fraught to carry out biopsies at the time of infection due to increased risk of patient harm (the few that were obtained during this study were done unintentionally). However, this study demonstrated that urine samples and vaginal douches could be taken reliably for peptide analysis and gave results in line

with the mRNA expression seen in the biopsies. Consequently, a future investigation could rely solely on peptide analysis due to the validation provided in this study.

The next significant weakness, relating to the issue outlined above is that the study was never powered for a genetic association study. The TLR5<sup>392Stop</sup> SNP is potentially a functionally relevant polymorphism and the data acquired in relation to it looks promising, but it needs to be recognised that with a population allele frequency of 5-10% with heterozygous dominant effect, a population of between 300 and 400 would need to be examined to find a genotype relative risk of 2 at 5% significance with power of 90% (Hall and Blakey 2005). The original population study described by Hawn *et al* in 2009 achieves such a figure by looking at 431 rUTI cases and 430 controls. This study does not have such numbers and should therefore be considered essentially as pilot data. The inferences and conclusions drawn from the sub-analysis of this small group should therefore be seen in that context and the evidence needs further confirmation in larger studies, similar in size to that described by Hawn *et al*. and ideally incorporating additional sites.

A third weakness in the study was the lack of any analysis relating to peptide function of the clinical samples. This study focussed on quantitative analysis but there was, effectively, an assumption that the peptides detected were functional, which may not have been the case. Defensin function, particularly BD2 is known to be salt sensitive (Bals *et al*, 1998), plus post-translation modifications resulting in alternate isoforms of BD1 and BD2 have been described in the bowel (O'Neil *et al*, 1999) and urinary tract (Valore *et al*, 1998). The primary reason for the lack of functional data was the lack of a reliable technique for separation of the peptides. Attempts were made by Dr Achim Treumann at the North East Proteome Analysis Facility (NEPAF) to develop such a

technique on our behalf but technical difficulties in peptide isolation could not be overcome in the time available for the study. Nonetheless, high performance liquid chromatography (HPLC) based separation techniques have been described (Selsted, 1997), and could potentially be used in functional studies.

Another element that could potentially be improved is the inclusion criterion for the control group. Rather than have recruited subjects that have not had any UTI in the past 2-years, a more appropriate control group may have been subjects that have had one UTI in the past year but no recurrence. Such an approach may have obviated the possibility of recruiting 'super controls,' but practically it may have been much more difficult to find and recruit such individuals.

Finally, a more longitudinal approach to sampling with repeat urine samples and douches taken over a two-year period would have given more information in relation to whether the findings in relation to BD2 were consistent over time or not. The comparison with the second douche went some way to achieving this but more sequential samples would have given a better picture although practically this is unlikely to have been possible.

## 7.4. Conclusions and Implications

The results of the study support the original hypothesis that:

**“Women suffering from recurrent urinary tract infection (rUTI) have altered tolerance to infecting bacteria related to differences in expression of endogenous antimicrobial peptides (AMPs) and that identification of this deficiency represents a potentially useful opportunity for design of preventive or adjunctive treatment.”**

Furthermore, from the *in vitro* studies, it can be concluded that:

- BD1 was constitutively expressed and secreted in urothelial and vaginal cells.
- *E. coli* flagellin induced BD2 expression and secretion via TLR5 activation and the NFκB transduction pathway.
- BD2 is a potent antimicrobial with significant activity against uropathogenic *E. coli*.
- BD2 expression and secretion in vaginal cells is positively influenced by estrogen.

From the clinical study, it can be concluded that

- Women with rUTI have a significantly greater burden of lower urinary tract symptoms than control subjects who don't have rUTI.
- Women with rUTI had significantly lower basal levels of vaginal BD2 expression and secretion than controls.
- During a UTI, women with the TLR5<sup>392Stop</sup> SNP showed significantly less BD2 expression and secretion in the bladder and vagina than other women with rUTI but no SNP.

- No significant difference was seen in BD1 expression between women with rUTI and controls.

The main implication of this work is the novel identification of the importance of flagellin induced BD2 in the urogenital innate immune response, which is reduced in a significant proportion of women with rUTI particularly the sub-cohort with the TLR5<sup>392Stop</sup> SNP. The identification of the critical role of this specific peptide potentially provides a rational explanation to bring together a number of separate observations from both mouse and population studies (Andersen-Nissen *et al*, 2007; Hawn *et al*, 2009). Although the data must be interpreted with caution as the study was not powered to specifically look at genetic associations.

It also provides a realistic new target for induction or supplementation as the estrogen experiments and observation in pre- and post-menopausal women have already demonstrated that BD2 expression can be modulated by endogenous factors. Furthermore work by others has demonstrated that exogenous factors such as hyaluronic acid can have a similar effect (Dusio *et al*, 2011).

## **7.5. Further work**

This study has raised a number of possibilities for future work. The most pressing need is to further validate the finding in relation to BD2 in a cohort with samples taken during a UTI. Due to the inducible nature of BD2, sampling at the time of infection is a logical step plus by focussing on peptide samples (urines and douches) rather than biopsies, there is the potential to take a less invasive approach, which would be more appealing to potential recruits and allow for a larger study.



The genetic associations between TLR5<sup>392Stop</sup> SNP and reduced BD2 secretion will need further validation in a larger cohort of patients suitably powered as a genetic association study to look for differences and ideally involving patients at a separate location. The data presented in this thesis could provide promising pilot data for such a study particularly as the SNP potentially has a functional effect.

It would be useful to corroborate the findings in relation to defensins with other innate immune effectors such as the cytokines, interleukin 6 and 8. Colleagues in the research group have already begun to do this. There is also a need to further validate the findings relating to estrogen and better delineate the mechanism by which estrogen can induce BD2 so that potentially other agents can be developed to achieve the same effect. Data from this study has already provided pilot data for such a project funded by Wellbeing of Women (BH102118).

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# Appendix A

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This appendix section contains examples of the printed material used in the clinical part of the study:

- Patient information sheet
- Subject data sheet
- UTI Symptoms Assessment (UTISA) symptom questionnaire
- EuroQol 5 Domain (EQ-5D) Health state questionnaire

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Freeman Hospital  
0191 213 7139

Department of Gynaecology  
Royal Victoria Infirmary  
0191 2825640

The Freeman Hospital  
High Heaton  
Newcastle upon Tyne  
NE7 7DN

Tel: 0191 233 6161  
Fax: 0191 213 1968

*Patient Information Sheet*  
*Version 4.0 (03.05.2010)*

We would like to invite you to take part in a research study called:

### **Innate Defence Mechanisms in Female Cystitis**

Before you decide, you need to understand why the research is being done and what it would involve for you. So please take time to read all the following information carefully. Talk to others about this if you wish.

The first part tells you the purpose of this study and what will happen if you take part. The second part gives you more detailed information.

Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

#### **Part 1**

##### **What are you studying and why are you asking me?**

We want to find out why some women are more prone to getting cystitis than others. Most women experience cystitis at some point in their life but some get repeated attacks which are uncomfortable and stressful. Although antibiotics may be initially effective, repeated courses are less helpful and can cause the bugs to become resistant. In some patients the symptoms and pain can occur even in the absence of infection. Lack of alternative treatments is distressing for patients and frustrating for doctors.

In this study we want to find out whether differences in the type, amount and activity of special chemicals made by the body that normally neutralise invading bugs explains why some women are troubled with recurrent cystitis. We think that this information will help us develop new ways to improve women's defences against urine infection and reduce the misery of recurrent cystitis.

To do the study we need to ask women with and without recurrent cystitis to take part. We are inviting you because you have been referred to either the urology or gynaecology departments at Newcastle upon Tyne Hospitals for assessment or treatment of urinary problems such as infection, incontinence, bladder pain or blood in the urine (haematuria).

##### **Do I have to participate?**

No. It is entirely up to you to decide whether or not to participate and your decision will not affect the care you will receive.

##### **What will happen to me if I take part?**

If you agree to take part in this study we will ask you for information about any urinary infections you may have had and collect from you samples of blood, urine, vaginal fluid, and bladder and vaginal tissue. These

samples will be stored and then analysed in the laboratory to look for the type and strength of anti-microbial activity. The research laboratory will not know your personal identity.

#### **What will I have to do?**

You will need to give your consent to take part and complete two short questionnaires. We will then like you to answer some specific questions about your experience of urine infections. We will use some of a routine clinic or pre-procedure blood sample for analysis and take tiny samples of the lining of the bladder and vagina during the telescopic bladder examination (cystoscopy) that you will have as part of your routine clinic assessment or surgical procedure. You will then need to collect two urine specimens and two vaginal washes (douches) at home and bring them to the hospital. Depending on the results of the first analysis, we may ask you to give further samples of urine about 12 months after the first set.

#### **What are the possible benefits of taking part?**

The information we will obtain from the laboratory analysis will not necessarily directly benefit you. The measurements we make on the samples will help us decide whether improvement of natural defences in women with recurrent cystitis is a potentially useful way to improve treatment of this troublesome condition which may help women (including yourself) in the future.

#### **If I am interested, what happens next?**

We will go through this information sheet with you when you come for your clinic appointment and we will then ask you to sign a consent form to show that you have agreed to take part. You will be given copies of this information sheet and the consent form to keep. A copy of the information sheet and the signed consent form will be retained in your hospital case notes.

You will be free to withdraw at any time, without giving a reason. This again would not affect the standard of care you receive.

## **Part 2**

If the information in Part 1 has interested you and you are considering participation, please read the additional information in this second part before making any decision.

#### **What the study is about**

This research project aims to measure the levels and activity of natural substances called anti-microbial peptides (AMPs) that are made by the lining cells of the bladder and vagina. We intend to compare the levels found in women who suffer repeated cystitis with those found in women without cystitis to see if there are any deficiencies. If we do find significant differences this will guide future studies to see if we can design new treatments to improve women's natural defense against urine infection. We know from previous studies that these substances are made by the lining cells of the bladder and vagina and that they appear in the urine and vaginal fluid. We also know that the amount of AMPs produced is affected by the level of female hormones.

#### **Who we need to take part**

To successfully carry out the study we need to recruit 40 women who suffer from recurrent infections, 40 women who do not and 20 women with painful bladders. The women who take part should be evenly split between those whose periods have stopped (post-menopausal) and those who are still having periods (pre-menopausal). To do this we will be asking women who are attending for either an assessment or treatment of their bladder because of problems with cystitis, incontinence, painful bladder/ or blood in the urine (haematuria) whether they would like to take part in the study.



### **The information we need**

To make sense of the laboratory experiments we need some basic details of the number, type and severity of infections that each woman who takes part has suffered. We also need some details of other health problems and the use of some medicines, particularly female hormones, that we think may affect the function of the anti-microbial peptides. We will also note down the relevant findings from the routine clinic or pre-operative assessment of your particular urinary problem.

### **The samples that are needed**

To get an overall measurement of the amount and activity of AMPs produced from the urinary tract in different women we need to analyse tissue samples from the lining of the bladder and vagina, urine samples and samples of fluid washed from the vagina (vaginal douche). Tiny tissue samples (5 mm) will be taken from the lining of the bladder and vagina during the telescopic examination of the bladder that women with haematuria, incontinence or recurrent cystitis usually undergo as part of their routine clinical assessment or treatment. We will ask you to collect the samples of urine and vaginal fluid (douches) later yourself in your own home. We will provide you with the necessary containers and instructions and tell you where to bring the samples.

We will also keep some of the blood sample taken routinely before your procedure and store it in the laboratory. Once we have the results from the urine and tissue analysis we will then use the blood samples to look for genetic reasons why some women are prone to recurrent cystitis by analysing their DNA (genetic material). Nobody in the laboratory will know which individual the samples came from.

### **Possible risks of taking part**

We will look after all the information very carefully according to current regulations; the laboratory researchers will not have access to any identity information. The samples will be taken with a small instrument passed down the telescope which removes a tiny piece of the bladder and vaginal lining. If you are having the procedure done under local anaesthetic, this may involve a slight pin-prick type pain in the bladder and is barely felt in the vagina. Many women have a small amount of bleeding in the urine after the telescopic bladder examination and this may be made slightly worse by taking the tissue samples but will settle after passing urine a couple of times. There is a small risk of about 1% (one in a hundred) of more prolonged bleeding but this most often settles by drinking plenty to flush the blood away. If there was a problem we would advise you of the appropriate action and let your GP know about what had happened.

In the unlikely event that you are harmed by taking part in this research project, there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay for it. Regardless of this, if you wish to complain or have any concerns about any aspect of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms should be available to you.

If you wish to learn anything else about this research or are not sure whether to participate, please ask any member of your healthcare team for further details or for advice or contact the responsible researcher:

Robert Pickard  
Professor of Urology / Consultant Urologist  
Department of Urology, Freeman Hospital  
Newcastle upon Tyne  
NE7 7DN  
Tel: 0191 213 7139  
Fax: 0191 213 7127  
E mail: [R.S.Pickard@ncl.ac.uk](mailto:R.S.Pickard@ncl.ac.uk)

Ased Ali  
Wellcome Research Fellow / Specialist Registrar  
Department of Urology, Freeman Hospital  
Newcastle upon Tyne  
NE7 7DN  
Tel: 0191 213 7139  
Fax: 0191 213 7127  
E mail: [ased.ali@ncl.ac.uk](mailto:ased.ali@ncl.ac.uk)

**Will my taking part in the study be kept confidential?**

Yes. We will follow ethical and legal best practice and all information about you will be handled in confidence. Also, your personal identity will not be passed on to the laboratory researchers.

**What will happen to the samples and results from the research?**

The samples that you provide and the results from studies on them will be stored safely and securely in the laboratory. Once research samples are no longer needed they will be destroyed and the identity of individuals contributing to the study will never be known by the researchers. The only people who will have access to your tissue samples will be the scientists and technicians working on this project. The samples will be processed entirely within Newcastle University and any tissue surplus to the requirements for this study will be destroyed.

We will write to each participant at the end of the research to tell them of the study results. It is possible that the main results will be mentioned in the media and the results will be published in scientific journals, without any names of patients who donated samples ever being revealed.

**Who is organising and funding the research?**

This research has been assessed, approved and funded by the Wellcome Trust, a large charity that funds medical research. The work is also partly funded by the Cystitis and Overactive Bladder (COB) Foundation who are also contributing funding.

The laboratory work will be carried out at Newcastle University and the study will form part of a PhD thesis on female urinary tract infection written by Mr Ased Ali who is a Wellcome funded Research Fellow and Specialist Registrar in Urological Surgery.

**Payment of expenses**

We hope to arrange the urine collections, questionnaire completion and biopsy procedures at the time of your routine appointments at the hospital. If you have to come to the hospital at other times because of the research project such as to deliver the specimens we are happy to refund any travelling expenses. To do this you will need to contact the research nurse – Either Wendy Robson (Urology Department) on 0191 244 8311 or Tracy Ord on 0191 2821739 (Gynaecology Department) and say you are on the 'cystitis study'.

**Who has reviewed the study?**

The project has been discussed and reviewed by scientists and lay members of the Wellcome Trust. This study, like all research in the NHS, has been looked at by an independent group of people, called a Research Ethics Committee. Their aim is to protect your safety, rights, wellbeing and dignity.

Independent advice and general information about involvement of the public in health-related research is available from:

- Patient Advice & Liaison Service for the Freeman hospital which is the: North of Tyne PALS, First Floor, Old Stables Greys Yard, Morpeth, Northumberland, NE61 1QD. Telephone: 08000320202. Email: [northoftynepals@nhct.nhs.uk](mailto:northoftynepals@nhct.nhs.uk)
- The Wellcome Trust – Public Involvement in Medical Research. <http://www.wellcome.ac.uk>
- Register showing the range of research projects undertaken within the NHS registered with the National Institute for Health Research Clinical Research Network (NIHR CRN) : [http://www.ukcmr.org.uk/index/clinical/portfolio\\_new/P\\_search.html](http://www.ukcmr.org.uk/index/clinical/portfolio_new/P_search.html)

If you decide to take part, please ensure you keep a copy of this information sheet.

Thank you very much for reading this.

Please discuss this information with your family, friends, or GP if you wish.



## Female Cystitis – Subject data sheet

Hospital/NHS Number:  
Date of Birth:  
Age:

Study ID number:

Subject Log - Date of entry:

### Subject Category

Recurrent UTI ( $\geq 3$  UTIs/yr in the last year or  $\geq 2$  UTIs/yr for 2 years)

No UTI (No symptomatic or microbiological UTI for 2 years)

### HISTORY

#### Presenting Complaint & Urinary Symptoms

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#### Menopausal Status

Is the patient,

Pre – menopausal (regular periods in last 6 months)?

Date of last period: \_\_\_\_\_

Post – menopausal (No period in last 6 months)?

Currently going through menopause?

Has the patient had a hysterectomy? Yes

No

**IF PATIENT'S PRESENTING COMPLAINT DOES NOT RELATE TO RECUURENT UTI, PLEASE ADVANCE TO PAGE 2 (ALL PATIENTS)**

### RECURRENT UTI CASES

For how long have you suffered recurrent UTIs?

\_\_\_\_\_ years          \_\_\_\_\_ months

How many symptomatic urine infections have you had during last 2-years? \_\_\_\_\_

What was the commonest predominant type of urine infection?

No symptoms

Mild - smelly/cloudy urine, frequency and pain

Moderate – some systemic flu-like symptoms

Severe - Fever/rigors and loin pain)

How many courses of antibiotics have you taken for UTI in the last 2 years? \_\_\_\_\_

Do the infections often occur after sexual intercourse?

Yes

No

**ALL PATIENTS**

**UTISA Questionnaire score (given to patient):** Symptoms: \_\_\_\_\_ Bother: \_\_\_\_\_

**Do you have any vaginal symptoms?**

Yes  - Details:  
No

**Have you used vaginal oestrogen treatment in the last 2 years?**

Yes  - Details:  
No

**Do you take any vitamin tablets?**

Yes  - Details:  
No

**Do you drink cranberry juice regularly?**

Yes  - Details:  
No

**Have you ever used an oral contraceptive ('the pill')?**

Currently using  - Details:  
Previously used  - Details:  
No

**Have you ever used hormonal replacement therapy (HRT)?**

Currently using  - Details:  
Previously used  - Details:  
No

**Have you ever had a sexually transmitted infection?**

Yes  - Details:  
No

**Have you had any gynaecological or urological illness or surgery?**

Yes  - Details:  
No

**Have you ever been pregnant?**

No  
Yes **No. of times?** \_\_\_\_\_ **Any cystitis during pregnancy?** Yes  No

**Please list any other medical conditions you have:**

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---

**What is/was your line of work?**

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**Have you ever smoked?**

Yes  - Details:  
No

**Any exposure to industrial chemicals / dyes?**

Yes  - Details:  
No

**Please list any medication you are on:**

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**Any allergies?**

---

**Info from clinic record**

**EXAMINATION**

**Abdomen**

Negative

Positive findings:

**Vaginal**

Negative

Atrophic vaginitis

Prolapse

Other

**TESTS**

**Urine**

pH

Dipstick:

\*Creatinine/albumin ratio

Microscopy / Culture

**Blood**

Creatinine

eGFR

Vitamin D

Other

**IMAGING**

Investigation:            USS  

IVU  

CT  

USS findings:

Other imaging:

**CYSTOSCOPY**

Surgeon: \_\_\_\_\_

LA  

GA  

Positive findings:

Additional procedure:

**ADDITIONAL COMMENTS**

## Cystitis and its Impact on Your Life

Please answer the following questions about any urine infections you may have had in the past year (12 months)

Hospital/NHS Number:.....

Study ID:.....

Date: .....

We next want you to think about what symptoms you get with the urine infections and how much these symptoms trouble you. For each symptom we would like you to tell us how bad the symptom is in the left hand column and then how much trouble it causes you in the right hand column.

Please indicate whether you have had the following symptoms during a urine infection by circling one number for each symptom.		SYMPTOMS				If you experienced these symptoms during a urine infection please indicate how much they troubled you by circling one number for each symptom.			
Did not have	Mild	Moderate	Severe		Not at all	A little	Moderately	A lot	
0	1	2	3	3	0	1	2	3	3
0	1	2	3	3	0	1	2	3	3
0	1	2	3	3	0	1	2	3	3
0	1	2	3	3	0	1	2	3	3
0	1	2	3	3	0	1	2	3	3
0	1	2	3	3	0	1	2	3	3
0	1	2	3	3	0	1	2	3	3
0	1	2	3	3	0	1	2	3	3
0	1	2	3	3	0	1	2	3	3
0	1	2	3	3	0	1	2	3	3



Hospital/ NHS Number: .....

Study ID: .....

Date: .....

By placing a tick in one box in each group below, please indicate which statements best describe your own health state today.

**Mobility**

- I have no problems in walking about
- I have some problems in walking about
- I am confined to bed

**Self-Care**

- I have no problems with self-care
- I have some problems washing or dressing myself
- I am unable to wash or dress myself

**Usual Activities** (*e.g. work, study, housework, family or leisure activities*)

- I have no problems with performing my usual activities
- I have some problems with performing my usual activities
- I am unable to perform my usual activities

**Pain/Discomfort**

- I have no pain or discomfort
- I have moderate pain or discomfort
- I have extreme pain or discomfort

**Anxiety/Depression**

- I am not anxious or depressed
- I am moderately anxious or depressed
- I am extremely anxious or depressed

To help people say how good or bad a health state is, we have drawn a scale (rather like a thermometer) on which the best state you can imagine is marked 100 and the worst state you can imagine is marked 0.

We would like you to indicate on this scale how good or bad your own health is today, in your opinion. Please do this by drawing a line from the box below to whichever point on the scale indicates how good or bad your health state is today.

**Your own  
health state  
today**

Best  
imaginable  
health state

100

90

80

70

60

50

40

30

20

10

0

Worst  
imaginable  
health state

# Appendix B

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This appendix section contains the various letters confirming funding and regulatory approval:

- Letter confirming funding by the Wellcome Trust
- Letter confirming Ethical Approval
- Letter confirming R&D Approval
- Email confirming Caldicott and data protection approval
- Letter confirming inclusion of the study in the National Institute for Health Research (NIHR) Clinical Research Portfolio

Private and Confidential

Mr Ased Ali  
Surgical and Reproductive Sciences  
The Medical School  
University of Newcastle  
Framlington Place  
Newcastle NE2 4HH

Tel: +44 (0)20 7611 8888 Direct: 8358

Fax: +44 (0)20 7611 8545 Direct: 8352

E-mail: a.ch'ng@wellcome.ac.uk

Our Ref: 085040

15 July 2008

Dear Mr Ali

**"Antimicrobial peptides and female recurrent urinary tract infection."**

Thank you for coming to the Wellcome Trust on 3 July 2008 for an interview for a Research Training Fellowship. I am pleased to tell you that the application was successful. An award letter will follow shortly.

Please find attached some comments from referees who reviewed your application. I hope that you will find the comments useful, I am sure you will understand that I am unable to discuss their content further with you.

I look forward to hearing about the progress of your research in the future.

With best wishes,

Yours sincerely



Amelia Ch'ng  
Grants Adviser  
Pathogens, Immunology and Population Health

Cc: Professor Jeff Errington, University of Newcastle  
Mr Robert Pickard, University of Newcastle  
Dr Judith Hall, University of Newcastle

Enc: Referees' comments



**County Durham & Tees Valley 1 Research Ethics Committee**

The Tatchell Centre  
The University Hospital of North Tees  
Piperknowle Road  
Stockton on Tees  
Cleveland  
TS19 8PE

Telephone: 01642 824279  
Facsimile: 01642 824164

10 March 2009

Mr Ased Ali  
47, Hollywood Avenue  
Gosforth  
Newcastle upon Tyne  
NE3 5BR

Dear Mr Ali

**Full title of study:** The role of altered innate antimicrobial activity in recurrent urinary tract infection  
**REC reference number:** 09/H0905/15

The Research Ethics Committee reviewed the above application at the meeting held on 26 February 2009. Thank you for attending to discuss the study.

After the committee's initial deliberations you were invited to join the meeting to clarify some issues. Thank you for agreeing to attend.

The Committee noticed that the study title keeps fluctuating from each document. The Committee requested that you ensure that the same title is recorded on all documents.

1. Dr Ali informed the Committee that he will stick to the most simplistic title.

The Committee noticed incorrect details relating to PALs within the participant information sheet.

2. Dr Ali informed the Committee that he will amend this information with the correct details.

The Committee have requested further information be included in the participant information sheet with regards to Dr Ali, in particular reference to the study being in part fulfillment of a PhD.

3. Dr Ali informed agreed to amend the participant information highlighting the above.

The Committee requested copies of CV's relating to Mr Pickard and Dr Hall.

4. A copy of Dr Hall's CV was presented at the meeting.

**Ethical opinion**

The members of the Committee present gave a favourable ethical opinion of the above research on the basis described in the application form, protocol and supporting documentation, subject to the conditions specified below.

**Ethical review of research sites**

The Committee agreed that all sites in this study should be exempt from site-specific

assessment (SSA). There is no need to submit the Site-Specific Information Form to any Research Ethics Committee. The favourable opinion for the study applies to all sites involved in the research.

#### Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission at NHS sites ("R&D approval") should be obtained from the relevant care organisation(s) in accordance with NHS research governance arrangements. Guidance on applying for NHS permission is available in the Integrated Research Application System or at <http://www.rdforum.nhs.uk>.

- It was agreed that the most simplistic title would be used on all documentation.
- It was agreed that details relating to PALs will be amended within the participant information sheet.
- It was agreed that the participant information sheet be amended highlighting details of PhD.

#### Approved documents

The documents reviewed and approved at the meeting were:

<i>Document</i>	<i>Version</i>	<i>Date</i>
Supervisor CV		06 February 2009
Participant Consent Form	V:1	01 February 2009
Participant Information Sheet	V:1	01 February 2009
GP/Consultant Information Sheets	V:1	01 February 2009
Letter of invitation to participant	V:1	01 February 2009
Questionnaire: non-validated	V:1	01 February 2009
Questionnaire: UTISA		
Questionnaire: EQ-5D		
Peer Review		15 July 2008
Summary/Synopsis	V:2	01 February 2009
Covering Letter		06 February 2009
Protocol	V:2	01 February 2009
Investigator CV		06 February 2009
Application	V:2.0	01 February 2009

#### Membership of the Committee

The members of the Ethics Committee who were present at the meeting are listed on the attached sheet.

#### Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

**After ethical review**

Now that you have completed the application process please visit the National Research Ethics Website > After Review

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

The attached document "After ethical review – guidance for researchers" gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

We would also like to inform you that we consult regularly with stakeholders to improve our service. If you would like to join our Reference Group please email [referencegroup@nres.npsa.nhs.uk](mailto:referencegroup@nres.npsa.nhs.uk).

09/H0905/15	Please quote this number on all correspondence
-------------	--

With the Committee's best wishes for the success of this project

Yours sincerely

**Dr John Drury**  
Chair

Email: [carol.cheesebrough@nhs.net](mailto:carol.cheesebrough@nhs.net)

Enclosures: List of names and professions of members who were present at the meeting.  
"After ethical review – guidance for researchers"

Copy to: Amanda Tortice, R&D Dept.,  
4<sup>th</sup> Floor, Leazes Building  
RVI, Queen Victoria Road  
Newcastle Upon Tyne NE1 4LP

County Durham & Tees Valley 1 Research Ethics Committee

Attendance at Committee meeting on 26 February 2009

Committee Members:

<i>Name</i>	<i>Profession</i>	<i>Present</i>	<i>Notes</i>
Mrs Plym Auty	Retired Lay Member	Yes	
Dr Richard Bellamy	Consultant Physician/Trust Epidemiologist	Yes	
Dr John Drury	Consultant Pathologist	Yes	
Mr Brian Houston	Senior Lecturer in Physiotherapy	Yes	
Mrs F Hutchison	Principal Teacher	No	
Mr Vivek Kuvelker	Community Pharmacist	No	
Rev. Malcolm Masterman	Lead Chaplain	Yes	
Mrs Christine McIlvenny	Specialist Nurse	Yes	
Dr Paul McKee	Consultant Neurologist	No	
Miss Sophie Meredith	University Administrator	Yes	
Dr Peter Morrell	Consultant Paediatrician	No	
Dr Peter Newman	Consultant Neurologist	No	
Mr Calum Polwart	Specialist Oncology Pharmacist	Yes	
Mr Michael Roff	Chartered Engineer	No	
Mr A Wadhvani	Consultant in Emergency Medicine (A&E Dept)	No	

Also in attendance:

<i>Name</i>	<i>Position (or reason for attending)</i>
Mrs Carol Cheesebrough	Committee Co-ordinator

# The Newcastle upon Tyne Hospitals

LRF/EB/196

NHS Trust

15 May 2009

The Freeman Hospital  
High Heaton  
Newcastle upon Tyne  
NE7 7DN

Mr R S Pickard  
Senior Lecturer & Honorary Consultant Urological Surgeon  
Institute of Cellular Medicine  
The Medical School  
Newcastle University

Tel: 0191 233 6161  
Fax: 0191 213 1968

Dear Mr Pickard

## Trust Approval for R&D Study 4841

**Title:** The role of altered innate antimicrobial activity in recurrent urinary tract infection  
**Principal Investigator:** Mr R S Pickard  
**Max No of Patients:** 100  
**Funder:** The Wellcome Trust  
**Sponsor:** Newcastle Upon Tyne Hospitals NHS Trust

The Trust grants approval for the above project, dependent upon:

- (i) you, as Principal Investigator, agreeing to comply with the Department of Health's Research Governance Framework for Health and Social Care, and understanding their responsibilities and duties (a copy of responsibilities prepared by the Trust R&D Office is enclosed)
- (ii) you, as Principal Investigator, ensuring compliance of the project with all other legislation and guidelines including Caldicott Guardian approvals and compliance with the Data Protection Act 1998, Health and Safety at Work Act 1974, any requirements of the MHRA (e.g. CTA, EudraCT registration), and any other relevant UK/European guidelines or legislation (e.g. reporting of suspected adverse incidents).

### **Sponsorship**

***The Newcastle upon Tyne Hospitals NHS Foundation Trust will act as Sponsor for this project, under the Department of Health's guidelines for research in health and social care.***

***In addition, the Trust has a Research Governance Implementation Plan, agreed with the Department of Health, in order to fully comply with Research Governance and fulfil the responsibility of a Sponsor.***

***As the Trust is acting as Sponsor for the research and where some of the research is taking place outside of Newcastle upon Tyne, then all costs must be met for research governance audit visits to those sites. It is the responsibility of the PI to provide confirmation to the Trust of who will pay these costs. Audit is required under the Research Governance Framework for Health and Social Care. (Please note that the Trust randomly audits 10% of all its active research annually.)***

You must notify the R&D Office if any changes to the protocol, etc. are agreed with the Ethics Committee or if there are any associated changes in cost relating from such alterations. It is imperative that the R&D Office retains a *complete* and up-to-date set of all such material.

It is also the Principal Investigator's responsibility to ensure that all staff involved have Honorary Contracts, where necessary, issued prior to commencing the research. Please be aware that Honorary Contracts will not be issued without a favourable ethical opinion and funding.

In addition, unless otherwise agreed with the Trust, the research will be covered for negligence under the CNST (Clinical Negligence Scheme for Trusts), however cover for no-fault harm is the responsibility of the Principal Investigator to arrange if required.

Please also note that for any NHS employee who generates Intellectual Property *in the normal course of their duties*, it is recognised that the Intellectual Property Rights remain with the employer and not the employee.

Yours sincerely



Sir Leonard R Fenwick CBE  
Chief Executive

Enc

Cc (by email only):

Graham Regan, Finance Department, FH  
Dr J S Tapson, Clinical Director, Urology & Renal Services, FH  
Dr A Ali, Wellcome Research Fellow & Specialist Registrar in Urology, Institute of Cellular Medicine  
Miss J Peacock, Research Management & Governance Manager, RVI

**From:** Mythen, Michael

**Sent:** 09 February 2009 17:14

**To:** Pickard, Robert

**Subject:** Acceptance of Your Caldicott and Data Protection Application

Dear Mr. Pickard your Caldicott and Data Protection submission has been approved.  
09/02/2009

Innate Defence Mechanisms In Female Cystitis(676)

Regards

Michael

Michael Mythen.

Deputy Head of IM&T

The Newcastle upon Tyne Hospitals NHS Foundation Trust.

Freeman Hospital 4th Floor Cheviot Court NE7 7DN

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19<sup>th</sup> June 2009

Mr Ased Ali  
Newcastle University  
Institute of Cellular Medicine  
3<sup>rd</sup> Floor William Leech Building  
Newcastle upon Tyne  
NE2 4HH

Dear Mr Ali

**Re: The role of altered innate antimicrobial activity in recurrent urinary tract infection IRAS Ref: 4075 (NIHR CRN Study ID 7018)**

Thank you for completing the minimum dataset for the above study. I can confirm that the study is eligible for, and has therefore been included on, the National Institute for Health Research (NIHR) Clinical Research Portfolio. The record for this study can be viewed on the Portfolio Database at [http://www.ukcrn.org.uk/index/clinical/portfolio\\_new.html](http://www.ukcrn.org.uk/index/clinical/portfolio_new.html).

***Benefit of inclusion in the NIHR Portfolio***

Inclusion in the NIHR Portfolio of studies ensures your study can access NHS service support and research infrastructure support in England (i.e. support to help with study promotion, approval, identification of eligible patients, recruitment, and follow up etc). This support is now flowing through the Comprehensive Clinical Research Network to the 25 Comprehensive Local Research Networks (CLRNs) across England. Funding allocations to the CLRNs include an activity-based component driven by the data which are held on the UKCRN Portfolio Database and it is therefore essential that your study record is kept up-to-date. Please contact us as soon as possible via email ([portfolio@nihr.ac.uk](mailto:portfolio@nihr.ac.uk)) if any changes are required.

***Collecting your accrual data***

In order to ensure that your study remains on the NIHR Portfolio and receives appropriate support through the relevant Comprehensive Local Research Network(s), the UKCRN Coordinating Centre must collect accrual data for the above study from April 2008 and then each month on an ongoing basis.

If you haven't already had the opportunity to send this data to us, we would be grateful if you could do so as soon as possible. Accrual data should be supplied via the UKCRN Accrual Upload System and we will be contacting you in the near future to talk you through this process. Further information and data templates for uploading accrual data can be found on the UKCRN website at: [http://www.ukcrn.org.uk/index/clinical/portfolio\\_new/P\\_accrual.html](http://www.ukcrn.org.uk/index/clinical/portfolio_new/P_accrual.html). Please contact us ([accrual@nihr.ac.uk](mailto:accrual@nihr.ac.uk)) if you have any queries about the process.

We would also encourage you to provide data on accrual prior to April 2008 in order to contribute to the CCRN "baseline" and to provide information on the overall level of recruitment into this study. This can be submitted in a simplified format, simply stating the total number of patients recruited prior to April 2008.

In partnership with



**Directors**  
Professor Peter Selby  
Professor Janet Darbyshire



*Additional and new studies*

Please note that some new studies funded by NIHR Partners (as defined in the Eligibility Criteria) might need to undergo a further adoption process prior to inclusion onto the Portfolio (e.g. if individual studies are part of a programme grant). All new "non-automatic" studies (those funded by non-UK governments, e.g. EU, NIH, and industry-supported, non-industry sponsored - IITs) will also need to undergo a full adoption process.

UKCRN is keen to ensure that all studies which are eligible for inclusion into the NIHR Portfolio are identified so that they can be supported through the Comprehensive Clinical Research Network. If you are aware of any other potentially eligible studies which are recruiting or actively following up patients from April 2008, and which have not yet been confirmed as being on the Portfolio, we would be very grateful if you would let us know. Further details are available at [http://www.ukcrn.org.uk/index/clinical/portfolio\\_new.html](http://www.ukcrn.org.uk/index/clinical/portfolio_new.html).

Thank you for your support in this exercise which will be critical to the successful development of the national Comprehensive Clinical Research Network. Our aim is to ensure the provision of high quality infrastructure to support clinical research in the NHS and support the delivery of your study.

Please do not hesitate to contact me if you require any further information.

Best wishes



Dr Sam Taylor  
Portfolio Lead  
NIHR Clinical Research Coordinating Centre (NIHR CRN CC)  
Fairbairn House  
71-75 Clarendon Road  
Leeds  
LS2 9PH

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