THE PHYSIOLOGICAL AND MICROBIOLOGICAL RESPONSE TO RENAL UPEC INFECTION

Keira Melican

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Nature composes some of her loveliest poems for the microscope and the telescope.

ABSTRACT

The pathological outcome of a bacterial infection depends on the interplay between the host’s defences and the virulence arsenal of the pathogen. Appreciation of this interplay is crucial to the understanding of pathogenesis and the development of efficient clinical treatments. In this thesis we wanted to study the dynamics of the early stages of renal bacterial infection. While sacrificial models and in vitro experimentation has given us a wealth of information, they lack the spatial and temporal resolution required to follow the crucial first hours of infection. To overcome this we developed a multiphoton based live animal infection model which allows for the visualization of infection progression in real-time. This model allows for cellular resolution visualisation of events occurring in the live kidney with the influences of the all physiological factors such as the blood, nervous, hormonal and immune systems intact. Our model utilizes micropuncture techniques to directly infuse bacteria into the renal tubules allowing for a well defined time-frame of infection.

What we achieved was a unique insight into the rapid physiological responses to infection. Physiological responses described in this thesis include ischemic and obstruction injuries. These injuries are both related to dynamic physiological functions for which real-time live imaging is particularly suitable. Within 3-4 hours of the first bacterial interaction, epithelial signalling lead to activation of the clotting cascade and shut-down of local peritubular capillaries. The clotting response was shown to be crucial to isolate the infection and prevent sepsis. A rapid and dramatic drop in local tissue oxygen tension was also recorded with the combination resulting in a local ischemic injury. This infection-induced ischemia resulted in the characteristic cellular actin and integrin re-arrangements, but lacked a re-perfusion stage, instead resulting in localised tissue damage. We also investigated the effect of bacterial infection on renal filtration, revealing total nephron obstruction within 8 h. Other physiological responses seen include the infiltration of immune cells including both neutrophils and other unidentified mononuclear cells. This work shows that the full pathophysiology of pyelonephritis is a combination of numerous physiological injuries.

Investigating the microbiological response to infection revealed that certain virulence factors affected the kinetics of both bacterial colonisation and the host response. Expression of the exotoxin α-haemolysin was shown to induce a more rapid host vascular response. A synergistic interaction between the adhesion factors P and Type-1 was shown to facilitate optimal kidney colonisation. P fimbriae were important for bacterial-epithelial interaction and in withstanding the sheer stress of filtrate flow, while Type 1 fimbriae expression becomes pertinent as the bacterial community expands into the lumen. This heterogeneous population allowed for the formation of an epithelial anchored biofilm which contributes to renal obstruction.

Our work reveals new findings from both the physiological and microbiological responses to renal UPEC infection. These findings were made possible by the development and utilisation of the multiphoton based live-animal imaging model. It is hoped that as these types of live models become more integrated into infection biology awareness of these dynamic interplays will allow for improved treatment regimes.
LIST OF PUBLICATIONS

This thesis is based on the following papers:


III. Melican K, Sandoval RM, Abdul K, Josefsson L, Tanner GA, Molitoris BA, Richter-Dahlfors A. Synergy between Type 1 and P fimbriae during in vivo renal colonization, *Submitted manuscript*

Other papers produced during my PhD but not included in the thesis:


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<th>Description</th>
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<tr>
<td>UPEC</td>
<td>Uropathogenic <em>Escherichia coli</em></td>
</tr>
<tr>
<td>UTI</td>
<td>Urinary Tract Infection</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>RBC</td>
<td>Red blood cell</td>
</tr>
<tr>
<td>HlyA</td>
<td>α-Haemolysin</td>
</tr>
<tr>
<td>Sat</td>
<td>Secreted Autotransporter Toxin</td>
</tr>
<tr>
<td>CNF-1</td>
<td>Cytotoxic Necrotizing Factor 1</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen Associated Molecular Pattern</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like Receptor</td>
</tr>
<tr>
<td>GFR</td>
<td>Glomerular Filtration Rate</td>
</tr>
<tr>
<td>TGF</td>
<td>Tubuloglomerular Feedback</td>
</tr>
<tr>
<td>JGA</td>
<td>Juxtaglomerular Apparatus</td>
</tr>
<tr>
<td>PMN</td>
<td>Polymorphonuclear Neutrophil</td>
</tr>
<tr>
<td>ABU</td>
<td>Asymptomatic Bacteriuria</td>
</tr>
<tr>
<td>IL-8</td>
<td>Interleukin-8</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin-6</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour Necrosis Factor - α</td>
</tr>
<tr>
<td>MPM</td>
<td>Multiphoton Microscopy</td>
</tr>
<tr>
<td>LSCM</td>
<td>Laser Scanning Confocal Microscopy</td>
</tr>
<tr>
<td>SDCM</td>
<td>Spinning Disk Confocal Microscopy</td>
</tr>
<tr>
<td>SHG</td>
<td>Second Harmonic Generation</td>
</tr>
<tr>
<td>PTC</td>
<td>Proximal Tubule Cell</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
</tr>
<tr>
<td>PO₂</td>
<td>Tissue Oxygen Tension</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony Forming Units</td>
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</table>
1. INTRODUCTION

Medical problems associated with the urinary tract have afflicted mankind since the beginning of history. Evidence of kidney stones, calculi which form in kidney tubules and cause obstruction, have been found in Egyptian mummies dating from 4800 B.C. Surgery to remove kidney and bladder stones ‘cutting for the stone’ is one of the earliest forms of elective surgery and it even garners a mention in the Hippocratic Oath:

"Neither will I cut them that have the stone but will leave this operation to those who are accustomed to perform it."

Infection of the kidneys and its subsequent complications are believed to be responsible for the deaths of many notorious historical figures. Oliver Cromwell, is widely believed to have died from urosepsis in 1658, a full 2 years ahead of his posthumous execution and be-heading. Despite being the subject of considerable research over the years, urinary tract infections (UTI) are still a significant public health issue with far-reaching social and economic ramifications. During their lifetime 30-40% of all adult women experience symptomatic urinary tract infections (UTI), usually as a result of invasion and colonization by bacteria from the intestinal flora. Reoccurrence of UTI can be problematic with approximately 25% of woman experiencing reoccurrence within 12 months of the initial infection. UTI infections continue to challenge and intrigue researchers the world over, and are the subject of this thesis.

1.1 THE EVOLUTION OF MICROBIOLOGY

The field of Microbiology was founded in the 19th century when it was demonstrated that a number of diseases were caused by infectious agents. The field focuses on understanding the infectious agent, the microbe. The study of their genetics, metabolism, lifestyles and pathogenesis has led to a massive stockpile of information regarding how microbes, including bacteria, function. The phrase ‘Cellular Microbiology’ was termed in 1996, in a Science paper describing this new field emerging at the interface of Microbiology and Cell Biology. This brief paper describes how the two fields intersect and are mutually beneficial. Cell Biology benefited from the tool-kits available from microbiology, such as bacterial toxins that can disrupt cellular pathways. Microbiology likewise benefited from understanding the effects of numerous bacterial proteins, toxins and mechanisms on mammalian cells. A decade later, another meeting of the minds, this time of Microbiology and Immunology, led to the coining of the term ‘Infection Biology’. When a bacterial pathogen comes into contact with its host, it triggers a veritable cascade of responses. These cascades are not restricted to the one specific cell and often involve complex intra-cellular signalling networks. As noted in a Nature editorial on the topic, infection had for too long been studied from two different angles. Microbiologists studied
the pathogen, while immunologists studied the host immune response. Infection Biology aims to understand these complex cascades and to find ways to manipulate them to help prevent the development of disease.

The step towards Infection Biology recognised that the human body is made up of millions of cells. In the host these cells are organized into tissues. These tissues form organs which are interconnected by a multitude of ‘systems’ including the vascular system, the central nervous system, the lymphatic system and the immune system. This complexity is impossible to mimic in the cell culture dish, meaning in vivo experimentation has become more important in Infection Biology. ‘Within the living organism’ or the Latin phrase ‘in vivo’, is a term frequently seen in Infection Biology. The scientific definition of ‘in vivo’ varies however between different fields. In Bacteriology, ribosomal translation within the bacterial cell may be referred to as ‘in vivo’, in contrast to in vitro assays using purified components. Some cell culture studies have also been termed ‘in vivo’, as they are performed within living cells. In Infection Biology ‘in vivo’ generally refers to studies performed within animal models. Even with animal models however there is ambiguity. Sacrificial ‘in vivo’ experiments involve a retrospect analysis of the infection in dead tissue. So while the experimental infection may be carried out ‘in vivo’, the analysis is not. These models are commonly used for histology, RNA analysis and quantification of bacterial virulence. The use of explanted organs and tissues is also on the rise in the study of infection. These models, while having the complex tertiary structures of the tissues, do lack the systemic influences of the vascular, immune and hormonal systems, and they have limited usable time spans. The use of explants is increasingly described using the term ‘ex vivo’ or ‘Outside the living’. It can therefore be appreciated that the terminology of many scientific papers can be confusing. The definition of ‘in vivo’ is also evolving somewhat alongside the available technologies. In our work, and this thesis, we define ‘in vivo’ work as experiments, with simultaneous analysis, performed within the live animal model. While the complexity of the model has both pro’s and con’s, it holds huge promise for the study of pathogenesis as it is possible to study infection progression with the full complement of host factors such as immune, hormonal, and vasculature responses, present and accounted for. Live models such as the one described here are facilitating the step from ‘Cellular Microbiology’ to ‘Tissue Microbiology’.

1.2 URINARY TRACT INFECTIONS
Urinary tract infections occur when pathogens, often originating from faecal flora, enter the urethra. From this site pathogens can migrate to the bladder causing symptomatic cystitis or asymptomatic bacteriuria (ABU). Further migration up the ureters leads to infection of the kidneys. Lower UTI often present with clinical symptoms such as pain and urgency of urination. Patients’ urine may appear cloudy due to presence of bacteria, pus and sloughed epithelial cells. Urine examination and culture is essential for diagnosis, and the infection is usually treated with antibiotics. Upper UTI infections are more difficult to diagnose. They show similar symptoms to lower UTI but are often
accompanied by a sudden increase in temperature and uni- or bilateral flank pain\textsuperscript{19}. Diagnostic tools include standard laboratory examination of pyuria, bacteriuria, and immune cell casts in blood and urine. In complicated or recurrent cases, diagnostic imaging may be used as a tool to evaluate the extent of damage and any underlying abnormalities that may prevent clearance of the infection. Computed tomography is the preferred imaging method for the diagnosis of acute renal bacterial infection\textsuperscript{20}.

Bacterial infection of the kidney is medically termed pyelonephritis, indicating that infection has reached the pelvis or \textit{pyelum} of the kidney (\textit{nephros}). Pyelonephritis is a serious infection associated with a greater level of inflammation than cystitis\textsuperscript{21}. Gross pathology includes abscess formation in the renal parenchyma and oedema, often leading to irreversible scar formation. Renal scar formation, the formation of fibrosis, can contribute towards the development of renal insufficiency\textsuperscript{21}. Pyelonephritis is commonly defined as a tubulointerstitial disorder, based on the pathologic picture observed in renal biopsies. This indicates that the tubules and interstitial tissues, are most commonly involved\textsuperscript{22}. The normal kidney is considered relatively resistant to infection but abnormalities in structure and function can increase susceptibility\textsuperscript{23}. Risk factors in children include voiding dysfunction and vesicoureteral reflux, while in adults genetic susceptibilities (discussed later) and behavioural risk factors are most relevant\textsuperscript{24}.

The ability of bacteria to bind to renal epithelium is essential to the initiation of pyelonephritis. The epithelium is however far from uniform. On ascent from the bladder microbes migrate through the renal tubules. The tubules are made up of different segments, constituted of epithelial cells which are highly specialized in both structure and function. These epithelial cells perform tubular uptake and secretion and subsequently alter the glomerular filtrate, the precursor to urine\textsuperscript{25}. Therefore bacteria do not only meet very different epithelial linings on their way up the nephron, but they do so while also being exposed to a continuously altered composition of glomerular filtrate.

Uropathogenic \textit{Escherichia coli} (UPEC) is implicated as the causative agent in up to 80\% of community-acquired UTI's, making it the leading urinary pathogen\textsuperscript{21}. Other Gram-negative bacteria associated with UTI include \textit{Klebsiella}, \textit{Entrobacter}, \textit{Pseudomonas} and \textit{Proteus mirabilis}\textsuperscript{21}. The latter accounts for more than 40\% of UTI infections in infant boys. Gram-positive bacteria implicated in UTI include the \textit{Staphylococcus} strains\textit{ epidermidis} and \textit{aureus} as well as \textit{Enterococcus faecalis}\textsuperscript{21}. Once bacteria enter the urinary tract they rapidly adapt to their new niche within the host. To enable this adaption UPEC express numerous proteins and structures. UPEC strains contain more genes than their non-virulent K-12 counterparts, and these extra genes have been thought to be horizontally acquired pathogenicity islands\textsuperscript{26-28}. More recently, genetics approaches have suggested that UPEC may indeed be the parental strain, with strains causing asymptomatic bacteriuria (ABU) having lost virulence to avoid immune detection\textsuperscript{29,30}.
1.3 THE OFFENSE - UPEC VIRULENCE FACTORS

However the genetics may look, or have evolved, it remains that pathogenic UPEC strains express proteins which are considered essential for virulence. These proteins, traditionally known as virulence factors, have been described as factors which characterise disease isolates 31. Early definitions of virulence factors came from the basic epidemiology practice of comparing properties of faecal strains from healthy controls with urinary isolates from patients 32. Some of these factors however may also be considered as ‘fitness factors’, factors which enhance the growth and colonisation of the bacteria but may not be absolutely essential for infection. Siderophores, which allow the bacterial to sequester iron have been annotated as fitness factors because while their expression is not essential to virulence, it is advantageous 33,34. Conversely has also been shown that the acquisition of certain traits such as antibiotic resistance, which would appear advantageous for virulence have a negative effect on bacterial fitness 35,36. The traditionally annotated UPEC ‘virulence factors’ include exotoxins, proteases, capsule, ion acquisition systems, lipopolysaccharide and adhesion factors 37,38. Much of the research on these factors has been carried out in vitro and the expression of certain adhesion factors is still defined by their in vitro agglutination abilities 32,38. In UPEC the most well described virulence factors are involved in bacterial adhesion and are known as fimbriae or pili 28. These organelles allow UPEC to bind to the uroepithelium and withstand the stress of filtrate and urine flow. UPEC express numerous different fimbriae including P, Type 1, F1C, S, and Afa/Dr adhesins 38. P and Type 1 fimbriae particularly have been the focus of much research and are well understood 39,40.

Type 1 fimbriae is expressed by numerous E. coli strains, including both pathogens and commensal intestinal bacteria 41. For UPEC strains, the role of Type 1 fimbriae in cystitis has been extensively described. After initial urethral colonization, UPEC ascend into the bladder where the stratified transitional bladder epithelium is covered with apical plaques of uroplakin 42. The uroplakin contains mono-mannose moiety to which FimH, the tip adhesin of Type 1 fimbriae, binds 38,43. These uroplakins therefore serve as binding sites for Type 1 fimbriae, allowing UPEC to gain a foothold in the bladder 44. Type 1 fimbriae bind to bladder epithelial cells and initiate the internalisation of UPEC to form intracellular bacterial communities (IBCs) at the same time as initiating the host inflammatory response leading to sloughing of infected cells among other responses 45-49. These IBCs are thought to be a possible bacterial reservoir for recurrent infection 50.

P fimbriae were one of the first virulence factor associated with UPEC and are designated P due to their ability to agglutinate red blood cells (RBCs) of the P blood group 32,51. The tip adhesin of the P fimbriae, PapG, mediates attachment to Galα1-4Galβ containing glycolipids which are often found on renal epithelium 52. PapG has been found to have at least three allele variants, classes I, II and III. Class II is primarily linked to human pyelonephritis and class III to cystitis 51,53,54. CFT073, the UPEC strain used in the papers included in this thesis, carries two pap gene clusters, both encoding for the PapGII allele 51,55. While P fimbriae has long been considered an important virulence factor in
UTI, they do not fulfil the Molecular Koch’s Postulates as laid down by Stanley Falkow in 1988. This is because a majority, but not all, clinical isolates express P fimbriae. The précis role of P fimbrial expression in vivo is a matter of some contention with mutational studies giving somewhat conflicting results. Expression of P fimbriae is known to vary depending on environmental conditions and is under the control of phase variation. The phase variation of P fimbriae is based on the initiation of pap operon transcription controlled by a reversible epigenetic switch.

Phase variation is a common feature of bacterial adhesins, allowing for rapid adaptation to infectious niches and the development of a heterogeneous population. Bacteria contain the genes for numerous different fimbriae and it has been shown that even within UPEC there is a redundancy between these fimbriae. Type 1 fimbriae expression is also controlled by phase variation and is under the control of the fim switch, an invertible element containing the main promoter for the structural subunits. Negative cross-talk between Type 1 and P fimbriae has been demonstrated, with PapB being shown to repress the FimB-promoted off-to-on inversion of the fim switch. This means that UPEC express either Type 1 or P fimbriae but it is unlikely that they can express both simultaneously.

Protein toxins are another family of UPEC virulence factors. A majority of UPEC strains, including CFT073, lack the Type III secretion system, utilising instead the Type I and V secretion systems. Recent work has also suggested Type II and the Type IV pili secretion systems as virulence determinants in renal infection. The Type I secreted toxin α-haemolysin (HlyA) has long been associated with UPEC and is encoded by ~50% of clinical UPEC isolates. High concentrations of HlyA rapidly leads to cell lysis via its ability to form membrane pores. Sublytic concentrations have been shown in our lab to induce periodic calcium oscillations in primary renal cells, inducing cellular expression of the pro-inflammatory cytokines IL-6 and IL-8. Others have shown sublytic concentrations of HlyA effect numerous host pathways such as MAP kinase signalling, inactivation of the serine/threonine kinase AKT, histone phosphorylation and acetylation patterns. The secreted autotransporter toxin (Sat) is also associated with pyelonephritic strains having been isolated from CFT073. Sat is a vacuolating cytotoxin which appears to particularly damage renal cells including the proximal tubule cells. Cytotoxic necrotizing factor 1 (CNF -1) is another toxin expressed by some UPEC strains, but not CFT073. Thought to be transported via outer membrane vesicles, CNF-1 activates Rho family GTPases leading to inflammatory signalling and actin re-arrangement.

The endotoxin lipopolysaccharide (LPS) is also considered an important virulence factor of UPEC. LPS of UPEC is an important Pathogen Associated Molecular Pattern (PAMP) which is recognised by Toll–Like Receptor 4 (TLR4 is discussed in detail later), leading to the initiation of pro-inflammatory signalling. LPS is composed of an O-linked polysaccharide and a core polysaccharide attached to the bacterial outer membrane via the lipid A moiety. The lipid A is the endotoxically active segment of LPS, with the acylation state a major determinant for immunogenicity. The variability of the O-
polysaccharide determines serologic diversity with certain serotypes e.g. O1, O2, O4, O6, O7 and O75 strongly linked to UTI. Classification of *E. coli* is based on the combination of somatic O antigens, K capsular antigens and H flagella antigens. Capsular antigens associated with UPEC include K1, K2, K3, K5, K12 and K13. The CFT073 isolate used in this work is classified O6:K2:H158. Despite the vast amounts of research into the role of UPEC virulence factors for the progression of infection, no one factor can be considered unique or essential for UPEC. With this in mind, *in vivo* study of the roles and cross-talk between these factors is essential.

**Table 1. Notable UPEC Virulence Factors**

<table>
<thead>
<tr>
<th>Virulence Factor</th>
<th>Main mechanisms</th>
<th>Studied in this thesis</th>
</tr>
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<tbody>
<tr>
<td>P Fimbriae</td>
<td>Adhesion</td>
<td>Paper III</td>
</tr>
<tr>
<td>Type 1 Fimbriae</td>
<td>Adhesion</td>
<td>Paper III</td>
</tr>
<tr>
<td>S Fimbriae</td>
<td>Adhesion</td>
<td></td>
</tr>
<tr>
<td>F1C Fimbriae</td>
<td>Adhesion</td>
<td></td>
</tr>
<tr>
<td>Afa/dr Fimbriae</td>
<td>Adhesion</td>
<td></td>
</tr>
<tr>
<td>Flagellum</td>
<td>Motility/biofilm</td>
<td></td>
</tr>
<tr>
<td>Curli</td>
<td>Adhesion, biofilm</td>
<td></td>
</tr>
<tr>
<td>LPS/Lipid A</td>
<td>Serum resistance/immunogenicity</td>
<td></td>
</tr>
<tr>
<td>HlyA</td>
<td>Cytotoxicity, cytokine induction</td>
<td>Paper I</td>
</tr>
<tr>
<td>CNF-1</td>
<td>Actin re-arrangement, inflammation</td>
<td></td>
</tr>
<tr>
<td>Sat</td>
<td>Vacuolating cytotoxin</td>
<td></td>
</tr>
<tr>
<td>Capsule</td>
<td>Immune evasion</td>
<td></td>
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1.4 THE DEFENSE - HOST IMMUNE MECHANISMS

The mammalian urinary tract is protected by numerous defence mechanisms which strive to maintain a sterile environment. The physical defence of the epithelial barrier is complemented by mechanical defences including the sheer stress of urine flow and chemical defence including the expression of pro-inflammatory cytokines and antimicrobial peptides. Colonisation of the urinary tract can either lead to symptomatic disease such as cystitis and pyelonephritis, or develop into ABU. How the infection develops depends on the pathogen and its set of virulence factors as described earlier, as well as the host immune response. The immune system can be divided into the innate and adaptive immune responses. The innate responses are those mechanisms which recognise and respond immediately to the bacterial threat. Innate responses are non-specific whereas the adaptive immune response contains a memory which can build a specific immunity to a pathogen. The adaptive response can take days, to weeks, to develop to its full capacity. Relatively few papers focus on innate immunity as compared to the body of work concerning the adaptive response. In this work we focus on the innate response concentrating on the first hours of infection.
One of the first stages of the immune response to epithelial infection is bacterial recognition. Key factors for bacterial recognition are the Toll-Like Receptors (TLRs). The TLRs are named as such for their similarity to the drosophila toll protein and are members of the Toll/Interleukin-1 receptor super-family. TLRs are pattern recognition receptors which recognise a specific PAMP. For the recognition of UPEC the TLRs of interest are TLR4, TLR5 and TLR11. TLR4 recognises the LPS endotoxin of Gram-negative pathogens and is well studied in UTI. The recently described TLR11 is found to play a role in mouse model UTIs but in humans the encoding gene is thought to contain a stop codon mutation. TLR11 has also recently been linked with profilin from Toxoplasma gondii. TLR5, most commonly associated with recognition of bacterial flagellin, has also recently been shown to regulate the immune response in UTI.

The role of TLR4 in UTI was discovered using the C3H/HeJ mouse line which carries a missense mutation in the gene encoding TLR4. These mice are considered resistant to endotoxin and fail to mount an innate immune response. They develop an ABU like condition following infection rather than symptomatic disease. Distribution and expression of TLR4 in the kidney has been a matter of some debate. While some studies have shown that kidney cells do not express the protein, others have shown that it does. An in vivo study revealed a predominant localisation of TLR4 on the apical surface of distal tubule, as opposed to proximal tubules which showed little TLR4 in un-infected animals. Upon induction of sepsis however, they show expression of TLR4 in all kidney segments, suggesting an up-regulation of TLR4 following inflammation. It is important to note in this study that the bacteria were located in the blood stream with the kidney tubule cells only exposed to filtered LPS. An individual's susceptibility to UTI is thought to be effected by their expression of TLRs, particularly TLR4. Children prone to ABU have been shown to have reduced TLR4 expression on neutrophils and those carrying the TLR4 A(896)G allele are prone to the development of recurrent UTI.

TLR signalling is very complex, but generally it occurs via the myeloid differentiation factor 88 (MyD88) to induce cytokine signalling, activating nuclear factor kappa-B (NF-kB). This cytokine signalling triggers the infiltration of innate immune cells such as polymorphonuclear neutrophils (PMNs). PMNs and other inflammatory cells follow this chemotactic gradient to the site of infection. Chemotaxis is mediated through the IL-8 receptor CXCR1 which has been implicated in migration of neutrophils across the uroepithelial barrier. Studies have identified disease-associated polymorphisms and mutations in the CXCR1 gene of patients prone to pyelonephritis, highlighting the importance of this receptor in a correct immune response. Neutrophils recruited to the site of infection kill invading bacteria on their way through the tissue, but in the process damage to tissue often occurs. Liberation of neutrophil granules which contain antimicrobial peptides, proteins, and proteolytic enzymes, can lead to the dissolution of extracellular matrix, harm cell structures or cell function, and induce acute and potentially
irreparable damage. The host immune response is a formidable adversary for invading bacteria, but it is one which they have evolved numerous mechanisms to tackle.

### 1.5 THE PLAYING FIELD – THE KIDNEY

The primary function of the kidneys is to maintain the body’s extracellular fluid in a stable physiochemical condition. They filter the blood to remove waste products as well as maintaining ion and water balances. The human kidney is made up of approximately 1 million individual filtering units called nephrons.

![Diagram of the nephron](image.png)

Each nephron consists of a vascular component and a tubular component. These components meet at the glomerulus where blood passing through the glomerular capillaries is filtered into Bowman’s space. Following Bowman’s space is the S1 or early segment of the convoluted proximal tubule, followed by the hairpin like Loop of Henle, the convoluted distal tubule and finally the collecting duct where filtrate passes out of the nephron. The glomerulus, proximal tubules and distal tubules are located in the cortex of the kidney with the loop of Henle and collecting ducts in the medulla. The renal tubules consist of a single layer of epithelium with unique structure and function depending on the segment. Bowman’s capsule is lined with thin squamous epithelial cells, whereas the proximal tubule consists of cuboidal/columnar epithelia covered with microvilli (~150 per µm² of cell surface) which function to increase surface area for tubule re-absorption. The proximal segment is leaky and highly endocytic, with a thick, apical brush border that faces the tubule lumen; 67% of filtered water, Na⁺, Cl⁻, K⁺ and many other solutes from the glomerular filtrate is reabsorbed within the proximal tubule. Cells of the distal tubule segments lack microvilli and constitute a tight epithelium displaying low endocytic capacity and low permeability to water.
From the vascular side, blood enters each kidney via a single renal artery, which branches off to form afferent arterioles, which in turn lead to the glomerular capillary tufts. From the glomerulus blood flows into the efferent arteriole which divides into the peri-tubular capillaries that are intertwined with the nephron tubules. The peri-tubular capillaries rejoin to form the renal vein through which blood exits the kidney.

1.5.1 Renal Blood Flow

The kidney receive 20-25% of the total cardiac output of blood, equivalent to approximately 1.2 l/min in an average human. As the kidney is such a highly vascularised organ, it is highly sensitive to fluctuations in blood supply. Drops in blood supply either locally or organ wide can lead to renal ischemia. Renal ischemia is a well-studied injury common in renal transplant surgeries and its effects on renal morphology are well described. The response of the single layer of tubular epithelial cells to ischemia includes rapid loss of cell polarity and cytoskeletal integrity through the disruption of the apical actin. Internalisation of microvilli and apical membrane proteins accompanied by blebbing of the apical membrane leads to loss of the proximal tubular brush border. A redistribution of adhesion molecules, including the integrin family, facilitates the detachment of proximal tubule cells from the basement membrane, leaving behind a denuded basement membrane. The basement membrane is a collagen rich layer through which the epithelial cells attach to the connective tissue. This membrane is often reepithelialised in the event of reperfusion, the return of blood supply. Ischemia has also been shown to affect the renal microvasculature, causing endothelium injury and leading to permeability defects. Inflammation contributes to the pathogenesis of ischemia/reperfusion injury with roles being assigned to both T cells and macrophages while the role of neutrophils is still unclear. Paper II in this thesis focuses on the effects of UPEC infection on local renal blood flow.

Figure 2: Stages of renal ischemia/reperfusion. 1: Normal conditions. 2: RBC flow begins to slow, beginnings of PTC microvilli blebbing and integrin re-arrangement. 3: Stoppage of blood flow, loss of PTC polarity, blebbing of PTC. 4: PTC sloughing and the beginning of re-perfusion. 5: Re-epithelialisation of basement membrane. 6: PTCs regain polarity.
1.5.2 Glomerular Filtration
Approximately 20% of all blood plasma entering the kidneys is filtered via the glomerular capillaries into Bowman’s capsule. The essentially protein-free filtered plasma varies greatly from the final urine which we excrete. The complex tubular formations of the nephrons carries out tubular re-absorption and tubular secretion altering the filtrate as it passes through. Physical obstruction of the nephron can rapidly lead to acute renal failure. Obstruction of the urinary tract is often caused by renal calculi and sloughed tissue as well as physical abnormalities and conditions such as cancer and even pregnancy. Obstruction of filtrate flow rapidly leads to changes in hydrostatic pressure and anatomic changes, such as dilation of tubules. The increases in intra-renal pressure can also effect renal filtration and lead to a drop/stop in glomerular filtration rates (GFR). Extended tubular obstruction can lead to arteriole vasoconstriction and a drop in renal blood flow. Studies from our collaborators have shown that obstruction of a single nephron induces local regulation of these effects. They show that single nephron obstruction leads to induction of an inflammatory response, tubular cell injury, changes in glomerular capillary pressure and eventual disuse atrophy. Feedback between the tubules and renal vasculature has been well studied. Tubuloglomerular feedback (TGF) centres around the juxtaglomerular apparatus (JGA) where the thick ascending limb of a nephron passes back in close proximity to its glomerulus. The JGA is made up of specialized distal tubule epithelial cells called the macula densa, the renin secreting Juxtaglomerular cells in the arterioles and the extra-glomerular mesangium. The macula densa responds to luminal NaCl concentration to trigger signalling via the JGA leading to arteriolar constriction or dilation and a subsequent change in GFR. Tubular obstruction occurring due to cast formation following ischemic injury and cellular blebbing is an example of the dynamics and intricacy of the pathophysiology of renal injuries. The association between renal UPEC infection and glomerular filtration is the subject of Paper III. Recent work has suggested that bacterial products such as LPS impair ion transport functions of renal tubules in a TRL4 dependent manner, indicating a strong link between infection and renal hydrodynamics.

1.6 WATCHING THE GAME
Bacteria and microscopy enjoy a history which reaches back as far as the 1676 when Antonie van Leeuwenhoek looked down his primitive microscope and described the ‘animalcules’ that he saw. These animalcules were in fact bacteria being visualised for the first time using light based microscopy.

“The 4th sort of creatures... which moved through the 3 former sorts, were incredibly small, and so small in my eye that I judged, that if 100 of them lay one by another, they would not equal the length of a grain of course Sand; and according to this estimate, ten hundred thousand of them could not equal the
dimensions of a grain of such course Sand. There was discover'd by me a fifth sort, which had near the thickness of the former, but they were almost twice as long.”

- Antonie van Leeuwenhoek in a letter to H. Oldenburg, 9 Oct 1676.

1.6.1 Fluorescence Microscopy

Fluorescence microscopy is based on the visualisation of targets specifically labelled with fluorophores. Fluorophores are the functional groups of a molecule which enable it to be fluorescent. Fluorescence can be defined as a three-stage process, excitation, excited-state lifetime and emission. Excitation occurs when the fluorophore is moved to its excited state by the delivery of a photon of energy from an external source, such as a laser. When excited a fluorophore undergoes conformational changes and emission occurs as the fluorophore returns to its ground state. The photon of energy that is emitted is lower in energy than that which caused excitation due to partial dissipation, resulting in a longer wavelength emission. Excitation and emission may also be considered as the capture and release of a photon by the fluorophore. Each fluorophore has a specific excitation and emission spectra with the difference between the two known as the Stokes shift.

Fluorophores are often conjugated to specific antibodies so that they can be used to specifically identify targets one wishes to image. In fluorescence microscopy the labelled specimen is excited with wavelengths of light specific to the chosen fluorophores. Different emission spectra can be detected by using different wavelengths emission filters and are often visualised as different colours in a multicolour image. Fluorescence microscopy has found some great uses in the study of bacterial infection. The ability to label the pathogen and certain aspects of the host cell simultaneously has allowed for many experiments looking at the mechanisms of host-pathogen interaction. One drawback of fluorescence microscopy can be the interference of out-of-focus light coming from fluorophores in different focal planes of the specimen.

Confocal microscopy is a major advance on standard fluorescent microscopy which overcomes the issues of out-of-focus light. Confocal microscopy uses an exit pinhole which only allows light emitted from the focal plane to pass to the detectors. This eliminates any out-of-focus fluorescence and also creates the possibility for ‘optical sectioning’ to create 3D renders. Both laser scanning confocal microscopy (LSCM) and newer developments such as spinning disk confocal microscopy (SDCM) allow for 3D imaging of pathogen-host interaction, providing the ability to address spatial aspects of tissue infection. The capture speed and resolution of SDCM has allowed for some informative live studies of the dynamics of superficial infections, such as in the skin. Limitations of confocal microscopy include the occurrence of photobleaching and photodamage from the excitation lasers as well as limited depth penetration, particularly into light-scattering tissue. Photobleaching results from the irreversible destruction of the excited fluorophore.
1.6.2 Multiphoton Microscopy

Multiphoton microscopy can be considered as the ‘next step’ in fluorescent microscopy techniques. It offers almost comparable resolution to confocal microscopy while avoiding many of confocal microscopy’s inherent limitations\textsuperscript{131}. The primary advantage of MPM is its ability to image deeper into tissue with reduced tissue damage. The principle of MPM lies in the use of a non-linear optical process \textsuperscript{132}. While it uses similar scanning techniques to LSCM, MPM uses a long wavelength (infrared) laser to excite the fluorophores only within a specific femtoliter sample volume \textsuperscript{123}. This excited sample volume is only at the determined ‘focal plane’, meaning that the majority of the tissue is unexcited, protecting it from photodamage \textsuperscript{130}. The lack of ‘out-of-focus’ signal eliminates the need for a confocal pinhole, as all emitted photons are generated from the focal plane and are therefore relevant. By using detection methods which capture as many emitted photons as possible, this can help increase the sensitivity of fluorescence detection \textsuperscript{130}.

The use of longer excitation wavelengths, often around 770-900 nm, has multiple benefits. These wavelengths are generally considered to be less photodamaging than their shorter cousins, resulting in better cell viability \textsuperscript{131}. A reduced Rayleigh scatter also means these longer wavelengths can penetrate further into biological tissue, allowing imaging up to 1mm deep \textsuperscript{133}. To produce sufficient excitation energy using these lower energy, longer wavelengths of light, the photons energies need to be ‘crowded’ in space and time to enable two photons of light to interact with the fluorophore simultaneously \textsuperscript{130}. Simultaneous means that the two photons must arrive within the duration of the intermediate virtual state of the electron, approximately an attosecond (\textasciitilde{}10\textsuperscript{-18} s) \textsuperscript{130}. To achieve spatial ‘crowding’ or concentration, the excitation laser can be focused through an objective with a high numerical aperture. Temporal concentration is achieved by using ultra short (< 1ps) laser pulses to create the required excitation intensities while maintaining a relatively low average power \textsuperscript{131,134}.

\textbf{Figure 3.} Jablonski diagram showing 1-photon (confocal/fluorescence excitation) and 2-photon excitation.
1.6.2.1 Fluorophores and labelling

The simultaneous absorption of the multiple photons of light excite the fluorophores in tissue from which they emit their characteristic Stockes-shifted wavelength. In MPM, multiple fluorophores can be excited and detected at the same time, allowing for multi-target imaging. To enable this multi-targeted imaging the multiphoton excitation of the chosen fluorophores must be taken into account. A general rule in two-photon excitation is that a fluorophore requires two photons of half the energy, so that an easy estimation is to double the required one-photon excitation. Specific measurements of the two-photon spectra have shown a tendency towards a blue-shift, this includes rhodamine and fluorescein, two commonly used fluorophores. This blue-shift, combined with a large two-photon excitation cross section has proven to be advantageous as it allows for the simultaneous excitation of common fluorophores, which have quite separate single-photon excitation. A number of proteins display an intrinsic autofluorescence when exposed to two-photon excitation. The autofluorescent properties of NAPDH has been used to study cellular metabolism. In our work, and our collaborators, the endogenous auto-fluorescence of kidney proximal tubules cells allows for ease of identification. Two-photon excitation can also lead to second harmonic generation (SHG), a process of non-linear light scattering which can be used for imaging. SHG occurs when certain proteins, such as those within the extracellular matrix, simultaneously interact with multiple photons to produce radiation at half the wavelength of the incident light.

Standard labelling techniques used in microscopy, such as fluorophore-tagged antibodies, cannot be used in vivo due to the risk of interfering with biological processes. Dyes and labels for in vivo imaging need to be inert, bright and deliverable. Fluorescently conjugated dextran, a hydrophilic polysaccharide, is an often used in vivo imaging marker. Due to their uncommon poly-linkages (α-D-1,6-glucose), dextrans are resistant to most cellular glycosides, are considered biologically inert, and have low immunogenicity. Dextrans of different molecular weights have been used as bulk-fluid phase markers as well as for visualisation of epithelial cell endocytosis. The DNA stain Hoechst 33342 can be used to label cellular nuclei in vivo, but can only be used for short-term experiments due to its DNA binding properties. Delivery possibilities of the chosen dyes and labels for MPM live animal imaging must also be taken into account. Dyes need to be deliverable to the site of interest either via the blood stream or if that is impractical using techniques such as micropuncture. The development of transgenic animals expressing fluorescently tagged cells is another useful tool for live animal imaging.

1.6.2.2 Limitations of MPM

While MPM provides many great advantages for live animal imaging, there are of course drawbacks and limitations which must be taken into account. While MPM can reduce the overall levels of photodamage, it has been shown that rather than an intensity-squared relationship between excitation power and the photobleaching rate, multiphoton excitation may demonstrate higher order photobleaching (< 2) at the focal site. Pigmented tissues
which contain single photon absorbers such as haemoglobin or melanin can suffer from linear absorption of the infra-red excitation light which can result in some level of tissue heating \(^{147,148}\). During our own experiments we have found that frequent imaging of a single site can sometimes lead to subtle changes in tissue homeostasis. The effects we observed were modest changes in the cellular endocytosis pathways (unpublished data), effects which are very difficult to see without specific markers. The speed of image capture can also be considered a limitation depending on experimental aim. A multi-focal, multiphoton set-up, in which the incoming laser-beam can be split into numerous separate beams, is an improvement upon the relative slow scanning speed of a single beam MPM \(^{149,150}\). The recently developed TriM microscope works to overcome speed issues by parallelizing the excitation process, increasing the number of fluorescence photons per time unit without increasing photo damage \(^{151}\). These limitations and drawbacks need to be carefully considered and controlled during the experimental process.

### 1.6.2.3 Live Animal Models

When choosing a model animal for infection work there is many factors which need to be taken into account. Suitability of the model in terms of ease-of-use, manipulability and species specificity is important. In the design of a MPM study accessibility to the organ of interest is another consideration. Organs which can be easily accessed or exteriorized without affecting structure and function are ideal \(^{138}\). Physiological processes such as respiration, peristalsis and heart beat can produce movement artefacts which can limit the usability of some organs. These issues can often be overcome through innovative surgical and experimental techniques. Organs such as the brain, kidney, lymph nodes and even gut are established intravital imaging models \(^{15,130,138,141,152-155}\), while other organs such as the liver and spleen are fast catching up \(^{156-158}\). The physiological state of the animal is also crucial for the success of a study. An anesthetised animal has difficulty maintaining its body temperature and this must therefore be monitored externally. In the kidney a drop in temperature of only a few degrees can reduce the renal blood flow, GFR and other renal functions \(^{159}\). The maintenance of blood pressure and hydration are also crucial when studying an organ such as the kidney, some researchers have used continuous infusion of isotonic saline \(^{138}\) whereas others have used donor plasma to address this issue \(^{160}\). The live animal model also enables the study of systemic and remote responses to a local infection, giving a more complete picture of infection. The presence of all physiological factors, such as the vascular, lymphatic and nervous systems in the live animal model means the influences and interplays of these systems can be accounted for.
2. AIMS
The overall aim of my thesis work was to explore the pathogenesis of UPEC infection in the kidney. To allow this evaluation to take place in the ‘natural’ infectious environment of the living organ we developed an in vivo real-time imaging model based on MPM microscopy. The aims of this work progressed alongside the results with the initial aims of being able to visualise the bacteria quickly evolving into questions regarding the complex interplay between the bacteria and physiological injuries which contribute to the full pathogenesis of renal infection.

I. Development of a system to visualise bacterial infection in the living kidney
II. Understand the relevant time-frames of an in vivo renal infection
III. To investigate the causes and effects of vascular dysfunction following renal infection
IV. To understand the roles of UPEC virulence factors during infection
V. To study the associations between infection and physiological injuries
3. RESULTS AND DISCUSSION

3.1 PAPER I - A REAL TIME MODEL OF PYELONEPHRITIS

Paper 1 describes the establishment of a MPM based live animal infection model and results seen using this technique. The MPM based renal physiology model had previously been established and used successfully by our collaborators in Indianapolis among others\(^{139,161}\). To adapt this technique to the study of infection we needed to be able to visualize the bacteria. For this we required a stable, bright fluorescent strain which could be visualised \textit{in vivo} without the need for antibiotic selection pressure. The fully sequenced clinical pyelonephritic strain CFT073 made an ideal candidate for this genetic work\(^{55}\). Our system was based on an enhanced version of the green fluorescent protein called GFP\(^{+}\)\(^{162}\). This variant carries the GFP\(_{uv}\) mutations F99S, M153T, and V163A along with the EGFP mutations F64L and S65T, resulting in better folding of the protein and an increase in the detection sensitivity of 320-fold compared to the wild-type GFP\(^{162,163}\). It was important to find an area on the CFT073 genome in which we could insert GFP\(^{+}\) without causing downstream polar effects or disrupting gene expression. The \textit{cobS} gene of CFT073 was chosen as this gene, which in \textit{Salmonella typhimurium} is involved in vitamin B12 synthesis\(^{164}\), is truncated and therefore inactive in \textit{E. coli}. Initial experiments were carried out using the gene encoding the promoter of the ribosomal protein RpsM\(^{165}\) to drive GFP\(^{+}\) expression. This promoter however did not show consistent expression during ascending infections (unpublished data) which may be due to variations in bacterial multiplication during infection affecting ribosomal protein expression. To overcome this issue we used the P\(_{\text{LactO-1}}\) tetracycline promoter cloned in the absence of its repressor (BD Bioscience, CA, USA). The resulting CFT073 \textit{gfp}\(^{+}\) strain, LT004, gave us consistent GFP\(^{+}\) expression in both \textit{in vitro} and \textit{in vivo} (Paper I, Fig. 1).

To allow for imaging, the left kidney of the rat model was gently exteriorize and placed in a cover-slip bottom plate mounted on the microscope. The plate was filled with body temperature isotonic saline. MPM imaging of infections which had ascended from bladder inoculations 4 days previously established that this construct was suitable for MPM live imaging (Paper 1, Fig 2.). The ascending infection model was however unsuitable for imaging of infection progression due to a lack of spatial and temporal resolution. To control both the spatial and temporal aspects of the infection we used a kidney micropuncture technique\(^{166,167}\) in which the bacteria or sham PBS was infused directly into the lumen of superficial proximal tubules. To allow for macro identification of the infection site, nearby tubules were infused with a heavy mineral oil. For microscopic identification of the infected tubule, a 10 kDa cascade-blue labelled dextran was co-injected with the bacterial suspension. This dextran was rapidly endocytosed by the proximal tubule epithelial cells, resulting in a clear lining of the infused tubule. Approximately \(10^5\) CFU of bacteria were infused at a rate corresponding to average filtrate flow (~40nl/min). This technique allowed for the visualization of infection beginning...
within an hour of the first bacteria-host interaction. To visualise kidney structure and the vasculature flow with the MPM microscope fluorescent labels were delivered via a venous cannula. Hoechst 33342 is filtered by the kidney and labelled all cell nuclei, allowing for ease of identification. A 500 kDa tetramethylrhodamine labelled dextran was used to visualise the vasculature. This dextran is too large to be filtered by the glomerulus and therefore remained in the blood, labelling the plasma phase.

Initial work with the MPM-microinfusion model determined that the relevant time-frame of infection for studying early tissue and bacterial events was far shorter than previously appreciated. The entire infection process appeared to be complete within 22 h with the infiltrating neutrophils clearing the infection causing oedema. Many host tissue responses could already be identified within the first 8 h. Bacterial colonisation expanded from very few bacteria at 1 h post-infusion to colonisation of the entire tubule lumen within 5-8 h. (Paper 1, Fig 3). The infection was also found to be highly localized with surrounding tissue functioning normally.

The beauty of the live animal infection model is that it allowed us to identify numerous dynamic physiological changes occurring in the tissue within these first hours of infection. One of the more eye-catching results was the rapid loss of blood flow in the peritubular capillaries surrounding the infected tubule (Paper 1, Fig 4). Together with other vascular events, such as perivascular leakage under the renal capsule, this showed that the infection was rapidly affecting the vasculature despite bacteria being maintained within the tubule epithelium. The initiation of immune cell recruitment could also be seen in this
model. Large black silhouettes in the blood stream could be seen as early as 4 h. Due to the lack of intravital dyes to specifically label incoming immune cells it was difficult to ascertain exactly which cells arrive at the site first. Analysis of tissue at 8 h post–infusion with ex vivo staining and confocal microscopy revealed that while neutrophils had arrived at the site, other unidentified mononuclear cells were also present. Neutrophils could also be identified in the tubule lumen, having passed both the endothelial and epithelial barriers (Paper I, Fig. 5).

The spatio-temoral control of the infection model also allowed for the dissection of the specific infection site for mRNA expression analysis. The specificity of the dissection meant that the infection signals were not as heavily diluted by healthy tissue as is the case with other models. This meant we could identify mRNA signals from both the host, showing up-regulation of numerous inflammation markers, and also from the bacteria, showing for the first time in vivo expression of the exotoxin HlyA (Paper I, Fig. 5). To study the effects of this toxin in this model an isogenic hlyA strain of LT004, named LT005, was used. This strain showed similar colonization kinetics to LT004, but the vascular response was significantly delayed (Paper I, Fig. 6). This data indicated that the HlyA toxin was active in vivo and strains expressing it elicited a more rapid host response. This finding fit well with previous work from our group showing that HlyA induces calcium oscillations in renal tubule epithelia cells resulting in increased expression of the pro-inflammatory cytokines IL-6 and IL-8 68. In conclusion this paper set the stage for future work, establishing the MPM based model and focussing our interests on the first 8 h of infection.

3.2 PAPER II - EPITHELIAL INFECTION INDUCES CLOTTING

Paper II followed-up on the initial finding reported in Paper I that bacterial infection induced a rapid stoppage in peri-tubular vascular flow in the immediate vicinity of the infected tubule. In Paper I we speculated that the cause of the dysfunction was congestion of the blood vessels, possibly by infiltrating immune cells. Due the very short time-frame of dysfunction (3-5 h) however we wanted to further clarify the causes and effects of vasculature shutdown.

Each kidney nephron has an intertwined vasculature, as discussed earlier, which is linked to a single glomerulus 19,99. It therefore stood to reason that the response we were seeing may have been related to shut-down of the glomerulus linked to the infected nephron. To investigate this we switched to Munich Wistar rats, a strain selected for their surface localized glomeruli 168. Bacteria were infused into the lumen of the S1 segment of a proximal tubule located close to a surface glomerulus. This meant that we could image the peri-tubular and glomerular capillaries of an infected nephron simultaneously. These experiments unexpectedly revealed that vascular dysfunction following infection was initially restricted to the peri-tubular capillaries with glomerular capillaries functioning well up to 8 h post-infusion (Paper II, Fig. 1). This implied that the vascular response occurs as a result of a local signalling event.
We then investigated the effect of this vascular dysfunction on renal tissue oxygen tension (PO$_2$). While it was reasonable to assume that vascular dysfunction would lead to a drop in PO$_2$, the limited area of the dysfunction implied a possibility of O$_2$ diffusion from other parts of the tissue. The progressing infection was monitored using Clarke-type microelectrodes, which measure dissolved oxygen \(^{169}\). A striking drop in PO$_2$ was seen within 10 min of infection and plunged to 0mm/Hg within 3.5 – 4 h. This drop was more dramatic than expected and we speculated as to the cause of this apparent O$_2$ consumption. The two obvious candidates were the bacteria themselves and the proximal tubular cells (PTC), as at this early time point they are the only cells in contact with the bacteria. The bacteria were not shown to consume any measurable O$_2$. Primary cell preparations revealed that PTC increased their oxygen consumption levels within 1 h of infection and up-regulated expression of the pro-inflammatory cytokines TNF-\(\alpha\) and IL-6 (Paper II, Fig. 2). These results suggest a role for PTC as a first line responder to infection triggering a state of ‘hyper-metabolism’ consuming O$_2$ and initiating pro-inflammatory signalling.

The combination of the PO$_2$ drop and blood flow shut-down indicated an ischemia/hypoxic tissue injury. Renal ischemia causes extensive tissue damage as discussed earlier. Confocal \textit{ex vivo} imaging of the tissue actin cytoskeleton revealed actin rearrangement, including loss of PTC microvilli, in the infected tubules indicative of ischemic injury (Paper II, Fig. 3). Ischemic actin rearrangement is normally followed by integrin rearrangement which disrupts PTC-extracellular matrix (ECM) interaction \(^{103,104,108}\). Evidence of this could be seen in the breakdown of the cascade-blue dextran labelled epithelial lining, and the appearance of rounded blue labelled cells in the tubule lumens (Paper II, Fig. 3). Further evidence of integrin re-arrangement and epithelial breakdown could be seen with \textit{ex vivo} confocal imaging, showing bacteria located para-cellularly as well as on the basal sides of the PTC. Despite the epithelial breakdown however bacteria were initially maintained within the tubule space. This was shown to be due to an intact tubular basement membrane, visualised by \textit{ex vivo} collagen IV staining (Paper II, Fig. 4). The basement membrane appeared to act as a hindrance to immediate bacterial spread, but did not completely prevent bacterial spread. By 24 h the basement membrane was also destroyed along with other tissue structures. It would be of interest to investigate what factors lead to the basement membrane break-down, is it a bacterial action, or is it infiltrating PMNs which compromise its integrity? In all likelihood it is a combination of factors which contribute to basement membrane breakdown and tissue destruction.

The effects of blood vessel dysfunction and ischemia could be seen within the tissues in a number of ways as described above. We then investigated the causes of this dysfunction. Analysis refuted immune cell occlusion as the causative factor with vasculature shut-down occurring prior to the arrival of immune cells. Areas in which the vessels were in the process of shutting down were identifiable by ‘plasma streaming’ where the red dextran fluid phase marker did not contain any RBCs. Looking into these areas revealed
large black masses within the vessels accompanied by numerous smaller silhouettes. These silhouettes were too small to be RBCs and it was proposed that they were in-fact platelets (Paper II, Fig 5A). This pointed to the possibility of a blood clot. RNA expression analysis from infected areas revealed the up regulation of several members of the clotting cascade, supporting this hypothesis (Paper II, Fig. 5B). These findings indicate that within hours of infection, while bacteria are still within the tubule lumen, signalling across the epithelial-endothelial barriers triggers initiation of clotting. The exact signal cascade is still unknown but is an obvious line for further research.

To investigate the physiological reason for the clotting we tried to disrupt the process by treatment with the anticoagulant heparin. Treatment delayed the onset of clotting and peri-tubular dysfunction by a few hours (Paper II, Fig 6). This delay was sufficient to allow a systemic spread of bacteria in all treated animals. These animals displayed a sudden drop in blood pressure followed by death within 8 h of infusion. Post-mortem examination revealed high CFU counts in the blood, heart, liver and spleen. The bacteria recovered were expressing GFP+, confirming they had originated from our infusion. Taken together this data suggests the initiation of clotting and the shut-down of peri-tubular capillaries functions to isolate the infection and prevent systemic spread. The subsequent tissue damage caused by the ischemia may be seen therefore as ‘collateral damage’.

The idea of isolating an infection via coagulation is not a new one, an oft-cited example demonstrating a primitive example of coagulation and isolation is that of hemolymph coagulation in the horse-shoe crab following injury and infection. There is also much information regarding the effect of injury and infection on the mammalian coagulation system. The difference between this body of work and our own is that the clotting in our system occurs in the absence of any obvious endothelial injury and without direct interaction between the bacterial pathogen and the blood. This implies a signalling cascade initiated at the epithelium which triggers endothelial activation and clotting, leading to isolation of the infection site. In conclusion this paper outlines a novel mechanism by which the mammalian system can isolate an epithelial infection by shutting down blood supply to the area. This appears to prevent systemic spread but does result in local tissue damage.

3.3 PAPER III - P AND TYPE 1 FIMBRIAE ACT SYNERGISTICALLY
The aim of Paper III was to investigate the in vivo microbiological behaviour following infection and study the effects of specific UPEC adhesion factors. Initially we were interested in investigating how bacterial attachment organelles functioned in vivo to allow bacteria to bind to the kidney tubule. The kidney tubule environment is hostile for a number of reasons but distinctively due to the mechanical stress of primary glomerular filtrate flow. Firm attachment to the epithelium is therefore crucial to renal UPEC colonisation. As mentioned in the introduction the renal epithelium is far from uniform with different segments of the nephron presenting very different epithelial characteristics. UPEC has been reported to attach throughout the nephron with the exception of the
glomerulus. The glomerulus, long promoted as the site of bacterial translocation to the blood stream, is rarely found to be infected in patient biopsies and we have not seen it in our experimental ascending UTI models (unpublished data). We hypothesised that this may be due to an inability for UPEC to bind to the flat squamous epithelia in vivo. Direct infusion of UPEC into this site however revealed that they can bind and colonise the glomerular capsule (Paper III, Fig. 1). This indicates that binding is possible in the glomerulus and another form of defence must exist which prevents glomerular colonisation in vivo. One strong candidate is expression of antimicrobial peptides such as cathelicidin.

From here we went on to investigate the roles of the UPEC attachment organelles, specifically Type 1 and P fimbriae and their role during infection.

Expression analysis of mRNA excised specifically from infection sites at 8 h revealed expression of both Pap_A2 and FimA, the major structural subunits of the fimbriae (Paper III, Fig. 2). This suggested that both fimbriae were being expressed and therefore relevant in vivo. A library of bacterial mutants was created which included K-12 strains as well as isogenic mutants of the CFT073. Real-time analysis of these strains within the live model gave some interesting results. The GFP+ expressing K-12 lab strain ARD42, which only expressed Type 1 fimbriae, did not colonise with the same initial kinetics as LT004 but was unexpectedly able to form the characteristic local tissue oedema at 24 h. Addition of plasmid-borne P fimbriae expression (creating ARD43) greatly enhanced the initial colonisation of K-12, allowing it to attach and multiply with similar kinetics to LT004. Despite the high bacterial loads the host response, in the form of vascular shut-down, was muted following infection with this strain (Paper III, Fig. 3). This indicated that P fimbriae does enhance initial attachment in vivo as well as showing that the host does not respond as quickly to K-12 strains of E. coli.

Using isogenic mutants of LT004 we could confirm that P fimbriae expression enhanced early colonisation, with the ARD41 strain, lacking PapG mediated binding, struggling to establish infections in a majority of infusions. ARD41 and ARD43, both lacking P fimbriae mediated attachment were both seen to be 'flushed' through the tubules along with the glomerular filtrate (Paper III, Movie S1). This suggested that P fimbriae mediate epithelial attachment in vivo and helps UPEC withstand filtrate flow. From this respect we can begin to think of P fimbriae as a ‘fitness factor’. We and many others have shown that P fimbriae expression is not essential for renal infection, and similarly many clinical isolates lack it. What we show is that there is an advantage to its expression within the kidney, helping UPEC to bind and colonise more rapidly, helping them gain a better foot-hold before the onset of ischemia and immune cell infiltration.

The majority of studies on UPEC Type 1 fimbriae have focussed on its role in bladder infection. Unsurprisingly ARD40, lacking FimH mediated binding, as able to attach and colonize in the early hours of infection, due to their P fimbriae expression. It was only at later time-points of infection, when the bacterial colony was filling the tubule lumen did any alteration become apparent. ARD40 struggled to colonise the central parts of the tubule.
lumen, forming a tube-like community around the epithelial linings (Paper III, Fig 3E-F). Type 1 has previously been implicated in biofilm formation and particularly when under the pressure of shear stress. We used standard *in vitro* biofilm assays to test the ability of ADR40 to produce biofilm. We could see that, in comparison to LT004, it had significantly reduced biofilm forming capabilities (Paper III, Fig. 4). This suggested that a role of Type 1 fimbriae and particularly FimH during renal infection involves inter-bacterial binding and biofilm formation within the centre of the tubule. This biofilm would allow bacteria to colonise away from the epithelia as well as helping them withstand the sheer stress of filtrate flow.

In combination it became apparent that P and Type 1 fimbriae functioned in synergy to withstand the hydrodynamic nature of the kidney tubule and enable colonisation. P fimbriae enhanced epithelial adhesion while Type 1 fimbriae mediated luminal colonisation. While the selection pressure of filtrate flow was apparent, we could also show an adverse influence of this heterogeneous bacterial community on filtration. We used small molecular weight dextrans to study nephron filtration (Paper III, Fig. 5, and accompanying Movies S3 and S4). By tracing the intensity of filtered dextran we could see that bacterial infection rapidly reduced filtration. By 8 h no dextran was filtered through the infected nephron, demonstrating how bacterial infection causes an obstruction injury.

This paper outlined some more specific roles for the bacterial adhesions P and Type 1 fimbriae during *in vivo* renal infection. It showed that they work in synergy to enable optimal colonisation. The formation of a biofilm within the tubule allows bacteria to withstand filtrate pressure but also functions to obstruct the nephron. Obstruction, like ischemia, induces tissue damage and inflammation. Obstruction can therefore be added to ischemia as a contributing factor to the overall pathophysiology of pyelonephritis.
4. CONCLUSIONS AND PERSPECTIVES

The papers which make up my thesis describe novel physiological and microbiological facets of kidney infection. It has opened up numerous new lines of research as we could finally study infection in its ‘natural habitat’ with all the accompanying physiological factors intact. To do this we developed an MPM-based live imaging platform to follow a live bacterial renal infection. One of the biggest advantages of the model has been to see the dynamic interplay between bacteria and host and the way in which each influences the outcome of infection. It is hoped that as this technology and others like it become more wide-spread in infection research that these interactions and their consequences will become fully understood.

4.1 THE PHYSIOLOGICAL RESPONSE

The study of UPEC infection within the live kidney has revealed various physiological responses to infection. One of the first significant findings related to the rapidity of the response with a majority of events occurring within the first 22 h of infection. Early changes occurring in the tissue included coagulation, epithelial breakdown, vascular leakage, immune cell recruitment and tissue destruction. Many of these events can be considered contributors to the umbrella terms ischemia and obstruction. Renal ischemia and obstruction are both well-studied physiological injuries and it is known that both cause inflammation and tissue destruction in their own right. Both are multifactorial and can vary in severity. Severe ischemia or obstruction can lead to end stage renal failure, as can pyelonephritis. What this works reveals is that the pathophysiology of pyelonephritis is in fact a combination of infection and physiological injuries such as ischemia and obstruction. It is with this in mind that we can begin to look at new treatment options. One idea from this work is that the treatment of a pyelonephritis patient with anticoagulants may in fact increase bacterial translocation into the blood stream. Anticoagulation therapy is an important aspect of sepsis treatment but in mild cases the risk of systemic coagulation may need to be weighed against increased bacterial spread. In terms of antibiotic treatment the findings of both ischemia and obstruction are relevant. As is well demonstrated in Paper III, Fig. 5C and movie S3, within 8 h of infection no blood or filtrate is entering the infection site. The site is completely isolated, which does not allow for antibiotic delivery. What these findings suggest is that we may need to look into other areas of treatment. We can see that the host immune response is in most cases well designed to handle this small infection. Perhaps new treatments need to look into ways to enhance this response, particularly in immune compromised or severely infected patients.

This work has opened up new avenues of investigation regarding how the host responds to infection. We have moved away from the classical ‘Cellular Microbiology’ model of bacteria meets host cell and begun to identify the comprehensive tissue responses
to infection. Studying infection in this ‘live’ environment is currently the only way to study these responses and as these techniques become more wide-spread greater understanding of the host-bacterial interplay will lead to better treatment.

4.2 THE MICROBIOLOGICAL RESPONSE

Bacteria migrating from other areas of the body into the urinary tract must adapt rapidly to this challenging and active environment. This environment also changes dramatically during the course of infection as the host responds. In this thesis we have looked at the roles certain bacterial virulence factors play in vivo. What we have found is that factors which have been well-described in previous models display unique features when studied in real-time under the dynamic pressure of the live kidney. In Paper I expression of the toxin HlyA affected the kinetics of the host response. This possibly links back findings showing how HlyA induces calcium oscillations in PTC, inducing cytokine production 68. Paper I therefore identifies the in vivo consequences of these in vitro findings, an un-expected effect on host vasculature. Paper III also described new roles for well-studied virulence factors, particularly revealing an important role or Type 1 fimbriae in kidney infection. This work was also able to show a difference between colonisation with and without P fimbriae expression, showing a fitness advantage which may be undetectable in other infection models. A synergy between the two fimbriae appeared to aid efficient colonisation of the tubule. The temporal resolution of an infectious niche was highlighted, with the interior and periphery of a single tubule lumen exerting different adaptation pressures. This work shows how bacteria can very rapidly adapt to the changing environment around them and how they have developed mechanisms to withstand numerous physical and mechanical defence mechanisms.
5. FUTURE PERSPECTIVES

This work has opened up many new pathways of investigation. The MPM-based infection model allows for an ‘all-inclusive’ investigation of infection. The application of these types of models will allow for high-resolution studies of the interplays of numerous hosts and pathogens. In the specific case of UTI, this work has brought up as many new questions as it has answered to date. Possible lines of investigation stemming from this work include:

- Elucidation of the signalling cascade and mechanism behind the initiation of the clotting cascade following infection
- Mechanism behind the immediate drop in PO₂ following infection
- What role does the basement membrane play in bacterial containment and by which mechanism is it breeched?
- Is the ischemic response to infection organ or pathogen specific?
- What role do other fimbriae play during renal infection?
- By what mechanism do UPEC enter the blood stream in urosepsis cases?
- Do biofilms play a role in infection of other hydrodynamic niches?

As can be seen there is much work still to be done. It is hoped that as these live animal models become more common, many of these questions will be answered. Answers to these questions would help in the development of new treatment regimes, regimes which take into account both the microbiological and physiological responses to infection.
6. **POPULAR SCIENCE ABSTRACT - ENGLISH**

Bacterial infection is an ongoing problem in today’s society. Despite many years of research and advancements in sanitation and treatment, these infections are still a major public health issue. Understanding the details of what happens when a bacteria enters and begins to infect the body is crucial for finding new ways of dealing with these problems. In this work we have investigated what happens during the first hours following bacterial infection of the kidney. Kidney infection occurs when a bacteria enters the urinary tract and migrates up through the bladder to the kidneys. Kidney infection is medically termed pyelonephritis and can lead to many serious consequences such as scarring of the kidneys and possibly even kidney failure.

To investigate these details we developed a new type of experimental model in which we use a special microscope to watch what is happening in a kidney of an anesthetised rat which has been injected with bacteria. This technology allows for a type of ‘infection reality-show’ as we watch how both the bacteria and the kidney respond to the infection. A unique aspect of this model, compared to many previously used, is that by using a live animal we can see the influences of dynamic physiological processes such as blood flow and the filtration of the kidney.

This work shows how the kidney responds to infection by identifying the bacteria and then sending signals to other cells to help protect itself and eliminate the bacteria. Some of these signals were shown to lead to clotting in the blood vessels around the infection. This clotting stopped the blood flow in the area to prevent bacteria passing into the blood stream and spreading throughout the body. This clotting does however cause injury to the kidney itself in the form of ischemia, a loss of vital oxygen and nutrients normally supplied by the blood stream. Another aspect we saw was how the mass of bacteria which could grow in the kidney prevented the flow of primary urine. This type of injury, when the kidney is prevented from filtering normally, is known as obstruction. Both obstruction and ischemia cause damage to the kidney tissue and this work shows how these injuries contribute to the overall damage caused by bacterial infection.

The bacteria were also shown to respond in a different way when inside the living kidney as opposed to what has previously been reported in the laboratory. The bacteria have numerous ‘hair-like’ structures on their outside, called fimbriae, which allow them to bind to the kidney cells. What we show is how two specific types of these fimbriae, the P and Type 1 fimbriae, work together to allow the bacteria to bind and grow in the kidney despite the fact primary urine is continually flowing past them. The ability to follow the progression of an infection in real-time has allowed us to identify new responses from both the bacteria and kidney. It has also allowed us to see the interplay between the two, with kidney filtration affecting which fimbriae the bacterial express and the bacterial growth conversely affecting the flow of filtrate. It is hoped that appreciation of these interplays can lead to a overall better understanding of pyelonephritis.
7. MY SCIENTIFIC CONTRIBUTION

Short of re-writing my abstract, I would like to think that this thesis contributes to the understanding of the dynamic interplay that occurs between bacteria and host during an infection. An ‘all-inclusive’ investigation of the first hours of infection has been made possible through a combination of technologies from both the visualisation and physiology fields. Collaborations with specialists from different fields such as renal physiology has been essential to the findings presented here. Aside from the specific findings related to vasculature, filtration and bacterial virulence factors I hope this work helps broadens the perspectives of the players, timeframes and dynamics involved in bacterial pathogenesis. Particularly I hope it can lead to a more wide-spread appreciation of the influences of physiological processes on infection. This work has led to the recognition many ‘new’ aspects of the pathogenesis problem and as the adage states ‘recognizing the problem is half the battle’.

Science is always wrong. It never solves a problem without creating ten more.

~George Bernard Shaw
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