The role of the bladder microbiome in lower urinary tract disorders.

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DECLARATION

I certify that this work has not been accepted in substance for any degree, and is not concurrently being submitted for any degree other than that of Doctor of Philosophy being studied at the Universities of Greenwich and Kent. I also declare that this work is the result of my own investigations except where otherwise identified by references and that I have not plagiarised the work of others.

Signed…………………………………..

Louise Krska

October 2017

"Success is a journey, not a destination"

For Mrs G Brown, Dr J Krska

and Elizabeth

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To my wonderful, beautiful mum, who convinced me to do a PhD and helped me through it every day; thank you for being my inspiration. And to the rest of my family for your support, love and patience while I continued to bum around as a student!

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And last but not least to Grandma Barnsley, to whom I never got to say goodbye to but this is for you. I hope I would have made you proud.

ABSTRACT

Lower urinary tract symptoms (LUTS) cumulate a multitude of syndromes and urinary tract infections (UTI). This thesis focuses principally on recurrent UTIs (rUTIs) in renal transplant recipients and overactive bladder (OAB). In many cases, the cause of OAB is unknown. Recent studies in bladder microbial investigation have proposed that low-level bacteriuria or alterations in the bladder microbiome may be a cause for the symptoms of this syndrome. This thesis sets out to investigate the types of bacteria in the bladder of women with OAB and renal transplant recipients compared to asymptomatic controls by culturing urine samples and using 16s rRNA gene sequencing to identify exact species in each sample. The data is analysed alongside clinical data to determine better biomarkers for both rUTI and OAB. The results highlight that current hospital tests for infection are underreporting low-level infection and many of the biomarkers for infection do not correlate with incidence of infection. Here, the use of clue cells as a biomarker for rUTI is investigated for the first time with optimistic results. Furthermore, the alterations of the bladder microbiome over time post-renal transplant highlight consistently reduced incidence of particular species indicating shifts in the bladder microbiome in these patients. Using similar methods to explore the microbiome of women with OAB, it was found that there is a significant reduction of *Lactobacillus*, a crucial probiotic in the urogenitary tract that may be key to shifts in the bladder microbiome in OAB. The final section attempts to explore the physiology behind the symptoms of OAB in light of recent findings associating bacteria to OAB by looking at pericytes (a peri-vascular cell type found on capillaries). Pericytes are capable of transdifferentiating into myofibroblasts, a fibrotic cell which may increase spontaneous contraction in the bladder. This section investigates pericytes behaviour in the bladder in response to ATP, angiotensin, lipopolysaccharide and pro-inflammatory cytokines in a series of DIC imaging and immunohistochemistry experiments. The data supports this theory since pericytes constrict vessels upon acute stimulation and pericyte numbers dwindle with prolonged exposure. In conclusion, this thesis highlights the importance of correct testing for bladder infections and suggests that shifts in the bladder microbiome may be a cause for some lower urinary tract symptoms and this could be caused by pericyte responses to ATP and downstream cytokines.

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αSMA	Alpha-smooth muscle actin
ACh	Acetylcholine
ANG II	Angiotensin II
ATP	Adenosine 5'-triphosphate
BTX-A	Botulinum neurotoxin A
CBA	Columbia blood agar
CFU	Colony forming unit
c-KIT	Receptor tyrosine kinase
CNA	Colistin and nalidixic acid agar
CNS	Central nervous system
CSU	Catheter sample urine
DAPI	4',6-diamidino-2-phenylindole
DO	Detrusor overactivitiy
EM	Electron microscopy
EQUC	Enhanced quantitative urine culture
GAG	Glycosaminoglycan
IB4	Isolectin B4
IBC	Intracellular bacterial colonies
IC	Intersticial cystitis
ICAM-1	Intercellular adhesion molecule 1
ICC	Intersticial cells of Cajal
IDO	Idiopathic detrusor overactivity
LPS	Lipopolysaccharide
LUTS	Lower urinary tract symptoms
MALDI-TOF MS	Matix-assisted lazer desorption/ionisation - time of flight mass spectrometry
MHC	Majoh histocompatibility complex
MSU	Midsteam sample urine
NG2	Neuroglial antigen 2
NGF	Nerve growth factor
NKA	Neurokinin A
NO	Nitric oxide
OAB	Overactive bladder
PDGF-β	Platelet-derived growth factor receptor beta
PGE-2	Prostaglandin E2
PI	Propidium iodide
RTR	Renal transplant recipient
rUTI	Recurrent urinary tract infection
SEM	Scanning electron microscopy
SUI	Stress urinary incontinence
TEM	Transmission electron microscopy
TLR	Toll-like receptor
TRPV	Transient receptor potential (vanilloid)
UPEC	Uropathogenic *Eschericia coli*
UTI	Urinary tract infection
UUI	Urge urinary incontinence
vSMC	Vascular smooth muscle cell

CHAPTER 1 -
GENERAL INTRODUCTION

1.1 OVERVIEW OF THE BLADDER

The bladder is a spherical organ that functions to store urine produced by the kidneys and subsequently eliminate it from the body. It is situated in the abdomino-pelvic cavity and is connected to the kidneys via ureters that enter the bladder in the trigone area. Once the bladder reaches a volume of approximately 300–400 ml, a sense of fullness begins, however the voluntary signal to begin voiding can be withheld until a suitable time and place when urine is then voided in a process known as micturition, via the urethra.

The function of the bladder is strictly regulated by molecular signalling, receptors, nerves and muscles. In many lower urinary tract symptoms, such as overactive bladder (OAB), this function has become disrupted leading to spontaneous uncontrolled activity. Currently, the exact cause of many cases of OAB is unknown, however many investigations have highlighted a possible role for specific cells found within the bladder such as interstitial cells of Cajal (ICC) (1), myofibroblasts (2), and pericytes (3). Furthermore, recent investigations into low-level infection within the bladder have resulted in the identification of a 'core' bladder microbiome (4), which may also play a valuable role in healthy bladder function. This introductory chapter shall discuss i) the structure of the bladder with highlights on the fore mentioned cells of interest, ii) the normal function of the bladder and how it is controlled, iii) various symptoms of bladder dysfunction with a highlight on OAB, iv) urinary tract infections and the role of the bacteria in OAB, and v) the bladder microbiome and its role in bladder dysfunction.

1.2 THE STRUCTURE OF THE BLADDER

The bladder is formed of multiple notably unique layers of different cells with differing functions, consisting of the innermost urothelium, followed by the basal membrane, the lamina propria, the detrusor muscle, a perivesicle fat layer, and finally the peritoneum (figure 1.1). Since the fat and peritoneum have little reported

importance in the function of the bladder, these shall not be discussed further. The urothelium comprises of superficial or umbrella cells, intermediate and basal cells. The umbrella cells are in the closest proximity to the bladder contents and as they are exfoliated, are replaced by those in the intermediate layer below. Urothelial cells have a half-life of approximately 3 to 6 months (5), however this is often reduced when the bladder is put under stress *e.g.* during infection, when they are shed more rapidly. The basal membrane attaches the urothelium to the lamina propria; a complexity of connective tissue containing collagen and elastin as well as a microvasculature network, sensory afferent nerves, fibroblasts, and myofibroblasts (6). The muscle layer or detrusor follows, which is innervated with somatic, sympathetic and parasympathetic nerves. The layers work in association with each other to control bladder filling and voiding via complex interactions between activator signalling molecules, receptors and nerves in both the bladder and the central nervous system (CNS). The structure and expression of markers and receptors in each layer as well as individual cells of interest in each, such as ICCs, myofibroblasts and pericytes shall be discussed first, followed by the physiological regulation of the bladder by activator signalling and the role of the CNS.
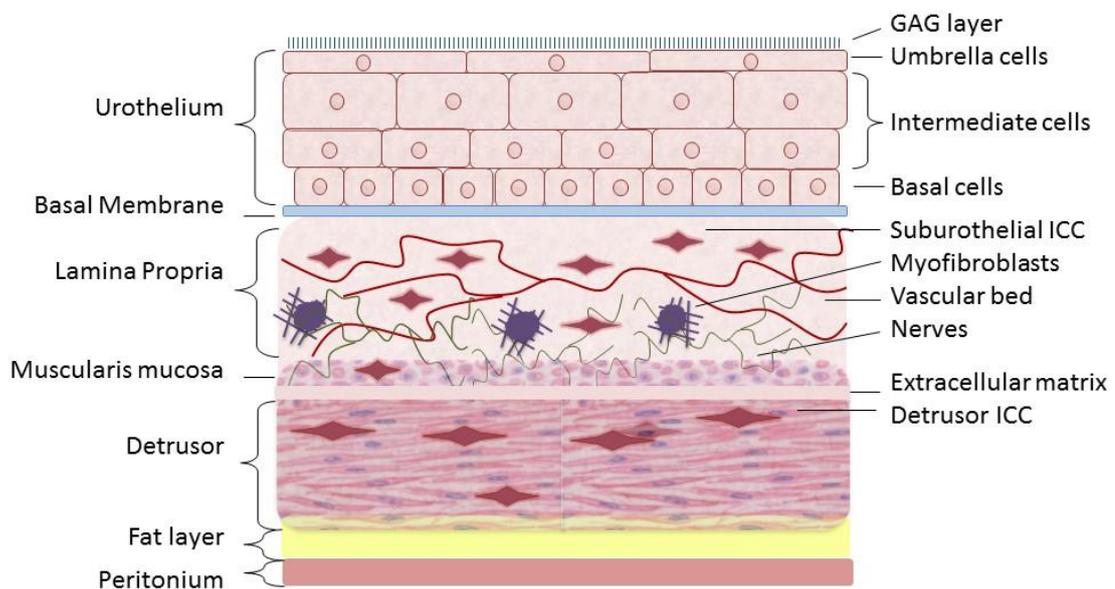
Figure 1.1: Cross section of bladder wall. The urothelial layer consisting of superficial umbrella cells, intermediate cells and basal cells. The basal membrane attaches the urothelium to the lamina propria, which consists of microvasculature, efferent nerves, myofibroblasts, suburothelial interstitial cells of Cajal (ICC), and microvasculature including pericytes. Below this a muscularis mucosa layer; a thin layer of muscle followed by the extracellular matrix and the detrusor; a thick layer of smooth muscle. The detrusor contains detrusor ICCs and is innervated with afferent parasympathetic and sympathetic nerves. The bladder is then contained in a fat layer and a peritoneum.

1.2.1 THE UROTHELIUM

The urothelium is the innermost layer of the bladder consisting of umbrella cells, intermediate cells and basal cells creating a urothelial layer. In humans there are up to 5 layers of intermediate cells however only one in mice (6). The replacement of umbrella cells occurs by fusion of basal cells into intermediate cells followed by fusion of intermediate cells into umbrella cells (5). Old umbrella cells are shed into the urine and discarded during micturition. Umbrella cells are so called due their shape and ability to open out and spread across the urothelial surface as the bladder stretches. The urothelium was historically known as a passive barrier and was generally renowned as the most impenetrable barrier in the body (7). This is due to the presence of hexagonal plaques on the umbrella cells called uroplakins, which are made up of crystalline proteins (8, 9). Tight junctions between the umbrella cells further prevents ion exchange between the urine and blood (10), and a glycosaminoglycan (GAG) layer on the outside of the umbrella cells prevents bacteria coming in too close contact with the vulnerable cell membrane (11) (figure 1.1). More recent investigations have discovered that the bladder urothelium is much more selective and sensory than previously thought. Various studies on the sensitivity of the bladder urothelium to stretch, temperature, capsaicin, pH, and various signalling molecules such as adenosine 5'-triphospahe (ATP), acetylcholine (ACh), and bradykinin, as well as infectious components such as pathogen associated molecular patterns (PAMPs *e.g.* lipopolysaccharide) and cytokines have highlighted the importance of the urothelium in sensing changes in the bladder environment during regular bladder function and disease states. Each of these individual sensory components shall be discussed in more detail in sections *1.3 Physiological regulation of the bladder* and *1.5 Bladder Infections.*

1.2.2 THE LAMINA PROPRIA

The lamina propria consists of an extracellular matrix containing multiple cell types such as adipocytes, myofibroblasts and ICC, as well as a wealth of microvasculature and nerves. The specialised cells in this region of the bladder have been shown to be vital for supporting and propagating the sensory mechanism required for healthy bladder function, therefore this section will discuss nerves, ICC

and myofibroblasts, their specific markers and how they have been associated to bladder dysfunction, particularly OAB.

1.2.2.1 SENSORY AFFERENT NERVES

Afferent nerves found in the lamina propria are most crucial for bladder function (12), and consist of myelinated fibres (Aδ) and unmyelinated fibres (C-fibres), which originate from neural cell bodies of the dorsal root ganglion. Aδ fibres are most important for sensing bladder filling (12), whereas C-fibres do not respond to increased bladder volume but are stimulated by high mechanical threshold and respond to noxious chemicals (13), and cooling by temperature or menthol (14). The sensory afferents are predominant in the trigone area of the bladder and less dense in the upper dome (15). Afferent nerves express receptors for chemical stimuli *e.g.* ATP of ACh originating from the urothelium or from proximal cells such as ICC or myofibroblasts and signal to the CNS to initiate the sensation of bladder fullness (3, 16). It is hypothesised by various research teams that in some forms of OAB these nerves are either being over stimulated by increased production of ATP or ACh (17, 18), or by the increased presence of interstitial cells in proximity to nerves (2)(discussed in sections *1.2.2.2 Interstitial cells of Cajal* and *1.2.2.3 Myofibroblasts*). Another theory suggests that increased production of NGF by mast cells, which has shown to be increased in individuals with OAB, may be leading to alterations in the autonomic innervation (19, 20)(discussed in sections *1.5.5.2 Mast cell infiltration and activation* and *1.5.5.3 Nerve growth factor and other biomarkers*).

1.2.2.2 INTERSTITIAL CELLS OF CAJAL (ICC)

ICC are found in organs innervated by peripheral nerves, in particular the intestinal tract (21) and bladder. Most ICC, but not all, are receptor tyrosine kinase (c-Kit) positive, which is the ligand for stem cell factor, and this marker is generally used to identify ICC. Staining for c-Kit positive cells in the bladder has identified ICC in the smooth muscle layers and the lamina propria, where they are named detrusor ICCs (dICC) and suburothelial ICCs (sICC), respectively (22).

In the intestine, ICCs act as a pacemaker stimulating autonomic rhythmical peristalsis by spontaneously generating Ca^{2+} transients, which produce depolarizing currents to initiate smooth muscle contraction (23). Interestingly, similar

spontaneous activity occurs in the bladder to adjust the smooth muscle during filling to accommodate for increased volume (24). There is much interest in spontaneous bladder activity as this movement can become overactive, termed detrusor overactivity (DO) and is, in some cases, the cause for OAB, a syndrome that can lead to urinary incontinence (25). Therefore, many studies have tried to identify the origin of spontaneous bladder activity with various conclusions, however one of particular solidity is the role of ICC (1, 3, 23, 26-28). The exact contribution of ICC to spontaneous activity is still debated as to whether they directly generate action potentials (26), or if they merely accentuate them (3), but it is certain that these are not contractile cells (26). An exciting contribution by Biers *et al* (1) found that numbers of dICCs are increased in individuals with OAB suggesting that if ICC are indeed the origin of spontaneous activity, then accentuation of this mechanism may be the cause of some cases of OAB (16). ICC have therefore been targeted by use of Glivec, an inhibitor of c-Kit, a marker for ICCs (27, 28) with positive results, further supporting the role of ICC in bladder dysfunction. Due to the location of sICCs, it is more likely that these cells act as signal transductors between urothelial cells and sensory nerves providing a sensory role rather than acting upon smooth muscle cells in the case of dICCs (29). The term sICCs is often used interchangeably with myofibroblasts due to the similarities of both the function and cell marker expression on these two cell types. However, the presence of certain cell markers on myofibroblasts, such as alpha smooth muscle actin (α-SMA), the multiple cell origins, and contractile properties of myofibroblasts does make these cells unique (discussed next). For that reason, myofibroblasts shall be taken as a separate cell type, unique to sICCs, and the focus shall be on this cell type rather than sICCs.

1.2.2.3 MYOFIBROBLASTS

Myofibroblasts are the principal cell form responsible for fibrosis in tissues; forming extracellular fibrotic matrices consisting of collagen, fibronectin and glycosaminoglycans in the surrounding tissue (30). Fibrosis is a physiological change that occurs after chronic injury or exposure to injury stimuli resulting in damaged endothelium/epithelium, cytokine production (predominantly tumour growth factor-beta 1 (TGF-β1)) (31), inflammatory cell infiltration (32), activation of myofibroblasts, and subsequent production of extracellular matrices (for a review see

Tomasek *et al*, (30)). The result is the thickening of tissue, scarring and, in extreme cases, organ malfunction (33). Myofibroblasts are usually derived from resident fibroblasts activated by various factors during inflammation and/or damage. Fibroblasts differentiate into proto-myofibroblasts after exposure to mechanical stress (34), and platelet derived growth factor (PDGF), which, as the name suggests, is released from activated platelets (30). These proto-myofibroblasts require TGF-β1 to become active myofibroblasts, which is released from various cell types including white blood cells and platelets, and induces α-SMA production and collagen type 1 (35, 36), making myofibroblasts phenotypically unique from fibroblasts (37). They also have actin bundles within their structure, which adhere to the fibronexus of the cell, allowing the internal structure of the myofibroblasts to be strongly attached to the extracellular fibronectin (38).

Identifying myofibroblasts in any organ is difficult due the absence of a unique marker for this cell type (table 1.1) (39). As discussed, myofibroblasts are distinctive from fibroblasts in that they express α-SMA. Unfortunately, expression of α-SMA is not unique to myofibroblasts; it is also expressed by smooth muscle cells (SMC) (40). C-Kit can be used to distinguish myofibroblasts from fibroblasts and SMCs as they are generally c-Kit negative and this marker has been widely used as a marker for myofibroblasts in mice. However, c-Kit is not expressed on all subtypes of myofibroblasts and not always in humans (41). Fortunately, myofibroblasts can be identified by other means. By using transmission electron microscopy (TEM) to identify the fibronexus, unique to this cell type, myofibroblasts have been identified in human bladder biopsies and their ultrastructure and exact location within the lamina propria determined. The images generated by Wiseman *et al* in 2003 show that myofibroblasts exist underneath the urothelial layer, in the lamina propria (42). Also noted is the proximity of these cells to nerve varicosities giving rise to speculation of the exact role of these cells in bladder function. It could be suggested that these cells, like sICC, may accentuate responses from exogenous ATP and other purines to act as an intermediary between the urothelium and afferent nerves (16, 42, 43). That is, myofibroblasts may act as an amplification step in the sensory process between the urothelium and nerves and thus may also be involved in contraction. This is further supported by the fact that increased numbers of myofibroblasts is

associated to increased spontaneous contraction (2). In respect to OAB, it has also been reported that thickening of the bladder wall is increased in individuals with OAB compared with asymptomatic individuals (44, 45), which may be due to fibrosis. Therefore, it could be suggested that increased numbers of myofibroblasts could be a cause for some cases of OAB. Recently, there has been interest in pericytes as a potential progenitor cell for myofibroblasts development alongside fibroblasts (46). As such, these cells are of equal interest but firstly however, it is important to discuss the role of pericytes more broadly, which will be discussed in the following section on microvasculature.

Cell type	c-Kit	α-SMA	PDGFRβ	NG2	Preferred method of identification
ICC	+	-	-	+/-	c-Kit
Fibroblasts	-	+/-	+	-	Vimentin
Myofibroblasts	+/-	++	++	+/-	Fibronexus by TEM
SMC	-	++	-	-	α-SMA
Pericytes	+/-	+/-	+	+	NG2

Table 1.1: Expression of receptors used to identify myofibroblasts and pericytes in the bladder. C-Kit is not unique to myofibroblasts as it is also expressed on interstitial cells of Cajal (ICC) and some pericytes (41). Alpha smooth muscle actin (αSMA) is also found on smooth muscle cells (SMC), some pericytes and fibroblasts (40). Neuro-glial 2 (NG2), the principal marker for pericytes, is also found on some myofibroblasts and fibroblasts (47). Platelet derived growth factor receptor (PDGFRB) is found on fibroblasts and pericytes and binding of PDGF assists in the activation of these cells to differentiate into myofibroblasts (47). Therefore, it is difficult to differentiate between these cells types, the preferred method is therefore suggested in the final column.

1.2.3 BLADDER MICROVASCULATURE

The microvasculature of the bladder supplies oxygen and nutrients to the bladder as well as removing metabolites and waste products. The bladder blood supply arrives via the iliac artery into the lower peritoneum and branches into the dorsal and ventral sides of the bladder and leaves by the iliac vein (48). The vasculature is predominantly found in the lamina propria but also has capillary extensions into the urothelium and detrusor muscle. The capillary arrangement in the bladder is winding so that bladder stretch during filling does not affect blood pressure or blood flow (49). The arterioles in the bladder are supported by smooth muscle cells and surrounded by nerve cells and fibroblasts. Capillaries and venules on the other hand have a thinner endothelial layer but are supported by pericytes, which likely regulate vessel diameter in these vessels replacing the need for SMCs (50, 51).

1.2.3.1 PERICYTES

Pericytes are peri-endothelial cells, which support the vasculature network and can be found throughout the body principally dotted along the microvessels such as capillaries, post-capillary venules and terminal arterioles (47). They are believed to play a valuable role in i) vasoconstriction and vasodilation in response to vasoactive compounds, ii) control of vascular permeability to both cells and molecules, iii) pluripotency, and iv) angiogenesis (each of these shall be discussed in more depth later in this section). Pericytes are derived from the mesenchymal stem cell lineage, the progenitor cell line for osteoblasts (bone cells), chondrocytes (cartilage cells), adipocytes (fat cells), and are closely related to vascular smooth muscle cells (vSMC) in that they both express αSMA, CD13, PDGFRβ, neuro-glial 2 chondroitin sulphate proteoglycan 4 (NG2), a marker for oligodendrocyte cells, and other various markers requiring further validation (47) (table 1.1). The similarity between pericytes and myofibroblasts is also evident by the shared markers αSMA and PDGFRβ. It is therefore difficult to stain for pericytes in tissue. The most effective marker is therefore, is NG2 since the structure of pericytes is significantly different from that of vSMCs so that they can be distinguished (figure 1.2A). Crawford *et al* used NG2 and isolectin B4 (IB4) to stain for pericytes and vasculature respectively in the medulla of rat kidney (52). Images from their 2012 publication highlight how pericytes can be identified along the vasculature based on their slightly bulbous morphology (figure 1.2B).
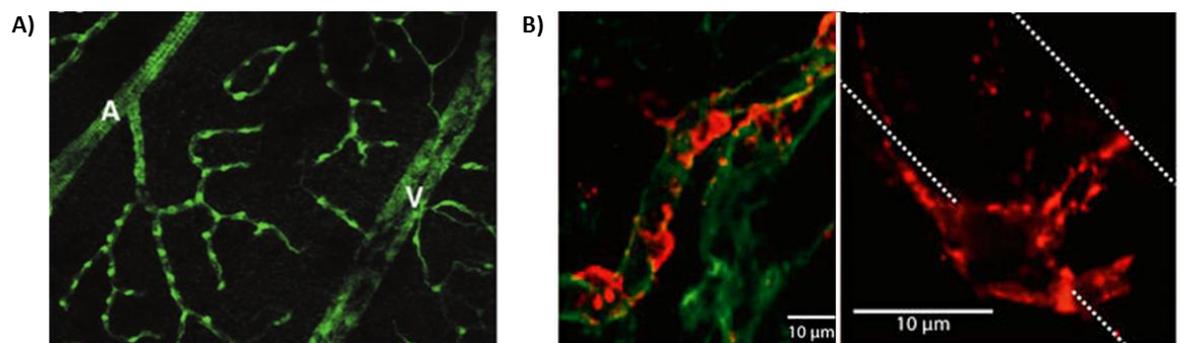
Figure 1.2: Staining of vascular smooth muscle and pericytes with neuro-glial 2 antibody and vasculature by isolectin B-4, A) (Adapted from Murfee, Skalek and Peirce (53)). Smooth muscle cells in both arterioles (A) and venules (V) express NG2 (green), the bulbous structures of pericytes can be seen in the capillaries **B) (Adapted from Crawford *et al* (52)).** Pericytes (NG2, red) are dotted along the microvessels and differ from endothelial smooth muscle in that they express a bulbous structure, which make them easy to identify on the vasculature (IB4, Green). They also have projections, which extend along the and around the vessel.

Vasoactive compounds can act directly at pericytes to alter the flow of blood through the vasculature by causing vasodilation or vasoconstriction. The control of blood flow can also have an effect on the permeability of the vascular bed to molecules and cells, which is essential during the inflammatory process. Perhaps the most relevant is by Crawford *et al* who used an array of vasoactive compounds in a live rat kidney slice model to investigate the function of pericytes on regulation of medullary blood flow. Pericytes responded to various vasoconstrictive agents such as angiotensin II, noradrenaline, endothelin, indomethacin, and ATP by constricting the vasa recta more significantly than at non-pericyte sites (52). The reverse was true for vasodilatory agents such as prostaglandin E2, NO, bradykinin, and carbachol which caused vasodilation. By facilitating vasodilation, pericytes are able to increase vascular permeability. They can also upregulate certain adhesion molecules such as intercellular adhesion molecule 1 (ICAM-1) in response to pro-inflammatory cytokines *in vivo* (54, 55). ICAM-1 binds to leukocyte integins such as LFA-1 and assists in leukocyte extravasation from the blood to tissues (56), which is the main cause of inflammation.

Pericytes are also pluripotent; that is, they can differentiate into other cell types under specific conditions. Pericytes appear to share pluripotency features with mesenchymal stem cells (from which they originate) in that they have been shown to differentiate into osteoblasts, chondrocytes, adipocytes, vSMCs and skeletal muscle cells *in vitro* (57, 58)*.* The pericyte marker PDGFRβ has been used in a variety of studies to confirm if the exact origin of vascular pluripotent cells is indeed pericytes by using techniques such as fate mapping and constitutive activation (59, 60). The problem in these experiments however is that PDGFRβ is also expressed by other cells of mesenchymal origin such as fibroblasts and some smooth muscle cells (table 1.1). Fate mapping (discussed in more detail in section *6.5.4 - Tracking pericyte transdifferentiation in the bladder*) using NG2 is also inconclusive for the same reason (59), nevertheless, there is still open debate as to whether pericytes contribute to a vascular niche of pluripotent cells.

Hashitani *et al* have described the morphological features of pericytes in the bladder by performing TEM and scanning electron microscopy (SEM) and immunohistochemistry using α-SMA, c-kit and NG2 in the suburothelial venules,

capillaries and arterioles of rat bladders (50). EM revealed pericytes adjacent to endothelial cells in the venules whereas arterioles had fewer pericytes and more arteriolar smooth muscle cells surrounded by nerve bundles. Pericytes were positive for α-SMA but negative for NG2 and c-Kit in the venules, however in the capillaries and suburothelial arterioles, both pericytes and smooth muscle cells were positive for NG2 and PDGFRβ. Therefore, the exact location of pericytes within the bladder structure has been identified, however the function of pericytes has not been as well characterised in the bladder as in other organs such as the kidney, lung and brain. Studies in other organs can inform us of the possible role that they might play in the bladder and highlight methods by which the function can be investigated. As mentioned in the section on myofibroblasts, pericytes have been associated to the development of fibrosis through their ability to differentiate into myofibroblasts. Fate mapping in mice has been performed to observe pericyte contribution to fibrosis in the kidney (59), CNS (61), lung (62), skin (63), and liver (64). Taking the results of these studies together, it can be summarised that in the presence of increased PDGF-B, VEGF-A and TGF-β1, pericytes increase expression of αSMA and regulators of G protein signalling (*e.g.* RGS5) and can transdifferentiate into fibroblasts and myofibroblasts (47). If this also occurred tin the bladder it may in turn lead to the increased spontaneous bladder contractions and fibrosis often seen in certain cases of OAB. Due to the importance of pericytes in regulating blood flow and the fact that they can differentiate into myofibroblasts, which have been associated to increased spontaneous contraction particularly in OAB (16), pericytes are therefore of much interests in the bladder and their function is investigated in *Chapter 6 – The role of pericytes in bladder infection*. This concludes the section on the urothelium and the lamina propria and the cells within it. The next section discusses the final layer of the bladder, the detrusor muscle.

1.2.4 THE DETRUSOR MUSCLE

The detrusor muscle is made up of smooth muscle cells whose contractions allow for urine to be stored safely by adjusting to increasing volume, and later allowing for controlled urine voidance. The smooth muscle cells in the detrusor are morphologically similar to that of other smooth muscle cells, containing actin and myofilaments attached to dense bodies under the cell membrane. These cells are

arranged in two unique orientations, longitudinally in the outer and inner layer of detrusor, and circularly in the central muscle layer (65). The detrusor is highly innervated with autonomic (parasympathetic and sympathetic) and somatic efferent nerves, predominantly parasympathetic nerves which are involved in the neural control of detrusor contraction and overall bladder function (discussed in more detail in the section *1.3 - Physiological control of the bladder*).

As discussed earlier, dICC appear to contribute to spontaneous bladder activity (1), which are small contractions of the smooth muscle that allow muscle to adjust during bladder filling. The strength and frequency of these contractions increases in the later phases of filling and may contribute to the sensation of bladder fullness (66, 67). The strongest detrusor contractions occur during voiding and are controlled by the CNS rather than local stimuli. The bladder has first to 'instruct' the CNS of bladder fullness, which involves complex physiological interactions between the urothelium, lamina propria, nerves and detrusor discussed in the following section.

1.3 PHYSIOLOGICAL REGULATION OF THE BLADDER

The urothelium comprises of specialised umbrella cells, which open out to accommodate increasing volumes of urine (68), and act as sensors to both chemical and mechanical changes in the bladder (for a review see Birder and Andersson (69)). As mentioned previously in section *1.2.1 The Urothelium,* urothelial cells express a variety of signalling receptors including i) muscarinic and nicotinic receptors for ACh, ii) purinergic receptors for ATP and other purines, iii) adrenergic receptors for adrenaline and noradrenaline (NA) and iv) transient receptor potential channels (TRP channels), of which there are multiple groups of differing functions; the most abundant in the bladder is the vanilloid receptor (TRPV). This allows the urothelium to detect changes in the bladder environment and signal to other cells (*e.g.* ICC, myofibroblasts, nerves and muscle cells) to respond in particular ways controlling bladder regulation for storing and voiding urine. This section shall focus on the types of signalling receptors that are expressed in the bladder, the cells that express these receptors and the cells which are able to release other signalling molecules in response to these stimuli.

The principal function of TRPV channels is to detect changes in heat (TRPV-1, 2, 4), capsaicin, found in chilli peppers (TRPV-1), osmolality and stretch (TRPV-4), and pH via H^+ protons (TRPV-1). Increases in H^+ (lowered pH), increased temperature, presence of capsaicin, and stretch induce TRPV channel activation in the bladder, which in turn may lead to an increase in intracellular calcium leading to release of ATP, ACh, nitric oxide (NO), neurokinin A (NKA), and nerve growth factor (NGF), all of which act upon other cells and structures within the bladder (70), and in a paracrine manner to urothelial cells (figure 1.3). The activation of TRPV channels in the urothelium initiates signalling to the lamina propria via these transmitters and cytokines to inform the brain that the bladder is filling up. The receptors for ACh, NO, ATP, the ATP by-products, bradykinin and NKA are expressed on a variety of cells including ICC, myofibroblasts, nerves and SMCs (table 1.2).

ATP is released from urothelial cells by exocytosis or ATP-binding cassettes (ABC) (71) when the cells are put under pressure from increased volume in the bladder (72), or as a response to other signalling molecules. This is thought to be controlled by intracellular Ca^{2+} mediated by carbochol (73). Released ATP and its by-products *i.e.* ADP, AMP and Adenosine, bind to P2 receptors (P2R) and P1 receptors (P1R) respectively. There are two kinds of P2R; - P2XR (ligand-gated ion channels - ionotropic) and P2YR (coupled to G protein - metabotropic). Both types of receptors can be homomeric or heteromeric and have various agonists and antagonists. The P2XRs most associated with bladder function are P2X1 (expressed on dSMCs), P2X2 and P2X3 (expressed of myofibroblasts) (42). P2X1 has been shown to induce an inward current of Ca^{2+} and Na^+ contracting the detrusor muscle. In the detrusor, ATP is released by efferent nerves to directly stimulate the smooth muscle cells (table 1.2). P2X3 on myofibroblasts could directly affect afferent nerves due to their proximity to each other; myofibroblasts also release ATP and therefore may be an intermediary cell accentuating ATP responses to afferent nerves (74). P2YR stimulation by ATP has been suggested to cause the release of IL-8, a pro-inflammatory cytokine, and increases spontaneous contractions (75).

Nicotinic (Nm and Nn) and muscarinic (M_{1-3}) cholinergic receptors are highly responsive to nicotine and muscarine, respectively, but all bind ACh. In the bladder, the muscarinic receptor M_2 is expressed throughout, however it is the M_3 receptor

that is responsible for contractions (table 1.2) (76). Another neurotransmitter released in the bladder, noradrenaline (NA), binds to β_3 adrenergic receptors expressed on all responder cells other than efferent nerves in the bladder. Other receptors do exist in the bladder for other stimulants; tachykinin receptors are stimulated by nerve growth factor (NGF) and bradykinin binds to B1 and B2 receptors.

Cell type	Purinergic receptors (ATP, UTP, ADP, Adenosine)	Cholinergic receptors (ACh)	Adrenergic receptors (NA)	Vanilloid receptors (heat, pH, stretch)	Chemical mediators released
Urothelial cells	P2X1, P2Y	M_2, M_3	β_3-AR	TRPA, $TRPV_{1, 2\ \&\ 4}$, $TRMP_8$	ATP, ACh, NO, NKA, NGF,
ICCs	$P2Y_{2, 4\ \&\ 6}$	M_2, M_3	β_3-AR	$TRPV_1$	
Myofibroblasts	$P2Y_{2, 4\ \&\ 6}$, $P2X_3$	M_2, M_3	β_3-AR	$TRPV_1$	ATP
Afferent nerve	P2X3	M_2, M_3	β_3-AR	$TRPV_1$	NKA
Efferent nerve	P2X	M_2, M_3			ATP, ACh
SMCs	P2X1	M_2, M_3	β_3-AR	$TRPV_1$	

Table 1.2: Table of signalling receptors and their expression on various cell types in the bladder. ATP is released by urothelial cells, myofibroblasts and efferent nerve cells and can stimulate all cells in the bladder via purinergic receptors $P2X_1$ in the urothelial cells, $P2Y_{2, 4}$ and $_6$ on myofibroblasts and ICCs and P2X3 on afferent nerves. SMCs respond to ATP from efferent nerves via P2X1. All cells express cholinergic receptors M2 and M3 however only receptor M3 stimulates contractions. All cells express and respond to noradrenaline via the $\beta3$-AR receptor except efferent nerves. The majority of TRP receptors are expressed in the urothelium to respond to heat, pH and stretch however other cells (bar efferent nerves) also express TRPV1 which responds to temperature and capsaicin.

Once the volume of urine in the adult bladder reaches 300 – 400 ml, the urothelium begins to stretch stimulating TRPV receptors and releasing ATP and ACh. Stimulation of TRP receptors increases intracellular calcium which can further stimulate release of ATP, ACh and NO in response to this. ATP and ACh are also released as a response to bladder stretch (reviewed by Lori Birder and Karl-Erik Andersson (69)). These signalling molecules are able to stimulate the various cells in the lamina propria *e.g.* ICCs, myofibroblasts and afferent nerves via M3 receptors, β_3-AR receptors and various P2X and P2Y receptors, respectively (table 1.2 and figure 1.3). Myofibroblasts may be able to influence/accentuate close proximity lamina propria sensory nerves in response to ATP, ACh and NO released from urothelial cells by releasing further ATP acting collectively as a stretch-receptor organ (42, 77). Work by Wu, Sui and Fry has developed a better understanding of the role of myofibroblasts in the bladder by looking at purinergic signalling. A study in 2004 showed that addition of ATP leads to calcium transients in isolated guinea pig myofibroblasts and observation of calcium

oscillation using various purine compounds and blockers gives insight into the types of purinoceptors expressed on bladder myofibroblasts (43). Authors suggest P2Y$_2$ as a most likely receptor, however this is inconclusive due to the difficulties in constructing pharmacologically unique profiles for receptors based on their agonists. A later study in 2008 by the same group further investigating the response of myofibroblasts to sensory triggers, highlighted that these cells can also respond to low pH in a similar manner to which they do ATP (16). Interestingly, these responses were attenuated with addition of capsaicin (a TRPV agonist) and the NO donor SNP suggesting the presence of sensory modulation mechanisms in this cell type. Furthermore, the proximity of myofibroblasts to each other in these stimulatory experiments (cell pairs compared with single cells) showed augmented responses suggesting a role for gap junctions and adherins in enhancing responses to stimuli. The stimulation of afferent nerves (potentially enhanced by myofibroblasts) signals to the CNS to instruct the individual of a need to void, which relays back to the efferent nerves to release ACh and NA to stimulate smooth muscle contraction and sphincter relaxation, respectively (figure 1.3). The mechanism for this is discussed in more detail in the next section *1.3.1 neural control of bladder function.*

A)

TRP receptors

B) *ATP* · · · *ACh* · · · NOS -> · · *NO*

P2Y, P2X$_1$ M3, M2, M5   β3-AR

C)

D) P2Y   P2X$_3$ M3   M3, M2/4   β3-AR   β3-AR

E) ATP

F) ACh   NA
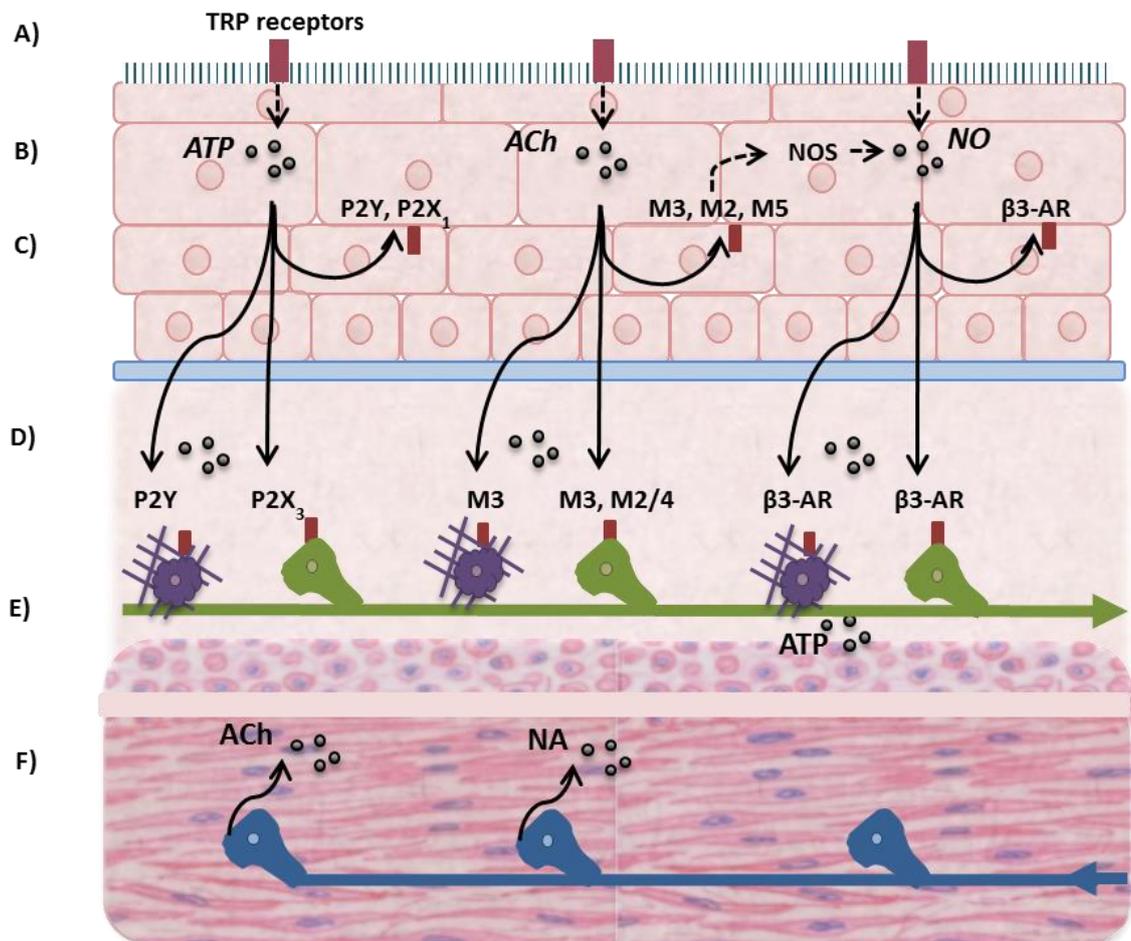
Figure 1.3: Schematic of physiological regulation of normal bladder function. A) Stretch, pH change, temperature change, vanilloid, capsaicin activate TRP receptors on urothelial cells as well as stretch induced ATP release by exocytosis, **B)** Urothelial cells release ATP, ACh and NO in response to stretch, **C)** ATP, ACh and NO can act in a paracrine manner to further stimulate urothelial cells via P2Y$_{2,4}$ and $_6$, P2X$_1$ for ATP; M3, M2 and M5 for Ach; and β3-AR for NO, **D)** ATP binds to P2Y receptors on myofibroblasts and P2X3 on afferent nerves. ACh binds to M3 on myofibroblasts and M3, M2/4 on afferent nerves. NO binds to β3-AR on both myofibroblasts and afferent nerves, **E)** Afferent nerves responding to ACh and NO signal to the CNS to induce a conscious desire to void, **F)** Once a suitable place to void has been found the pontine micturition centre sends a signal via the somatic nervous system to override the sympathetic nervous system (noradrenaline, NA) and stimulate the parasympathetic nervous system (ACh). NA relaxes the sphincters and ACh causes constriction of the detrusor.

1.3.1 NEURAL CONTROL OF BLADDER FUNCTION

So far, local bladder sensitisation has been discussed, however these sensations and the way they affect bladder function are regulated by the CNS. Bladder filling and voiding is controlled by both the autonomic and somatic pathways of the nervous system. The autonomic nervous system is involuntary and controls smooth muscles in the body. This branch of the nervous system can be divided into the parasympathetic system which promotes active bodily function such as digestion, salivation, defecation and urination, and the sympathetic system which redirects

away from the parasympathetic system, in many cases stopping active bodily function, particularly during stressful or adrenaline induced reactions. During storage and filling of the bladder the hypogastric nerve (sympathetic) functions by release of noradrenaline to activate β_3 adrenergic receptors relaxing the detrusor muscle and α_1 adrenergic receptors to contract the urethral smooth muscles (table 1.3), and inhibit the parasympathetic nerve presynaptically. During micturition, the pelvic nerve (parasympathetic) functions by release of ACh to activate M_3 muscarinic receptors on the bladder smooth muscle. The pudendal nerve (somatic) functions by release of acetylcholine to relax the external urethral sphincter by stimulating nicotinic receptors (70, 78, 79) (table 1.3).

Nerve System	Innervation	Neuro-transmitter	Receptor	Effect	Resulting control
Autonomic (involuntary)	Parasympathetic (pelvic nerve)	ACh	M_3 receptor (detrusor)	Contracts the detrusor muscle	Micturition
	Sympathetic (hypogastric nerve)	NA	β_3 receptor (detrusor) α_1 receptor (urethra)	Relaxation of the detrusor and contraction of the bladder neck	Bladder storage
Somatic (voluntary)	Pudendal nerve	ACh	Nicotinic receptor (external sphincter)	Urethral sphincter and pelvic floor	Micturition

Table 1.3: Neural control of the bladder. During bladder storage the detrusor is relaxed and the bladder neck and sphincters are contracted as a result of noradrenaline release via the sympathetic nervous system. The noradrenaline binds to β_3 receptor in the detrusor and α_1 receptor in the urethra to keep urine stored until a suitable time and place to void is found. Once a decision to void has been made, the somatic nervous system signals via the pudendal nerve to relax the external sphincter. At the same time the sympathetic nervous system is overridden by the parasympathetic nervous system. During voiding, acetylcholine contracts the detrusor muscle and relaxes the sphincters via binding to M3 and nicotinic receptors, respectively, to induce micturition.

When the bladder fills up with urine from the kidneys, constant pressure is maintained in the bladder with a higher pressure in the urethra. As the bladder fills, the stimulus of stretching stimulates TRPV receptors as well as releasing ATP from urothelial cells triggering low frequency afferent ACh signalling along the pelvic nerves to the sacral region of the spinal cord, which in turn stimulates the parasympathetic preganglionic neurons that control the detrusor muscle. At the same time, increasing afferent signalling of the sensory neurons ascend up to the pontine micturition centre in the periaqueductal grey matter of the cerebellum which results in a conscious desire to urinate. Here the information is integrated and transferred to two sets of neurons. If an appropriate time and place to urinate is not found signals

are transferred back down the spinal cord to inhibit the parasympathetic preganglionic neurons controlling the detrusor and the somatic motor neurons (pudendal nerve) controlling the external urinary sphincter. Once a voluntary decision to void has started the somatic motor neurons inhibit the external sphincter relaxing it and the detrusor muscle contracts as discussed in the following section (65, 79).

The pelvic floor muscle also plays a valuable role in bladder function. Although not part of the bladder itself, the pelvic floor muscle supports the organs directly above it which includes the bladder and can be consciously controlled and therefore trained. During later stages of filling and storage, when the bladder is under the most pressure, the pelvic flood muscles can consciously support the bladder until voiding. Furthermore, by contracting the pelvic floor muscles in the opposite way, micturition can be forced, expelling urine at an increased rate (80).

1.3.2 BLADDER CONTRACTION MECHANISM

The function of contraction of the detrusor muscle depends upon release of stored Ca^{2+}. ACh released from afferent neural cells bind to receptors in the sarcoplasmic reticulum, which depolarises the membrane of the muscle fibre releasing calcium from calcium stores in the sarcoplasmic reticulum. The calcium binds to troponin on actin filaments altering the shape of troponin and rotates the actin fibre towards the myosin fibre. Hydrolysis of ATP to ADP and inorganic phosphate (Pi) on the myosin head extends it upwards to attach to binding sites on the actin filament to form a cross-bridge. Cross-bridge formation triggers a power stroke, which pulls the actin filament along the myosin and releases ADP and Pi. A new ATP molecule attaches to release the myosin and actin. This cycle is repeated and the myosin and actin filaments slide across each other contracting the muscle (81). Increased pressure in the bladder due to detrusor contraction opens the internal sphincter. The pressure in the urethra is then reduced and the pelvic floor muscle relaxes forming a funnel in the bladder neck as urine is voided (65, 78, 79).

Both the physiological and neurological control of the bladder can become impaired through infection, disease, injury, or unknown means, leading to bladder dysfunction. The following sections discuss how dysregulation can affect the normal function of

the bladder and with OAB as a particular focus for this thesis since in many cases the exact cause of this common bladder disorder is unknown.

1.4 Bladder Dysfunction

In many cases of bladder dysfunction, the cause lies in the CNS rather than the bladder itself. For example, bladder dysregulation can occur in autonomic neuropathological diseases where control of muscles is impaired such as in Parkinson's, multiple sclerosis, brain tumours or syphilis. Uncontrolled firing along parasympathetic nerves or a lack of firing along sympathetic nerves can lead to incontinence. Bladder dysfunction can also occur when the CNS has been damaged, in particular, by injury to the spinal cord. In these cases, the sense of fullness of the bladder may not signal to the brain, and/or the conscious somatic control may not be communicated back to the bladder. For the sake of this thesis however, the focus shall remain on the physiological dysfunction of the bladder rather than dysregulation by the CNS. For a good review article on CNS-related bladder dysfunction see a review by Kennelly and DeVoe (82).

Lower urinary tract symptoms (LUTS) is a term given to difficulties in filling, storing or voiding urine (as opposed to upper urinary tract symptoms which refers to disorders of the kidneys and ureters). LUTS include urinary tract infections (UTI), interstitial cystitis (IC), stress urinary incontinence (SUI) and urge urinary incontinence (UUI). This section will differentiate between these common disorders, their symptoms and the causes of each, and explain how OAB is, in itself, a separate and unique syndrome.

1.4.1 Stress Urinary Incontinence (SUI)

Stress urinary incontinence (SUI) is a form of incontinence that mainly affects post-natal or post-menopausal women. They find it difficult to hold in urine when stress or pressure is applied to the bladder during strenuous exercise or when coughing, laughing or sneezing for example. It is easily diagnosed with a stress-test, where the patient's bladder is filled up with fluid and then they are asked to cough. In women with SUI the pelvic floor muscles are not strong enough to control the bladder properly which may be due to muscle rupturing during delivery of a baby,

hypermobility of the urethra, or lowered detrusor pressure or lowered oestrogen levels post-menopause for examples. SUI patients show predominantly physical changes due to weak or damaged pelvic floor and sphincter muscles (rather than physiological changes such as increased ATP release or inflammation as seen in other LUTS), and therefore the symptoms can commonly be rectified with pelvic floor exercises, structural pessaries, vaginal cones or surgical insertion of a tension-free transvaginal support (communication with Dr Aswini Balachandran at Medway Maritime Hospital).

1.4.2 BLADDER PAIN SYNDROME/INTERSTITIAL CYSTITIS

Patients with bladder pain syndrome, or interstitial cystitis (IC) as it is better known, present with symptoms similar to bacterial cystitis *e.g.* dysuria, increased frequency of urination and urgency, however IC is typically characterised by inflammation in the absence of infection (83). If a patient's microbiology results are negative (that is they have less than 10^5 colony forming units per millilitre (CFU/ml) of a single species of bacterium in their urine when plating midstream urine), IC is diagnosed following a cystoscopy, whereby areas of inflammation, broken skin, glomerulations and Hunner's ulcers within the bladder are observed (83). Causes of IC are multiple and could include autoimmunity, mast cell infiltration, viral infection, or production of toxins in the urine (84). The symptoms of IC cannot be treated with antibiotics due to a lack of an apparent infection. Instead pentosan polysulfate sodium is the only prescribed treatment for IC however a restricted diet, anti-depressants and anti-histamines have been reported to relieve symptoms (85).

1.4.3 OAB AND DETRUSOR OVERACTIVITY

OAB is a syndrome characterised by an array of symptoms such as urinary urgency, urge incontinence, increased frequency whereby the patient feels the need to urinate more than seven or eight times per day, and nocturia (increased need to urinate at night), in the absence of a bacterial infection (86). The symptoms of this syndrome are similar to that of IC however in there is an absence of inflammation in OAB (observed through cystoscopy) and it is often pain free which informs a diagnosis away from IC (83). There are various suggested causes for OAB but in some cases the cause is unknown and is termed idiopathic detrusor activity (IDO). OAB is

extremely common, affecting up to 41.9% of men and 31.3% of women over 75 (87), and given that many cases of OAB are idiopathic, there is much research interest into other possible causes in order to find prospective new treatments for this particular LUTS. This section shall discuss i) the epidemiology of OAB, ii) how IDO is diagnosed by ruling out other possible causes for the symptoms, and iii) current and prospective treatment of OAB.

1.4.3.1 EPIDEMIOLOGY AND ECONOMIC BURDEN OF OAB

OAB affects both men and women of all ages and prevalence increases with age. There have been various studies investigating the demographics of OAB based on age, gender, body mass index, smoking and other health related issues however there are discrepancies in each of the studies. A study based on a population-based study of 5 European countries (including the UK) by Milsom *et al* in 2001 reported a clear increase in incidence of OAB with increased age, which has been widely supported (87), however whether OAB is more predominant in women than in men has since been debated (88, 89). Milsom's study was however the largest (n=16,776) reporting OAB to be more common in women (17.4%) than men (15.6%) and increases with age up to 41.9% in men and 31.3% (87).

It is estimated that around 16.6% of the total population of the UK suffer with OAB (87), however this is likely to be a significant underestimate due to the nature of the condition which means people are embarrassed to talk about it, and it goes undiagnosed and untreated (90). Patients with OAB tend to feel trapped, unwilling to go out in case of an accident, leading to depression and loneliness, which can, in older patients, lead to admission into nursing homes (91, 92). When the urge to urinate occurs, sufferers are more likely to rush to the toilet increasing risk of accidents, trips, falls and fractures (92). Along with the cost of treatment of OAB, these accidents and nursing home admission cost the health service around £1 billion per year in the UK alone (93).

1.4.3.2 DIAGNOSIS OF OAB

OAB is usually diagnosed by excluding other LUTS first; for example, OAB is not a UTI based on microbiological results nor is it IC based on cystoscopy and a lack of pain (table 1.4). Certain additional tests can help diagnose whether a particular

patient's symptoms are caused by an infection, neurological disease, bladder outlet obstruction, or IDO. Knowing the cause enables the consultant to prescribe the correct treatment for each case *e.g.* antibiotics, surgery, or pharmaceuticals.

LUTS	Symptoms	Diagnosis	Causes	Treatments
UTI	Dysuria Urgency, Increased frequency	$>10^5$CFU/ml	Bacterial infection	Trimethoprim, Nitrofurantoin, Norfloxacin
IC	Dysuria, Urgency, pain Increased frequency	$<10^5$CFU/ml , Cystoscopy	Unknown	Dietary restrictions, Pentosan polysulfate sodium
SUI	Leakage during increased pressure on the bladder	Stress-test	Weak pelvic floor muscles	Bladder training, Structural pessary, TVT
OAB	Urgency, Increased frequency, Nocturia	$<10^5$CFU/ml, ICIQ, Not SUI or IC	Neurological disease, blockage, UTI, DO.	Bladder training, Anti-muscarinics, BTX-A
IDO	Urgency, Increased frequency, Nocturia	All other tests negative. Urodynamics indicate DO.	Neurological, Unknown	Bladder training, Anti-muscarinics, BTX-A

Table 1.4: Summary of the symptoms and causes of various lower urinary tract symptoms and their respective treatments. Urinary tract infections (UTI) are diagnosed when a patient has $>10^5$ CFU/ml of a single species of bacteria in a midstream sample of urine. This results in symptoms such as urgency and increased frequency and is treated with antibiotics. Interstitial cystitis (IC) is diagnosed when an individual does not have an infection ($<10^5$ CFU/ml) and has observed inflammation by means of a cystoscopy. Certain dietary restrictions and pentosane polysulfate sodium can be used to treat IC symptoms which often include urgency, pain and increased frequency. Stress urinary incontinence (SUI) is caused by weakened muscles and diagnosed with a stress test. Bladder training is the initial treatment followed by pessaries and tapes to support the muscles. OAB is diagnosed when an individual has no infection ($<10^5$ CFU/ml), no inflammation and positive responses to the international continence questionnaire (ICIQ) and may be caused by neurological disease such as multiple sclerosis or Parkinson's, blockages in the urethra, low-level infection ($<10^5$ CFU/ml) or detrusor overactivity. Idiopathic OAB is diagnosed when an individual has detrusor overactivity and the cause is unknown (IDO). For both OAB and IDO, the treatments include antimuscarinics and Botox (BTX-A).

In the case of male patients with OAB, the cause can often be benign prostatic hyperplasia, which is a enlargement of the prostate, which increases with age, putting pressure on the bladder (reviewed by Roehrborn (94)). However, since this thesis shall focus on female patients with OAB only, the possible causes in women only shall be discussed here forth. Patients suffering with neurological diseases such as neuropathy, dementia, spinal cord injury, multiple sclerosis or Parkinson's disease can also experience OAB symptoms due to loss of muscle control as discussed in the previous section on bladder dysfunction (95). Specific tests on a patient's neurological status will diagnose one of these as a potential cause for OAB. Background medical checks and routine urinalysis testing for infection, diabetes, and

blood or protein in the urine, which could be a sign of a kidney problem, can exclude infection and diabetes as possible causes for overactivity (96). This is often then followed by fitting a specific description dictated by international validated questions from the International Consultation on Incontinence Modular Questionnaire (ICIQ) (see Appendix 1.3). By obtaining a score of 1 or above on certain questions such as "Do you suffer with a sudden need to urinate?" a patient is then diagnosed with OAB (communication with Dr Balachandran, Medway Maritime Hospital). It is then essential to look at post-void residual volume to see if the bladder is emptying properly which might indicate a blockage in the urethra. A final test is urodynamics, which involves filling the bladder with fluid and measuring pressure over time. If the pressure fluctuates involuntarily this indicates detrusor overactivity (DO), which can either be due to a neurological condition, or in many cases, cannot be explained at all in which case it is diagnosed as IDO (96). Current treatment of OAB can range from lifestyle changes, to pharmacological intervention and in severe cases, botox injections or insertion of a small device that generates electrical stimulation of the post-tibial or sacral nerve (discussed in more detail in the next section).

1.4.3.3 CURRENT AND PROSPECTIVE TREATMENTS OF OAB

There are a numerous lines of management for OAB depending upon the severity of an individual's symptoms and their responsiveness to treatment. For the first line of treatment, consultants will usually encourage the patient to adapt their lifestyle and partake in bladder control exercises (97). If these are not successful in curbing symptoms, medical intervention is the next line of treatment, however bladder exercises are always recommended in conjunction with this. Alternative therapies have recently been introduced which are more invasive such as botox injections, however these are reserved for severe refractory OAB, that is, individuals who have failed to respond to all other treatments (98). In this section, each of these treatments is discussed in more detail.

 Lifestyle changes involve adaptations to an individual's diet and pelvic floor exercises. The symptoms of OAB can be exacerbated by increased consumption of diuretics such as alcohol, coffee, tea and fizzy drinks, therefore patients with OAB are encouraged to reduce their intake of these drinks as well as reducing the quantity of liquid consumed in the evenings in order to moderate nocturia. Much of the time, like

with SUI, pelvic floor exercises can be used to reduce the symptoms of OAB (99). Bladder training is always advised as a first line of treatment (communication with Dr Aswini Balachandran at Medway Maritime Hospital). These involve the patient trying to retain urine for as long as possible when the urge to urinate occurs. Another method is stop-start training where the patient starts to void and then attempts to stop and hold the urine in the bladder for as long as possible (97). Over time increasing the interval between voiding can significantly reduce the urge to urinate in patients with OAB but this does not work with DO.

If pelvic floor exercises are not found to be helpful, consultants will follow the next line of treatment; prescription of antimuscarinic drugs, which block the muscarinic acetylcholine receptors preventing smooth muscle contraction. There are 7 antimuscarinic drugs currently on the market in the UK for OAB and they all have slightly different effects on patient symptoms dependent on responsiveness to the drug (98). Since antimuscarinics are not specifically designed to target solely the detrusor muscle there are a multitude of unwanted side effects associated with these types of drugs including: - inhibition of salivation, reduced intestinal mobility, cognitive dysfunction, and memory impairment (100). In many cases the side effects cause the patient more distress than the OAB symptoms themselves and the medication is stopped by patient or changed by the consultant. Another setback is that responsiveness to the drug often subsides after a matter of weeks and thus the patient may be prescribed a new type of antimuscarinic. When multiple drugs have been unsuccessful, the patient's syndrome is termed refractory and alternative therapies may be considered.

The neurotoxin botulinum A (BTX-A) produced by the bacterium *Clostridium botulinum* has recently been introduced as a treatment for OAB and has proven to be fairly successful (101). BTX-A is injected directly into the wall of the bladder wall at multiple points where it blocks the local vesicular release of neurotransmitters (98). Unfortunately, in some cases patients find they cannot urinate at all after treatment and may need to self-catheterise until the BTX-A has subsided after approximately 3-7 days allowing the patient to regain control again (102). Furthermore, this line of treatment requires repeat administration every 6 - 12 months initially, increasing in

frequency as treatment progresses, which will also carry further financial implications (103).

  As previously mentioned, patients with OAB, by definition, do not present with detectable bacteria during routine microbiology assessment. However, in 2011 James Malone-Lee of University College London presented an abstract at the International Continence Society reporting results of an antibiotic treatment trial for patients with OAB symptoms (104). Group one (n=147) presented with OAB and pyuria and was prescribed antibiotics straight away, group two (n=212) presented with OAB and no pyuria and was not treated, and the third group (n=81) had OAB and late onset of pyuria at which point they were prescribed antibiotics. There was clear evidence of antibiotic efficacy demonstrated by the clearance of all symptoms in all groups prescribed the antibiotics. Those who started antibiotics later in the study period took significantly longer to recover than those who started antibiotics straight away. The results of this study have not yet been published beyond the abstract and the numbers of patients included are relatively low, however they do suggest that infection is being missed in a large number of these patients (the reasons for this are discussed in the next section) and that antibiotics could be a valuable treatment for some types of OAB in the near future but this study needs expanding upon in order to determine which, if any, particular species are relevant in the treatment of OAB with antibiotics.

1.5 URINARY TRACT INFECTIONS

A UTI is a bacterial infection occurring anywhere along the urinary tract from the urethra to the kidneys. The main symptoms of a lower UTI (acute bacterial cystitis) include increased frequency of urination, urinary urgency, dysuria (painful urination), and discoloured or foul-smelling urine (105). When an infection reaches the kidneys, symptoms may also comprise of pain or pressure in the back and a fever. UTIs are generally diagnosed by the NHS when a patient has greater than 10^5 CFU/ml of a single species of bacterium in their urine when plating midstream urine (105, 106). Most UTIs are caused by uropathogenic *Escherichia coli* (UPEC; 85%) but also by bacterium such as *Enterococcus faecalis, Proteus mirabilis, Klebsiella pneumoniae, Pseudomonas aerogenosa,* and *Serritia marcenscens* (107, 108). UTIs are treated with oral antibiotics such as nitrofurantoin or trimethoprim, taking into consideration the local antibiotic resistance patterns (109).

UTIs are defined as recurrent UTIs (rUTI) when a patient has a UTI three to four time within a 12-month period (105). The reasons for rUTI occurrence could be due to genetic predisposition (110), the patient being immunocompromised, antibiotic resistance or intracellular capabilities of particular bacterial species allowing them to be protected from antibiotic targeting and immune responses (111)(discussed in section *1.5.2.4 - Intracellular bacterial communities in OAB*). In cases of rUTI, alternative antibiotics are prescribed and often for longer-term, which can further develop multi-drug resistant strains and increase risk of rUTI. Recurrent UTIs are of particular interest when caring for individuals who have received a renal transplant as they are particularly predisposed to rUTIs due to the immunosuppressant and antibiotic medications they are required to take post-transplant. These drugs predispose renal transplant recipients (RTR) to rUTI since they reduce the effect of the immune system in order to prevent organ rejection. Furthermore, these patients also receive antibiotics as a prophylactic for infections, particularly pneumonia and UTIs, which may occur as a result of immunosuppression.

1.5.1 RECURRENT UTI IN RENAL TRANSPLANTATION

Renal transplantation is the surgical removal of a healthy kidney from a living or deceased individual and implanting it into a patient with significantly reduced kidney function. Chronic kidney disease is measured by the estimated glomerular filtration rate (eGFR) which indicates how well an individual's kidneys are functioning to filter the waste from the bloodstream. When a patient has low eGFR, dialysis is often required to perform blood filtration, however in more severe cases, renal transplantation is a real alternative to renal dialysis. This is usually a required surgical procedure for patients suffering with end-stage renal disease, which can be brought on by a number of factors including diabetes, hypertension, pyelonephritis (upper urinary tract infection), or autoimmune diseases such as autoimmune glomerular nephritis (112). Finding an appropriate organ donor is more complex than finding an appropriate blood donor since cells within the organ express major histocompatibility complexes (MHC) for which there are thousands of haplotype variants across the population (56). Foreign MHC is a target for immune cells, therefore it is essential to try to match the MHC haplotypes of the donor and the recipient as closely as possible, and is the reason why family members are often the best option for organ transplantation. Difficulty in finding a good match also means that when a kidney donor match is found and the kidney is transplanted it is essential to maintain the health of the kidney and the recipient, and monitor their health for many years post-operation. Success rates are very good in the UK for renal-transplantation with approximately 93% kidney survival and 96% patient survival within the first year, and 86% kidney survival and 89% patient survival after 5 years (113). The post-operative care costs approximately £17,000 for the first year and approximately £5,000 for every subsequent year, whereas dialysis costs an estimated £30,800 per year per patient (NHS Standard Contract 'For adult kidney transplant service (114). Therefore, if a kidney donor match can be found, a renal transplant is the preferred method as it offers enhanced quality of life, enhanced survival rates and reduced cost implications.

During the procedure, neither of the two kidneys within the recipient are removed, but rather the new kidney is connected to the external iliac artery and external iliac vein in the extra-peritoneal left or right iliac fossa. The donor ureter is

connected to the bladder and is lined with a stent to offer support and good urine flow as the graft establishes. Immunosuppressants and antibiotics are prescribed immediately to suppress immune responses and act as a prophylactic against infection, respectively (discussed in more detail in the following sections).

1.5.1.1 POST-OPERATIVE CARE FOR RENAL TRANSPLANT RECIPIENTS

As mentioned, finding a good match that will reduce graft-versus-host disease and graft rejection is difficult and therefore maintaining kidney health is absolutely essential post-transplant. There are 19 transplant centres in the UK where transplants are performed, however patients are required to attend local hospital renal outpatients for check-ups and medication to monitor, prevent and treat graft rejection and infection. Immunosuppressants and antibiotics are continually prescribed for 3-12 months depending upon the stability of the graft and the patient (115). The first few weeks are intensely monitored since acute-graft rejection is the most common form of rejection and can occur within the first week to three months, but if caught early can be treated. Routine monitoring is performed weekly within the first 3 months and then monthly for the remaining year. Monitoring strategies includes blood tests, electrolyte and glucose measurements, liver function tests and blood pressure measurements, to ascertain general health (116). Further tests for inflammation, infection, rejection and renal failure may be performed via blood tests, scans and in some cases, a kidney biopsy (116). Rejection can subsequently be treated if caught early enough. The stent is usually removed six to ten weeks post-transplant to reduce risk of infection (116).

Immunosuppression is essential to graft success as it reduces the systemic immune responses and allows the graft to become established quickly with reduced risk of rejection. Initially, basiliximab is prescribed for the first few weeks to prevent acute graft rejection during the induction period. Basiliximab is an IL-2 receptor monoclonal antibody that blocks the IL-2 receptor and prevents maturation and activation of T-cells and B-cells, which are able to recognise foreign MHC (117). Immunosuppression is acheived thereafter by use of a calcineurin inhibitor. Calcineurin is essential for signalling in T-cells and when blocked prevents T-cell proliferation. Two particular calcineurin inhibitors are used post-renal transplantation; tacrolimus and cyclosporine (used in rare cases *e.g.* diabtetes), which

have similar side effects, however the choice of drug depends upon the side-effects of the patient. Side effects can include hypertension, renal dysfunction and nephrotoxicity, and risk of infection, due to the inability of the weakened immune system to fight opportunistic pathogens (118).

Since patients must be prescribed immunosuppressants for life post-transplant, this renders individuals at high risk from opportunistic infections, notably bacterial infections such as pneumonia, MRSA, UTIs and pyelonephritis; viral infections such as herpes, hepatitis, cytomegalovirus, and Epstein-Barr virus; and yeast infections such as candiditis (119). For this reason a cocktail of antibiotics is prescribed as a prophylactic for 3-6 months usually consisting of co-trimaxazole, a combination of sulfmethoxazole and trimethoprim, which are particularly effective at protecting against *Pneumonocystis jirovaci* pneumonia (116). Oral anti-fungals are advised to prevent oral, vaginal and urinary candiditis. Unfortunately, UTIs are still common in RTR and are therefore of particular interest as they can significantly reduce graft function (120).

1.5.1.2 INCIDENCE OF rUTI IN RENAL TRANSPLANT RECIPIENTS

Reported prevalence of UTI incidence in RTR varies greatly between 23% and 75% depending on the study. The most prevalent species which are found to cause UTI in RTR is *E.coli* in 34.5% of cases, followed by *Enterococcus* (19.5%) and *Pseudomonas aeruginosa* (12.5%). Strong correlations have been recorded between incidence of rUTI and intensity of immunotherapy suggesting that most infections are opportunistic (121).

1.5.1.3 CONSEQUENCES OF UTI POST-RENAL TRANSPLANT

The correlation with incidence of rUTI and graft rejection has been widely accepted. A study by Pelle *et al* comparing 255 patients with chronic rejection and 351 patients with no rejection showed a strong correlation between UTI and chronic rejection. Furthermore, Pelle's investigations have confirmed that UTI increases the risk of acute pyelonephritis (APN) detected by increased numbers of infiltrating immune cells, which decreases risk of survival (122). Increased acute rejection episodes carry high risk of developing complicated chronic rejection which is harder to control and often results in graft and host morbidity (122). Kamath *et al* suggested

a theory that pro-inflammatory cytokines produced during a UTI could lead to inflammation in the graft that promote and support graft-versus-host reactions (123), which has since been supported by a report showing that TNF-α and IL-1β significantly reduce graft acceptance (124).

Furthermore, increased use of antibiotics, both as a prophylactic and as a treatment carry high risk of developing antibiotic resistance. El Amari at al found that antibiotic resistance develops in 78% of treated *E.coli* and *E.faecalis* infections in RTR (125). Another investigation using co-trimoxazole as a prophylactic for women with rUTI found that 100% of women treated with this antibiotic cocktail developed resistant forms of UPEC (126). Although this study was not performed in immunosuppressed RTRs, it highlights the high probability of antibiotic resistance development in vulnerable patients with this particular antibiotic cocktail. As resistant strains are able to colonise the urinary tract, treatment becomes harder in the individual and it causes further risk to hospital and community health. It is therefore essential to be able to diagnose UTIs early before they develop into chronic or recurrent infections, which can increase the risk of rejection and morbidity or the need for further treatment with antibiotics. Unfortunately, current routine urinalysis techniques do not always pick up low-level opportunistic infections until they are deeply manifested (127).

1.5.2 DIAGNOSIS OF UTIS

UTIs are diagnosed when a patient has 10^4 colony forming units (CFU) or more when plating midstream urine (Public Health England (128)). Anything less than this and patients are considered negative for infection by most diagnostic laboratories (105, 129). In the late 1950's a Harvard nephrologist, Dr Edward H. Kass, derived this traditional diagnostic criterion as a threshold to define UTI (106). According to the study, 95% of patients had $>10^5$ CFU/ml of a single species in voided urine. A major flaw with this with Kass's findings was that his study was only based on patients with severe infections of the kidney (pyelonephritis) therefore the CFU count in the bladder was undoubtedly higher than in patients with a lower tract UTI. In 1964, Gallagher *et al* conducted a study that showed that approximately one-third of women with an acute UTI had bacterial counts between 10^2 and 10^4 CFU/ml of a single species discrediting Kass's diagnostic assumption (130). Later in 1984, Stamm

et al concluded that urine culture with more than 10^2 CFU/ml of one species among acutely symptomatic women is the best criterion to be used to establish diagnosis of UTI (127). Since then, the Infectious Diseases Society of America (IDSA) (131), and the European Association of Urology (EAU) (132), have revised the guidelines to include a cut off of 10^3 CFU/ml for the diagnosis of bacterial cystitis. However, most diagnostic laboratories still follow Kass's criterion interpreting urine culture with $<10^5$ CFU/ml as probable absence of infection (129). This could arguably be in order to reduce prescription of antibiotics in order to prevent antibiotic resistance and for financial consideration. For this reason, many low-level infections could be being missed in many cases of LUTS. Even at low level, certain species could potentially lead to the symptoms of UTIs; increased frequency, nocturia and urgency, which are similar symptoms to OAB. As such, reduced bacterial threshold and low-count bacteriuria has been of recent interest to a number of research groups who believe that OAB may be caused by a low-level infection missed by diagnostic laboratories due to the high threshold of 10^5 CFU/ml.

1.5.3 BACTERIURIA IN OVERACTIVE BLADDER

1.5.3.2 'LOW COUNT' BACTERIURIA IN LOWER URINARY TRACT SYMPTOMS

In the past 5 years, a handful of research groups including those headed by Professor Kate Moore (University of New South Wales, Australia), Professor James Malone-Lee (University College London, UK) and Professor Vik Khuller (Imperial College London, UK) have reported bacterial cystitis (bacteriuria with or without pyuria) in approximately one third of patients with refractory OAB, that is patients who do not respond to treatment (129). Malone-Lee and collaborators published the first indication that 'low count' bacteriuria may be relevant in OAB in 2008 (133). When culturing midstream urine (MSU) samples from patients with OAB they discovered that by reducing the threshold to 10^2 CFU/ml more than 30% of patients had bacteriuria compared to 12% with a threshold of 10^5 CFU/ml. A later study by Moore and collaborators supported these findings and further reported 39% bacteriuria with 10^2 CFU/ml versus 6% in individuals with no OAB symptoms (134). They also revealed the predominant species found to cause low-level bacteriuria in OAB to include *E.coli, Streptococci, Klebsiella, Pseudomonas and Enterococci.* Both of these studies used MSU samples, which have since been argued to be highly

contaminated with perineal organisms (129), as such catheter specimens of urine (CSU) are regarded as more accurate in providing insight into the species within the bladder itself (129). Due to the invasive nature of the collection method, CSU sampling is harder to recruit for and limits obtainable sample numbers. However, a study by Walsh *et al*, again led by Moore, performed similar experiments in 2011 using CSU samples from women with OAB and asymptomatic women (135). Their work revealed two thirds of women with OAB to have low count bacteriuria, which is higher than reported with MSU (30-39%) (133, 136), however the comparison with the control group was insignificant.

In 2013, Malone-Lee *et al* set out to fully investigate the diversity of urinary species in OAB using the 16s rRNA gene sequencing technique, which provides more accurate species identification than biochemical techniques used in previous studies (137). Their work showed a hugely diverse range of bacterial species found in both MSU and CSU sample urine with the most predominant species from CSUs identified as *Staphylococcus*, *Streptococcus*, *Lactobacillus*, *Escherichia coli* and *Enterococcus*. Their results however showed a larger diversity of bacteria from CSU than MSU contradicting the well-accepted notion that MSU samples contain more contaminants that CSU samples. This was however an excellent bridging publication between previous work on 'low count' bacteriuria and the most up to date report on the range of bacteria residing within the bladder published a year later by Pearce *et al* (138) (discussed in more detail in the introduction to *Chapter 5 - Improved methodology for urine culture*).

1.5.3.2 UPEC AND A POSSIBLE ROLE IN THE PATHOPHYSIOLOGY OF OAB

Most UTIs are caused by UPEC (85%), but also by bacterium such as *Enterococcus faecalis, Proteus mirabilis, Klebsiella pneumoniae, Pseudomonas aerogenosa,* and *Serritia marcenscens* (107, 108), all of which have also been isolated from patients with OAB (136-138). Many research studies looking at the pathogenesis of bacteria in UTIs have been performed with UPEC. The findings from these studies could be applied to OAB considering recent evidence on the importance of 'low count' bacteriuria in some individuals with OAB. UPEC enter the bladder via the urethra and establish themselves by invading the urothelium, which induces immune responses such as antimicrobial factors and activation of mast cells and macrophages. The

ability of UPEC to do this relies heavily on lipopolysaccharide (LPS) in the cell wall, as well as pili and fimbrae, which are used for attachment to host cells (139, 140). UPEC and many other Gram-negative bacteria are capable of forming biofilms (strongly adhered groups of cells in an extracellular polymeric matrix) through LPS binding and quorum sensing (reviewed by Flores-Mireles (141)). Furthermore, UPEC release toxins and metabolites that can severely affect the function of host cell. For example, the production of the toxin cytotoxic necrotizing factor 1 (CNF1) provokes actin filamentation in the host cells by acting upon GTPases (142), and α-haemolysin triggers pore-formation in target cells and disrupts cell adhesion (143). Altered structural integrity of the bladder urothelium as a consequence of UPEC adhesion and invasion will understandably have repercussions on the functionality of the bladder as a whole.

1.5.3.3 INTRACELLULAR BACTERIAL COMMUNITIES IN OAB

FimH on the type 1 pilus found on *E.coli* can induce intracellular targeting allowing this bacterium to live inside host epithelial cells in intracellular bacterial communities (IBC) (139, 142, 144-148). Despite inducing immune responses, both biofilms and IBCs are resistant to targeting by host immune defences and water-soluble antibiotics (142, 149-152), providing a reservoir of bacteria, which can lead to reinvasion and rUTIs. Incidence of IBCs prompts exfoliation of infected superficial cells exposing naïve transitional cells underneath. IBCs have been observed in patients with OAB (137, 152-154) and could therefore be implemented in triggering symptoms of urgency in OAB.

The field of IBCs in the bladder has been particularly well investigated by Professor Scott Hultgren's and collaborators at Washington University, St Louis, USA, however their focus is mainly on IBCs in UTIs and research is often performed in mice rather than humans. Hultgren's resrach has shown that adhesion of UPEC to the host cell membrane is led by the binding of FimH adhesion to mannosylated uroplakins on the bladder epithelium (145, 147). Three main post-invasion stages have been described in the acute lifecycle of intracellular UPEC (in murine models) using SEM, TEM and immunohistochemistry (144, 145). Early IBC; - individual bacilli divide within lysosomes of the host urothelial cell, Middle IBC; - coccoid bacterium form tightly packed biofilm-like pods (144), Late IBC; - peripheral bacteria regain bacillus

formation and efflux from the host cell to re-infect adjacent cells (145, 149). Fluxed bacteria often take the form of filamentous bacilli, which allows them to evade phagocytosis by neutrophils (149). A later study by the same group using similar techniques on samples of urine from women with cystitis and rUTI, but not OAB, showed the presence of biofilm-like pods and filamentous bacteria in human urothelial cells also (111). They identified other bacteria in IBCs including *Enterobacter aerogenes, Klebsiella pneumoniae* and *Proteus mirabilis,* and expectedly patients with infections caused by Gram-positive bacteria (LPS and FimH negative) did not present with IBCs. They concluded that the presence of filamentous bacteria indicates presence of IBCs in the epithelium and could be useful in predicting rUTIs.

The work by Hultgren *et al* focuses on UTIs and cystitis but not OAB, however most of the fore mentioned groups working on bacteriuria in OAB have also demonstrated a presence of intracellular bacteria in their findings on OAB. Cheng *et al*, led by Moore, noted IBCs in 56% of samples that were negative for routine microbiology by observing cells with confocal microscopy (154)-cited in (129). Malone-Lee *et al* have also shown that by performing an antibiotic protection assay on MSU samples and then lysing urothelial cells with Triton X-100, there are more colony forming units post-lysis than pre-treatment suggesting a reservoir of intracellular bacteria within host cells of species *E.coli, Enterococcus faecalis, Streptococcus anginosus* and *Proteus mirabilis* (137). Taken together these studies suggest that intracellular bacteria may be important in OAB however only the latter study compared CFU numbers from patients with LUTS with asymptomatic individuals. The differences between IBC CFU/ml were not significantly different between OAB and asymptomatic adults (137) suggesting that IBCs may occur in healthy individuals as well, but it is the pathogenicity of the bacterium in these reservoirs that is the cause for LUTS.

In a separate publication in the same year, Malone-Lee and collaborators investigated the invasiveness of bacteria by confocal microscopy and showed that *Enterococcus faecalis* are more invasive than *E.coli* by performing confocal analysis of cultured cells infected by either *Enterococcus* or *E.coli.* (152). This introduces an interesting alternative to the heavy focus on UPEC and highlights the importance of investigating other uropathogenic bacterium.

Of course due to the proposed lifecycle process of intracellular bacteria there is likely a transient production of filaments, therefore it is essential to sample urine more often in order to increase the likelihood of detecting them. It also implies that antibiotic treatment ought to be prolonged to ensure efficacy. Hultgren *et al* has publicised results on an exciting alternative to antibiotics. They showed that by treating mice burdened with multidrug resistant UPEC with a single oral dose of a FimH agonists bacterial load was reduced by >1000-fold (155, 156). Taken together these studies suggest that the species of intracellular bacteria are of more relevance than the presence of IBC themselves, however they may be a potential means of detecting low-level bacteriuria, explaining rUTI and a possible cause for OAB in some patients. Furthermore, in cases where IBCs are present, alternatives to antibiotics could be used which are more effective at targeting pathogenic intracellular species, thus preventing development of antibiotic resistance with over use of antibiotics. .

1.5.4 IMMUNE RESPONSES TO BACTERIA IN THE BLADDER

Despite the bladder being at risk of colonization, it has numerous innate immune defences that prevent UTI. For example, as urine is voided, the force of the fluid passing through the narrow urethra removes many bacteria from within it. Hence, midstream samples of urine are less sterile than catheter samples. Furthermore, the GAG layer (figure 1.1) prevents bacteria from reaching the exposed surface of the urothelial cells. Other innate defences consist of secretory immunoglobulin A (sIgA) production (157), antimicrobials and defensins produced in the kidney and the vagina. Finally, innate immune cells, again similar to those found in the gut, can also be found within the urothelial walls of the bladder. Such cells include of macrophages, dendritic cells (158, 159) and γδ T cells (160). γδ T cells are so called as their T cell receptor is composed of different glycoproteins (a γ chain and a δ chain) compared to the standard glycoproteins found on αβ T cells such as helper T cells, cytotoxic T cells and Treg cells (161). The exact role of these unique cells is generally unknown, however they are associated with innate functions, and since they do not require major histocompatibility complex II for activation, they presumably act as danger sensors in mucosal areas and skin.

As previously mentioned, many of the bacterial species found in patients with OAB express LPS in their cell wall, which is extremely potent activator of immune

responses. The pathophysiology of UPEC in UTIs has been extensively studied in relation to how they initiate immune responses in the bladder. Considering the recent findings in regards to colony count, this information could be extrapolated to OAB. Furthermore, there are increased populations of mast cells in patients with OAB and IC compared with controls indicating a possible role for these immune cells in exacerbating symptoms in these syndromes (162).

1.5.4.1 LIPOPOLYSACCHARIDE AND TOLL-LIKE RECEPTORS

In 1993 a study by Svanborg *et al* showed that epithelial cell lines grown in culture with UPEC have increased intracellular IL-1α, IL-8 and IL-6 concentrations which may in turn be involved in the recruitment of polymorphonuclear cells (PMNs) (163). A later study revealed exposure of renal epithelial cells to the exotoxin α-haemolysin of UPEC increased intracellular Ca^{2+}, which correlated with increased IL-6 and IL-8 production from these cells (164). As previously mentioned, *E.coli* express LPS and type 1 pili in their cell wall which are extremely potent activators of innate immune responses acting via binding to receptors such as toll-like receptor 4 (TLR4), CD14, sCD14 and LPS-binding protein (LBP) (159). Bladder cell lines (*e.g.* 5637 epithelial cells, T24 epithelial cells, J82 epithelial cells, and A498 epithelial cells, all derived from bladder carcinoma) express CD14 and TLR4 and TLR2 and activation of bladder cells is dependent on TLR signalling, p38 MAP kinase, and the transcription factor NF-κB which transcribes the afore mentioned pro-inflammatory cytokines IL-6 and IL-8 (159). A later study by Song *et al* revealed that another distinct pathway involving Ca^{2+} and cyclic adenosine monophosphate (cAMP) exists in bladder epithelial cells, which can also trigger IL-6 production (165). Taken together these studies illustrate that UPEC bind to CD14 and TLRs in the bladder urothelium via LPS to activate p38 MAP kinase, Ca^{2+} and cAMP signalling which in turn triggers IL-6 and IL-8 production (figure 1.3). IL-6 is associated with activation of the acute phase response, which increases production of C-reactive protein from the liver and IL-8 acts as a chemotactic factor for neutrophils. Interestingly, mast cell and neutrophil infiltration, C-reactive protein, IL-6, and IL-8, have all been shown to be increased in patients with OAB (166-169).

Neutrophils, mast cells and macrophages are all increased in the bladder of patients with OAB compared with asymptomatic individuals (162, 169, 170). These cell types are associated with innate immune responses. Neutrophils are the first cells to reach a site of infection by chemo-attraction to IL-8 released by activated macrophages. Mast cells are often recruited during parasitic infections and allergic reactions and release nerve growth factor (NGF), prostaglandins, histamine, and tyrosine, which can in turn provoke smooth muscle contraction (figure 1.4) (171-173). A study by Liu *et al* showed that mast cells are increased in both OAB and IC however the tight junction protein, zonula occluden-1 (ZO-1), and adherence protein, E-cadherin, are only decreased in IC indicating a breakdown of urothelial integrity associated with mast cell infiltration occurs in IC but not in OAB (162). Interestingly, antihistamines can be used to ease the symptoms of IC (85), however have not been trialled for treatment of OAB symptoms despite the similarities in these two LUTS disorders in regards to mast cell infiltration and prostaglandin increase.

1.5.4.3 NERVE GROWTH FACTOR AND OTHER BIOMARKERS

Many studies have associated NGF with OAB, and proposed it to be a biomarker of OAB (174-176). NGF is a molecule that promotes differentiation, maintenance and repair of sensory and sympathetic neurons (20). Within the bladder, it is produced from the urothelium and smooth muscle cells, however it has also been shown that mast cells produce NGF. It has been widely publicised that NGF is higher in urine from in patients with OAB compared to asymptomatic controls (n=51 maximum) (174-176)However increased NGF is not restricted to OAB and has been indicated other LUTS including IC (177) and mixed urinary incontinence (175). NGF is however reduced post anti-muscarinic and BTX-A treatment (178) but also post antibiotic treatment (179), therefore it could be worthwhile investigating the correlation between urinary NGF and presence of pathogenic and/or intracellular bacteria to better understand the potential effects of low-level infection and how OAB may manifest as a result.

Of course a vast array of other cytokines have been analysed in the urine of patients with OAB using ELISA's and Luminex™ technology. The results of these tend to show increased macrophage inflammatory protein (MIP-1B), monocyte chemotactic

protein-1 (MCP-1), IL-10, soluble IL-2 receptor-α, tumour necrosis factor (TNF) and epidermal growth factor (EGF) in samples of urine from individuals with OAB compared to asymptomatic individuals suggesting a possible inflammatory role (166-168). Interestingly MCP-1 is known to provoke mast cell activation (180), which correlates with increased mast cell numbers in these patients. However the results of these studies looking at cytokines in urine have all been very contradictory indicating that samples size may be an issue (n=20 maximum) or perhaps that cytokine variation between individuals is too varied to make conclusions at this stage. Testing the same individual at various times on a wider number of patients would clarify this.

1.5.5 ACTIVATOR SIGNALLING AND BACTERIURIA

Another potential biomarker for OAB is ATP. Increased ATP in urine has been well documented in individuals with OAB and IC compared to asymptomatic individuals (75, 181-183). Furthermore, a study by Conterez *et al* investigating ATP release from biopsies taken from individuals with OAB with pyuria (n=15), OAB without pyuria (n=33) and asymptomatic individuals (n=9) showed that biopsies with pyuria have a higher basal level ATP than the other groups, suggesting that infection leads to increased ATP release (182). It was also been shown that P2Y receptor expression is increased in patients with OAB with pyuria and ATP acts in a paracrine/autocrine manner. The authors concluded this research hypothesising intracellular bacteria as a cause for the observed increase in ATP and receptor expression. This investigation does however lack accompanying culture, which would have given more support to the idea that bacteria are at the centre of this hypothesis.

A study by Save *et al* has shown that bladder urothelial cells infected with intracellular UPEC in culture increase ATP levels in the supernatant of cells (75), however UPEC themselves contributed to this increase which was investigated by comparing ATP levels in bacterial, cell culture and co-culture medium. Studies by the same group have also shown that ATP release by cells infected with UPEC induces IL-8 release via P2 receptor signalling (75, 184, 185). Furthermore, ATP activation of P2 receptors directly affects neutrophil migration to the source of IL-8, acting in a chemokine manner (186). Taken together these findings highlights the importance of extracellular ATP and P2 receptor signalling in bladder bacterial infection and their combined role in generating immune responses. Additionally, since sensory nerves

and myofibroblasts express P2 receptors it is hypothesised that ATP released during infection can directly affect the afferent nerves inducing contractions of the detrusor muscle as part of the micturition reflex (43, 71) (figure 1.4).
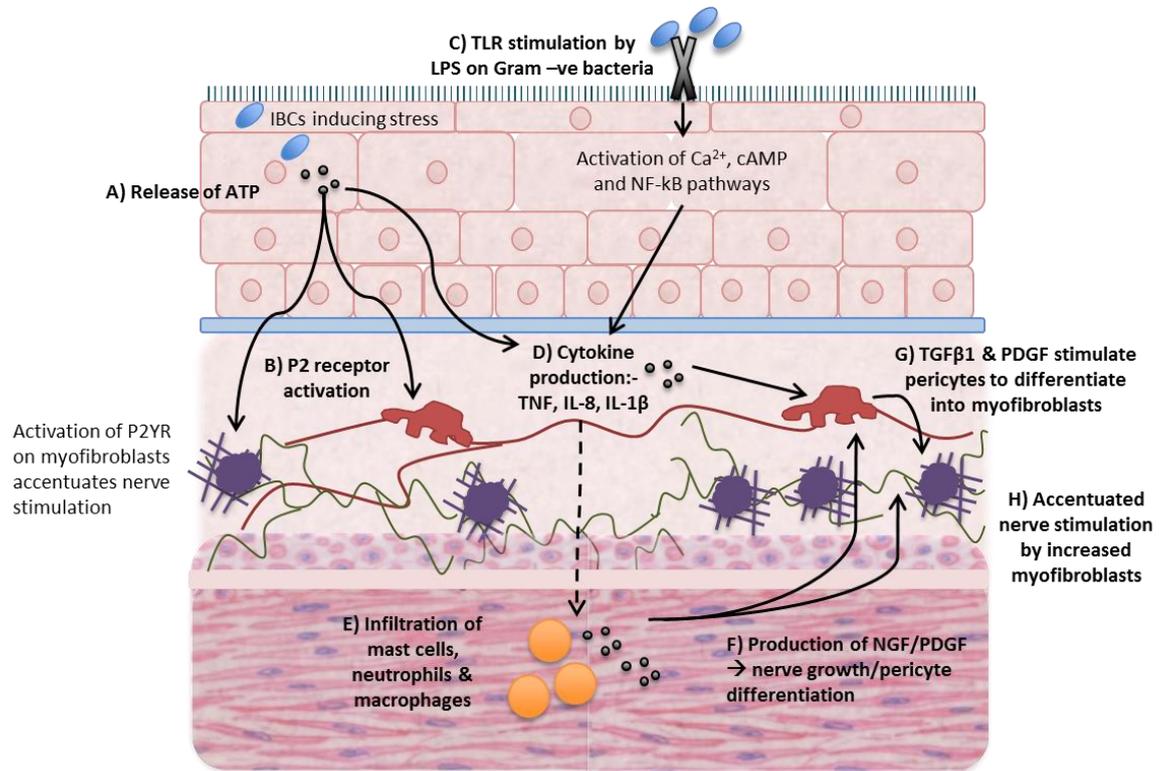
Figure 1.4: Schematic of how bacteria can alter the physiological function of the bladder A) ATP is released from stress-induced urothelial cells which may occur due to invasion by urothelial bacterium such as UPEC **(75)**. ATP is also released by the bacteria themselves, **B)** ATP release binds to P2Y receptors on urothelial & suburothelial cells and myofibroblasts (42). Myofibroblasts may in turn induce signalling of suburothelial sensory nerves (16, 42, 43). Excess ATP presence can activate cytokine production and directly induce neutrophil infiltration **(75, 184, 185)**, **C)** LPS on Gram-negative bacteria induce activation on Calcium, cyclic AMP and NF-kB pathways to release cytokines including IL-6, IL-8 and TNF **(159, 164, 165)**, **D)** Cytokines lead to infiltration of mast cells and neutrophils **(186)**, **E)** Mast cells release NGF which stimulates nerve development, maintenance and repair . They also release histamine, prostaglandins and tryptase which can cause smooth muscle contraction **(171-173) F)** Immune cell infiltration leads to production of more cytokines including NGF **(162, 178)** and PDGF which can lead to altered innervation and pericytes transdifferentiation, respectively, **G)** Stimulation of pericytes by TGFB1 and PDGF stimulates pericytes to differentiate into myofibroblasts (47). **H)** Increased myofibroblasts presence in turn increases the signal strength received by afferent nerves, which will in turn instruct the CNS of a sudden need to urinate (2).

1.6 THE BLADDER MICROBIOME

The work on low count bacteriuria in OAB by Moore and Malone-Lee discussed in *Section 1.5.3.2 'Low count' bacteriuria in lower urinary tract symptoms* suggests a possible role for pathogenic bacteria in the pathology of OAB. Interestingly, all of these studies made comparisons to asymptomatic individuals and in all cases the reports show presence of bacteria in asymptomatic individuals as well as in OAB (134, 136, 137). This supports the notion that urine is not sterile and that the bladder is normally colonised with bacteria. Furthermore, advanced methods comprising of enhanced culturing techniques and mass spectrometry have described a 'microbiome' present in bladders of asymptomatic individuals. The term microbiome refers to a niche of bacteria and their genes present in a particular environment. There is a well-described microbiome in the human gut which is tolerated by the immune system and the metabolites of which can prevent gut infection. Alterations in the gut microbiome have been associated with some gut diseases such as Crohn's disease and inflammatory bowel disease, where there is no obvious bacterial infection but the lack of some key 'good bacteria' or probiotics has led to dysfunction of the immune system (187). The notion of a potential bladder microbiome lends itself to the hypothesis that alteration may be a cause for some LUTS in much the same way. The following section discusses key bacterial species in the microbiome of the healthy bladder and the role that these bacteria play in regulating the immune system and protecting against pathogens. Alterations in the bladder microbiome can be caused by a number of factors, which will also be discussed, as well as the evidence to date about the potential effects this may have on LUTS.

1.6.1 A 'CORE' BLADDER MICROBIOME

Microbes isolated from the bladder were not included in the Human Microbiome Project based on the theory that urine is sterile (188). Research published in the last 6 years has rectified this misconception in both the scientific and general community. Many bacterial species are noncultivatable, that is, they cannot be cultured by routine microbiological techniques. Various alternative techniques have been adopted for identifying species including culture independent molecular PCR on the 16s rRNA gene and expanded quantitative urinary culture (EQUC). Multiple investigations led by Fortenberry *et al* (189-191), Jakobsen *et al* (192, 193),

and Wolfe *et al* (138, 194-197), have employed the former of these methods to identify bacterial species in the urine of healthy men and women. They each collected clean catch urine and extracted bacterial genomic DNA directly from the samples followed by next generation sequencing and bioinformatics which provides quantitative information on all species present within each sample. Taken together these studies have identified notable taxa which could make up the core bladder microbiome including: *Actinobaculum, Aerococcus, Anaerococcus, Atopobium, Bifidobacterium, Burkholderium, Corynebacterium, Finegoldia, Fusobacterium, Gardnerella, Lactobacillus, Jonquetella, Megasphera, Prevotella, Proteobacterium, Ralstonia, Sneathia, Staphylococcus, Streptococcus* and *Veillonella.* Hilt *et al*, led by Professor Wolfe, used EQUC which involved culture of CSU samples from OAB and asymptomatic individuals on blood agar, chocolate and colistin agar, and nalidixic acid agars in the following conditions: - aerobic at 35°C, aerobic at 30°C, 5% CO_2 at 35°C, anaerobic at 35°C and in campygas (6% O_2, 10% CO_2) at 35°C followed by species identification by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) (195). They identified and tabulated all the species that were cultured in each of these conditions affirming the most prevalent as *Lactobacillus* (15%), *Corynebacterium* (14.2%), *Streptococcus* (11.9%), *Actinomyces* (6.9%), and *Staphylococcus* (6.9%). Pearce *et al*, again led by Wolfe, recognised that even with EQUC some species were still missed when comparing to the spectrum of species found by high-throughput sequencing. Therefore they combined the results from high-throughput sequencing of the 16s rRNA gene with those from EQUC as well as MALDI-TOF MS to obtain the most complete description of bladder bacterial species to date (138). In this same study they observed the differences between the normal bladder and that of women with urinary incontinence. They concluded that there may not be key distinct differences between the two groups but rather that each individuals microbiome is unique and dysbiosis in the individual is a more likely cause of urinary symptoms. By understanding how the bladder microbiome serves to protect the bladder, it can be better understood how dysbiosis can possibly lead to dysfunction.

1.6.2 THE PROTECTIVE ROLE OF THE MICROBIOME

The bladder, much like the gut, is a mucosal tissue and is exposed to the exterior environment especially in women who have a shorter urethra than men, and therefore it is prone to colonisation by microorganisms. Many species of bacteria that colonise the mucosal tissues are tolerated by the host's immune system and live in symbiosis providing protection against pathogens and preventing overactivity of the immune system. The immune system's principal role is to protect against infection by recognition of foreign antigen within the body. The cells of the immune system undergo a selection check before they enter the peripheral body so that they recognise and tolerate self-antigen; this is known as central tolerance (for a review see Hogguist *et al* (198)). Since humans are obligate symbionts, that is, they rely on commensal microorganisms for survival, another level of tolerance is required to prevent elimination of necessary microbes which is known as peripheral tolerance (for a review see Mueller (199)). As microorganisms begin to colonise the host, which occurs straight after birth, the peripheral immune system begins to recognise commensal bacteria and develops tolerance towards them. In part, an individual's microbiome and their peripheral tolerance develop together. In the gut, where the biomass of microorganisms can reach up to 3% of total body mass, a unique mucosal immune system consisting of specialised cells such as microfold (M) cells, regulatory T (Treg) cells and tolerant antigen presenting cells (APC) help to develop tolerance to the commensal species. M cells obtain antigen from commensal bacteria in the gut lumen and transport it to other immune cells and Treg cells inhibit immune responses through the production of particular suppressive cytokines such as TGF-β and Interleukin 10 (IL-10). Activation of these cells by commensal bacteria helps to prevent autoimmunity and immune overactivity. Interestingly, in some autoimmune diseases such as Crohn's disease dysregulation of the immune system has been associated to alterations in the numbers of M cells and regulatory T cells (200). Therefore, commensal bacteria play a vital role in shaping, maturing and maintaining a balanced immune system, at least in the context of the gut.

As in the gut, constitutively secreted IgA (SIgA) is produced in the bladder (157), which acts to constantly inhibit bacteria for which it has a high affinity. IgA does not only act to eliminate pathogenic bacteria but can control signals generated by commensal bacteria for which it has a lower affinity. In the gut, dendritic cells 'taste'

the exposed environment and present commensal bacterial antigen to B cells which undergo class switching of the immunoglobulin genes to produce IgA in the presence of suppressive cytokines such as TGF-β (201). Notably, germ free mice have significantly reduced SIgA in their gut, therefore the presence of commensal bacteria is essential for the production of IgA (202). Although the same has not been proven in the bladder the presence of dendritic cells and SIgA suggests a similar mechanism may exist.

Many probiotic species are able to protect against pathogens directly, by producing metabolites and proteins such as lactic acid and various bacteriocins by *Lactobacillus* species. These species are also acid tolerant so they cannot be affected by their own production of lactic acid. *Lactobacillus crispatus* has been identified as the species that produces the highest concentrations of hydrogen peroxide of all *Lactobacillus* species in the bladder followed by *L.jensenii* (203)*. Other species have been shown to produce hydrogen peroxide; *L. delbrueckii, L. acidophilus, L. johnsonii and L. gasseri* (204)*. Together, this taxa contributes highly to the antimicrobial proteins and organic acids found in the body which can keep pathogenic bacteria at bay. Furthermore, *Lactobacillus* is of particular interest in the gut and vagina, where they have not only been shown to prevent infection in various clinical trials but have also been shown to modulate homeostasis and support a healthy nervous system and emotions (205), the circadian cycle (206), and prevention of bacterial vaginosis during pregnancy (207).

The commensal bacteria of the microbiome are therefore essential for preventing autoimmunity and maintaining production of antimicrobials and IgA by manipulating the host immune system. Also, key species appear to be able to contribute to the production of antimicrobials and can themselves directly affect bodily functions. This gives a strong foundation to advocate that dysbiosis of the bladder microbiome would be detrimental and inevitably lead to dysfunction.

1.6.3 ALTERATIONS IN THE BLADDER MICROBIOME

As with any microbiome, the symbiotic balance between microbes and host, and other microbes is ever changing and evolving as the environment changes. Certain bacteria will not be able to survive with alterations in temperature, pH, salinity and substrates allowing those that can to thrive and become more populous

by natural selection (208). Since the bladder functions as a storage for urine prior to voiding, the unwanted metabolites from kidney filtration will be present in the urine. Variations in diet can alter these metabolites and in the gut have been shown to reduce the diversity of the microbiome (209, 210). Drugs, both medicinal and recreational, can have a profound effect on the gut microbiome (210, 211), and this is likely to include the bladder microbiome too since both the gut and the bladder are exposed to downstream metabolites.

As previously mentioned, each individual's microbiome is unique. Ones exposure to microbes will evidently have an effect on the diversity of their microbiome. As an individual develops their hormones change, *i.e.* during puberty, pregnancy and menopause. Changes in hormones have in fact been shown to have an effect on the microbiome of the vagina, particularly in reducing *Lactobacillus* incidence in postmenopausal women (212). Furthermore, sexual exposure can have a profound effect on the urogenital microbiome (190, 191). Since each individual's microbiome is unique, unprotected sex can introduce new species, especially with oral and anal sex. Due to the proximity of the bladder to the vagina in women, changes in the vaginal microbiome will evidently lead to alterations in the bladder also. A most significant cause of alteration of the microbiome is the use of antibiotics, which can in some cases only target specific taxa leaving room for other species to grow. Furthermore, use of antibiotics can allow survival of bacteria with beneficial genetic mutations inferring antibiotic resistant leading to the development of antibiotic resistant niches within individuals and populations. Therefore it is inevitable that the microbiome will change as one is exposed to various factors.

1.6.4 MICROBIOME ROLE IN LUTS

Many of the aforementioned researchers who employed new methods to identify the core microbiome, furthered their studies by comparing samples from asymptomatic individuals with samples from individuals with LUTS, including IC (n=8) (192), OAB (n=41, 60, 11) (138, 194, 195), exposure to STIs (n=10, 10) (190, 191), and post renal transplant (n=60) (213). Of these that investigated the differences between the microbiota in asymptomatic individuals and women with OAB, increased incidence of *Gardnerella, Actinobaculum, Actinomyces, Aerococcus, Arthrobacter, Cornybacterium,* and *Oligella,* and reduced incidence of *Lactobacillus,*

Streptococcus, and *Bifidobacterium* were observed. Interestingly, Siddiqui *et al* noted a reduction in microbiome diversity in women with IC with increased incidence of *Lactobacillus* and reduced *Bacteriodes*, *Bifidobacterium* and *Clostridium* (192). These differences not only demonstrate that the microbiota from patients with OAB and IC both differ from asymptomatic individuals but that they are also unique from each other, suggesting that these syndromes are perhaps more distinct from each other than previously thought.

One particular study of interest observed changes in individual microbiomes of patients post renal transplant (213). These patients were treated with sulfmethoxazole-trimethoprim as a prophylactic antibiotic. The key species that decreased in prevalence between 1 and 6 months post renal transplant were *Proteobacteria*, *Escherichia* and *Porphyromonas,* likely due to the use of antibiotics. These studies indicate a significant alteration in the microbiome of the bladder with LUTS suggesting that bacteria may indeed play a role in development of bladder dysfunction however this is not proven. There could be numerous other factors affecting changes in the microbiome observed between comparable groups.

1.7 CURRENT RESEARCH QUESTIONS

There is an irrefutable amount of evidence that supports the fact that urine is not sterile, and rather that there is a bladder microbiome, which is unique to individuals and populations, and can be altered through lifestyle changes and disease. What is still unknown however, is whether alterations in the bladder microbiome are a direct cause of bladder dysfunction, and if so, how this alters the physiological function of the bladder. There are various research questions that have arisen from the discussions in the introduction. The research in this thesis will aim to determine if changes in the bladder microbiome may; i) be a cause for rUTI in renal transplant recipients in the first results chapter; ii) be a cause for some idiopathic cases of OAB in the second results chapter; and iii) lead to bladder dysfunction by investigating the role of pericytes during bladder infection in the final results chapter.

The first results chapter tries to determine whether changes in the bladder microbiome may be a cause for rUTI in RTRs. Alterations in the microbiome can be due to a variety of factors but one of the most devastating of these is the use of

antibiotics. The use of antibiotics is often acute and temporary as one to two weeks of treatment is sufficient to control infections. However, in the case of recurrent infections of the mucosal areas it is necessary to repeat antibiotic use and change the types of antibiotics prescribed as antibiotic resistance develops. Another example of extended antibiotic use is as a prophylactic during organ transplantation to counteract the use of immunosuppressants, which are essential for foreign organ tolerance. Recurrent infection as a result of antibiotic and immunosuppressants use is no more relevant to organ survival than in kidney transplantation, where patients are highly susceptible to rUTI post-transplantation. It is essential therefore to determine how use of prophylactic antibiotics and immunosuppressants alters the bladder microbiome post-renal transplant and if changes can indicate predisposition to rUTI and thus prevent infection before it manifests. The first results chapter takes a closer look at the exact species found in the bladder of renal transplant recipients over time to determine if the bladder microbiome does indeed change over time in these patients and whether it correlates with any clinical changes which can be used to predict UTIs and rUTIs.

The second results chapter tries to determine whether changes in the bladder microbiome may be a cause for OAB. OAB is an incredibly common syndrome costing millions to health services across the globe on treatments, many of which are unsuccessful due to the existence of idiopathic forms of OAB. The cause of IDO has recently been associated to low level bacteriuria however no real conclusions have been formed as to how this could lead to the symptoms of OAB. Many of the pathogenic bacteria associated with UTI, and more recently OAB, are able to invade host cells to form IBCs capable of avoiding antibiotics and immune responses, in turn leading to recurrent infections. A handful of studies have proposed that there may be an intracellular component to OAB, however a detailed description of both extracellular and intracellular bacterial species found in OAB and asymptomatic individuals does not yet exist. The second results chapter aims to confirm if there is indeed a role for IBC in OAB by performing an established isolation technique followed by genetic identification of bacteria. Furthermore, by determining if presence of IBC or particular pathogens correlates with any alterations in clinical characteristics it would be possible to understand exactly how infection could affect

the function of the bladder in OAB. In particular by looking at cytokines, ATP, and white blood cell count in order to link the observed increase in these biomarkers in OAB with infection. Additionally, any significant biological and clinical differences between OAB and asymptomatic individuals could potentially be developed further with an aim to better diagnosing this syndrome.

The third results chapter tries to determine whether changes in the bladder microbiome can be more easily detected. If changes in the bladder microbiome are significant to rUTI in post-renal transplant, or OAB, it would be essential to develop a more cost effective means of determining low-level infection than is currently employed by many diagnostic laboratories. In most laboratories, the criterion for infection is $10^4 – 10^5$ CFU/ml of a single species to determine infection, however it is clear that low-levels of bacteria are also capable of causing bladder dysfunction. Many of the methods used to determine exact species are costly and time consuming; therefore with the results from the clinical studies, we would like to develop a means of finding a more effective method of investigating the bladder microbiome in individuals.

The final results chapter tries to determine how changes in the bladder microbiome may lead to the physiological symptoms observed in OAB. Many studies have confirmed that significant differences in the microbiome exist between asymptomatic individuals and various LUTS, but it is not clear how alterations in the microbiome could lead to dysfunction. Increased numbers of pathogenic bacteria, even at less than 10^5 CFU, may interfere with the complex physiological and neurological control of normal bladder function by cytokine and ATP signalling for example. Many pathogenic bacteria found in UTI and OAB are Gram negative and therefore have LPS in their cell walls which is a potent activator of immune responses. Downstream cytokines of these responses can directly affect vascular permeability increasing immune cell infiltration, inflammation, ATP release and fibrosis. Furthermore, bacteria and infected/stressed cells release ATP, which cannot directly affect the afferent nerves and the detrusor but can diffuse to the lamina propria and stimulate P2 receptor expressing cells such as myofibroblasts and pericytes. Since pericytes in the bladder are already α-SMA positive and express PDGFRβ it suggests that these cells have the potential to develop into myofibroblasts in the presence of PDGF. The

research by Hashitani was concluded by suggesting "the pluripotency of pericytes may contribute to the remodelling of the bladder suburothelium that is commonly seen in an overactive bladder" (50). With this in mind, and the role that myofibroblasts play in bladder function as potential intermediary cells in the sensory process, the research in this thesis will aim to determine if infection could lead to pericyte changes.

1.8 HYPOTHESES

1. The use of antibiotics and immunosuppressants for renal transplant recipients leads to changes in the microbiome which predisposes these patients to rUTI.
2. The microbiome of women with OAB is different to that of asymptomatic women, potentially caused by increased age, predisposing older women to OAB.
3. Intracellular bacteria may be a reservoir in both renal transplant recipients and women with OAB leading to rUTIs and induced smooth muscle contraction in each case, respectively.
4. A cost effective, quick method for determining low-level infection needs to be developed which can be adapted by diagnostic laboratories in order to better identify potential causes for LUTS.
5. Bacteria in the bladder can lead to loss or differentiation of pericytes, altered activity of pericytes, remodelling of the suburothelium and accentuated sensory responses to bladder filling caused by an increased myofibroblast presence.

1.8.1 Aims and Objectives

In order to begin to test these hypotheses the following objectives will be carried out:-

1.

- Determine how the bladder microbiome changes over the course of 6 months post-renal transplant.
- Compare microbiome changes with clinical and biological observations to determine if there are any potential biomarkers for predisposition to rUTI in renal transplant.

2.

- Investigate the differences in the microbiota in the urine between patients with OAB and asymptomatic individuals in various age groups.
- Analyse and compare all clinical and biological information from patients with OAB and asymptomatic individuals to determine if there are any potential biomarkers for OAB.

3.

- Determine if there is an intracellular component to renal transplantation and OAB and which species are most prevalent in each case.
- Can presence of IBC be a predictor for rUTI post-renal transplantation?

4.

- Investigate various methodologies for identifying urinary species of bacteria and their adaptabilities for diagnostic laboratories.
- Determine how these methodologies can be used in conjunction with other diagnostic tests to potentially better diagnose OAB.

5.

- Observe pericytes in the urinary bladder of mice.
- Investigate the function of bladder pericytes by observing the responses to various vasoactive compounds.
- Determine the responsiveness of bladder pericytes to LPS and downstream cytokines.

CHAPTER 2 –

GENERAL METHODOLOGY

2.1 COLLECTION AND PROCESSING OF HUMAN SAMPLES

Collection of samples was individual for each project and is described in detail in the methods for each results chapter; sections *3.3* and *4.3 – Project specific methodology*.

2.1.1 PATIENT RECRUITMENT AND ETHICAL APPROVAL

Patients visiting renal outpatients clinic at Kent and Canterbury Hospital for renal donor and recipient check-ups provided midstream sample urine (MSU) samples for the renal transplant prospective cohort study (chapter 3). All recipients were prescribed co-trimoxazole 80/400 mg tablets (80 mg Trimethoprim and 400 mg Sulfamethoxazole) for 12 months post-transplant as a prophylactic for pneumonia. Upon being consented, patients were invited to provide an MSU at 1 month, 3 months and 6 months post-transplantation. On each visit they also fill in in a questionnaire designed by East Kent Hospital University Foundation Trust to determine symptoms and impact on life (*see Appendix 1.1 – Diagnosing urine infection in kidney transplant patients, EKHUFT, 2015).* Also, 2 MSU samples were taken from the renal donor patients before removal of the donor kidney, and acted as the asymptomatic control group. These were taken during donor check at approximately 1 month and 2 weeks pre-transplant and are therefore here forth named the 'donor baseline' group.

For the OAB study (chapter 4), MSU and CSU samples were collected from patients who fitted a specific description of OAB dictated by International Validated Questions from the International Consultation on Incontinence Modular Questionnaire – Female Lower Urinary Tract Symptoms Long Form (ICIQ-FLUTS-LF) (*see Appendix 1.3 - ICIQ-FLUTS-LF).* Patients were all women clinically diagnosed with OAB after an assessment of their history, cystoscopy, urinalysis, and urodynamics. All patients also indicated a positive response to Question 3a of the ICIQ-FLUTS-LF (Do you have a sudden need to rush to a toilet to urinate?). All patients were female aged 18 and over and suffered moderate to severe OAB with urgency, frequency and nocturia. Any patients with symptomatic UTI, IC, predominant SUI symptoms, or previous pelvic

radiation were excluded from the cohort. Asymptomatic control samples were also all from females, taken from women who had scored 0 on the ICIQ-FLUTS short form to ensure that they did not suffer with any OAB symptoms. All sampling methods met the ethical approval and each patient gave consent on every occasion before providing a sample. MSU samples were obtained from patients attending outpatient clinics at Medway Maritime Hospital, Gillingham, Kent. For CSU samples, patients with OAB partaking in a cystodistension clinical trial (214) at the Sunderland Day Case Clinic at Medway Maritime Hospital were requested to provide a CSU sample and bladder biopsies on the day of their surgery. The biopsies were taken via a cystoscopy (telescopic examination of the inside of the bladder) and were placed in sterile saline to be processed as according to the protocols for microbial culturing, electron microscopy or ATP assay (described later in this chapter). Asymptomatic control samples of catheter specimen urine were obtained from patients attending the same day surgery for non-bladder surgeries such as hysterectomy, myomectomy and laparoscopy. All samples were collected under general anaesthetic in a sterile operating theatre prior to the scheduled operation.

2.1.2 ROUTINE URINALYSIS AND NHS MICROBIOLOGY

Samples were immediately analysed with a urinalysis dipstick (Multistix, Seimens) for leukocytes, nitrites, protein, pH, blood, specific gravity, ketones and glucose (128, 215). Dipstick analysis is used as a rapid test for possible infection whereas traditional cell counting methods must be performed within 2 hours of sample collection and repeated three times for accuracy. Leukocyte esterase is released from leukocytes, usually present in urine during an infection. Nitrites are another sign of infection. Protein and glucose in the urine signifies kidney failure and diabetes, respectively. A small sample of urine was also mixed with Sternheimer-Malbin stain (Fisher Scientific, Loughborough, UK) in a ratio of 1:1 and loaded onto a standard haemocytometer (216). Shed urothelial cells, white blood cells and red blood cells (figure 2.1) were counted per µl and multiplied by a dilution factor of 2 since urine was diluted with stain in a 1:1 ratio.
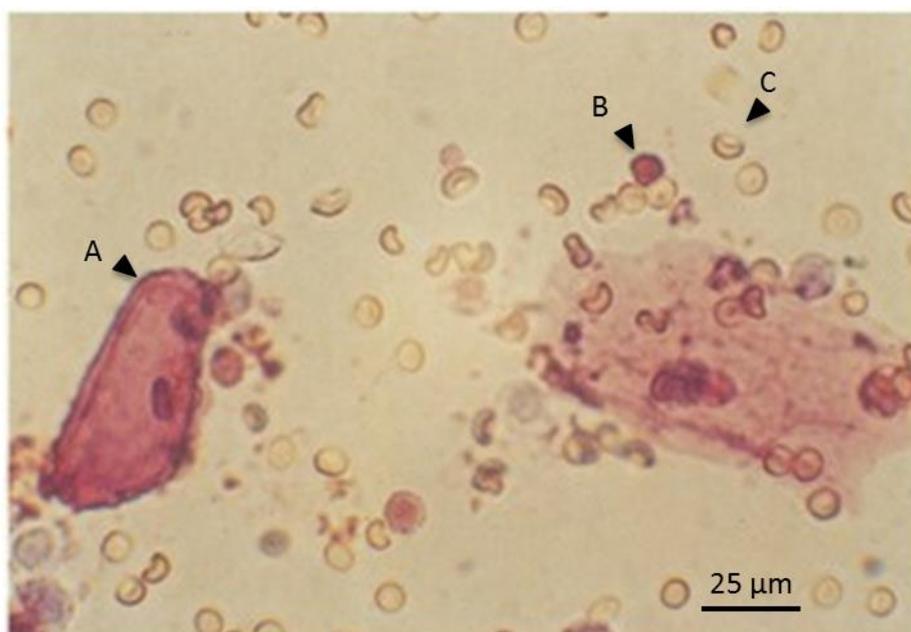
Figure 2.1: Image of Sternheimer-malbin stained urine. **A)** Urothelial cells are identifiable as large squamous cells with a dark pink central nucleus **B)** white blood cells are identifiable as small, compact, dark pink cells with a minimal cytoplasm, **C)** red blood cells are identifiable as nucleus-free, concave cells with a lighter colouration.

Urine samples were sent to hospital pathology for routine culturing and biochemical testing of cultures. Each hospital had its own protocol for culturing samples. Medway hospital was not able to provide a protocol for their urine culture however the protocol lfor East Kent Health Trust can be found in the appendix (*appendix 1.2 - MSU and CSU culture interpretation, EKHU NHS Foundation Trust, 2015*). Results of these were compared with our own (chapters 3 and 4).

2.1.3 PROCESSING OF FRESH URINE SAMPLES

Urine was kept on ice or in the fridge until processing, which was performed within a 4 hour window post-collection in order to reduce cell death, particularly of white blood cells (215). The following tests were performed on all samples and are discussed below; - i) urine was stained with DAPI (4',6-diamidino-2-phenylindole) to determine the presence of possible intracellular bacteria in urothelial cells (217), ii) any samples with prospective intracellular bacteria are then stained with acridine orange and crystal violet to determine if the bacteria are indeed intracellular (218), iii) urine was plated to culture microbes which were then identified using 16s rRNA

gene sequencing (137)(discussed in section *2.1.3.5 Identification of isolates by 16s rRNA gene sequencing*).

2.1.3.1 DAPI & ACRIDINE ORANGE/CRYSTAL VIOLET STAINING OF CYTOSPUN URINE

   Both MSU and CSU samples were stained with DAPI, which binds to DNA (figure 2.2B). This allowed the identification of urothelial cells with associated bacteria (clue cells), which may or may not be intracellular. Fresh samples were diluted with 4% v/w paraformaldehyde (PFA) in 0.4M phosphate buffered saline (PBS), in a ratio of 1:1 to fix them, then 100 μl was loaded into a Cytospin™ funnel attached to a standard microscope slide. The Cytospin™ centrifuge was run at 800 RPM for 5 mins depositing a thin-layer cell preparation on the microscope slide (figure 2.2A). The slides where then mounted with a small droplet of Vectasheild mounting medium with DAPI (Vector Laboratories, Peterborough, UK) and imaged with an x40 air objective on a Nikon eclipse 50i fluorescent microscope (217). DAPI was excited by a mercury bulb at 350 nm and emission was visualised via a DAPI emission filter at 470 nm (blue). Presence of urothelial cells with closely associated bacteria indicated that bacteria could be intracellular (figure 2.2B). Since DAPI can penetrate both eukaryotic and prokaryotic cells it was necessary to conduct a separate test to determine if the associated bacteria were indeed intracellular.
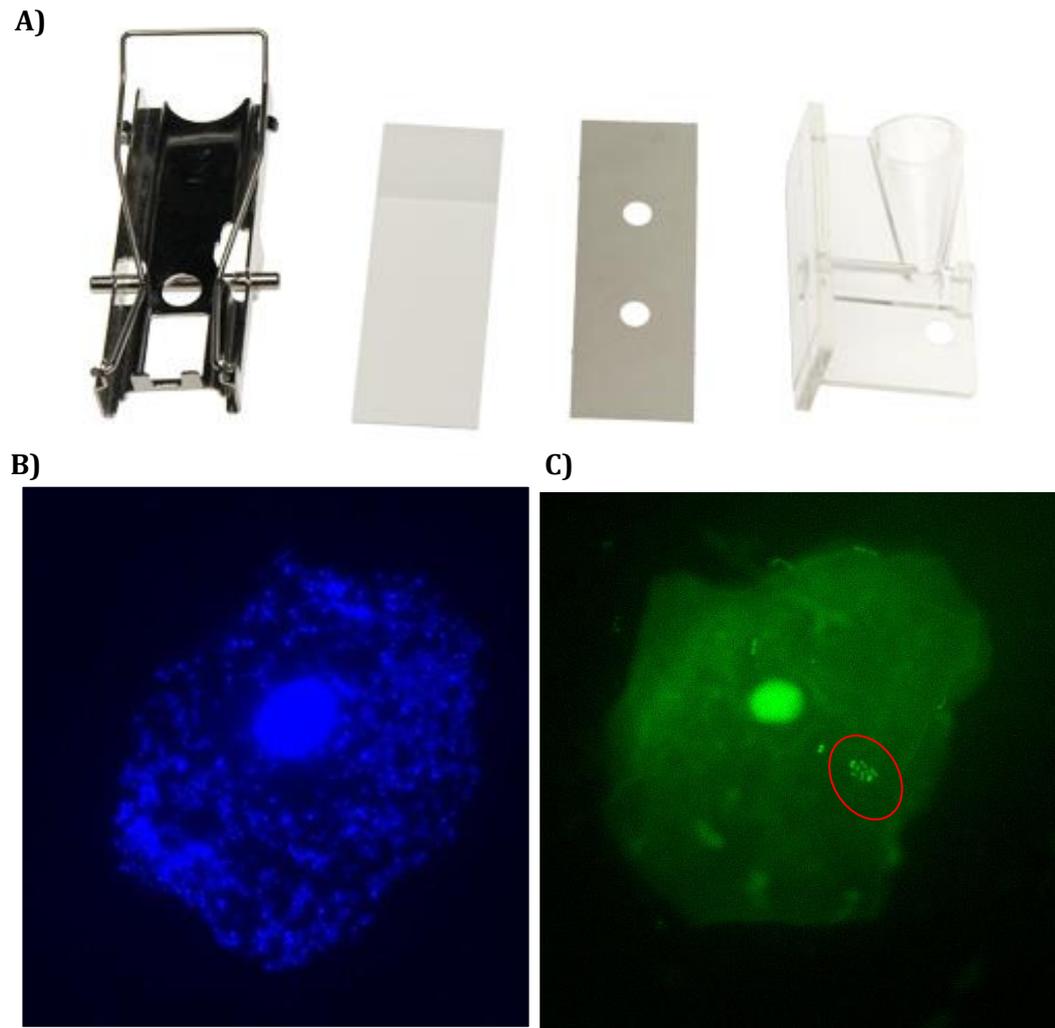
Figure 2.2: Image of cytospin setup and images of urothelial cells stained with DAPI and Acridine orange and crystal violet. A) Cytospin setup consisting of the metal holder, microscope slide, filter and funnel. Urine is pipetted into the top of the funnel and spun in a cytospin centrifuge. Cells are deposited through the filter holes on to the microscope slide. Excess liquid by the filter, which is discarded after use. **B)** DAPI stained urothelial cell with associated bacteria, which could be intracellular or extracellular. **C)** Acridine orange and crystal violet stained urothelial cell with intracellular bacteria indicated inside the red circle.

The subsequent procedure for staining urine was with acridine orange and crystal violet. Acridine orange binds to DNA and is able to penetrate eukaryotic cells and stain urothelial cell nuclei and all bacteria. Crystal violet however is not able to penetrate eukaryotes therefore would only stain extracellular bacteria by quenching the colour of the acridine orange from these particular cells (218). Cytospin™ funnels were loaded with fresh urine without PFA and run at 800 RPM for 5 mins depositing a thin-layer of urothelial cells on the microscope slide (figure 2.2A). Small DAKO™ pen circles were made around the cell preparations to allow the fluids to stay

concentrated during the staining process. The slides were washed 3 times with Hanks balanced salt solution (Sigma Aldrich, Gillingham, UK) on a rocker for 5 mins each time. Acridine orange (0.5%) (Sigma Aldrich, Gillingham, UK) was prepared in Gey's balanced salt solution (Sigma Aldrich, Gillingham, UK). This stain was added to the slides and kept covered to protect from light for 25 mins on the rocker at room temperature. The slides were then washed 3 times for 5 mins with Hanks balanced salt solution to remove excess acridine orange. The final stain was 0.1% crystal violet (Sigma Aldrich, Gillingham, UK) in 0.15 M NaCl. This solution was left for 25 mins on the rocker followed by a final 3x 5 min washes with Hanks. The slides were mounted with Citifluor AF1 (Agar Scientific) and analysed with an x40 air objective on a Nikon eclipse 50i fluorescent microscope. Acridine orange bound to DNA was excited by a mercury bulb at 502nm and emission was visualised via a FITC emission filter at 526nm (orange). One slide was prepared per patient and the presence or absence of intracellular bacteria was noted (qualitative) (figure 2.2C).

2.1.3.2 STREAK PLATING AND ANTIBIOTIC PROTECTION ASSAY

Fresh urine (5 ml, if available), was centrifuged in a 15 ml falcon tube at 800 RPM for 5 mins. The sediment was resuspended in 100 μl 0.1M PBS and 5 μl was streak plated on 2 chocolate agar plates per patient sample (E&O Labs) with a sterile single-use plastic 5 μl inoculation loop (Better Equipped, Wrenbury, UK) as shown in figure 2.3.
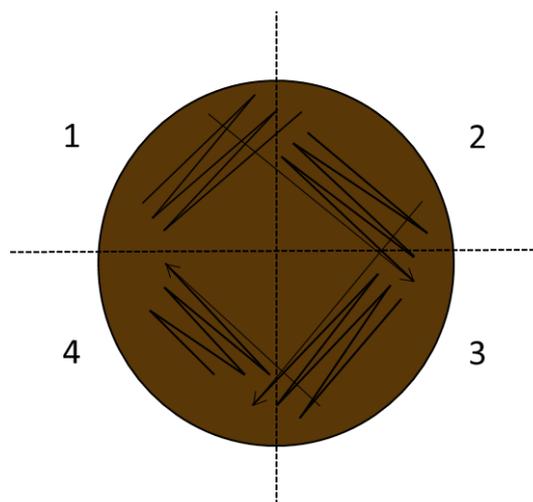
Figure 2.3: Diagram of method used for streaking urine on agar plates. Centrifuged urine (5 μl) was transferred to the chocolate plate in section 1, this was streaked back and forth in section 1, and a new inoculation loop was used to streak from section 1 across into section 2 and repeated through to section 4. In each section the bacterial concentration is reduced allowing for individual colonies to be isolated from sections 3 and 4.

One plate was placed in 5% CO_2 at 37°C for 48 h and one was placed in a 2.5 l anaerobic jar with anaerogen (Fisher Scientific, Loughborough, UK) and anaerobic indicator, resazurin (Fisher Scientific, Loughborough, UK) and kept at 37°C for 7 days (figure 2.4). Anaerobic bacterial growth is slower than in bacterial growth in 5% CO_2 therefore the plates in anaerobic jars were left for longer to ensure growth was optimised.

For samples taken from Medway Maritime Hospital, Gillingham, Kent, UK an antibiotic protection assay protocol was designed in order to identify bacteria that were antibiotic resistant and intracellular. This protocol was adapted from Khasriya *et al* (137). In cases where an antibiotic protection assay was performed, the remaining resuspension was treated with 700 μl Minimum Essential Medium Eagle (Sigma Aldrich, Gillingham, UK) containing L-glutamine, 5% filtered fetal bovine serum (Life Technologies, Paisley, UK), 200 ng/ml Amoxicillin, 200 ng/ml Gentamicin and 200 ng/ml Linezolid (all Sigma Aldrich, Gillingham, UK) overnight in order to kill any extracellular bacteria (figure 2.4). (These antibiotics were chosen specifically since they have poor intracellular penetration would therefore be less likely to affect intracellular bacteria. The concentrations were optimised by Khashriya in his PhD thesis, University College London, UK (219). After 16 hours, this was spun down at 800 RPM for 5 mins, the supernatant discarded and the pellet resuspended in the small amount of fluid remaining after centrifugation (approx. 100 μl) and from this 5 μl was streaked onto 2 chocolate agar plates each using a sterile 5 μl inoculation loop (figure 2.4). The antibiotics were then washed off by adding 5 ml 0.1M PBS to the falcon tube and centrifuging again. By performing a wash step, the antibiotics are removed and prevent any intracellular bacteria released in the following step from being killed by the antibiotics. The resuspended pellet was plated on 2 chocolate agar plates. Finally, 150 μl 0.01% Triton X-100 was added to the falcon and left for 5 mins at room temperature to lyse any urothelial cells and release intracellular bacteria. This was plated on 2 chocolate agar plates as detailed previously (figure 2.4). For each step, 1 plate was placed in 5% CO_2 at 37°C for 48 hours and 1 was placed in a 2.5 L anaerobic jar with anaeogen (Fisher Scientific, Loughborough, UK) and anaerobic indicator, resazurin (Fisher Scientific, Loughborough, UK) and kept at 37°C for 7 days. The number of intracellular bacterial CFU can be identified from the antibiotic

resistant ones by deducting the number of CFU from the third plating from the final plating (figure 2.4).
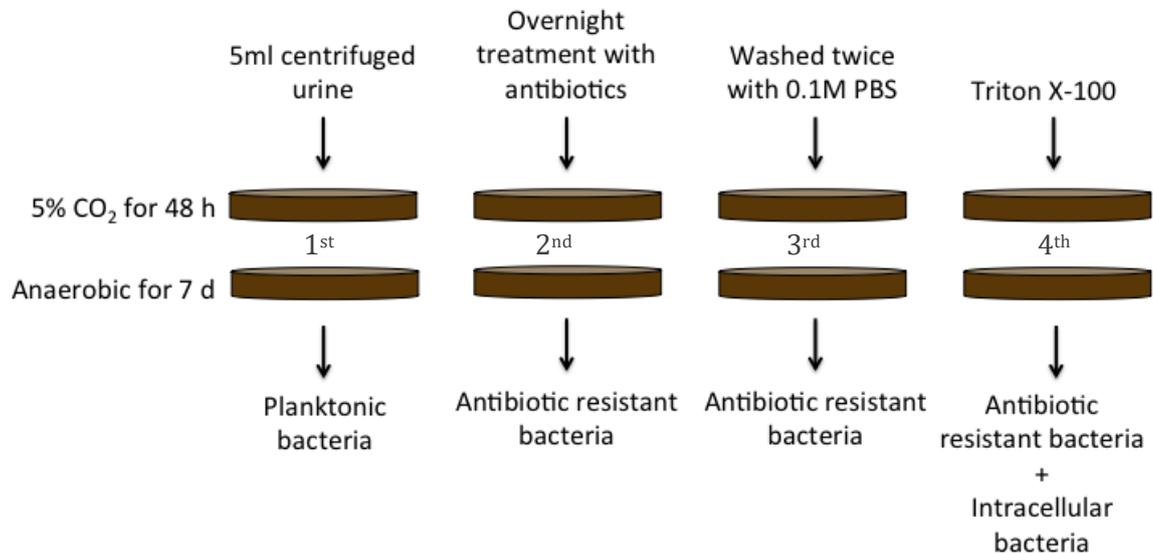
Figure 2.4: Illustration of antibiotic protection assay protocol, adapted from Khasriya *et al* (137). 5 ml of urine is centrifuged and the sediment plated on 2 plates to culture planktonic bacteria. The remaining sediment has an antibiotic cocktail added consisting of 700 μl Modified essential medium eagle containing L-glutamine, 5% filtered fetal bovine serum, 200 ng/ml Amoxicillin, 200 ng/ml Gentamicin and 200 ng/ml Linezolid. After 16 Hours this is plated a 2nd time on 2 plates to culture antibiotic resistant strains. The sediment is then washed with 0.1 M PBS and plated a 3rd time to culture antibiotic resistant bacteria which may have remained stuck to urothelial cells. Finally the sediment is treated with 150μl 0.01% Triton X-100 and plated a 4th time to culture both antibiotic resistant and intracellular bacteria. For each step 1 plate is placed in CO_2 and 1 in anaerobic conditions and incubated at 37oC for 48 hours and 7 days, respectively.

2.1.3.3 PURITY PLATING

At the end of the respective growth periods, plates were checked for growth and all colonies were identified by their appearance, counted and purity plated on chocolate agar. Purity plating was performed by inoculating individual colonies with a sterile inoculation loop (Better Equipped, Wrenbury, UK) and streak plated as in figure 2.3 on fresh chocolate plates. The purity plates were allowed to grow in 37oC for a further 48 hours/7 days in their respective environments. Once pure plates were obtained for each isolate, the isolates were Gram stained (discussed next), and the remaining bacterial growth from the purity plates was placed in cryogenic storage at -80oC in 1.5ml Nunc® CryoTubes (Sigma Aldrich, Gillingham, UK) containing Brain Heart infusion broth (Sigma Aldrich, Gillingham, UK) and 10% Glycerol (Sigma Aldrich, Gillingham, UK) to prevent cell lysis during freezing.

2.1.3.4 GRAM STAINING

      A Gram staining kit was obtained from Sigma Aldrich, Gillingham, UK. A thin emulsion of bacteria mixed with sterile dH_2O was prepared on a microscope slide then fixed by passing over a flaming Bunsen burner. Gram's crystal violet solution (100%) was flooded over the microscope slide and left for 1 min. Crystal violet enters the peptidoglycan layer of bacteria, of which there is more in Gram-positive bacteria. Excess crystal violet was then rinsed off with tap water and then the slide flooded with Gram's iodine for 1 min to trap crystal violet in the peptidoglycan. The iodine was rinsed off with tap water and a few drops of 50% acetone, 50% ethanol decolourisation solution added for 3-4 seconds to remove crystal violet and iodine from the thinner layers of peptidoglycan found in Gram-negative bacteria. A counter stain of Gram's safranin was flooded over the slide for 1 min to stain Gram-negative bacteria and rinsed off with tap water and allowed to dry. Slides were covered with immersion oil (Sigma Aldrich, Gillingham, UK Aldrich), then a cover slip and a second drop of immersion oil. Bacteria were identified as Gram-positive (violet) or -negative (pink) as well as their shape (*e.g.* bacillus, coccus) using a standard light microscope with a x100 oil objective

  In cases where the Gram stain was questionable, bacteria were regrown from cryopreservation and a fresh stain performed. If bacteria were particularly small, x100 focus was not sufficient to determine the exact shape of the bacteria. The Gram stain result for these isolates was noted as 'Inconclusive'

2.1.3.5 IDENTIFICATION OF ISOLATES BY 16S rRNA GENE SEQUENCING

      Isolates were identified by sequencing of the 16s rRNA gene. Isolates were revived from cryogenic storage by replating on chocolate agar. A small scraping of frozen crybroth containing the cryogenically preserved bacteria was taken from the CryoTubes with an inoculation loop and streaked onto chocolate plates and incubated in $37^{O}C$ for a 48 hours in the environment in which the isolates were originally discovered (5% CO_2 or anaerobic). Any isolates that had contaminated or unsuccessful regrowth were noted and later replated using more of the cryopreserved broth and more of the chocolate plate. The initially colony description was referred to in order to identify the correct isolate in cases of contaminated

regrowth. Where it was not possible to resuscitate the isolate, 'Failed regrowth' was noted.

If resuscitated culture was pure, a 1 μl inoculation of each isolate was suspended in 100μl sterile nuclease free water (Thermo Scientific, Paisley, UK) in a PCR reaction tube and lysed using an Eppendorf Mastercycler™ (Eppendorf, Stevenage, UK) set to 94°C for 5 mins to extract genomic DNA. A polymerase chain reaction was set up for each isolate in a sterile PCR reaction tube. Each tube contained 5 μl genomic DNA (bacterial lysate of unknown concentration), 12.5 μl Q5 Polymerase Mastermix (New England Biolabs, Hitchin, UK), 1.25 μl forward primer (DG74Fwd - 5'-AGG AGC TGA TCC AAC CGC A-3'), 1.25 μl reverse primer (RDR080Rev - 5'-AAC TGG AGG AAG GTG GGG AC-3') (Eurofins, Ebersberg, Germany) and 5 μl sterile nuclease free water (Thermo Scientific, Paisley, UK). Primers were chosen based on their correspondence to highly conserved areas in pathogenic bacteria (220). The reaction was run on an Eppendorf Mastercycler™ at the following temperatures and times based on the primer and gene size: - 1) 98°C for 30 seconds (denature), 2) 98°C for 10 seconds (denature), 3) 55°C for 20 seconds (anneal), 4) 72°C for 20 seconds (extend) [Steps 2-4 repeated 37 times], 5) 72°C for 2 mins (final extension). An extra reaction was prepared using 5 μl sterile nuclease free water instead of 5 μl bacterial lysate to act as a negative control and another reaction containing *E.coli* lysate, which had previously been successfully identified by 16s rRNA gene sequencing, to act as a positive control. Each PCR product was checked for reaction success and purity by loading on a 1% agarose gel containing ethidium bromide (Fisher Scientific, Loughborough, UK) and running at 120V for 90 mins alongside the negative control and a 50 basepair ladder to determine band size. Successful PCR reactions were identified based on the presence of a band at the correct size for the 16s rRNA gene which is around 300 basepairs (figure 2.5). Any PCR products that did not show a band at the correct size based on the 50kb ladder or had no band at all had PCR performed again using an alternative reverse primer (RW01 - 5'-AACTGGAGGAAGGTGGGGAT-3')(taken from the PhD of Alberto Conterez-Sanz, supervised by Scott Wildman at The Royal Veterinary College, University of London (221)). If by checking with gel electrophoresis PCR had failed using both sets of primers the isolate was noted as 'Failed PCR'.
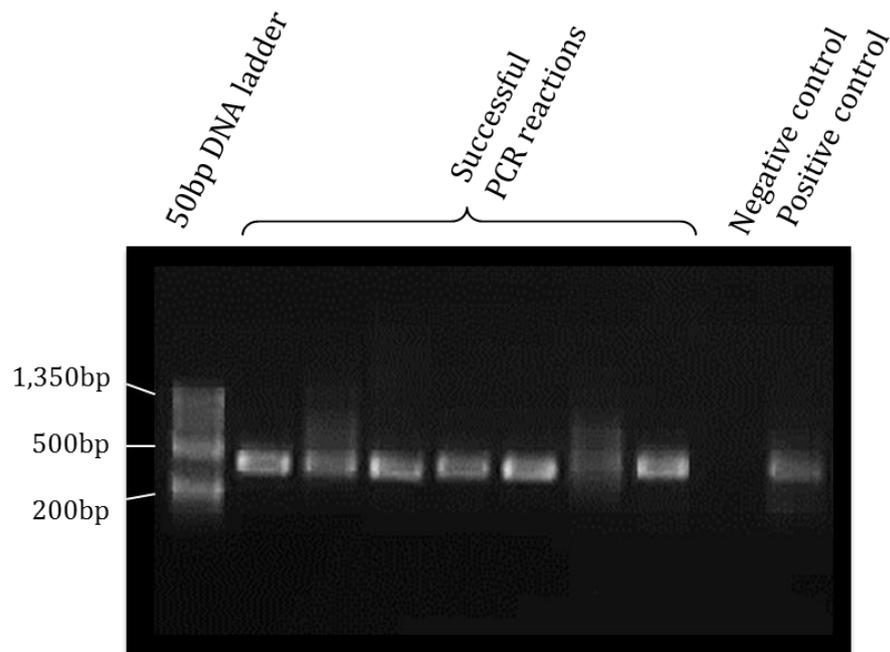
Figure 2.5: Example of a gel image of successful PCR products with a 50 base pair ladder. Ladder is used to determine the size of bands in PCR products. A negative control with no bacterial lysate indicates that there is no contamination in the PCR reaction mastermix. A positive control using *E.coli* lysate shows how bands should be seen in successful PCR reactions.

Successful PCR reactions were purified using a PCR Purification Kit (Qiagen, Manchester, UK). The 15 µl of PCR product remaining after the gel electrophoresis is mixed with 75µl Buffer PB and transferred into a QIAquick spin column insert (figure 2.6) and centrifuged at 13,000 RPM for 45 seconds. At this stage, the DNA binds to the white filter of the column. The flowthrough was discarded and 750 µl Buffer PE was added and centrifuged at 13,000 RMP for 45 seconds. This contains ethanol and acts as a washing step which removed excess dNTPs, buffers, polymerase and magnesium salts but kept the DNA bound in the filter. A second centrifugation step ensured all the Buffer PE had been removed from the column and the column insert was then transferred to a labelled Eppendorf tube™. 50µl Buffer EB was added to the column insert and left for 1-2 mins to allow the DNA to elute from the filter. A final centrifugation step drew the purified DNA into the Eppendorf tube™.
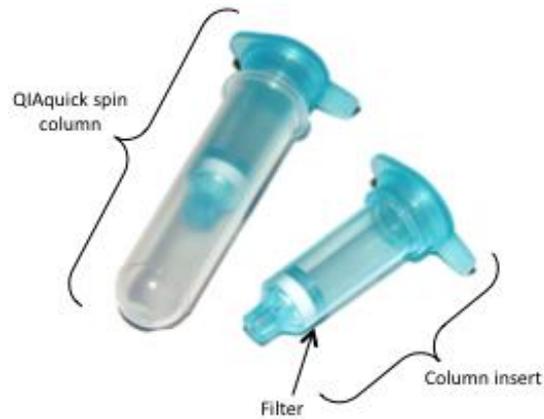
Figure 2.6: Image of QIAquick spin column used to purify DNA from successful PCR reactions. DNA is bound in the filter of the column insert when mixed with buffer PB. A wash step keeps DNA in the filter and removes excess buffers, polymerase, nucleotides and magnesium into the receptacle of the spin column. A final step adding buffer EB to the column insert elutes DNA from the filter into an Eppendorf.

The concentration of each product was recorded using a Nanodrop 2000 (Thermo Scientific, Paisley, UK) and adjusted to 5 ng/μl using sterile nuclease free water. The purified PCR product (15 μl, 5 ng/μl) was added to a Mix2Seq tube (Eurofins, Ebersberg, Germany Genomics) with 2 μl 10 pM forward primer (used for the original PCR reaction) and sent to Eurofins Genomics, Ebersburg, Germany for Sanger sequencing. If the returned sequence was very short, consisting of only 5-10 nucleotides, or had no bases at all (shown as 'NNN'), a BLAST could not be performed. In these cases, the purified PCR product was run again on a gel in order to check that there was indeed a band of the correct size. If no band was seen, a PCR was run again, checked using gel electrophoresis, purified and sent for sequencing. If a band was seen the sample was sent again for sequencing with no further modifications. Most often, the sequencing would generate a usable sequence which could be BLASTed to determine a species ID, however if the sequencing had failed twice those isolates were noted as 'Failed sequencing'. A microbe genomic BLAST (Basic Local Alignment Search Tool, NCBI) was performed on the returned sequences and the microbial identity with the highest percentage genetic match with the lowest indent was taken as the species and recorded in a database and compared with the Gram stain for accuracy.

It was important to compare the observed Gram stain/shape of the sequenced isolates with the true Gram stain/shape of the bacterium proposed by BLAST. If the Gram stains were not concordant these isolates were omitted from the final comparisons and noted as 'non-concordant'.

2.1.4 ASSAYS ON FROZEN URINE

A variety of assays were performed on patient urine to analyse nucleosides *e.g.* ATP and two particular cytokines of interest: - IL-8, and MCP-1 alongside creatinine to standardise all the assays.

2.1.4.2 CREATININE ASSAY

Creatinine is a breakdown product of creatine phosphate in the muscles and is normally produced at a constant rate. It is excreted through glomerular filtration of the kidneys into the ultra-filtrate which forms urine. Since water is excreted and reabsorbed in the kidneys, the concentration of creatinine can vary depending upon the quantity of urine produced. It is therefore necessary to standardise all of our assays to creatinine levels. A creatinine assay (R&D Systems, Abingdon, UK) was performed on all samples (each patient was done in duplicate) before the ELISA assays and luciferin/luciferase assay. In order to measure the creatinine concentration in urine, the samples first had to be defrosted and required a 20-fold dilution in dH_2O. Following this, mixing 2.5 ml of NaOH with 12.5 ml of picric acid made an alkaline picrate solution. A standard of 100 mg/dl creatinine was initially diluted 1 in 5 to 20 mg/dl in dH_2O then serially diluted 1 in 2 to 10 mg/dl, 5 mg/dl, 2.5 mg/dl, 1.25 mg/dl, 0.62 mg/dl and 0.31 mg/dl. 50 μl of standard, control (dH_2O only), and prepared urine was plated out in triplicate. 100 μl of the alkaline picrate solution was added to all wells and incubated for 35 mins at room temperature. The light absorbance was measured on an Infinite M200 Pro set at 490 nm using Tecan i-control software. Graphpad Prism 5.0a was used to create a standard curve constrained to go through zero (figure 2.7) and the concentration of creatinine extrapolated for each patient urine sample.
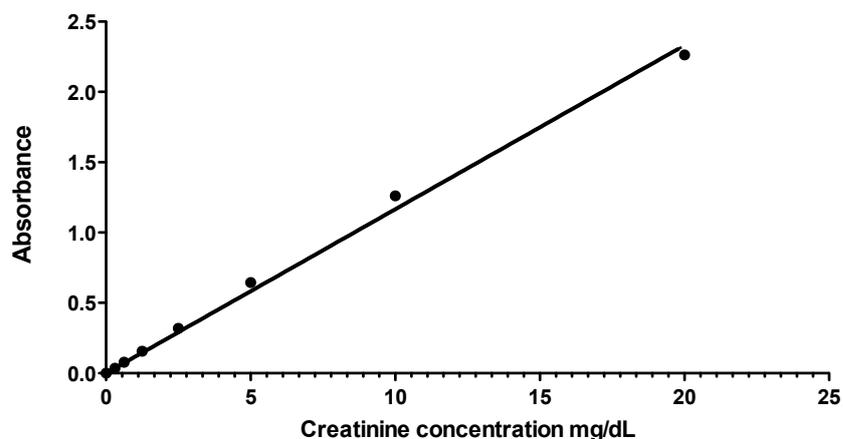
Figure 2.7: Example of standard curve for creatinine. Standard curves were created in GraphPad Prism and then used to extrapolate concentrations of creatinine in urine samples from.

2.1.4.2 LUCIFERIN/LUCIFERASE ASSAY

An adenosine 5'-triphosphate (ATP) Bioluminescent Assay kit was purchased from Sigma Aldrich, Gillingham, UK. Standards of ATP were prepared by performing a 10 fold serial dilution of stock 10^{-2} M of ATP to give 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8} M. Into a white 96 well plate 50μl of 0.01M PBS was added into each of the required wells. 1 μl of the 10^{-3} M standard was added into the first 3 wells. This gave a concentration of 2×10^{-5} M. The same was performed for the other standards as shown in figure 2.8 below and a negative control of just 0.01M PBS was included at the bottom. 1μl of urine was added into the other wells in triplicate. ATP is rapidly degraded by freeze-thaw cycles and therefore these were avoided for all urine samples.
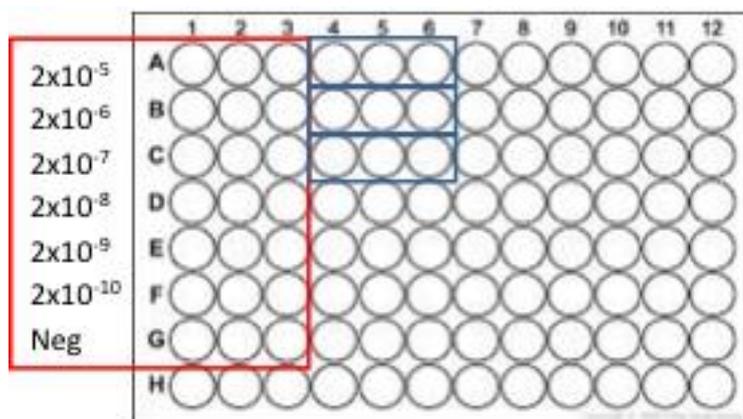
Figure 2.8: Diagram of ATP standard concentrations in Luciferin/luciferase assay. Areas where standards were added to a white 96 well plate in triplicate and the respective concentrations at the side (RED) and the wells used for measuring the concentration of ATP in patient urine samples (BLUE)

The ATP Assay Mix (FLAAM) and ATP Assay Mix Diluent (FLAAB) were mixed fresh for each assay. Firstly, the required volume for the total number of wells was calculated; - FLAAB and FLAAM were added together in a ratio of 25:1 therefore 60 μl FLAAM was added to 1440 μl FLAAB. The reaction mixture was protected from light pollution since luciferin is rapidly degraded by light. The final mixture (50 μl) was added to each well and the luminescence was read immediately on a BioTek Plate Reader with Gen 5 software. Graphpad prism 5.0a was used to create a standard curve (figure 2.9) and the concentration of ATP extrapolated for each patient urine sample. These values were divided by the creatinine concentration for each patient in order to standardise the results.
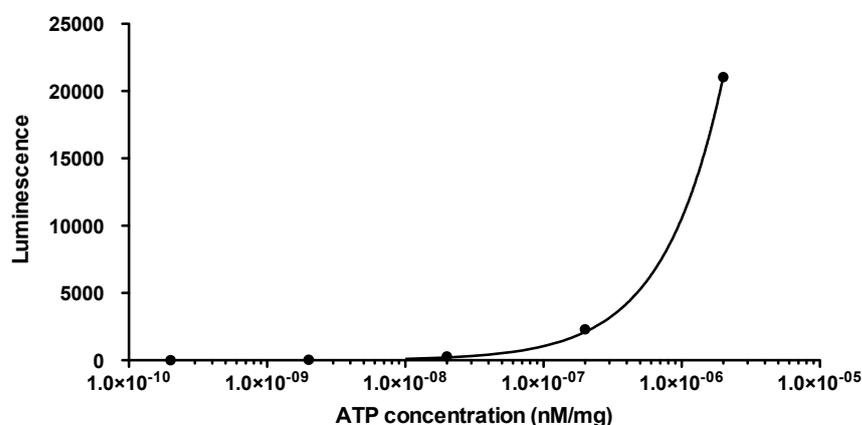
Figure 2.9: Example of standard curve for ATP. Standard curves were created in GraphPad Prism and then used to extrapolate concentrations of creatinine in urine samples from.

ELISA assays were performed on samples of urine from individuals with OAB and asymptomatic controls to measure the concentration of IL-8 and MCP-1 in the urine.

For the IL-8 ELISA (Invitrogen, Paisley, UK), the standard was first reconstituted to 10 ng/ml with standard diluent buffer which was then diluted 10-fold in standard diluent buffer to create an initial standard concentration of 1000 pg/ml. This was followed with 2-fold serial dilutions in standard diluent buffer to generate concentrations of 500 pg/ml, 250 pg/ml, 125 pg/ml, 62.5 pg/ml, 31.2 pg/ml and 15.6 pg/ml. Standard diluent buffer with no IL-8 standard added acted as a negative control. Urine samples were defrosted from -20°C storage and 50 µl was added to the relevant wells. The IL-8 standards (50 µl) were also added to the relevant wells. IL-8 binds to anti-IL-8 antibodies bound to the bottom of the wells. This was followed by 50 µl of biotinylated anti-IL-8 solution and then left at room temperature for 90 mins. The anti-IL-8 solution contains anti-IL-8 antibodies that bind to the IL-8 in a sandwich (figure 2.10). After the incubation time, all the liquid was decanted from the wells and washed 4 times with working wash buffer (25x wash buffer diluted 25-fold in dH$_2$O). Any unbound anti-IL-8 solution was discarded in this step. After washing 100 µl of streptavidin-horse radish peroxidase (HRP) working solution (10 µl of 100x concentrated solution with 1 ml streptavidin-HRP diluent) was added to each well and incubated at room temperature for 30 mins. In this step, the presence of anti-IL-8 antibodies allowed the streptavidin to bind to it, which only occurs in the presence of IL-8. Post-incubation, the wells were decanted and washed 4 times as previous. The final step was to add 100 µl of stabilised chromogen to each well. Chromogen turns blue in the presence of HRP. With higher concentrations of IL-8, there was a higher concentrations of HRP and therefore a deeper blue colour. After 30 mins, 100 µl of stop solution was added to each well. This turns the blue solution to yellow. The light absorbance of the wells was measured using a BioTek Plate Reader set at 450nm with Gen 5 software. Prism 5.0a was used to create a standard curve (figure 2.10) and the concentration of IL-8 extrapolated using Graphpad prism for each patient urine sample. These values were divided by the creatinine concentration for each patient in order to standardise the results.

For the MCP-1 ELISA (Life Technologies, Paisley, UK), the standard was first reconstituted to 10,000 pg/ml with standard diluent buffer which was then diluted 10-fold in standard diluent buffer to create an initial standard concentration of 1000 pg/ml. This was followed by a 2-fold serial dilution using standard diluent buffer to generate working concentrations of 500 pg/ml, 250 pg/ml, 125 pg/ml, 62.5 pg/ml, 31.2 pg/ml and 15.6 pg/ml. Standard diluent buffer with no MCP-1 standard added acted as a negative control. Samples were defrosted and diluted 4-fold in standard diluent buffer (50 μl urine in 150 μl buffer). Incubation buffer (50 μl) was added to all required wells followed by 50 μl of standard and prepared samples in triplicate. On top of this 50 μl of human MCP-1 biotin conjugate was added to each well except to the chromogen blanks. The plate was left to incubate at room temperature for 2 hours. MCP-1 binds to anti-MCP-1 antibodies bound to the bottom of the wells. The MCP-1 biotin conjugate solution contained anti-MCP-1 antibodies that bind to the MCP-1 in a sandwich (figure 2.10). After the incubation time, all the liquid was decanted from the wells and washed 4 times with working wash buffer (25x wash buffer diluted 25-fold in dH_2O). Any unbound MCP-1 biotin conjugate solution is discarded in this step. After washing 100 μl of streptavidin-horse radish peroxidase (HRP) working solution (10 μl of 100x concentrated solution with 1ml streptavidin-HRP diluent) was added to each well except the chromogen blanks and incubated at room temperature for 30 mins. In this step, the presence of MCP-1 biotin antibodies allows the streptavidin to bind to it, which will only occur in the presence of MCP-1. Post-incubation, the wells were decanted and washed 4 times as previous. The final step was to add 100μl of stabilised chromogen to each well and incubated for 30 mins at room temperature in the dark. Chromogen turns blue in the presence of HRP. With higher concentrations of MCP-1, there will be higher concentrations of HRP and therefore a deeper blue colour. After 30 mins, 100 μl of stop solution is added to each well. This turns the blue solution to yellow. The light absorbance of the wells was measured using a BioTek Plate Reader set at 450 nm with Gen 5 software. Graphpad prism 5.0a was used to create a standard curve (figure 2.10) and the concentration of MCP-1 extrapolated using Graphpad prism for each patient urine sample. These values were divided by the creatinine concentration for each patient in order to standardise the results.
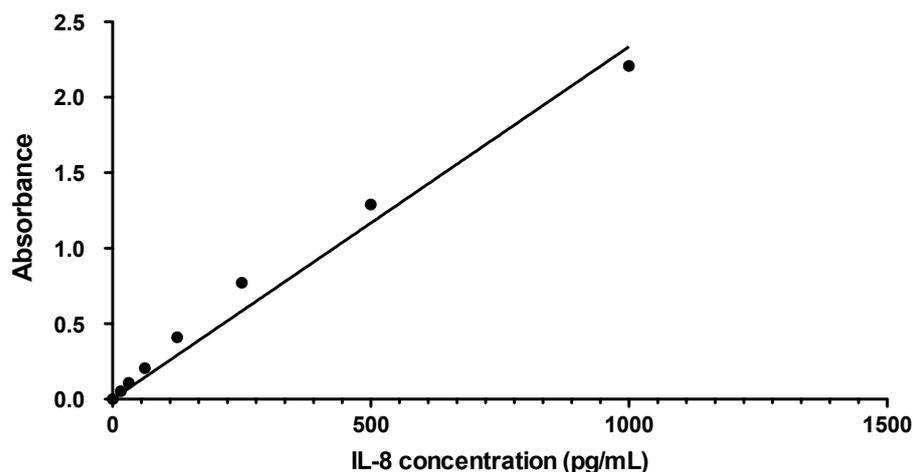
Figure 2.10: Example of standard curve for IL-8 and MCP-1. Standard curves were created in GraphPad Prism and then used to extrapolate concentrations of creatinine in urine samples from.

2.1.5 BLADDER BIOPSIES

Bladder biopsies were taken from patients with pure OAB as diagnosed by the ICIQ-FLUTS long form (*appendix 1.3*) attending the Sunderland Day Care Centre at Medway Maritime Hospital for a cystodistension clinical trial (214). The patients were asked if they would be willing to provide a CSU sample and biopsies of bladder for research purposes during their cystodistension. Patients were given a general anaesthetic and the samples taken prior to the cystodistention. A total of 4 biopsies were taken per patient; 2 were sent to hospital pathology for clinical diagnosis and 2 were provided to the university for either bacterial culture or electron microscopy, the details of which are described in the following sections. Biopsies were collected from the hospital and transported back to the university in Eppendorf tubes™ containing a sterile saline solution provided by the hospital on ice.

2.1.5.1 BACTERIAL CULTURE FROM BLADDER BIOPSIES

The antibiotic protection assay was performed on the biopsies as discussed previously, however prior to this the biopsies had to be broken up. The Eppendorf tubes™ containing the biopsies were shaken and gently vortexed to detach bacteria and urothelial cells then the protocol for the antibiotic protection assay was followed (details in section *2.1.3.2 – Antibiotic Protection Assay*), followed by purity plating,

Gram staining and identification of isolates by 16s rRNA gene sequencing as discussed in section *2.1.3.5 - Identification of isolates by 16s rRNA gene sequencing.*

2.1.5.2 ELECTRON MICROSCOPY ON BLADDER BIOPSIES

Some biopsies were used for imaging using TEM to look for intracellular bacteria (111). The biopsies were immediately placed in gluteraldehyde overnight to fix them. They were then washed 3 times in 0.01M PBS to remove the gluteraldehyde. Each biopsy was transferred into a 7 ml glass vial and submerged in a 4% stock osmium tetroxide (OsO_4, Sigma Aldrich, Gillingham, UK) in water (4 ml H_2O, 2 ml OsO_4, 4 ml 2x PBS) and left for 1 hour at room temperature in order to stain the biopsies to provide contrast for imaging. After this incubation time, the OsO_4 was washed off with dH_2O (x2) for 5-10 mins each time. Then the biopsies were further fixed by incubation at room temperature in a series of increasing concentrations of ethanol:- 30% for 10 mins, 50% for 10 mins, 70% overnight, 100% for 10 mins (x2). The biopsies were next transferred to propylene oxide (Sigma Aldrich, Gillingham, UK) for 15 mins (x2) to further dehydrate them then a 50:50 solution of propylene oxide mixed with epoxy embedding medium (Sigma Aldrich, Gillingham, UK) for 30 mins to gradually infiltrate the embedding medium. This was then followed by 30 mins in pure epoxy embedding medium (x2) assuming orientation of the biopsy within. The embedded biopsies were transferred to foil embedding capsules to retain the shape and stored in a 60°C oven for 24 hours to harden the resin.

In order to cut the embedded biopsy into 0.5 μm thin slices, a glass knife was prepared by cutting a fine edge into a small square of glass. The edge was then used to form a boat with silver tape (figure 2.11A). The sample was placed in a chuck of a microtome slicer and the resin was then trimmed to size before thin 0.5μm slices were captured in the boat of the glass slicer and transferred to a coverslip. Each slice was then air dried and stained with 1% toluidine blue to stain for individual structures in the cells. These slices were then transferred to gold holders (figure 2.11B) to be inserted into the electron microscope. Images were obtained of potential intracellular bacteria and autophagosomes within the urothelial cells of the bladder biopsies.
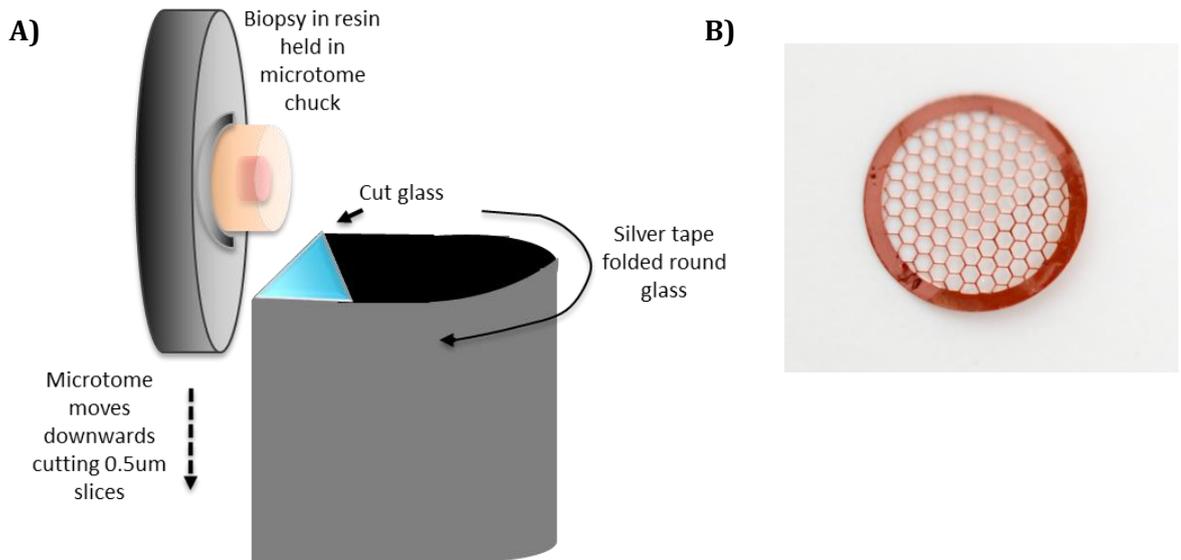
Figure 2.11: **Diagram of microtome used to slice biopsy and holders used to mount slices for TEM. A)** Diagram showing how glass cut at a 45° angle is used to cut resin bound bladder biopsies. The biopsy is held in the chuck of a microtome which moves downwards cutting 0.5μm thick slices on the glass. The slices are collected in a boat created by looping silver tape around the glass block. **B)** An example of the 3mm diameter gold holders used to mount the toluidine blue stained slices for imaging in the electron microscope.

2.2 BLADDER PERICYTE OBSERVATION AND FUNCTIONAL EXPERIMENTS

2.2.1 TISSUE PREPARATION

Physiological saline solution (PSS) was prepared fresh prior to each experiment containing: - 100mM NaCl, 5mM KCl, 0.24mM NaH_2PO_4, 0.96mM Na_2HPO_4, 10mM Na acetate, 1mM $CaCl_2$, 1.2mM $MgSO_4$, 5mM glucose, 25mM $NaHCO_3$, and 5mM Na pyruvate (all Sigma Aldrich, Gillingham, UK) as suggested by Crawford *et al* for *ex vivo* observations of kidney slices (52). This was bubbled with 95% O_2/5% CO_2 to conserve tissue viability. pH was adjusted to 7.4 with 10M NaOH and maintained via the bicarbonate buffering system by presence of HCO_3, H_2CO_3 and CO_2.

9 week old male JAX C57/Black 6 mice of an average weight of 21-26 g (Charles River UK Ltd, Kent, UK) were culled by cervical dislocation in accordance with the Animals Scientific Procedures Act of 1986. The bladder was immediately removed and placed in PSS bubbled with 95% O_2/5% CO_2. The bladder was then transferred to a petri dish containing fresh PSS, also being bubbled with 95% O_2/5% CO_2, under a dissection scope and the urothelium was carefully removed using forceps, tweezers

and a fine bristle brush. The urothelium was either used for functional experiments, or stained with fluorescent antibodies for tissue viability and pericyte location (described in the respective sections below).

2.2.2 TISSUE VIABILITY

Live mouse urothelium was prepared as described in previous section and placed in Nunc® tissues culture dishes (Fisher Scientific, Loughborough, UK) before being treated with propidium iodide (Thermo Fisher, Paisley, UK) and DAPI for dead cells and all cells, respectively as performed by Crawford *et al* on rat kidney slices (52). The bladder urothelium was cut into four sections and incubated in PSS being aerated with 95% O_2/5% CO_2 before being stained for cell viability for each of the following time points: - 15 mins, 30 mins, 1 hour and 2 hours. Post incubation, the tissue was stained with 5 μM propidium iodide (PI) (Sigma Aldrich, Gillingham, UK Aldrich) for 15 mins. PI is taken up by cells with compromised cell membranes and is therefore used to stain for nuclei of dead cells. 3 x 10 min washing steps with PSS followed to remove excess PI then a 15 min fixing step using 4% v/w paraformaldehyde (PFA) made up in 0.4M PBS. After this step, oxygenated PSS was no longer required. 3 more washing steps followed using PBS to remove excess PFA. The fixed tissue was then mounted on to microscope slides with Vectasheild mounting medium containing DAPI (Vector Laboratories, Peterborough, UK) to stain for nuclei of all cells, both live and dead and covered with a coverslip. Slides were analysed using an x40 air objective on a Nikon eclipse 50i fluorescent microscope. DAPI was excited with a mercury bulb at 350nm and emission visualised via a DAPI emission filter at 470nm (blue). PI was excited at 536nm and emission visualised via a Texas Red emission filter at 617nm (red). Separate images were taken using both filters and then overlapped using Image J software. Cells were counted manually per image using the Image J cell counter plug-in. By counting the number of DAPI stained cells (figure 2.12A) and PI stained cells (figure 2.12B) in a field per view (220 μm x 165 μm) it was possible to obtain a ratio of live to dead cells for each time point by the following equation: -

$$\% \text{ cell viability } = \left(\frac{(\text{Number of DAPI cells}) - (\text{Number of PI cells})}{\text{Number of DAPI cells}} \right) x\ 100\%$$

84

A) 15 min DAPI cell count
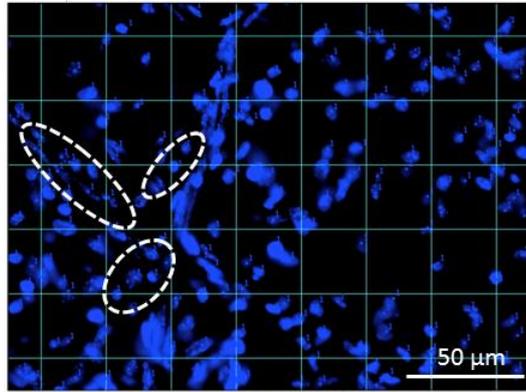
B) 15 min PI cell count
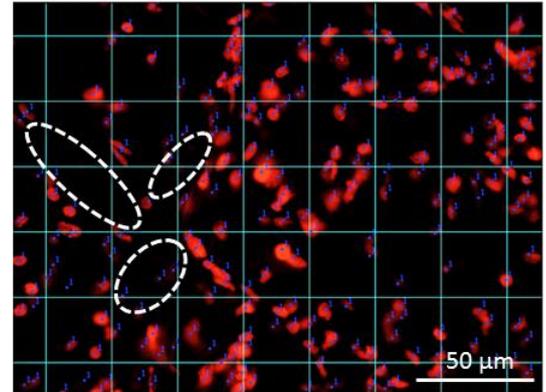
Figure 2.12: Cell counting method for staining with DAPI and propidium iodide. A) DAPI cell count example for tissue left in physiological saline for 15 mins indicates 218 total cells. **B)** Propidium iodide cell count example for tissue left in physiological saline for 15 mins indicates 179 dead cells. Therefore (218-179/218) x 100% = 17.9% live cells.

2.2.3 IMMUNOHISTOCHEMISTRY NG2/IB4

Mouse urothelium was prepared as previously described and placed in Nunc® tissues culture dishes (Fisher Scientific, Loughborough, UK) with 300 µl PSS. All subsequent incubations were performed at a volume of 300µl. Each slice was pre-incubated at room temperature for 4 h with 10 ng/ml TNF-α, 10 ng/ml of IL-1β or 50ng/ml of LPS (R&D Systems, Abingdon, UK) or PSS alone (to act as a control) in order to stimulate pericytes prior to immunohistochemistry (52). After the 4 h incubation 50µg/ml Alexa-488 conjugated isolectin B$_4$ (IB$_4$) was added to the wells to stain for perivascular cells, made up in PSS for 45 mins. After this initial staining, the tissue was washed by incubating slices in normal PSS bubbles in a chamber with 95% O$_2$/5% CO$_2$ for 15 mins. Tissue was then fixed with 4% paraformaldehyde prepared in 0.4 M PBS by incubating for 15 mins. After this step, oxygenated PSS is no longer required. Paraformaldehyde is washed off in PBS 3 times for 10 mins each time. Next the tissue is permeabilised using 0.1% Triton X-100 (in 0.1M PBS) for 10 mins on a rocker table at room temperature. Tissue was then blocked with 10% serum made in 0.1% triton X-100 solution for 2 hours on the rocker table at room temperature.

Blocking prevents non-specific binding of donkey-raised secondary antibodies added later. The primary antibody was rabbit anti-neuro-glial 2 (NG2), to stain for pericytes, made up to 1mg/ml in 10% donkey serum/0.1% Triton X-100. Tissue was incubated in the fridge overnight with this antibody mixture. The following day, after 3x 10 min washes in 0.1M PBS, the donkey anti-rabbit Alexa 555 (secondary antibody) was prepared in 10% Donkey serum/0.1% Triton X-100 and the tissue incubated in the solution for 2 hours. 3 final wash steps with PBS followed and then using Citifluor AF1 (Agar Scientific) tissue was mounted on glass slides and sealed with coverslips and nail varnish.

The tissue was analysed using an upright Nikon eclipse 50i fluorescent microscope with x40 air objective. Alexa-488 was excited with a mercury bulb at 488nm and emission was captured using a XXX emission filter at 520nm (green). Alexa-555 was excited with a mercury bulb at 555nm and emission was captured using a XXX emission filter at 580nm (red). Multiple images were taken and later analysed using Image J software. The distance between pericytes was measured in control urothelium and compared to those in urothelium pre-treated with cytokines and LPS. The mean number of pericytes on a length of capillary was calculated and compared to that of urothelium pre-treated with cytokines and LPS.

2.2.4 DIC IMAGING OF LIVE TISSUE

For experiments in which pericyte-mediate changes in capillary diameter were recorded in response to chemical stimuli (52), bladder urothelium was dissected as discussed in section *2.2.1 – Tissue preparation*. The urothelium was mounted with the superficial urothelium facing upwards on a bath and secured with a harp made of platinum and nylon threads (figure 2.13A). This assembly was then transferred to the stage of an upright Olympus BX51WI microscope and continually superfused with oxygenated PSS using a Watson Marlow 120S peristaltic pump at a rate of ~2.5 ml min^{-1} (1.5 ml dish volume) (figure 2.13B). Capillaries with both pericyte and non-pericyte sites were identified and DIC images were captured using an Olympus x60 water immersion objective, an attached Rolera XR CCD camera (QImaging, Surrey, Canada) and Image Pro Plus 7.0 software (Media Cybernetics, Cambridge, UK). Multiple DIC images were taken at 1 frame per second for a total of 1200 frames to generate a real-time video of the changes in capillary diameter.
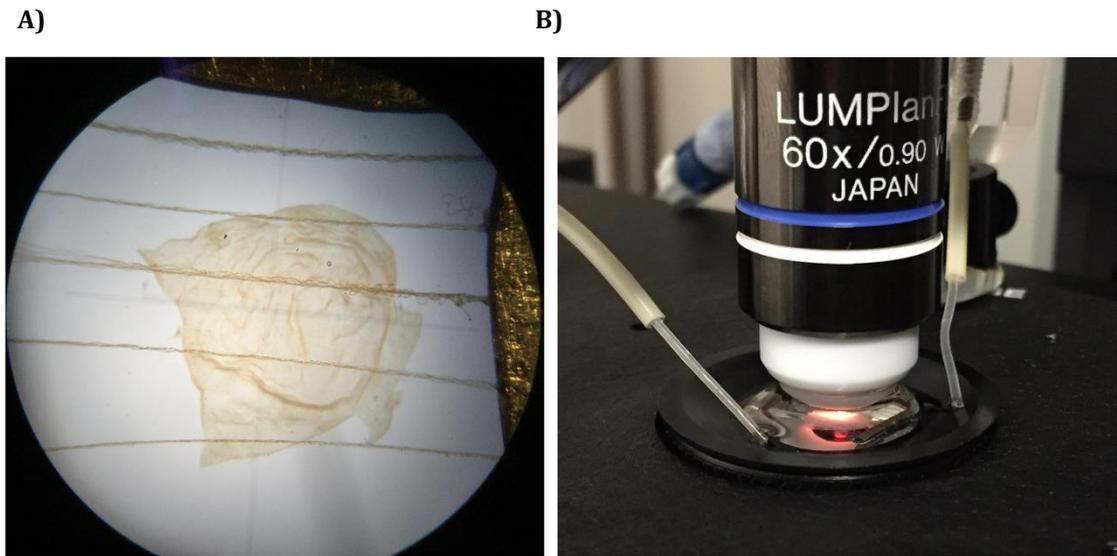
Figure 2.13: Images of mouse bladder urothelium under harp during DIC imaging. A) Half a mouse bladder urothelial layer held in place in a bath with a platinum and nylon harp**, B)** The urothelium in the bath with the harp placed under a x60 water objective being superfused with PSS oxygenated with 95% O2/5% CO2. Vasoactive drugs were also superfused across the tissue using this set up.

A number of experiments were performed using a range of vasoactive drugs, at varying concentrations, to determine pericyte activity in the bladder. In each case, baseline images were taken during superfusion with PSS and then the drug was added and the changes recorded in video format. The following drugs were used to evoke vasoconstriction: 1 nM, 10 nM and 100 nM angiotensin-II (Ang-II), and 10 mM and 1 mM ATP. The following drugs were used to evoke vasodilation: NO donor, 100 µM S-nitro-N-acetyl-D, L-penicillamine (SNAP), 100 µM and 10 µM sodium nitroprusside (SNP) and 100 nM, 50 nM, 10 nM and 1 nM Bradykinin. All drugs were purchased from Sigma Aldrich, Gillingham, UK and prepared into stock aliquots as per the data sheets provided. When required, working concentrations were prepared in oxygenated PSS.

Analysis of video images was performed offline using Image J software. Straight-line measurements were taken from one side of the inner capillary wall to the other at both pericyte and non-pericyte sites for each experiment. The length of these lines was measured in pixels and translated into µm (47 pixels converts to 10µm), and repeated every 5 frames for the duration of the video. Resting baseline measurements were taken at the start of each experiment for approximately 100 seconds. These

measurements acted as the baseline diameter for each capillary; expressed as 100% diameter. All subsequent measurements were calculated and expressed as a percentage change from this baseline value as seen in the equations below. Data were compared using a paired Student T-test and unpaired T-test where applicable.

$$\% \, \Delta \text{ vessel diameter } = \left(\frac{\text{Measured diameter}}{\text{Mean baseline diameter}} \right) x \, 100$$

$$\% \text{ constriction or dilation} = 100\% \text{ (baseline)} - \% \, \Delta \text{ vessel diameter}$$

2.3 STATISTICS

A chi-squared test was used to compare incidence of one species of bacteria, incidence of intracellular bacteria, incidence of Gram negative bacteria, or incidence of positive culture between disease state and controls in each of the studies. This was done by first creating a 2x2 contingency table of incidence of bacteria versus no incidence. For example, if 45 of disease state patients (n=60) and 20 of control patients (n=50) cultured positive for *Staphylococcus* the contingency table below would be generated (table 2.1). From this a chi-squared test is performed by inputting the data into GraphPad prism to determine a p value for the difference in incidence between groups for each bacterial species.

	Staphylococcus	No *Staphylococcus*	
Disease state	45	15	**60**
Control	20	30	**50**
	65	**45**	

Table 2.1 Example of 2x2 contingency table for Chi squared test.

An unpaired Student's T test (or a one way ANOVA where applicable) was performed to compare ages, BMIs, mean number of species, pH and concentrations of ATP and IL-8, between disease state and controls. This test was also used to compare the distance between pericytes post-incubation with drugs in immunohistochemistry experiments.

A paired Student's T test was performed to compare capillary distance pre- and post- drug application in DIC imaging experiments.

P<0.05 was considered as statistically significant for all tests. Sample size (n) represents 1 patient (unless otherwise stated as 1 sample in the case of multiple samples per patient) or 1 pericyte in 1 mouse.

CHAPTER 3 – RESULTS 1: PREDICTING rUTIS POST-RENAL TRANSPLANT

3.1 INTRODUCTION

As discussed in the General Introduction, renal transplant recipients are particularly predisposed to rUTI due to their post-transplant care management scheme, which includes immunosuppresants and antibiotics, which allows for opportunistic infections and antibiotic resistant species to thrive. In this chapter, we will investigate the species of interest in our cohort of patients and whether intracellular bacteria could be a niche for rUTIs in RTRs in order to better understand how a bacterial infection manifests itself and how these patients develop recurrent infections despite being on antibiotics.

3.1.1 SPECIES OF INTEREST IN UTI IN RENAL TRANSPLANTATION

Reported prevalence of UTI incidence in RTR varies greatly between 23% and 75% depending on the study, but this is most likely due to the variation in criterion for diagnosing infection and intensity of antibiotic and immunosuppressant treatment. The largest study to date analysed medical reports from the US Renal Data System database where Medicare were the primary source of medical insurance for renal transplants; a total of 23,924 RTR records were analysed (120). It emerged that within the first year post-transplant 17% of both men and women developed a UTI, increasing to 60% of women and 47% of men within 3 years (120). A later study by Pelle *et al* in 2007 found 75.1 % of RTRs (n=177) develop an early UTI within the first 6 months and 18.7% develop acute pyelonephritis (APN) (122). This is significantly higher than the previous study, however does highlight that UTI can be extremely common in RTRs. Also from the Pelle study it was found that the highest prevalence of first UTI was caused by *E.coli* (28.4%) followed by *Enterococcus* spp. (23.6%) then *Pseudomonas aeruginosa* (14.9%) and *Staphylococcus non-aureus* (11.5%)(122). Other studies, (summarised in Table 3.1), have found very different percentages of causative species of UTI. For example, one study by Valera *et al* found that 71% of infections were caused by *E.coli* (222), whereas the study by Chuang *et al* found only 29% of infections to be caused by this pathogen (223). It can be seen however that

the predominant species across all studies are *E.coli, Enterococcus, Pseudomonas aeruginosa,* and *Klebsiella* (table 3.1). A systematic review on this area by Parasuraman *et al* has cumulated the percentages from Chuang, Pelle, Memikoglu and Di Cocco to determine an overall view of the microbiology of UTIs in RTRs summarised at the bottom of table 3.1 (121).

Study	Patient numbers	Majority Taxa of UTIs in RTRs
Chuang *et al*, 2005 (223)	n = 500	29% *E.coli*, 24% *Enterococcus*
Valera *et al*, 2006 (222)	n = 161	71% *Enterococcus*, 10% *Pseudomonas aeruginosa*
Dantas *et al*, 2006 (224)	n = 163	30.4% *Enterobacter*, 13.4 % *Pseudomonas aeruginosa*
Pelle *et al*, 2006 (122)	n = 177	28.4% *E.coli*, 23.6 % *Enterococcus spp.*
Memikoglu *et al*, 2007 (225)	n = 136	59.1% *E.coli*, 16.9% *Klebsiella spp.*
Di Cocco *et al*, 2008 (226)	n = 218	66.3% *E.coli*, 10.1% *Enterococcus*
Gołębiewska *et al*, 2014 (227)	n = 209	58% *E.coli*, 36% *Enterococcus spp.*
Parasuraman *et al* 2013 (121) (Systemic review of Chuang, Pelle, Memikoglu, and Di Cocco)	**n = 1,519**	**34.5% *E.coli*, 19.5% *Enterococcus* 12.5% *Pseudomonas aeruginosa* 9.5% *Staphylococcus spp.* 9% *Klebsiella spp.***

Table 3.1: Studies characterizing predominant species causing UTI in renal transplant recipients (RTR): Each study has similar patient numbers ranging from 136 to 500 RTRs however there are significant differences between the predominant species causing UTIs in each study. E.coli has been found in one study to be the cause of 71% of UTIs (Valera *et al*) but only 29% in another (Chuang *et al*). Overall, the four main causes of UTI in RTRs are *E.coli, Enterococcus, Pseudomonas aeruginosa,* and *Klebsiella.*

3.1.2 PREDISPOSING FACTORS FOR UTI POST-RENAL TRANSPLANT

Pelle's 2007 study also analysed correlations between UTI and APN incidence and a number of other clinical features to identify predisposing factors for infection in RTRs (discussed next) (122). They found that the risk of developing APN was higher in women (64%) than men (36%) and correlated strongly with frequency of episodes of acute rejection. Furthermore, they suggested that the intensity of immunotherapy may be a cause for rapid onset of APN, although other investigations have concluded

dose is not a relevant factor in UTI incidence (122). Together these strong correlations suggest that there is no direct precursor for increased incidence of rUTI in RTRs but rather there could be a multitude of risk factors of varying influence. The fore mentioned systematic review by Parasuraman also generated odds ratios (OR) for various risk factors where ORs of greater than 1 represent true risk (121) Risk factors for UTI and their ORs which are notably greater than 1 were being female (OR 5.8), obtaining a kidney from a deceased donor (OR 3.64), and increased immunosuppression dose (OR 17.04). Increased immunosuppression dose is proposed here as the main determinant of UTI incidence and is calculated from a review of 7 publications in the field and is therefore likely to be more reliable than individual studies. Interestingly, risk factors for UTI and their ORs also found women to be at greater risk (OR 5.14) and those with increased frequency of acute rejection episodes (OR 3.84), which correlates with Pelle's finding (122). Other factors include inclusion of stents post-operation, which increases the likelihood of biofilm formation (discussed in section *1.5.4 Bacteriuria in overactive bladder*), since stents offer an ideal growth surface for biofilm forming bacteria such as *Proteus* and UPEC. For this reason they are removed as soon as possible, after 6 weeks. Fayek *et al* reported that presence of a stent increased incidence of UTI to 14.2% compared to incidence with no stent at only 7.9% (228). Furthermore, there are insensitivities with the other tests involved in monitoring RTRs discussed in the next section,

3.1.3 UNREPORTED ROUTINE URINALYSIS

As mentioned in the section *1.5.3 Diagnosis of UTIs,* many diagnostic laboratories diagnose UTIs when a patient has $10^{4\text{-}5}$ colony forming units (CFU) of a single organism or 10^5 CFU/ml mixed growth with one predominant organism when plating midstream urine (128). Despite the Infectious Diseases Society of America (IDSA) and the European Association of Urology (EAU) revising the guidelines to include a cut-off of 10^2 CFU/ml for the diagnosis of bacterial cystitis (229), most laboratories still follow Kass's criterion interpreting urine culture with $<10^5$ CFU/ml as probable absence of infection (129). For this reason, many potentially dangerous opportunistic infections could go unreported in RTRs. By reducing the threshold, risk of rejection can be minimised by catching opportunistic infections early before they cause physical damage and stimulation of immune attack of the graft.

Another difficulty with current urinalysis techniques is that they often miss the low-grade inflammation associated with low-level bacteriuria. Even though dipstick leukocyte esterase has been validated against the higher threshold of 10^5 CFU/ml, the readings can very often be incorrect. Leukocyte esterase is released from neutrophils during localised inflammation whereby a positive result would indicate pyuria associated with an infection. Unfortunately, false positive results can occur in women when samples are contaminated with vaginal discharge. Elevated glucose or oxalic acid in the urine may also reduce efficacy of the dipstick reading (230). Furthermore, in the clinical laboratory, counting the numbers of white blood cells (WBC) in unspun urine either by automated cell counting or my microscopic methods has been discredited as a marker for UTI since WBCs die rapidly post-voidance and positive results do not always correlated with infection (215). Since pyuria is used in many hospital pathology labs to determine if a patient has a possible UTI, many infections could be going unreported if culture is not performed on samples due to negative pyuria and no symptoms.

Many quiescent bacteria also go unreported as they are often classified as contaminants at low-level when detected in a mixed culture (129). The concept of a bladder microbiome introduces the idea that opportunistic bacteria may already be residing in the bladder and use of immunosuppressants and antibiotics allows them to develop into infections. Antibiotics can target particular genus of bacteria and not others allowing the non-susceptible species to grow opportunistically and the use of immunosuppresants reduces the immune system's efficacy at dealing with alterations in bacterial growths (*i.e.* infections). Furthermore, certain quiescent bacteria living in the bladder urothelium may be able to form intracellular bacterial communities, which can make identifying potential infections harder (as discussed in section *1.5.2.4 - Intracellular bacterial communities in OAB*) (111, 144, 145, 153).

3.1.4 INTRACELLULAR BACTERIAL COMMUNITIES POST-RENAL TRANSPLANT

Kelley *et al* have previously discovered that 44% of MSU samples taken from 53 RTRs within the first 2-4 weeks post-transplantation and before stent removal presented with intracellular bacteria within shed urothelial cells (153). However, only one of these patients was diagnosed with a UTI by routine microbiological culture (see *Appendix 1.2 - MSU and CSU culture interpretation, EKHU NHS Foundation*

Trust, 2015). This suggests that intracellular bacteria may be an underlying cause for rUTIs since their life cycles involve invasion and replication followed by efflux to naïve nearby host cells. This cycle creates a niche of bacteria that are able to evade antibiotics and immune responses and may be responsible for the recurrence observed in these patients (111, 144). From this discovery, a grant proposal was drafted by Medway School of Pharmacy Urinary System Physiology Unit in collaboration with Canterbury and East Kent NHS Renal Unit and accepted by Kidney Research UK. The grant was based on the hypothesis that quiescent bacteria residing intracellularly in the bladder urothelium are responsible for the rUTI and sub-clinical UTIs experienced by patients post-renal transplant. The following chapter is based on the data generated from the aims and methods outlined within that grant proposal.

3.2 Hypothesis and Aims

We hypothesise that intracellular bacteria in the bladder urothelium are responsible for many of the rUTI experienced by patients post-renal transplant.

In order to begin to test these hypotheses the following objectives will be to:-

1. Investigate alterations in bladder bacterial species of interest over a period of 6 months post-transplant compared to baseline controls.
2. Observe intracellular bacteria in shed urothelial cells and compare to incidence of low-level infection to determine whether intracellular bacteria could be a predictor for rUTI.
3. Analyse and compare all clinical and biological information from RTRs and controls to determine if there is any potential for development of improved biomarkers to better predict rUTI in RTRs.

3.3 METHODOLOGY

3.3.1 DISCLAIMER

Renal transplants were performed at Guy's Hospital London, UK. Collection of clinical data, including patient age, BMI, form completion and urine collection was performed by clinical staff and clinicians at Kent and Canterbury Hospital, Kent, UK. Dipstick urinalysis and Sternheimer-Malbin analysis was performed by myself and the research nurses at Kent and Canterbury Hospital. Hospital pathology was performed by clinical staff in the pathology department at William Harvey Hospital, Ashford, UK. University culture, sequencing and analysis was performed by myself.

3.3.2 ETHICS

Ethical approval for this research was granted by NHS Health Research Authority committee for South East Coast, Kent. All participants were over the age of 18, fully briefed and consented before being recruited into the study.

3.3.3 PATIENT SAMPLE COLLECTION

This was a prospective cohort study, which analysed mid-stream urine samples obtained one month, three months, and six months after transplantation from RTRs. Most patients received renal transplant surgery at Guy's Hospital, London and attended the renal outpatients department of Kent and Canterbury Hospital for monitoring. All patients were prescribed co-trimoxazole 80/400 mg tablets (80 mg Trimethoprim and 400 mg Sulfamethoxazole) for 12 months post-transplant as a prophylactic for opportunistic infections. Upon being consented, patients were invited to provide 3 mid-stream samples of urine at each time point after transplantation; 1 month, 3 months and 6 months. On each visit they also completed a questionnaire designed by East Kent Hospital University to obtain a symptoms score and determine impact on life (*see Appendix 1.1 – Diagnosing urine infection in kidney transplant patients, EKHFT, 2015).* Mid-stream urine samples were also taken from the kidney donors before donation, which acted as the control group. This study was blinded by non-disclosure of the origin of each sample until after the isolated bacteria were identified.

3.3.4 ROUTINE URINALYSIS AND NHS MICROBIOLOGY

Fresh midstream samples were taken from patients upon visiting the renal outpatients clinic and immediately analysed with an automated cell counter and automated dipstick urinalysis for epithelial cells, white blood cells, red blood cells, leukocyte esterase, nitrites, protein, pH, specific gravity, ketones and glucose as per standard hospital protocol. To compare with the automated systems, a 10μL sample of urine was mixed with 10μL Sternheimer-Malbin stain (Fisher) and loaded onto a standard haemocytometer within 2 hours of sample collection. Shed urothelial cells, white blood cells and red blood cells were counted per μL and multiplied by a dilution factor of 2 to account for the 1:1 dilution with Sternheimer-Malbin.

Urine samples were sent to hospital pathology for routine culturing and biochemical testing of cultures *(see Appendix 1.2 – MSU and CSU culture interpretation, EKHU, 2015)*. Results of these were compared with our own. In total, 329 samples were collected from 72 patients. Of these, 36 were donors, which provided up to 2 samples, the remaining 36 were RTRs providing samples at various time points post-transplant. All 329 samples underwent routine hospital culture, automated cell counting, dipstick analysis, microscopic cell counting and DAPI staining.

3.3.4 UNIVERSITY CULTURE

In some cases, multiple samples per patient were collected per time point due to additional patient monitoring, however only 1 sample per time point per patient was also cultured using expanded culture methods at the university (university culture) in order to reduce cost and workload. Therefore, 145 RTR samples and 18 donor samples were frozen at -20°C (for later culture if required). As such 127 RTR samples and 39 donor samples of urine were used to perform university culture as per the methods discussed in the General Methodology, section *2.1.3 - Processing of fresh urine samples* (figure 3.1). The results of both university and hospital culture could be compared for each sample and patients in respect to clinical data.
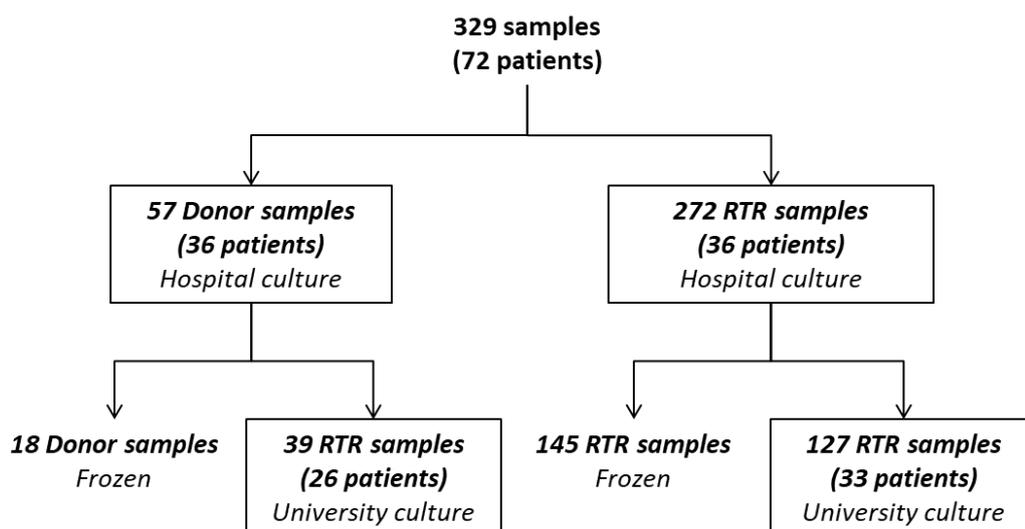
Figure 3.1: Number of samples cultured by hospital and university culture. A total of 329 samples from 72 patients were collected. Of these 57 samples were from 36 donors pre-donation and 272 samples were from 36 RTR patients at various time points from 1 week to 6 months post-transplant. All samples underwent hospital culture, hospital dipstick and cell counting and DAPI analysis. Due to time restraints on sample processing, 18 donor samples and 145 RTR samples were frozen and therefore university culture was not performed on these samples. All remaining samples were cultured by university methods.

3.3.5 UNBLINDING THE STUDY

Until all sequencing was completed, Kent and Canterbury Hospital renal research staff blinded this study from the university. Post-sequencing the details of whether the sample had come from a donor or recipient, the patients' gender, age, prophylactic and immunosuppressant treatment programme and whether the patient had been treated for a UTI at any stage were released for analysis.

Analysis was performed by two notably different means – one method comparing individual samples to compare the presence of specific biomarkers and determine the efficacy of hospital testing on a sample by sample basis, the results of which are discussed in section *3.4.3 – Incidence of UTIs, 3.4.4 – Trends associated with UTIs and rUTIs* (culture), *3.4.5 – Hospital biomarkers for UTIs* and *3.4.7 - Changes in the bladder microbiome post-RT*. The second method involved comparing patients; at any one time point and over the series of time points, the results of which are discussed in sections, *3.4.4 – Trends associated with UTIs and rUTIs* (age, sex and recurrence), and *3.4.6 Alternative biomarkers to predict UTIs and rUTIs.*

3.3.6 SPECIFICITY AND SENSITIVITY TESTING

In cases where it was necessary to test the specificity and sensitivity of hospital tests, culture and biomarkers, a 2x2 contingency table was created. On the left was the gold standard test or presence of infection and on the top was the biomarker or test being scrutinised (table 3.2). Where both the gold standard and test are positive (a) or negative (d) this is classified as a true positive or true negative, respectively. In cases where the gold standard is positive but the test is negative (c), this is classified as a false negative. In cases where the gold standard is negative but the test is positive (b), this is classified as a false positive. Sensitivity and specificity are calculated as follows: -

$$\text{Sensitivity of biomarker} = \frac{a}{a+b} \qquad\qquad \text{Specificity of biomarker} = \frac{d}{c+d}$$

Confidence intervals were calculated using the Clopper-Pearson method. Tests or biomarkers with a sensitivity of 90% and a specificity of 95% are classified as excellent.

		Positive biomarker	Negative biomarker
Gold standard	Positive culture	**a** True positive	**c** False negative
	Negative culture	**b** False positive	**d** True negative

Table 3.2: Example of 2x2 contingency tables used for the calculation of sensitivity and specificity of tests. Sensitivity is calculated as number of samples with a true positive/(number of samples with a true positive + number of samples with a false positive). Specificity is calculated as the number of samples with a true negative/(number of samples with a true negative + number of samples with a false negative).

3.4 RESULTS

3.4.1 ISOLATES OMITTED FROM ANALYSIS

In 30 cases it was not possible to resuscitate the isolate therefore 'Failed regrowth' was noted (n=30/1492, 2.01%, table 3.3). 28 isolates were contaminated with *E.coli*, probably from contaminated cryobroth. Due to the rapid growth rate of *E.coli* it was not possible to isolate the slower growing original isolates from beneath the *E.coli* and these samples were noted as 'Contaminated cryobroth' (n=28/1492, 1.88%, table 3.3). 80 isolates had failed PCR using both sets of primers the isolate was noted as 'Failed PCR' (n=80/1492, 5.36%, table 3.3). 30 isolates were not able to be sequenced on two separate occasions and therefore these isolates were noted as 'Failed sequencing' (n=30/1492, 2.01%. table 3.3). In 29 cases it was not possible to identify the Gram stain of an isolate due to mixed staining, or if bacterium were particularly small and 100x focus was not sufficient to determine the exact shape of the bacterium, the Gram stain result for these isolates was noted as 'Inconclusive Gram stain' (n=32/1492, 2.14%, table 3.3).

Therefore, of a total 1492 cultured isolates from 230 samples, 1140 (76.41%) were successfully sequenced. For 153 isolates the originally recorded Gram stain was not concordant with the Gram stain of the BLASTed sequenced and these isolates were omitted from the final comparisons (n=168/1140, 11.26%, table 3.3).

	Total isolates	Percentage of total isolates
# of Isolates identified	1492	100%
Failed regrowth	30	2.01%
Contaminated cryobroth	28	1.88%
Failed PCR	80	5.36%
Failed sequencing	30	2.01%
Inconclusive Gram stains	32	2.14%
Non-concordant Gram stains	168	11.26%

Table 3.3: Total number of isolates from each cohort missing or omitted from analysis: Total of 1492 isolates collected from all samples. 2.01% of total isolates failed to regrow upon resuscitation. 1.88% of total isolates were too contaminated to perform PCR. 5.36% of total isolates failed PCR twice with different primers on each occasion. 2.14% of total isolates could not be identified by the Gram stain. 11.26% of total isolate BLAST results indicated a species that were not in concordance with the original Gram stain noted.

3.4.2 DEMOGRAPHICS OF PATIENT COHORTS

A total of 329 MSU samples of urine were collected from 72 patients; 57 from 36 control patients and 272 from 36 RTRs, with 22 controls patients providing 2 samples, and RTRs providing between 1 and 10 samples each over a period of 3 to 241 days post RT with most patients providing between 2 and 4 samples each. All samples underwent hospital culture. 39 control samples and 127 of the RTR samples were cultured in our laboratory (university culture) as per the methods discussed in the General Methodology, section *2.1.3 - Processing of fresh urine samples*. The remaining samples were frozen for further investigation at a later date if necessary (figure 3.1).

3.4.3 INCIDENCE OF UTIS

In order to identify the percentage incidence of UTI in RTRs and controls, the number of patients who presented with a UTI at any time point was calculated. Of the samples which were sent for hospital microbiological culture. 2 control patients (2 samples, 5.5%, n=36) and 12 RTRs (17 samples, 33.3%, n=36) were reported to have positive culture $>10^4$ CFU/ml and therefore presented with a clinical UTI at any time point (figure 3.2A). These are statistically different by a Chi squared test (p=0.0074).

Of the total 72 patients, 59 patients (166 samples) were also cultured by university culture methods in our laboratory; 26 donor patients (39 samples) and 33 RTRs (127 samples), where a CFU count of $>10^3$ CFU/ml identified positive 'low-level infections'. From the 59 patients cultured at the university, 4 donors (5 samples, 15.4%, n=26) and 12 RTRs (28 samples, 35.3%, n=33) were reported to have positive culture $>10^3$ CFU/ml (figure 3.2B). These are not statistically different by a Chi squared test (p=0.13).

A) Hospital culture

72 patients (329 samples)

36 donors (57 samples)

36 RTRs (272 samples)

2 donors (2 samples) with positive hospital culture **(5.7%)**

34 donors (55 samples) with negative hospital culture **(94.3%)**

12 RTRs (17 samples) with positive hospital culture **(33.3%)**

24 RTRs (255 samples) with negative hospital culture **(66.6%)**

B) University culture

59 patients (166 samples)

26 donors (39 samples)

33 RTRs (127 samples)

4 donors (5 samples) with positive university culture 15.4%)

22 donors (34 samples) with negative university culture **(84.6%)**

12 RTRs (28 samples) with positive university culture **(35.3%)**

21 RTRs (99 samples) with negative university culture **(64.7%)**

Figure 3.2: Culture results of samples cultured by hospital and university culture. A) A total of 72 patients provided 329 samples. 36 donors provided 57 samples and 36 renal transplant recipients (RTR) provided 272 samples. All of these samples were cultured by hospital methods and it was found that 2 samples from 2 different donor patients (5.7%) and 17 samples from 12 different RTRs (33.3%) were positive for UTI. **B)** Of the total 329 samples, 166 samples from 59 patients were cultured by university methods also. From these, 5 samples from 4 different donor patients (15.4%) and 28 samples from 12 different RTRs (35.3%) were found to have low-level bacteriuria by university methods.

In order to compare the effectiveness and reliability of university culture, the incidence of positive hospital culture and incidence of positive university culture was compared in a contingency table where hospital culture is the gold standard. All samples were compared in the same calculation. The percentage confidence, discrepancy, sensitivity and specificity was calculated as discussed in *Section 3.3.6 Sensitivity and sensitivity testing*. A total of 8 true positives and 125 true negatives were found leaving 8 false negatives and 25 false positives, which is an 80.1% concordance between the two culture methods. The sensitivity is calculated as 50% and specificity is calculated as 83.3% (table 3.4).

All samples

n= 166 samples (127 RTR + 39 donor)

	Positive university culture	Negative university culture	
Positive hospital culture	8 samples	8 samples	Concordance = 80.1 % Discrepancy = 19.9 %
Negative hospital culture	25 samples	125 samples	Sensitivity = 50.0 % Specificity = 83.3 %

Table 3.4: **Sensitivity and specificity of university culture compared to hospital culture.** Hospital culture is taken here as the gold standard of testing for UTI in RTRs. University culture has a concordance of 80.1% with hospital culture. There are 25/166 false positives and 8/166 false negatives, a sensitivity of 50.0% and a specificity of 83.3%.

Another means of analysing reliability of hospital and university culture was to compare incidence of UTI with symptom score. A positive symptom score is taken as a score of 1 or more to the questionnaire found at *Appendix 1.1 – Diagnosing urine infection in kidney transplant patients, EKHFT, 2015.* Symptom score was compared against hospital culture (n=272 samples, table 3.5A) and university culture (n=127 samples, table 3.5B). The resulting % concordance of symptom score to hospital culture is low at 54.8% for hospital culture; sensitivity of this test of infection is 58.8% and specificity is 54.5% (table 3.5A). The resulting % concordance of symptom score to university culture is lower still at 42.5% for university culture; sensitivity of this test of infection is 33.3% and specificity is 45.4% (table 3.5B).

A) Hospital culture and symptom score

n=272 samples

	Positive symptom score	Negative symptom score
Positive hospital culture	10 samples	7 samples
Negative hospital culture	116 samples	139 samples

Concordance = 54.8 % Sensitivity = 58.8%
Discrepancy = 45.2 % Specificity = 54.5%

B) University culture and symptom score

n= 127 samples

	Positive symptom score	Negative symptom score
Positive university culture	9 samples	18 samples
Negative university culture	54 samples	45 samples

Concordance = 42.5 % Sensitivity = 33.3%
Discrepancy = 57.5 % Specificity = 45.4%

Table 3.5: Positive culture compared with positive symptom score. Symptom score of 1 or more to the questionnaire found at *Appendix 1.1 – Diagnosing urine infection in kidney transplant patients, EKHFT, 2015* compared with culture result. **A)** Concordance of symptom score and hospital culture is 54.8%. The sensitivity of this as a test of infection is 58.8% and specificity is 54.5%. **B)** Concordance of symptom score and university culture is 42.5%. The sensitivity of this as a test of infection is 33.3% and specificity is 45.4%.

3.4.4 TRENDS ASSOCIATED WITH UTIS AND RUTIS

Numerous studies have shown that UTI occurrence is higher in women, and data from this study shows that this is the true for both those identified by hospital culture and university culture. Of the control patients included in the study (n=36), 52.8% were female and 47.2% male. Of the RTR patients (n=36), 55.5% were female and 44.5% male (p>0.05, figure 3.3A). The age of donors (n=36) and RTR patients (n=36) follows a normal distribution ranging from 22 to 69 years for donors and 18 to 77 years for RTRs. Both have a mode age of 41 to 50 years (fig 3.3B). Upon observing the sex of those RTRs with positive hospital culture (n=12) and positive university culture (n=12), 66.6% were female and 33.3% male in both cases (p>0.05, figure 3.3C). These differences are not considered to be significant by Chi squared test as p>0.05. The age of RTRs with positive culture shifts towards the higher ages however the mode age for RTRs with infections remains as 41 to 50 years (figure 3.3D).

A) All patients

B) All patients

C) RTR patients
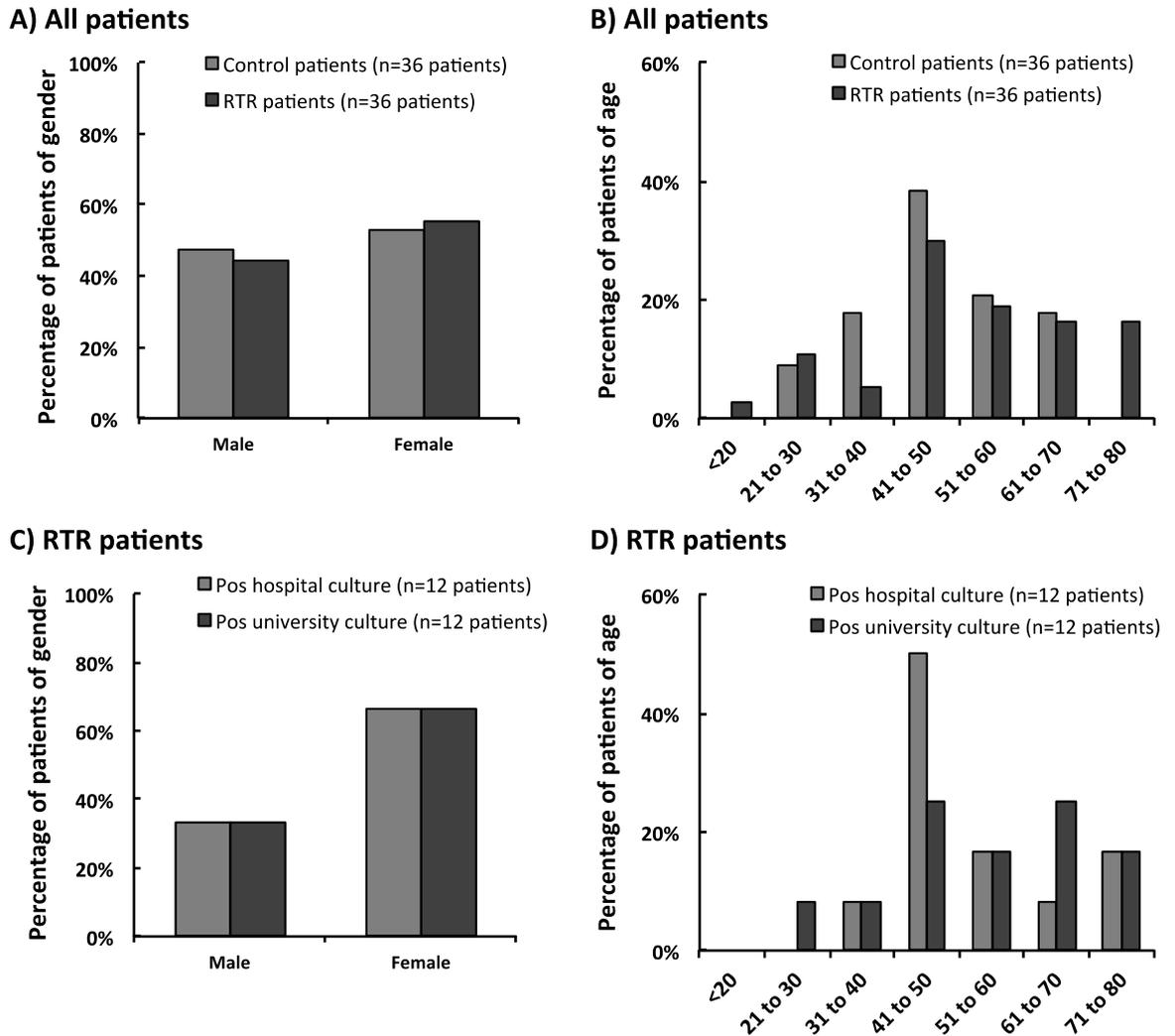
D) RTR patients

Figure 3.3: Sex and age of control and RTR patients and RTR patients with positive hospital and university culture. A) Sex of donors (n=36) are 52.8% female and 47.2% male, and sex of RTR patients (n=36) are 55.5% female and 44.5% male. **B)** Age of donors ranges from 22 to 69 years with a mode age of 41-50 years. Age of RTR patients ranges from 18-77 with a mode age of 41-50 years. **C)** Sex of RTR patients with infections are 66.6% female and 33.3% male for both methods of infection identification. **D)** Age of RTR patients with infections trends to be higher than in fig 3.3B however still has a mode age of 41to 50 years.

Incidence of UTI post-transplantation reportedly increases with time post-transplant. From this study, incidence of UTI does increase slightly from 11.1% of RTRs having a hospital grade UTI within the first month (>1month, n=27) to 13.4% after one month (n=23), then 25% after 3 months (n=24) (figure 3.4A). After 6 months this drops slightly to 20% (n=20). By analysing the percentage of RTRs with low count bacteriuria by university culture methods, an increase is also observed overtime from <1 month at 18.5% to 21.7% after 1 month, and then further to 25%

after 3 and 6 months (figure 3.4A). These data are not significant by a one-way ANOVA (p<0.05).

A sample from each patient for each time point was not possible in all cases and 4 patients only gave 1 sample due to time restraints on the study. From the remaining 33 patients it was possible to determine the percentage of RTRs who had incidence of rUTIs; that is ≥2 UTIs within 6 months post-transplant. From the 33 RTRs, 4 were diagnosed with a UTI more than twice or more within the 6-month period post-transplant by hospital culture (12.1%, n=33) (figure 3.4B). By university culture, 7 RTRs had low count bacteriuria more than twice within the 6 month period post-transplant (21.2%, n=33) (figure 3.4B). These are not significant by a Chi squared test (p=0.5). Interestingly, only 2 patients of the 4 patients with rUTIs diagnosed by hospital culture had rUTIs by university culture also (data not shown).

A) RTRs at given time points (n=33 patients)

B) RTRs across all time points (n=33 patients)
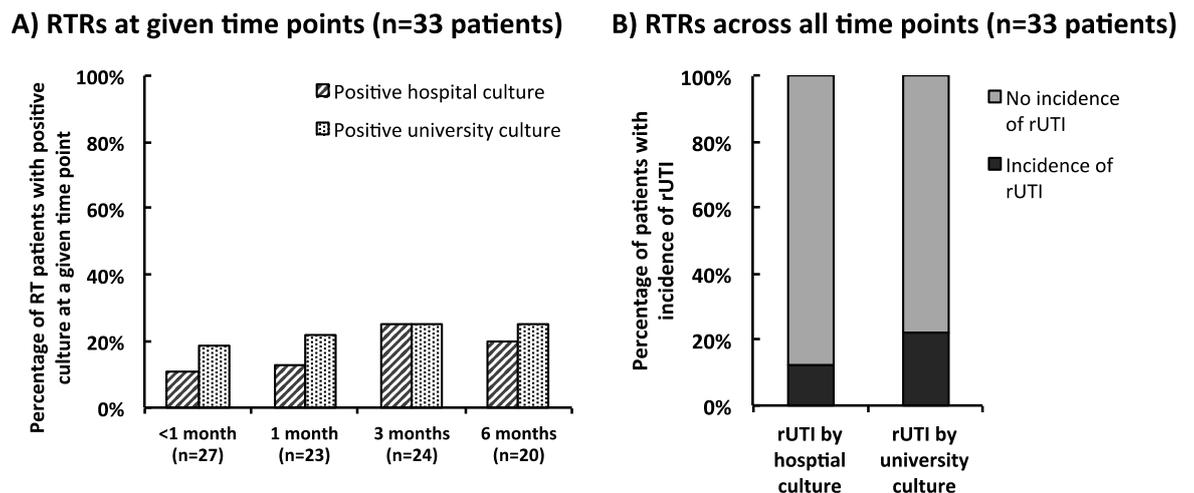
Figure 3.4: Trends of UTIs identified by hospital and university culture. A) Incidence of UTI increases slightly with time post-transplant. At less than one month, 11.1% of all RTRs were diagnosed with a hospital grade a UTI (n=27), and this increases to 13% at 1 month (n=23), then to 25% at 3 months (n=24) and drops slightly to 20% at 6 months (n=20). With university culture these percentages are expectedly higher at 18.5% (1 month), 21.7% (1 month), and 25% (3 and 6 months) (p<0.05 by ANOVA). **B)** Of 33 patients who supplied more than one sample post-transplantation, 4 (12.5%) were diagnosed with rUTI (≥2 UTI in less than 6 months) by hospital culture and 7 (21.8%) expressed low count bacteriuria by university culture. P>0.05 by Chi squared test.

If samples grown by hospital culture were found to have >10^4 CFU/ml of pure growth then species identification was performed by culturing on a range of growth media, which select for particular types of bacteria. For 6/17 (35.3%) positive hospital cultures the causative species was identified as *E.coli* and 1/17 (5.8%) of infections were caused by *Klebsiella,* 1/17 by *Enterococcus,* 1/17 by *Citrobacter,* and 1/17 by *Staphylococcus* (figure 3.5A). If >10^4 CFU/ml mixed growth was found then this was reported as mixed growth, likely due to contamination, and the individual species were not identified. This was the case for the remaining 7/17 infections identified by hospital culture, therefore, the species were not identified for a large percentage of positive samples, but it could be determined by looking at the species of bacteria identified by 16s rRNA sequencing and number of CFUs of bacteria grown by university culture. Of the 7 samples identified by hospital culture as mixed growth, only one had positive university culture which could be used to identify possible causative species. In this single sample, >10^3 CFU/ml of *Lactobacillus* and *Streptococcus* were identified, which may have been the reason for the hospital observing mixed culture (data not shown).

By identifying the species of all culturable isolates which grew at >10^3 CFU/ml by university culture by performing 16s rRNA sequencing it is possible to get a wider view of the species which could be causing UTI in RTRs. The most common species growing at >10^3 CFU/ml in urine of RTRs (n=29) is identified by 16s rRNA sequencing as *Lactobacillus* (34.5%), followed by *E.coli* (24.2%)*, Corynebacterium* (13.8%)*, Atopobium (10.3%), Streptococcus, Gardnerella,* and *Bifidobacterium* (8.3%) (figure 3.5B).

A) RTRs with positive hospital culture (n=17 samples)
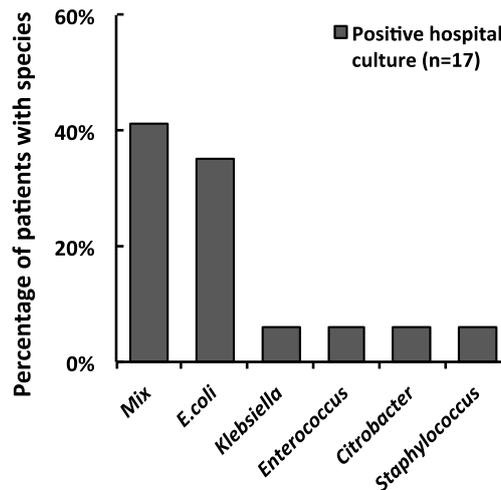
B) RTRs with positive university culture (n=29 samples)
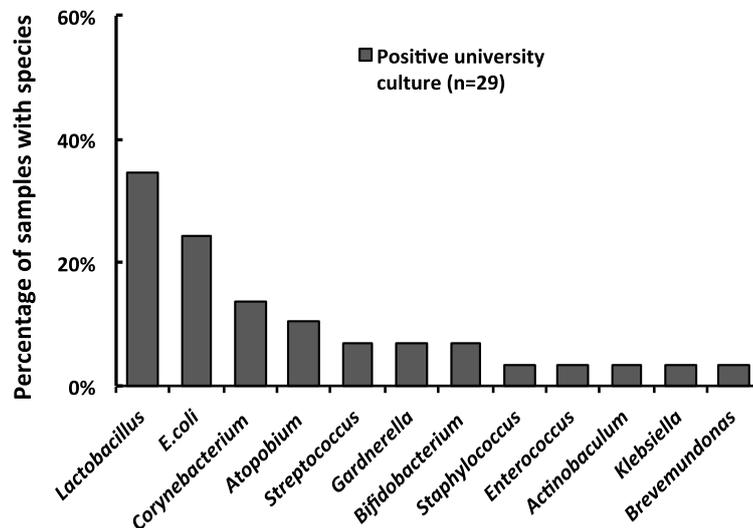
Figure 3.5: Trends in UTI from positive hospital cultures and university cultures. A) Infections identified by hospital culture (grey bars) were mainly identified as mixed cultures, which are classified as contaminated samples, followed by 35.3% caused by *E.coli*. **B)** Infections identified by university culture (black bars) were mainly identified as *Lactobacillus* (34.5%), which is generally classified as a probiotic species and non-pathogenic, followed by 24.2% caused by *E.coli*, then 13.8% by *Corynebacterium* and 10.3% by *Atopobium.*

3.4.5 HOSPITAL BIOMARKERS FOR UTIS

Many UTIs are pre-detected by hospital markers such as dipstick analysis and WBC count, therefore in order to determine if pyuria or nitrites can be effectively used as a surrogate biomarker for infection in these samples, the positive infections were correlated with incidence of positive leukocyte esterase (a dipstick marker for leukocytes), nitrites (a dipstick marker for bacterial growth), and positive WBC count performed by automated and manual methods. In this case, individual samples were

analysed rather than patients. The sensitivities and specificities were calculated for each biomarker with both hospital culture (table 3.6A) and university culture (table 3.6B) as the gold standard in the contingency tables.

Leukocyte esterase is highly specific (99.2% to 99.7%) but not very sensitive (6.06% to 10.5%) when compared with either hospital culture or university culture (table 3.6A and B). Manual WBC count is also highly specific (96.9% to 97.7%) but not very sensitive (0.0% to 21.05%) when compared with either hospital culture or university culture. Automated WBC count is much more sensitive when compared with manual WBC count with a sensitivity of 68.4% in hospital culture and 57.6% with university culture. Automated WBC count does not have as high a specificity as leukocyte esterase of manual WBC count however, at only (48.4% to 67.7%).

A) Biomarkers with hospital culture
n= 329 samples (RTR and donor patients)

	Sensitivity	Specificity
Nitrites	42.1%	77.5%
Leukocyte esterase	10.5%	99.7%
Automated WBC count	68.4%	67.7%
Manual WBC count	21.05%	97.72%

B) Biomarkers with university culture
n= 166 samples (RTR and donor patients)

	Sensitivity	Specificity
Nitrites	45.4%	56.9%
Leukocyte esterase	6.06%	99.2%
Automated WBC count	57.6%	48.4%
Manual WBC count	0.00%	96.92%

Table 3.6: Sensitivity and specificity of common biomarkers of infection compared to hospital and university culture. A) LE in the most specific biomarker when used with hospital culture (99.7%) however it is not very sensitive (10.5%). Manual WBC is also specific (97.7%) but not sensitive (21.05%). Automated WBC count in the most sensitive biomarker test at 68.4%. **B)** LE in the most specific biomarker when used with university culture (99.2%) however it is not very sensitive (6.06%). Manual WBC is also specific (96.9%) but not sensitive (0.0%). Automated WBC count in the most sensitive biomarker test at 57.6%.

3.4.6 ALTERNATIVE BIOMARKERS TO PREDICT UTIS AND RUTIS

Since automated cell counting for pyuria is the most accurate method of detecting pyuria (table 3.6) and has the strongest correlation with positive culture, it can be hypothesised that positive pyuria could be used to predict an infection before it becomes sufficiently established to be detected. By looking at patient clinical history, the number of patients with previous positive pyuria prior to an infection was determined. The number of patients with a hospital detected infection (n=10) who had previously high pyuria was only 1 (figure 3.6A). Applying this same method to patients who had infection detected by university culture methods (n=13), the number of patients with previous positive pyuria was also only 1.

Another hypothesis could be that by reducing the threshold of CFU count to 10^3 CFU/ml, an infection could be detected before is has become established enough to be detected by hospital culture. By looking back at patient university culture history, the number of patients with previous positive culture detected by university culture prior to an infection was counted. The number of patients with a hospital detected infection (n=12) who had previously positive university culture was only 1 (figure 3.6A). This suggests that high CFU count infections establish rapidly and unless more frequent culture testing is performed then this method cannot be used to predict an infection. Applying this same method to patients who had infection detected by university culture methods (n=12), the number of patients with previous positive university culture was 5.

Since IBCs have been of previous interest to UTI as they could protect bacteria from immune responses and antibiotics and allow for rUTI occurrence when bacteria re-emerge from within urothelial cells. Using the same analysis theory as before, it was hypothesised that observance of 'clue cells' (cells with closely associated bacteria detected by DAPI staining (General Methodology 2.1.3.1)) could be used to predict an infection. By looking back at clue cell observations in patients, the number of patients with previous clue cell observations prior to an infection was counted. The number of patients with a hospital detected infection (n=12) who had previously positive clue cell observation was 5 (figure 3.6A). Applying this same method to patients who had infection detected by university culture methods (n=12), the number of patients with

previous clue cell observations was 6, suggesting that clue cells could be used as an indicator of potential UTI.

Since clue cells show bacteria in close proximity to urothelial cells and could represent intracellular species, the samples with positive infection and clue cells observations were correlated to determine which species could be on the clue cells. It was found that of the 6 samples with positive university culture and clue cells, 4 contained >10^3 CFU/ml *Lactobacillus* (66.6%), 2 out of 6 contained >10^3 CFU/ml *Corynebacterium*, and 1 out of 6 contained *E.coli, Klebsiella, Atopobium, Streptococcus,* and/or *Gardnerella* (figure 3.6B)
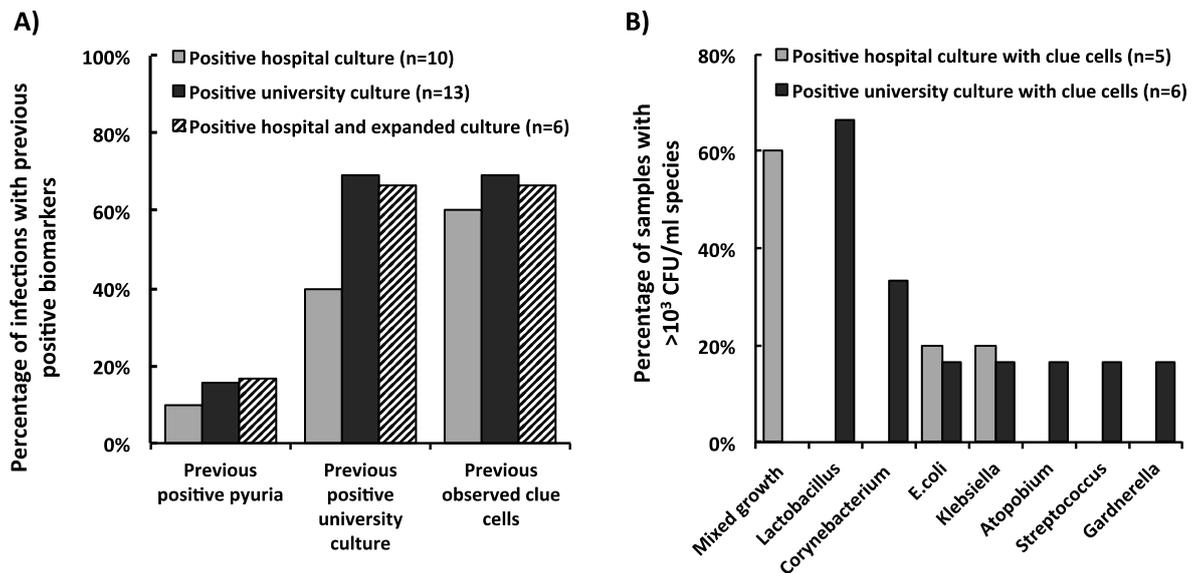
Figure 3.6: History of positive biomarkers as a predictor of infection. A) Of RTRs who had an incidence of infection identified by hospital culture (grey bars, n=12) and RTRs who had infection identified by university culture (black bars, n=12), only 1 of each had previous positive pyuria suggesting pyuria likely only occurs once an infection has established and cannot be used to predict UTI. Of 12 RTR who had an incidence of infection identified by hospital culture, only 1 had incidence of previous positive university culture suggesting high level infection is spontaneous and establishes rapidly, therefore cannot be predicted unless sampling is more regular. 5/12 RTRs who had infection identified by university culture had previous positive infection so low-level infection is more stable than severe infection identified by hospital culture and may be caused by quiescent bacteria. Incidence of clue cells is more effective as a predictor of infection since 5/12 RTRs with positive hospital culture and 6/12 RTRs with positive university culture were found to have previous observations of clue cells in the urine. **B)** Of patients with clue cells and infection identified by university culture (grey bars, n=6), 66.6% of infections were caused by *Lactobacillus*, a non-pathogen, followed by 13.5% by *Corynebacterium*. Of patients with clue cells and infection identified by hospital culture (black bars, n=6), 60% of infections were caused by mixed cultures followed by 20% by *E.coli and Klebsiella*.

3.4.7 CHANGES IN BLADDER MICROBIOME POST-RT

By looking at individual species of interest identified by 16s rRNA sequencing which are found in each patient at each time point it is possible to analyse the incidence of the most predominant species, not just those that are pathogenic or greater than 10^3 CFU/ml. The donor patients acted as normal healthy controls and the species found in each RTR were pooled per time point; <1 month, 1 month, 3 months and 6 months post-renal transplant. From figure 3.7A it can be seen that *Staphylococcus* is the most common species in both controls (74.2%) and RTRs across all time points (59.1% to 80.95%) and therefore its incidence is not affected by co-trimaxazole use. *Streptococcus* is the second most common genus in controls (61.8%), however incidence of *Streptococcus* is significantly reduced in RTRs at <1 month post transplantation (11.1%, $p < 0.0001$***) and remains reduced across all time points (13.6% at 1 month to 27.8% at 6 months, $p < 0.01$* to ***) (figure 3.7B).

Interestingly, similar trends can be found for *Streptococcus*, *Corynebacterium*, *Peptoniphilus* and *Actinomyces* whereby incidence reduces from controls within the first month but increases again after 3 months. As previous mentioned, *Streptococcus* incidence reduces from 61.7% in controls to 11.1% in RTR within the first month 11.1% ($p < 0.0001$***), however the incidence increases to 33.3% after 3 months ($p < 0.05$, figure 3.7B). *Corynebacterium* and *Peptoniphilus* incidence in controls was at 47.1% and is reduced within the first month to 14.8% for both species ($p = 0.012$*, figures 3.7C and 3.7D). By month 3, *Corynebacterium* has repopulated in RTRs to the 47.6% again, similar to what was found in the controls (figure 3.7C). However, *Peptinophilus* remains significantly lower in RTR compared to controls ($p < 0.05$*, figure 3.7E)

Actinomyces incidence is initially reduced in RTRs compared to controls from 35.3% to 7.4% ($p = 0.014$*, figure 3.7E) within the first month post-transplant suggesting these genus are sensitive to co-trimaxazole In the following months, incidence of *Actinomyces* fluctuates however generally remains lower than in controls (4.7% to 13.5%, figure 3.7E). These data suggest that *Streptococcus* and *Actinomyces* remains sensitive to co-trimaxazole whereas *Corynebacterium* and *Peptoniphilus* are able to develop resistance and repopulate the urinary tract. All other differences between bacteria in controls and RTRs at all time points are not significant.

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Incidence of *Enterococcus, Lactobacillus* and *E.coli* remain unaltered in RTRs compared to controls (figure 3.7F-H). Incidence of *Fusobacterium* is reduced to 0% in RTR patients suggesting this species is sensitive to co-trimaxazole, however it can be pointed out the incidence in controls was not high initially (14.7% figure 3.7I). All p values are calculated by Chi squared tests comparing incidence at time point to incidence in controls.
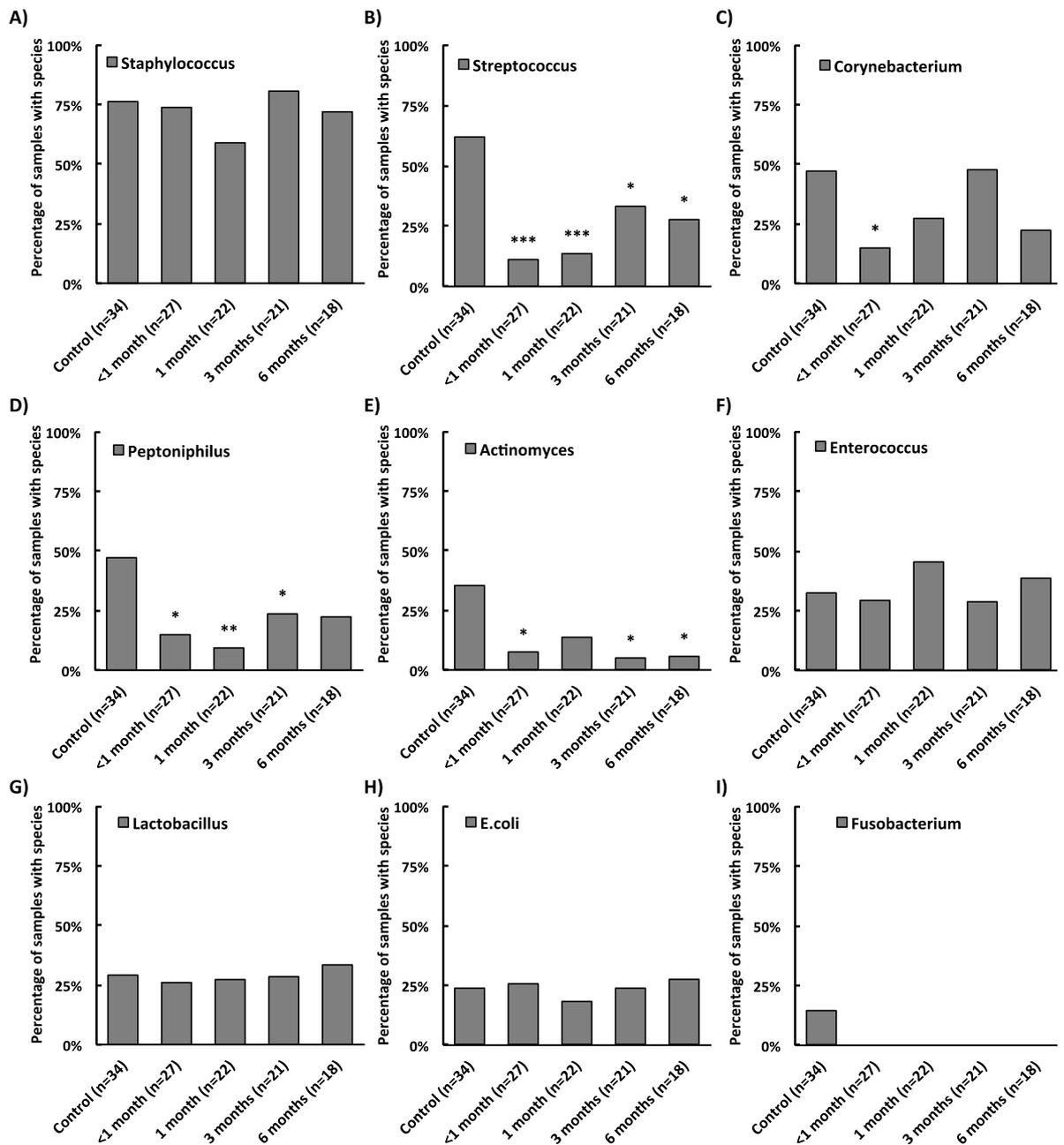
Figure 3.7: Changes in the microbiome of renal transplant patients over time. Donor samples are used as controls (n=34) as they have not received any immunsuppressants or antibiotic treatment. Isolates from patients who provided more than one sample in any given time point are pooled and counted as n=1. The same control group is used for each bar chart. All p values are calculated by Chi squared tests comparing incidence at time point to incidence in controls. **A)** The most predominant species in controls is *Staphylococcus*, found in 81.0% of control patients and remains relatively unaffected in RTRs (59 to 80.9%, p>0.05). **B)** Incidence of *Streptococcus* in controls is 61.8%. Within one month, incidence drops significantly from to 11.1% post-transplant (P<0.001***). Incidence is still significantly lowered in RTRs at 30 days (13.6%, p=<0.001***), 3 months (33.3%, p<0.01*) and 6 months (27.8%, p<0.01*). **C)** Incidence of *Corynebacterium* in controls is 47.6%. Within one month the incidence of *Corynebacterium* drops significantly from 47.6% in controls to 14.8% (p= 0.012*). Interestingly, incidence increases again after 1 month (27.3%) and further increases after 3 months (47.6%). **D)** Incidence of *Peptoniphilus* in controls is 47.1%. This is reduced in the first month in RTRs to 9.1% and remains lowered across all time points (14.8 to 23.8%). **E)** Incidence of *Actinomyces* in controls is 35.3%. This is reduced in the first month in RTRs to 7.4% and remains lowered across all time points (4.7 to 13.6%). **F)** Incidence of *Enterococcus* in controls is 28.6% and remains relatively unaffected in RTRs (28.6 to 45.4%, p>0.05). **G)** Incidence of *Lactobacillus* in controls is 28.6% and remains relatively unaffected in RTRs (25.9 to 33.3%, p>0.05). **H)** Incidence of *E.coli* in controls is 23.5% and remains relatively unaffected in RTRs (18.2 to 27.8%, p>0.05). **I)** Incidence of *Fusobacterium* in controls is 14.7% and this is reduced to 0% in RTRs. Taken together these data suggest that within the first month *Streptococcus, Corynebacterium, Peptoniphilus,* and *Actinomyces* are susceptible to antibiotic usage however *Corynebacterium* and *Peptoniphilus* are able to overcome this over time, probably by developing antibiotic resistance.

3.5 DISCUSSION

The aims of this chapter were to determine key bacterial species in rUTIs in RTRs, to investigate alternative biomarkers for rUTIs in RTRs, and to determine if there is an intracellular bacterial component to rUTIs in RTRs. Following investigation, the 5 main findings are: - i) hospital diagnosis of UTIs could be missing key low-level bacteriuria, ii) clue cells could be used as a prospective biomarker for rUTIs in RTRs, iii) there are significant reductions in *Actinomyces, Corynebacterium* and *Peptoniphilus* in RTRs.

3.5.1 INCIDENCE OF INFECTION IN RTRS REFLECTS PUBLISHED DATA

Our data has shown that the incidence of infection in RTRs reflects that of data published by others. The age of our cohort is similar to that of other reports of the average age of patients receiving renal transplants. From the US Renal Data System database sturdy where a total of 23,924 RTR records were analysed, 17,358 of these were men (60% and the remainder were women (40%) (120). Our data had a higher percentage of female recipients (55.5%). This could be due to the fact that the US study has a very large n number. Our data was however in keeping with this study in that the more female RTRs (40%) developed UTIs than men (22.2%, figure 3.3). In the large US study 38.2% of recruited males had had a UTI and 47% of females had had a UTI. These figures are slightly higher than ours, but this could be due to the fact that the patients recruited in our study had only recently had a RT. Finally, our data reflects the systemic review by Parasuraman *et al* (121) in that *E.coli* is the most prevalent pathogen causing UTIs in RTRs (figure 3.5).

3.5.2 HOSPITAL CULTURE AND IDENTIFICATION IS LIMITING

The percentage of infections identified from 272 samples sent for hospital culture was 6.2% (figure 3.2), whereas the percentage of infections identified by university culture out of 127 patients was 22% (figure 3.2). This is nearly a four-fold increase in the percentage of infections detected, indicating that the university culture method is able to detect low-level infections due to the lowered threshold of CFU count. It would therefore be expected that all positive hospital cultures would also be positive university culture methods, however this was only the case in 5.7% of

samples. University and hospital positive culture had an 80.1% concordance, a sensitivity of 50% and a specificity of 83.3% suggesting that some infections could also be being missed by university culture methods and therefore they may need reconsideration.

By performing 16s rRNA sequencing our method was more effective at identifying a greater range of species of pathogenic bacteria which could be causing UTI's in RTRs; identifying particular species such as *Atopobium, Corynebacterium, Streptococcus, Gardnerella,* and *Bifidobacterium* (figure 3.5). These species are not the same as those identified by Parasuraman's systemic review, which identified the highest prevalence of first UTI to be caused by *E.coli (*34.5%), *Enterococcus* (19.5%)*, Pseudomonas aeruginosa* (12.5%), *Staphylococcus spp.* (9.5%)*, and Klebsiella spp.* (9%), however their study cumulated results from 1,517 RTRs whereas we were only able to recruit 36 in the time of this study, which could be a reason for the differences in the types of species found (121). The species of interest from this study are not usually identified by hospital culture since they are generally not considered to be pathogenic, however they are notable emerging pathogens that have been associated with UTIs and should therefore not be dismissed from further investigation (231). Furthermore, university culture methods also do not prejudice for multiple growths greater than 10^4 CFU/ml from the same sample, which hospital culture regards as contamination. Therefore, although some positive infections by hospital culture were missed by university culture methods, the university method was over all better at detecting a range of bacterial species and was not prejudice for single cultures. Taken together this indicates that UTIs can best be identified by the reduced threshold of 10^3 CFU/ml since more infections can be identified.

3.5.3 HOSPITAL BIOMARKERS FOR INFECTION ARE LIMITING

Often, an infection is accompanied by symptoms of increased frequency of micturition, pain and nocturia. In some cases, this in itself can be a marker of infection, however our data shows that symptom score and hospital culture only has a concordance rate of 54.5%, a sensitivity of 58.8% and a specificity of 54.8%, therefore other biomarkers of infection must be used to identify infection.

Hospital automated cell counting appeared the best test for detecting pyuria compared to counting using a haemocytometer, since it had the best combined sensitivity (68.4%) and specificity (67.7%, figure 3.6A). Manual WBC count and nitrite dipstick had excellent specificities (>90%) however had very low sensitivities (<11%) Interestingly, the protocol for hospital culture must take into consideration positive pyuria by automated cell counting, however our analysis has shown that 42.4% of low-level infections identified by university culture did not have accompanying positive pyuria suggesting that pyuria does not always accompany low-level infection (data not shown). Our results do reflect the conclusions made by Malone-Lee *et al* they discredit the use of microscopic pyuria and LE as biomarkers for infection (215).

3.5.4 USING UNIVERSITY CULTURE AND CLUE CELLS AS BIOMARKERS FOR INFECTION

By analysing patient clinical history it was possible to identify whether previous incidence of pyuria, low-level infection, or clue cell observation could be used to predict an infection. The number of patients with positive pyuria prior to detecting either low-level or clinical UTI was only 1 suggesting that positive pyuria con only be detected once an infection has become established and cannot be used to predict an infection. It was found that 69.2% of patients with a low-level infection (n=13) and 40% of patients with a clinical grade infection (n=10) had had previous low-level infections, indicating that low-level infections can be pre-determinant for clinical UTI (figure 3.5A). Therefore, by reducing the threshold of CFU/ml count, hospitals could put patients with low-level infections under more intense monitoring in order to catch any hospital grade infections sooner.

Presence of clue cells were observed in 17 out of 36 RTR patients at any particular time point and 9 of these developed a low-level infection by university culture (53%) and 6 developed a hospital grade infection (35%) (figure 3.5B). By looking at the samples containing clue cells and the species which were identified at $>10^3$ CFU/ml it was found that most of the samples with a positive infection containing clue cells were caused by *Lactobacillus,* which is generally regarded as a non-pathogenic, probiotic species in the urogenitary tract. Other species such as *E.coli, Corynebacterium* and *Klebsiella* were also identified however, and are regarded as pathogenic. Although these numbers are low, this is still a promising finding and

could be easily improved upon and adapted into hospital pathology methods in order to use clue cell observations as an indicator of potential UTI (discussed in more detail in section *3.5.7 –Future work and ideas*).

3.5.5 SPECIES OF INTEREST IN RTRS

By pooling the species found in each patient at each time point it was possible to determine the percentage incidence of each species at any time point. Interestingly, the incidence of *E.coli* remained relatively the same at each of the four time points compared to controls, signifying that although this species is the most common cause of UTI in RTR, the numbers of UTI's recorded were not high enough to alter the difference in incidence of this species between controls and RTRs (figure 3.7H).

There is a reduction in the incidence of *Corynebacterium, Streptococcus, Peptoniphilus* and *Actinomyces* (figure 3.7B-E) at all time points, which could be due to high susceptibility of these genus to co-trimaxazole. By consulting James McCormack's antibiotic sensitivity chart it is possible to see the range of bacteria that are susceptible to co-trimaxazole, which includes *Staphylococcus aureus*, some *Streptococcus spp.* and Gram negative bacilli except *Pseudomonas aeruginosa* (232). Co-trimaxazole is generally used a prophylactic against pneumonia, which in renal transplant patients is most often caused by *Staphylococcus aureus, Streptococcus pneumoniae,* and Gram-negative bacilli, and therefore rationalises the use of co-trimaxazole (233). The use of this particular antibiotic does not explain however the observed reduction in *Corynebacterium, Peptoniphilus* or *Actinomyces,* since these genus are not expected to be affected by co-trimaxazole (232). The fact that *Staphylococcus* incidence remains the same, despite use antibiotics, can be explained by the fact that *S.aureus* but not *S.epidermidis* are susceptible to co-trimaxazole. *Corynebacterium, Peptoniphilus* or *Actinomyces* are not generally recognised as probiotic or protective, but rather as emerging pathogens, however reduced incidence of these species may allow for other opportunistic pathogens to grow. First, susceptibility of these genus to co-trimaxazole needs to be determined to begin to confirm this theory, followed by co-culture. Antibiotic susceptibility testing could also explain increased incidence of *Corynebacterium* and *Actinomyces* 3 to 6 months post-transplantation which suggest repopulation, perhaps due to antibiotic resistance.

The incidence of clue cell observations was compared with positive university culture to determine if the species of bacteria associated to urothelial cells could be identified this way. Many of the samples positive for both clue cells and low-level infection were found to be caused by *Lactobacillus*, which is renowned for its probiotic properties in the genitourinary tract (234). If clue cells were to be used for predicting infections, it would have to be taken into consideration therefore that many observations might not be pathogenic. Fortunately, *Lactobacillus* cultures are easily identifiable on chocolate culture plates as small, green, dry colonies with mild beta-haemolysis. Therefore, positive clue cell observations followed by observations of these colony types could be regarded as normal healthy growth.

3.5.6 METHODOLOGY ERRORS AND LIMITATIONS

Due to the slower than expected rate of RTRs coming through Kent and Canterbury Hospital and the time constraints of this doctorate, it was not possible to collect the complete number of samples and patients suggested in the grant for this study. All of the observations made in this study would benefit from increased patient numbers in order to better support the findings and conclusions.

An issue with the university method of determining low level infections appeared when comparing the samples with positive hospital infections with those identified by culture. One would expect positive hospital infections to also be positive by university culture since the threshold for CFU/ml is lower (figure 3.2A). It can therefore be concluded that university culture is able to detect more infections but still miss others. This could be due to bacteria dying in the fridge or in transit between sample collection and plating. Alternatively, the use of selective media in hospital culture allows for certain species to grow better than on the chocolate agar utilised in university culture.

A limitation of observing clue cells and correlating it with identified infections by expanded culture is that one is assuming that the bacteria on the clue cells are the same as those identified by university culture. Furthermore, clue cells do not indicate that bacteria are intracellular. In order to ascertain if these are the same species and

if they are intracellular it would be essential to perform an antibiotic protection assay (137). This technique uses non-penetrable antibiotics to kill susceptible planktonic bacteria on urothelial cells, and is followed by a lysing and culture step to grow any intracellular bacteria which can then be identified by 16s rRNA sequencing. For future studies, this protocol should be performed to determine with certainty that infections are caused by species observed on clue cells.

The purpose behind this study was inform the next study on OAB in the following chapter. As discussed in the introduction, OAB is not considered an infection, however there have been recent advances in clinical research which suggest that alterations in the microbiome may be a cause for this syndrome (138, 193, 195, 235, 236). Alterations in the microbiome are most evident when taking antibiotics since they can reduce incidence of probiotics and allow for opportunistic infections. Hence, RTRs were used to represent high-risk patients in a preliminary study. The idea being that any potential changes in the microbiome leading to urinary tract symptoms is more likely to occur in those receiving a renal transplant due to risk of opportunistic infections. The data from this study on RTR patients who are all taking prescribed antibiotics and immunosuppresans is that significant reductions in certain bacteria is evident however this doesn't correlate with incidence of infection (data not shown). This suggests that the microbiome can be manipulated but it does not suggest that it is a cause for infection in this subset of patients. This suggests that any differences which may be seen in the following OAB study ought to be more rigorously investigated. Furthermore, inclusion of an antibiotic protection assay in the next study could further inform of the identification of intracellular species.

3.5.7 FUTURE WORK AND IDEAS

It was concluded that university culture methods were able to detect low-level infections which are not regarded as UTIs by hospital culture. This is likely due to the reduced threshold of CFU/ml count utilised by the university. Also observed was the fact that low-level infections were found in 40% of patients prior to diagnosis of a clinical grade UTI. Therefore, a large scale study aiming to better determine types of infection in RTRs could inform hospitals of ways they could introduce lower threshold CFU count as a means to monitor patients post-transplant. In cases where

low-level UTIs are diagnosed, these patients could then be put under more frequent monitoring in order to catch any potential complicated infections sooner.

Also observed was an increased range of bacterial species identified by university culture methods, some of which are notable emerging uropathogens. Many of these species could be being missed by hospital culture due to the restricted protocol for identifying pathogenic species. Expanded culture methods cannot easily be adapted in to hospital culture methods due to the cost and time implications of performing 16s rRNA sequencing, other than reducing the threshold of CFU/ml to 10^3 CFU/ml and extending growing times. However, some hospitals have recently begun using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI TOF-MS) to identify infections that cannot be identified by culturing on species-specific agar (237). Although expensive to install, this system can be used as a service by the entire hospital and, in some hospitals in the UK where it has already been implemented, has allowed for significantly faster, more effective, and more accurate patient diagnosis and treatment.

It was previously mentioned that the presence of clue cells does not infer intracellular bacteria. There are various methods which can be used to determine whether bacteria are intracellular upon detection of clue cells in a sample; i) an acridine orange and crystal violet stain on Cytospun urine to visualise intracellular bacteria, and ii) culturing bacteria obtained from within urothelial cells by performing an antibiotic protection assay. The second of these methods is more informative as it also allows for genetic identification by 16s rRNA sequencing post culture. This method will be adapted from Khasriya *et al* (137) for future investigations in this thesis.

Although this study has identified some species of interest that appear to be significantly reduced post-transplant, it is not known if these species are reduced due to antibiotic sensitivity or other means. There is very little literature on the effects of co-trimaxazole and co-trimaxazole resistance in *Corynebacterium, Actinomyces* and *Peptinophilus,* therefore determining the sensitivity of these genus to this antibiotic would be of great value. This can be done by performing a spread culture on chocolate agar then placing paper disks soaked in co-trimaxazole on the surface. If

these genus are sensitive to co-trimaxazole, an area of no growth would appear around the paper disk. Determining co-trimaxazole sensitivity would answer the question of whether the reduced observation is due to antibiotic use and therefore, if alterations in the bladder flora could be a reason for increased incidence of UTI in RTRs.

3.6 CONCLUSIONS

1. Percentage incidence of hospital diagnosed UTI in RTRs was 33.3%, in keeping with many studies.

2. University culture is able to detect more infections and has a high sensitivity, however this method of testing has a low specificity and misses some infections.

3. Automated WBC counting is the most effective way of detecting pyuria, however pyuria and infection do not always correlate and cannot therefore be used to diagnose infection without culture as well.

4. Species of bacteria identified by 16s rRNA sequencing differ from the cumulated findings of 4 similar studies, notably we have identified some emerging pathogens including *Atopobium, Corynebacterium, Streptococcus, Gardnerella,* and *Bifidobacterium.*

5. Reductions in *Actinomyces, Corynebacterium* and *Peptoniphilus* in RTRs could be due to antibiotic susceptibility and reduced incidence may be a cause for infection.

6. Clue cells could be used to predict future infections, however most clue cell observations were associated with high *Lactobacillus* growth, which is not a pathogen, but a probiotic species.

7. The methods used in the next study need to include antibiotic protection assay, include more rigorous measurements of changes in the microbiome and compare these to clinical factors.

CHAPTER 4 - RESULTS 2:
THE BLADDER MICROBIOME IN OVERACTIVE BLADDER

4.1 INTRODUCTION

Numerous studies have highlighted that the current criterion for diagnosing UTI is flawed and that by reducing the threshold of CFU/ml of a single species from 10^5 to 10^3 can reveal infection where it may have previously been missed (129, 134, 136). Application of this to OAB has indicated that there may be a possible 'low count bacteriuria' in OAB that could be causing the symptoms of OAB (134, 136, 137, 219). Positive results from a preliminary, small scale abstract by Malone-Lee *et al* have shown that treating OAB with antibiotics can reduce the symptoms of this syndrome (104). Interestingly, most UTIs are caused by the following species: - UPEC, *Enterococcus faecalis, Proteus mirabilis, Klebsiella pneumoniae, Pseudomonas aerogenosa,* and *Serritia marcenscens* (107, 108)*.* However, there are some studies which have performed EQUC and culture-independent 16s rRNA sequencing; the most advanced methods of exploring the microbiome to date, that have not reported these species to be significantly increased in OAB compared to controls (138, 194, 195, 197). They have however found increased incidence of species such as *Gardnerella, Actinobaculum, Actinomyces, Aerococcus, Arthrobacter, Cornybacterium,* or *Oligella* (138, 197)*.*

In recent years, the research groups that have applied these advanced techniques have moved away from the theory of a low-level infection and have proposed the concept of an imbalance in a bladder microbiome that is unique to every individual. Due to the proximity to the anus and vagina in women, this is more than feasible, and what with the capability of so many of the species identified in the 'bladder microbiome' to form biofilms and intracellular communities, the likelihood of bladder colonisation is strengthened. One investigation has proposed that the microbiome changes over time (236) and another, in concordance, stated that each individual should therefore act as their own control (236). Interestingly, Gram-positive UTI's such as those caused by *Aerococcus, Corynebacterium, Actinobaculum,* and

Gardnerella, are more prevalent in the elderly and are often over-looked as uropathogens (138, 231, 238, 239). *Aerococcus* is often misclassified or regarded as a contaminant in microbial culture (240), and therefore 16s rRNA sequencing could confirm species ID and increase awareness of this uropathogen. *Corynebacterium* and *Actinobaculum* are very slow growing species on blood agar and are therefore also occasionally regarded as contaminants (231). *Gardnerella* is better known as the causative species of bacterial vaginosis, however this species is slowly becoming recognised as a uropathogen as well (231). Once again this species requires extended culture of 48-72 hours. Taken together it could be hypothesised that microbiome imbalances may allow for increased levels of emerging pathogens and could play a part in OAB if less limiting culture assays are used to diagnose infection.

Other studies have proposed an intracellular component to OAB (17, 153, 241), whereby bacteria reside within the urothelium stimulating low level inflammation, which may in turn lead to detrusor muscle contractions. Furthermore, re-emerging bacteria from IBC may cause recurrent UTIs, which could be missed if they are at low level. There have been no published investigations which have been able to identify a spectrum of the exact species which are capable of forming IBC in the bladder. This chapter will investigate the microbiome of individuals with OAB by identifying all species of bacteria cultured from urine and comparing the microbiome to those of controls. Furthermore, by sequencing all species following an antibiotic protection assay it will give us insight into any intracellular species and determine if there is a role for intracellular bacteria in OAB aetiology. Along with the clinical data obtained from the hospital, this project aims to identify potential biomarkers of OAB with an aim of better diagnosing this syndrome.

4.2 HYPOTHESIS AND AIMS

We hypothesise that the microbiome, including incidence of intracellular bacteria colonies, changes with age, predisposing (in this case) women to OAB.

In order to begin to test these hypotheses the following objectives will be to:-

1. Investigate the differences in the microbiota in the MSU and CSU sample urine between patients with OAB and controls in various age groups irrespective of CFU/ml.
2. Determine whether there is an intracellular bacteria component to OAB and, if so, which species are most prevalent.
3. Correlate incidence of species of interest and differences in clinical data in order to determine any potential biomarkers for OAB.

4.3 METHODOLOGY

4.3.1 DISCLAIMER

The collection of clinical data, including patient age, parity (number of children), BMI, and patient diagnosis was performed by clinical staff and clinicians at Medway Maritime Hospital, Kent, UK. ICIQ form completion, urine collection, dipstick urinalysis and Sternhiemer-Malbin analysis was performed by myself and the clinicians at Medway Maritime Hospital. Hospital pathology was performed by clinical staff in the pathology department at Medway Maritime Hospital. University culture and sequencing was performed predominantly by myelf with assistance from clinicians from Medway Maritime Hospital. All analysis was performed by myself.

4.3.2 ETHICS

Ethical approval was obtained from the NHS Health Research Authority (NRES Committee South East Coast - Kent); IRAS 24056.

4.3.3 PATIENT SAMPLE COLLECTION

Midstream (MSU) and catheterised (CSU) samples were taken from patients who fitted a specific description of OAB dictated by International Validated Questions from the International Consultation on Incontinence Modular Questionnaire – Female Lower Urinary Tract Symptoms Long Form (ICIQ-FLUTS-LF) (see Appendix 1.3). Patients were clinically diagnosed with OAB following assessment of history and examination. All patients also indicated a positive response to Question 3a of the ICIQ-FLUTS-LF (Do you have a sudden need to rush to a toilet to urinate?). All patients were female aged 18 and over and suffered moderate to severe OAB with urgency, frequency and nocturia. Any patients with symptomatic urinary tract infections, interstitial cystitis, predominant stress incontinence symptoms, or previous pelvic radiation were excluded from the cohort. Control samples were also taken from women visiting out-patient clinics for non-urinary problems, who were asked to participate in the study and scored 0 on the International Consultation on Incontinence Modular Questionnaire – Female Lower Urinary Tract Symptoms Short Form (ICIQ-FLUTS-SF) to ensure that they did not suffer with any OAB symptoms. All sampling methods met the ethical approval and each patient gave consent on each occasion before providing a sample.

OAB MSU samples were collected from a weekly clinic held on Wednesday mornings run by Professor Jonathan Duckett at the department of Obstetrics and Gynaecology at Medway Maritime Hospital, Kent, UK. Control MSU samples were collected from a clinic on Monday mornings run by Dr. Peri Krishna-Moorthy, for women with non-urinary gynaecological queries at Medway Maritime Hospital, Kent, UK.

OAB CSU samples were collected from patients undergoing a clinical trial for cystodistension to treat OAB symptoms. Control CSU samples were collected from patients undergoing surgeries such as hysterectomy, myomectomy or laparoscopy. All samples were collected under general anaesthetic in a sterile operating theatre prior to the scheduled operation. Urine was analysed by dipstick once surgery was completed. Some of the urine was placed in boric acid and sent for hospital pathology The remaining urine was kept in a fridge until collected or delivered to the university within 4 hours after collection, where it was analysed as discussed in *Section 2.1.3 - Processing of fresh urine samples..*

Patients with OAB who provided a CSU sample before their cystodistension also provided two biopsies of bladder tissue. These biopsies were taken via a cystoscopy (telescopic examination of the inside of the bladder) and were placed in sterile saline to be processed as according to the protocols for microbial culturing, electron microscopy or ATP assay discussed in *Section 2.1.5 - Bladder biopsies.*

For age related analysis, patients were split into groups by age. The first age group, 20-44, is the average age at which women are between puberty and pre-menopause. The second age group, 45-60 is the average age at which women are post-menopause and pre-elderly and the final age group 65+ is the average age at which women reach elderly age.

4.4 RESULTS

4.4.1 DEMOGRAPHICS OF PATIENT COHORTS

Four main cohorts of patients were derived from collections from clinics and day surgeries; MSU samples from patients with OAB (OAB MSU, n=69), MSU samples from controls (control MSU, n=84), CSU samples from patients with OAB (OAB CSU, n=60), and CSU samples from controls (control CSU, n=14)(table 4.1). Descriptive statistics were used to describe the features of the individual cohorts when possible; age and BMI from hospital records; leukocyte esterase, nitrites, and pH from dipstick results, epithelial and white blood cell count from hospital pathology; UTI diagnosis from hospital pathology; epithelial and white blood cell count from Sternheimer staining; and incidence of observed 'clue cells' in DAPI stained urine (urothelial cells with closely associated bacteria). Not all data was able to be recorded for all samples due to clinician oversight in cases of age and BMI, difficulty accessing facilities after surgery and not performing a CFU count for the primary samples collected. The total n numbers for each data set is summarised in table 4.1

Most OAB and control CSU samples were not dipsticked or cell counted in the hospital due to difficult access to facilities after theatre collection (table 4.1). Control MSUs and CSUs were not sent for pathology as these individuals were not suspected of an infection and therefore the costs incurred were not justified (table 4.1).

	OAB MSU	Control MSU	OAB CSU	Control CSU
Total samples collected	**n=69**	**n=84**	**n=60**	**n=14**
Age	n=65	n=79	n=54	n=12
BMI	n=43	n=73	n=52	n=12
Dipstick analysis	n=66	n=68	N/A§	N/A§
Hospital pathology	n=59	N/A§§	n=53	N/A§§
University culture	n=60	n=82	n=42	n=14
University culture with CFU count	n=28	n=25	n=26	n=12
Sternheimer-Malbin	n=65	n=47	n=28	n=12
DAPI stain	n=69	n=80	n=48	n=12

Table 4.1: n numbers for clinical and biological data sets for each group of patients. A total number of 69 OAB MSU samples, 84 Control MSU samples, 60 OAB CSU samples, and 14 Control CSU samples were collected for this study. In each case, incomplete data was collected for the various data sets. This table summarises the n numbers for each of the following data sets: - patient age, patient BMI, urinary dipstick analysis, hospital pathology, university CFU count, Sternheimer-Malbin staining, and DAPI staining.

§ = OAB and control CSU samples were not dipsticked in the hospital due to difficult access to facilities after theatre collection. §§ = Control MSUs and CSUs were not sent for pathology as these individuals were not suspected of an infection and therefore the costs incurred were not justified.

4.4.1.1 AGE AND BMI

For the OAB MSU group, age ranged from 14 to 87 with a mean age of 59.9 (n=65) (figure 4.1A). In some cases patients BMI was not recorded due to clinician oversight, so for this reason only 43 patients BMI's are included in the statistics. BMI ranged from 17.3 to 45.6 with a mean of 29.4 (n=43) (figure 4.1B). Of the control MSU samples were collected, age ranged from 17 to 83 with a mean age of 39.6 (n=79) (figure 4.1C). BMI ranged from 15.5 to 41.1 with a mean of 26.3 (n=73) (figure 4.1D). Of the OAB CSU samples collected, age ranged from 17 to 83 with a mean age of 56.5 (n=54) (figure 4.1E). BMI ranged from 16.5 to 45.7 with a mean BMI of 29.6 (n=52) (figure 4.1F). Of the control CSUs were collected, age ranged from 22 to 79 with a mean age of 36.3 (n=12) (figure 4.1G). BMI ranged from 18.6 to 31.0 with a mean BMI of 23.7 (n=12) (figure 4.1H).
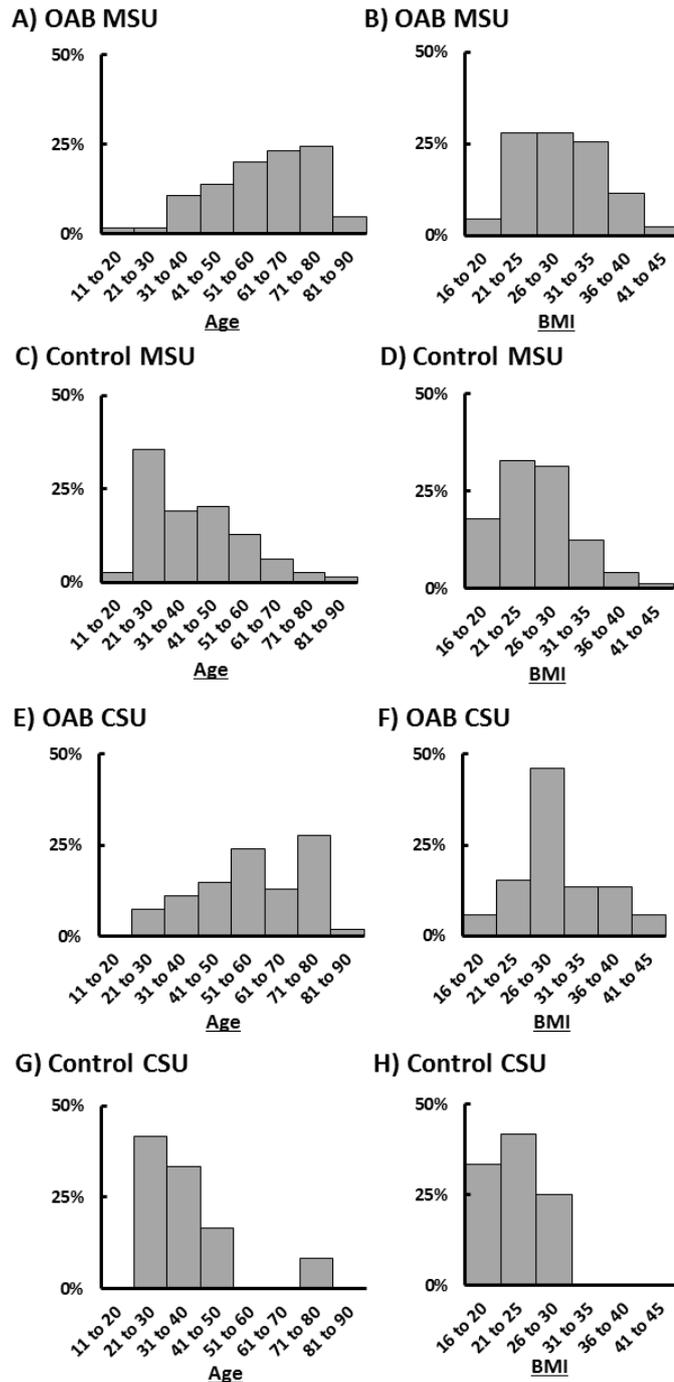
Figure 4.1: Demographics of age and BMI of OAB MSU, Control MSU, OAB CSU and Control CSU cohorts. A) 69 OAB MSU samples were collected from patients with OAB attending day clinics. Age of this cohort ranged from 14 to 87, mean age of 59.9 (n=65). **B)** BMI ranged from 17.3 to 45.6, mean of 29.4 (n=43). **C)** 84 Control MSU samples were collected from control patients attending day clinics for non-urinary gynecological queries. Age of this cohort ranged from 17 to 83 years, mean age 39.6 years (n=79). **D)** BMI ranged from 15.5 to 41.1, mean BMI of 26.3 (n=43). **E)** 60 OAB CSU samples were collected from patients with OAB undergoing cystodistension clinical trial in day theatre. Age of this cohort ranged from 17 to 83 years, mean age 56.5 years (n=54). **F)** BMI ranged from 16.5 to 45.7, mean BMI of 29.6 (n=52). **G)** 14 Control CSU samples were collected from control patients undergoing non-urinary gynaecological surgery in day theatre. Age of this cohort ranged from 22 to 79 years, mean age 36.3 years (n=12). **H)** BMI ranged from 18.6 to 31.0, mean BMI of 23.7 (n=12).

4.4.1.2 DIPSTICK ANALYSIS

From the OAB MSU group, dipstick results, leukocyte esterase was predominantly negative (50.0%, n=66), with 24.2% presenting with a trace reading and the remainder with +1, +2, or +3 (figure 4.2A). The mode (non-continuous data) result for nitrites was 'negative' with 93.94% of patients having this result (n=66) (figure 4.2B). pH of urine ranged from 5.0 to 8.5 with a mode pH of 6.0 and a mean of 6.1 (n=66) (figure 4.2C).

From the control MSU samples collected, dipstick results, leukocyte esterase was predominantly negative (64.7%, n=68), with 2 the remainder with +1 (10.3%), +2 (8.8%), or +3 (13.2%) (figure 4.2D). The mode result for nitrites was 'negative' with 97.06% of patients having this result (n=68) (figure 4.2E). pH of urine ranged from 5.0 to 8.5 with a mode of pH 5.0 (41.2%) and a mean of pH 6.0 (n=68) (figure 4.2F).

Many OAB and control CSU samples were not dipsticked or cell counted due to difficult access to facilities after theatre collection.
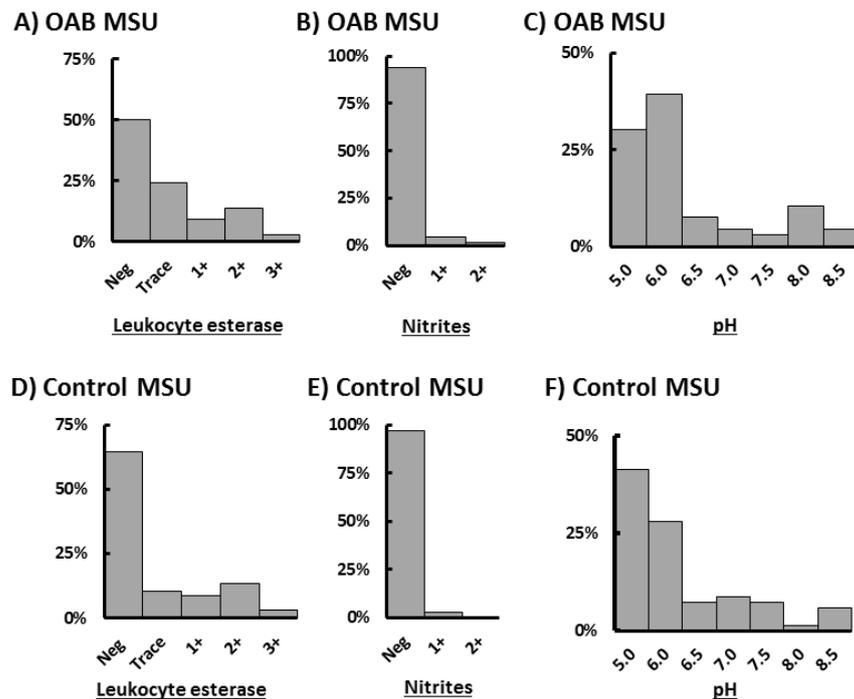
Figure 4.2: Demographics of dipstick analysis of OAB MSU and Control MSU cohorts. CSU samples were not able to be dipsticked due to difficult access to facilities after theatre collection. **A)** For the OAB MSU cohort, leukocyte esterase was predominantly negative (50.0%, n=66), with 24.2% presenting with a trace reading and the remainder with +1, +2, or +3. **B)** Nitrites were predominantly negative (93.94%, n=66). **C)** pH ranged from 5.0 to 8.5, mean pH 6.1, mode pH 6.0 (39.4%, n=66). **D)** For the Control MSU cohort, leukocyte esterase was predominantly negative (64.7%, n=68). **E)** Nitrites were predominantly negative (97.1%, n=68). **F)** pH ranged from 5.0 to 8.5, mean pH 6.0, mode pH 5.0 (41.2%, n=68).

4.4.1.3 Hospital pathology

In some cases, it was not possible to send samples for pathology due to the small volumes obtained. Also, in cases where patients were already diagnosed with idiopathic OAB and showed no symptoms of UTI, a pathology test was not required. Of the samples sent for pathology (n=59), 8.47% (n=5) of OAB MSUs were diagnosed as having a UTI by hospital pathology (figure 4.3A). These individuals were removed from further analysis as their diagnosis was no longer 'pure' OAB. The mode result for WBC count from hospital pathology was 10^1 to 10^2, with 76.3% of patients showing low numbers compared with only 1.7% having more than 10^3 (figure 4.3B). Hospital pathology reported 76.3% of patients (n=59) to have '+1' epithelial cells, 11.9% with none and the remainder with '+2' or '+3' (figure 4.3C).

Of the 60 OAB CSU samples collected, 5.66% (n=3/53) of patients were diagnosed with a UTI from hospital pathology (figure 4.3D). These individuals were removed from further analysis as their diagnosis was no longer pure OAB. The mode result for WBC count from pathology was 1 to 10, with 67.9% of patients showing low numbers compared with only 1.9% having more than 10^3 (figure 4.3D). Hospital pathology reported 81.1% of patients (n=53) to have '+1' epithelial cells, 15.1% with none and the remainder with '+2' or '+3' (figure 4.3E).

Control MSUs and CSUs were not sent for pathology as these individuals were not suspected of an infection and therefore the costs incurred were not justified.

A) OAB MSU
B) OAB MSU
C) OAB MSU

D) OAB CSU
E) OAB CSU
F) OAB CSU
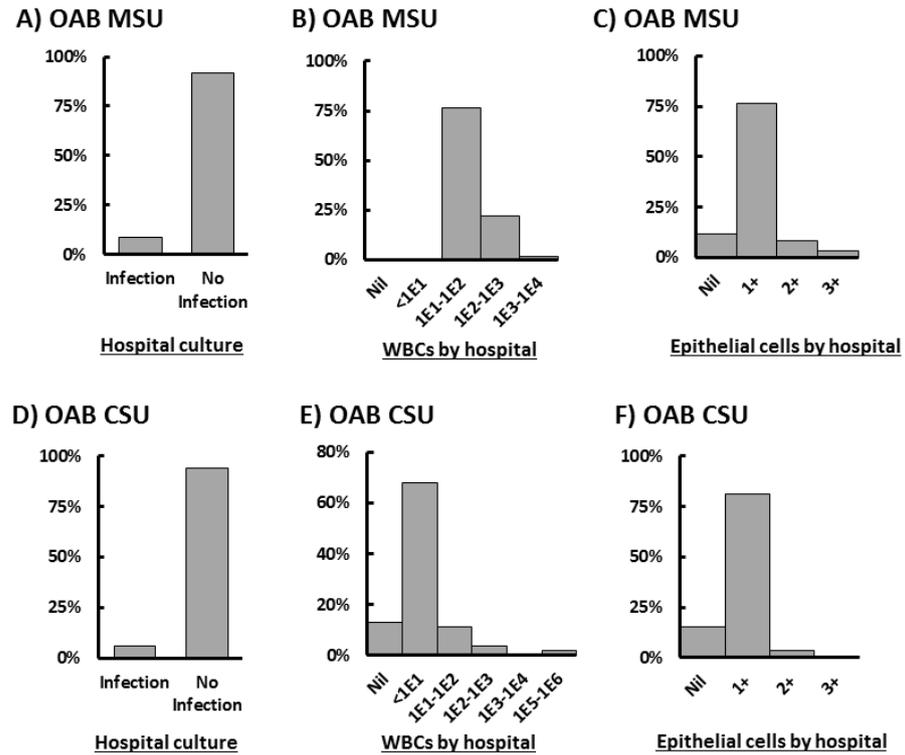
Figure 4.3: Demographics of hospital pathology of OAB MSU and OAB CSU cohorts. Control MSUs and CSUs were not sent for pathology as these individuals were not suspected of an infection. **A)** 8.5% of patients in the OAB MSU cohort (n=59) were diagnosed with infection by hospital pathology and removed from further analysis. **B)** Mode result for WBC count performed by hospital pathology was 1 to 10 cells/ml (%, n=59). **C)** Mode result for epithelial cell count performed by hospital pathology was '+1' (76.3%, n=59). **D)** 5.66% of patients in the OAB CSU cohort (n=53) were diagnosed with infection by hospital pathology and removed from further analysis. **E)** Mode result for WBC count performed by hospital pathology was 1 to 10 cells/ml (67.9%, n=53). **F)** Mode result for epithelial cell count performed by hospital pathology was '+1' (81.1%, n=53).

4.4.1.4 UNIVERSITY PATHOLOGY

For the OAB MSU group a total of 69 samples were collected. By decreasing the threshold of bacteria to 10^3, the number of infections identified by university culture performed in the laboratory was 21.4% (n=28) (figure 4.4A). The range of epithelial cells identified by Sternheimer staining and counting using a haemocytometer was 0 to 20 cells with a mode of 0 cells and a mean of 3.1 cells (n=65) (figure 4.4B). WBC count from this method ranged from 0 to 100 cells with a mode of 0 cells and a mean of 80 cells (n=65) (figure 4.4C). Staining cytospun urine with DAPI showed that the majority of samples contained either clean cast cells (shed urothelial cells with no associated bacteria, 31.9%) or clue cells only (39.1%, n=69) (figure 4.4D).

A total of 84 control MSU samples were collected. By decreasing the threshold of bacteria to 10^3, the number of infections identified by university culture performed in

the laboratory was 36.0% (n=25) (figure 4.4E). The range of epithelial cells identified by Sternheimer staining and counting using a haemocytometer was 0 to 30 cells with a mode of 0 cells (46.2%) and a mean of 3.6 cells (n=47) (figure 4.4F). WBC count from this method ranged from 0 to 100 cells with a mode of 0 cells (78.4%) and a mean of 3.5 cells (n=47) (figure 4.4G). DAPI observations on cytospun urine showed that 32.5% of samples contained clean cast cells (n=80), 23.8% contained clue cells only and 20.0% contained clue cells and polymorphonuclear cells (PMNs) (figure 4.4H).

A total of 60 OAB CSU samples were collected. The number of infections identified by university culture is 11.34% (n=26) (figure 4.4I). The range of epithelial cells identified by Sternheimer staining and counting using a haemocytometer was 0 to 15 cells with a mode of 0 cells (85.7%) and a mean of 0.9 cells (n=28) (figure 4.4J). WBC count from this method ranged from 0 to 500 cells with a mode of 0 cells (78.4%) and a mean of 18.7 cells (n=28) (figure 4.4K). DAPI observations on cytospun urine showed that 50.0% of samples contained clean cast cells (n=48) and 37.5% contained no cells (figure 4.4L)

A total of 14 control CSUs were collected. The number of infections identified by university culture is 18.2% (n=12) (figure 4.4M). The mode number of epithelial cells identified by Sternheimer staining and counting using a haemocytometer was 0 cells with 91.7% (n=12) and only 1 sample with 2 epithelial cells/ml (figure 4.4N). All samples (n=12) contained 0 WBC by Sternheimer staining (figure 4.4O). DAPI observations on cytospun urine showed that 33.3% of samples contained clean cast cells (n=12) and 66.7% contained no cells (figure 4.4P).
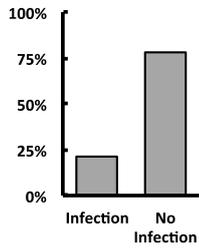
A) OAB MSU — University culture
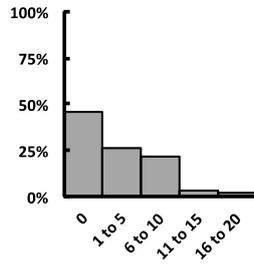
B) OAB MSU — Epithelial cells by Sternheimer
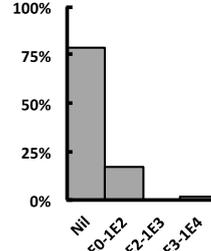
C) OAB MSU — WBCs by Sternheimer
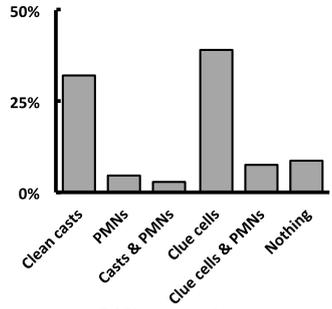
D) OAB MSU — DAPI observations
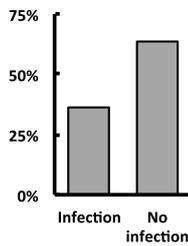
E) Control MSU — University culture
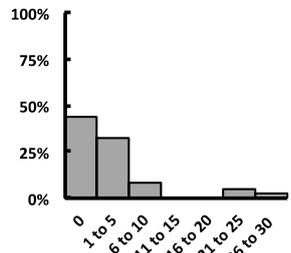
F) Control MSU — Epithelial cells by Sternheimer
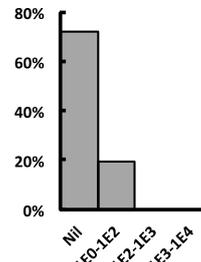
G) Control MSU — WBCs by Sternheimer
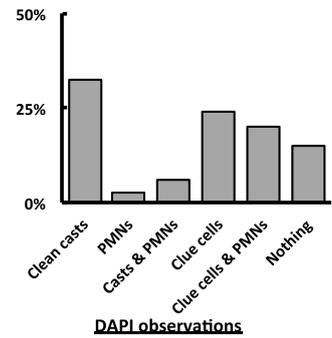
H) Control MSU — DAPI observations
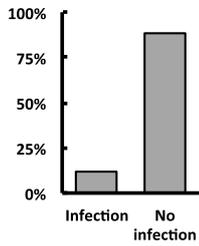
I) OAB CSU — University culture
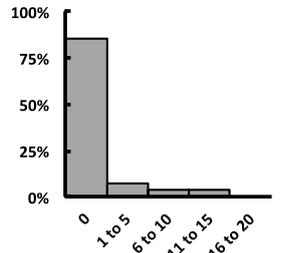
J) OAB CSU — Epithelial cells by Sternheimer
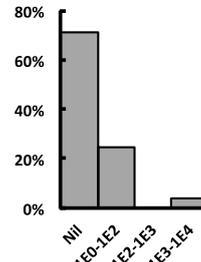
K) OAB CSU — WBCs by Sternheimer
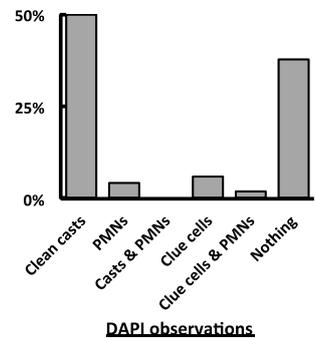
L) OAB CSU — DAPI observations
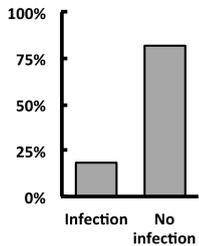
M) Control CSU — University culture
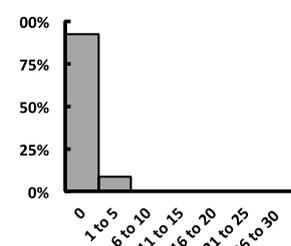
N) Control CSU — Epithelial cells by Sternheimer
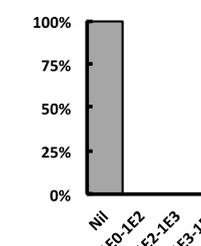
O) Control CSU — WBCs by Sternheimer

P) Control CSU — DAPI observations

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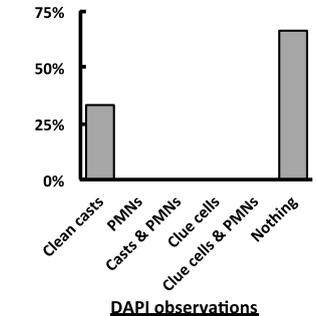
Figure 4.4: Demographics of university testing of OAB MSU and OAB CSU cohorts. A) 21.4% of OAB MSU cohort (n=25) were identified with potential infection by reduced threshold of 10^3 CFU/ml and expanded culture. **B)** Range of epithelial cell count performed by Sternheimer staining and haemocytometer from 0 cells/ml to 20 cells/ml with a mode result of 0 cells/ml (46.2%) and mean result 3.1 cells/ml (n=65) **C)** Range of WBC count performed by Sternheimer staining and haemocytometer from 0 cells/ml to 100 cells/ml with a mode result of 0 cells/ml (78.5%) and mean result 80 cells/ml (n=65) **D)** DAPI observations on cytospun urine shows 31.9% of samples contain clean casts and 39.1% contain clue cells (n=69). **E)** 36.0% of Control MSU cohort (n=25) were identified with potential infection by reduced threshold of 10^3 CFU/ml and expanded culture. **F)** Range of epithelial cell count performed by Sternheimer staining and haemocytometer from 0 cells/ml to 30 cells/ml with a mode result of 0 cells/ml (46.2%) and mean result 3.6 cells/ml (n=47). **G)** Range of WBC count performed by Sternheimer staining and haemocytometer from 0 cells/ml to 100 cells/ml with a mode result of 0 cells/ml (78.4%) and mean result 3.5 cells/ml (n=47). **H)** DAPI observations on cytospun urine show 32.5% samples contain clean cast cells and 43.7% contain either clue cells or clue cells with PMNs (n=80). **I)** 11.34% of the OAB CSU cohort (n=26) were identified with potential infection by reduced threshold of 10^3 CFU/ml and university culture. **J)** Range of epithelial cell count performed by Sternheimer staining and haemocytometer from 0 cells/ml to 15 cells/ml with a mode result of 0 cells/ml (85.7%) and mean result 0.9 cells/ml (n=28). **K)** Range of WBC count performed by Sternheimer staining and haemocytometer from 0 cells/ml to 500 cells/ml with a mode result of 0 cells/ml (74.2%) and mean result 18.7 cells/ml (n=28). **L)** DAPI observations on cytospun urine shows 50.0% of samples contain cast cells and much of the remainder contain nothing (37.5%, n=48). **M)** 18.2% of the Control CSU cohort (n=12) were identified with potential infection by reduced threshold of 10^3 CFU/ml and university culture. **N)** Range of epithelial cell count performed by Sternheimer staining and haemocytometer from 0 cells/ml to 2 cells/ml with a mode result of 0 cells/ml (91.7%) and mean result 0.1 cells/ml (n=12). **O)** Mode result for WBC count performed by performed by Sternheimer staining and haemocytometer was 0 cells/ml (100%, n=12). **P)** DAPI observations on cytospun urine shows 33.3% of samples contain clean cast cells and 66.7% contained no cells (n=12).

4.4.2 ISOLATES OMITTED FROM ANALYSIS

A total of 577 isolates isolated and cryopreserved from OAB MSU, 801 from Control MSU, 63 from OAB CSU and 38 from Control CSU giving a total number of 1479 isolates collected (table 4.2).

Of a total 1479 cultured isolates from 227 samples across all cohorts, 1354 (91.54%, n=1479) were successfully sequenced, 40 had failed regrowth (2.7%, n=1479), 18 failed PCR (1.2%, n=1479), 43 failed sequencing (2.9%, n=1479). 87 inconclusive Gram stains (5.8%, n=1479). Of the successfully sequenced isolates, 87 had inconclusive Gram stains and therefore could not be confirmed by Gram stain concordance (6.4%, n=1479), and 221 had non-concordant Gram stains (14.9%, n=1479) (table 4.2).

In three cases, a successful sequence was generated however there was no significance found upon BLASTing.

	OAB MSU	Control MSU	OAB CSU	Control CSU	Total isolates	Percentage of total isolates
# of Isolates identified	**577**	**801**	**63**	**38**	**1479**	**100%**
Failed regrowth	17	22	0	1	40	**2.7%**
Contaminated regrowth	10	8	0	0	18	**1.2%**
Failed PCR	4	11	0	0	15	**1.0%**
Failed sequencing	14	24	2	3	43	**2.9%**
Inconclusive Gram stains	37	40	10	0	87	**5.8%**
Non-concordant Gram stains	96	125	0	0	221	**14.9%**

Table 4.2: Total number of isolates from each cohort missing or omitted from analysis. A total of 577 isolates collected from OAB MSU, 801 from Control MSU, 63 from OAB CSU and 38 from Control CSU giving a total number of 1479 isolates collected. 2.7% of total isolates failed to regrow upon resuscitation. 1.2% of total isolates were too contaminated to perform PCR. 1.0% of total isolates failed PCR twice with different primers on each occasion. 5.8% of total isolates could not be identified by their Gram stain. 14.9% of total isolate BLAST results indicated a species that were not in concordance with the original Gram stain noted.

4.4.3 HOSPITAL TEST ACCURACY

Since the threshold of bacteria is set to 10^5 CFU for hospital pathology, many possible infections could be missed. By reducing this threshold to 10^3 CFU, it is possible to determine any possible low-level infections from each sample. Results show that by decreasing the threshold of bacteria to 10^3, the number of infections identified in OAB MSU is increased from 8.47% by hospital culture (n=59, table 4.3) to 21.43% by university culture (n=28, table 4.4). This is more than a two-fold increase. A two-fold increase is also identified in OAB CSU from 5.66% to 11.53%. Interestingly, although control samples were not sent for hospital culture, data shows that university culture identifies 35.0% of control MSU samples to have a low level infection, higher than in OAB (21.43%)(table 4.4).

Hospital test accuracy was determined by comparing dipstick analysis with traditional methods of cell counting and identification of infection by both hospital methods and university culture. Since control MSUs were not sent for pathology and there were restrictions on tests which could be performed on OAB CSUs in theatre, only OAB MSU were used for comparison of hospital culture, and only control MSU and OAB MSU were used for university culture. Leukocyte esterase dipstick results were positive in 4/5 cases of positive infection by hospital culture in OAB MSU samples (table 4.3). Interestingly, of infections identified by reduced threshold of bacteria in OAB MSU (n=6), only 1 of these samples had positive leukocyte esterase, suggesting higher bacterial presence is required to have measurable leukocyte esterase. These positive leukocyte esterase incidences do not however correlate with positive leukocyte count. Furthermore, many positive leukocyte esterase results do not correlate with infection (n=17/66) (data not shown).

Nitrite dipstick results are also used to indicate an infection by presence of bacteria. From both hospital culture and university culture methods, positive nitrites do not correlate with positive infection with 0.0% of all identified infections having positive nitrites (table 4.3 & 4.4). In fact, of all samples tested with a dipstick (n=134), only 6 had positive nitrites (data not shown).

	Control MSU	OAB MSU	OAB CSU
Infections identified by hospital culture	N/A	**5/59 (8.47%)**	**3/53 (5.66%)**
Infections with +ve leukocyte esterase	N/A	4/5 (80.0%)	N/A
Infection with +ve nitrites	N/A	0/5 (0.0%)	N/A
Infections with +ve leukocyte cell count	N/A	0/5 (0.0%)	N/A

Table 4.3: Infections identified by hospital culture and respective dipstick findings. Control MSU samples were not sent for hospital pathology as they were not suspected of having an infection. Of 59 OAB MSU samples sent for hospital pathology, 5 (8.47%) were identified as having an infection and withdrawn from further analysis. Of the 5 samples which were positively identified as having an infection, 4/5 (80.0%) had positive leukocyte esterase however 0.0% had a positive leukocyte cell count (>10/mL) or positive nitrites. Of 53 OAB CSU samples sent for hospital pathology, 3 (5.66%) were identified as having an infection and withdrawn from further analysis. OAB CSU samples were not able to be dipsticked so this data is not available.

	Control MSU	OAB MSU	OAB CSU
Infections identified by university culture	**9/25 (36.0%)**	**6/28 (21.4%)**	**3/26 (11.5%)**
Infections with +ve leukocyte esterase	1/9 (11.11%)	1/6 (16.67%)	N/A
Infection with +ve nitrites	0/9 (0.0%)	0/6 (0.0%)	N/A
Infections with +ve leukocyte cell count	1/9 (11.11%)	0/6 (0.0%)	N/A

Table 4.4: Infections identified by university culture and respective dipstick findings. Of 25 Control MSU samples cultured in the laboratory, 9 (36.0%) were identified as having a potential infection by reducing the threshold to 10^3 CFU/ml. Of the 9 samples that were positively identified as having an infection, 1/9/ (11.1%) had positive leukocyte esterase and a positive leukocyte cell count (>10/mL) however 0/9 (0%) had positive nitrites. Of 28 OAB MSU samples cultured in the laboratory, 6 (21.43%) were identified as having a potential infection. Of the 6 samples that were positively identified as having an infection, only 1/6 (16.7%) had positive leukocyte esterase however 0.0% had a positive leukocyte cell count (>10/mL) or positive nitrites. Of 26 OAB CSU samples cultured in the laboratory, 6 (21.43%) were identified as having a potential infection. OAB CSU samples were not able to be dipsticked so this data is not available.

4.4.4 Cultured isolates from urine samples

By identifying the species of all culturable isolates by 16s rRNA sequencing it was possible to map the differences in the microbiome of patients with OAB compared to controls to identify any potential species of interest. Percentage incidence of all species was compared between OAB MSU (n=60) and Control MSU (n=82). Species with non-concordant Gram stains, and individuals with confirmed UTI by hospital pathology (n=5) were excluded. Figure 4.5 shows decreased incidence of *Staphylococcus* from 65.9% in controls to 50.0% in OAB (p=0.084), *Lactobacillus* from 54.9% to 18.3% (p<0.0001****), *Peptoniphilus* from 26.8% to 20.0% (p=0.456), and *Fusobacterium* from 25.6% to 13.3% (p=0.114). Also observed is increased incidence of *Escherichia coli* from 11.0% in control to 30.0% in OAB (p=0.008**), *Bifidobacterium* from 7.3% to 15.0% (p=0.232), *Actinobaculum* from 2.4% to 11.7% (p=0.104), *Proteus* from 2.4% to 20.0% (p=0.001**) and *Clostridium* from 1.2% to 8.3% (p=0.097). Statistical significance on incidences of bacterial presence was calculated using chi-squared test for all species (figure 4.5).

From the introduction, it was mentioned that species such as *Aerococcus, Arthrobacter, Corynebacterium* and *Gardnerella* are underreported Gram-positive uropathogens more prevalent in the elderly. From this data there is no major difference between the incidence of *Corynebacterium* (36.6% in Control and 33.3% in OAB) or *Gardnerella* (18.3% in Control and 11.7% in OAB). There is a slight increase in incidence of *Aerococcus* (from 1.2% in controls to 3.3% in OAB) however frequency is still extremely low (figure 4.5).
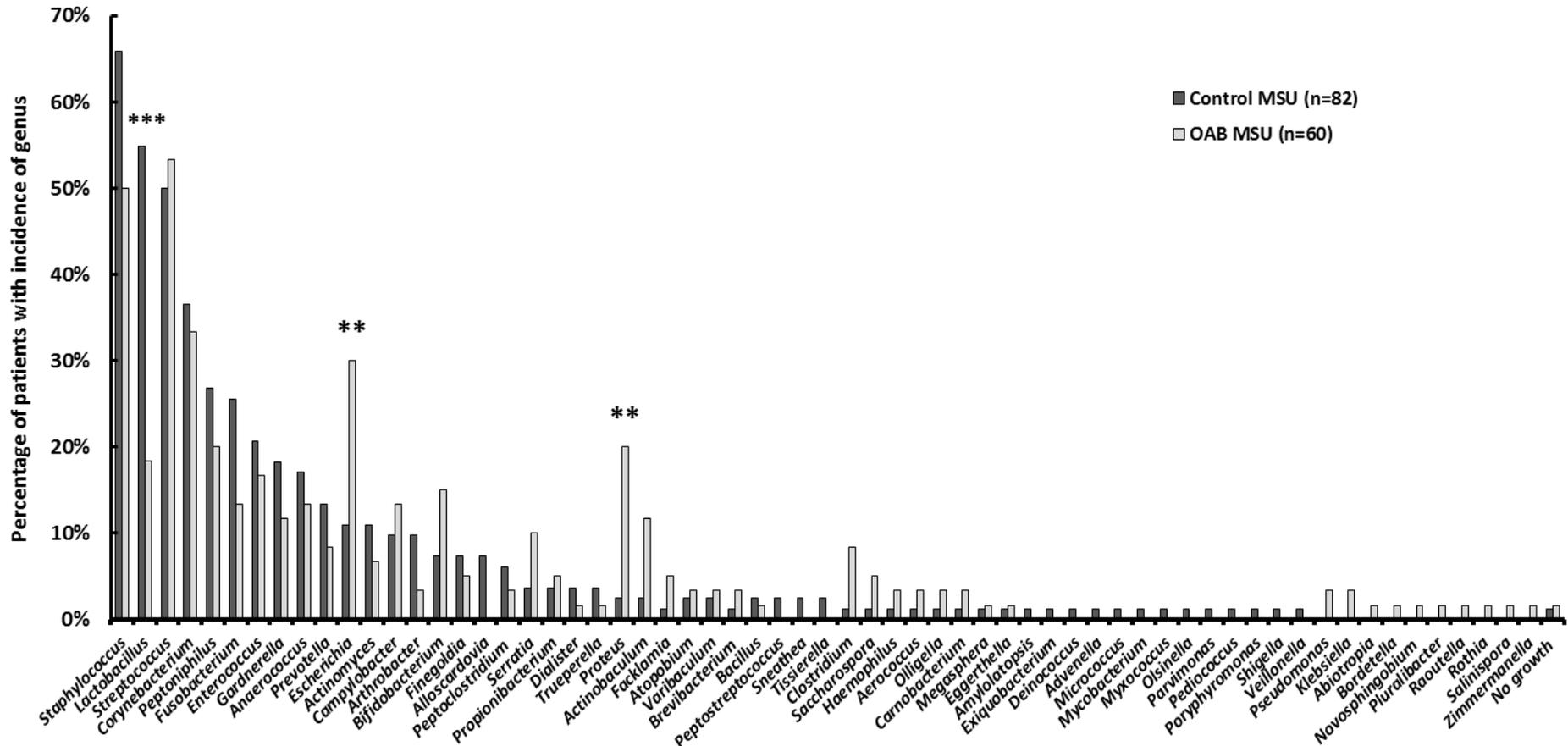
Figure 4.5: Percentage of patients with incidence of genus grown from OAB and Control MSU samples of urine. A total of 970 isolates cultured from MSU samples grown from 82 Control MSU samples and 60 OAB MSU samples were successfully identified by sequencing of the 16s rRNA gene. Incidence of each species is expressed as a percentage of samples with genus from the total number of samples. Decreased incidence from controls to OAB can be seen with *Staphylococcus* from 65.9% to 50.0% (p>0.05), *Lactobacillus* from 54.9% to 18.3% (p<0.0001***), *Peptoniphilus* from 26.8% to 20.0% (p>0.05), and *Fusobacterium* from 25.6% to 13.3% (p>0.05). Increased incidence from controls to OAB can be seen with *Escherichia coli* from 11.0% to 30.0% (p<0.01**), *Bifidobacterium* from 7.3% to 15.0% (p>0.05), *Actinobaculum* from 2.4% to 11.7% (p>0.05), *Proteus* from 2.4% to 20.0% (p<0.01**), and *Clostridium* from 1.2% to 8.3% (p>0.05).

By comparing the total number of species per patient it can be observed that the mean number of species is significantly greater in both Control MSU (n=82) (4.61 species per patient) and OAB MSU (n=60) (4.32 species per patient) than Control CSU (n=14) (1.79 species per patient) and OAB CSU (n=42) (0.95 species per patient) (p<0.0001*** by T test) (figure 4.6A).

By comparing Control CSU (n=14) and OAB CSU (n=42), again omitting non-concordant Gram stains, it can be seen that there are some differences between the species of bacteria from each cohort. There is a decreased incidence of *Lactobacillus* (28.6% to 14.3%, p=0.378), *Acidovorax* (21.4% to 7.1%, p=0.318), *Serratia* (21.4% to 0.0%, p=0.016*), *Staphylococcus* (14.3% to 4.8%, p=0.549), and *Gardnerella* (14.3% to 4.8%, p=0.549) from Control to OAB (figure 4.6). There is also an increased incidence of *Streptococcus* (7.1% to 14.3%, p=0.816) and *Escherichia coli* (0.0% to 11.9%, p=0.417). Of these differences, only that of *Serratia* is significant (p=0.017*). Statistical significance on incidences of bacterial presence was calculated using chi-squared test for all species (figure 4.6B). All p values for incidence of bacteria calculated using Chi squared test.

Analysing CSU data for underreported emerging Gram-positive uropathogens, there is again no significant increase in OAB of any of the species of interest in the data, but rather incidence is lower in OAB than controls. *Gardnerella* incidence is reduced from Controls (14.3%) to OAB (4.8%) (p=0.549), as is *Corynebacterium* (from 7.1% to 2.4%, p=0.405), *Arthrobacter* (from 7.1% to 0.0%, p=0.56) and *Aerococcus* (from 7.1% to 0.0%, p=0.56) (figure 4.6B).
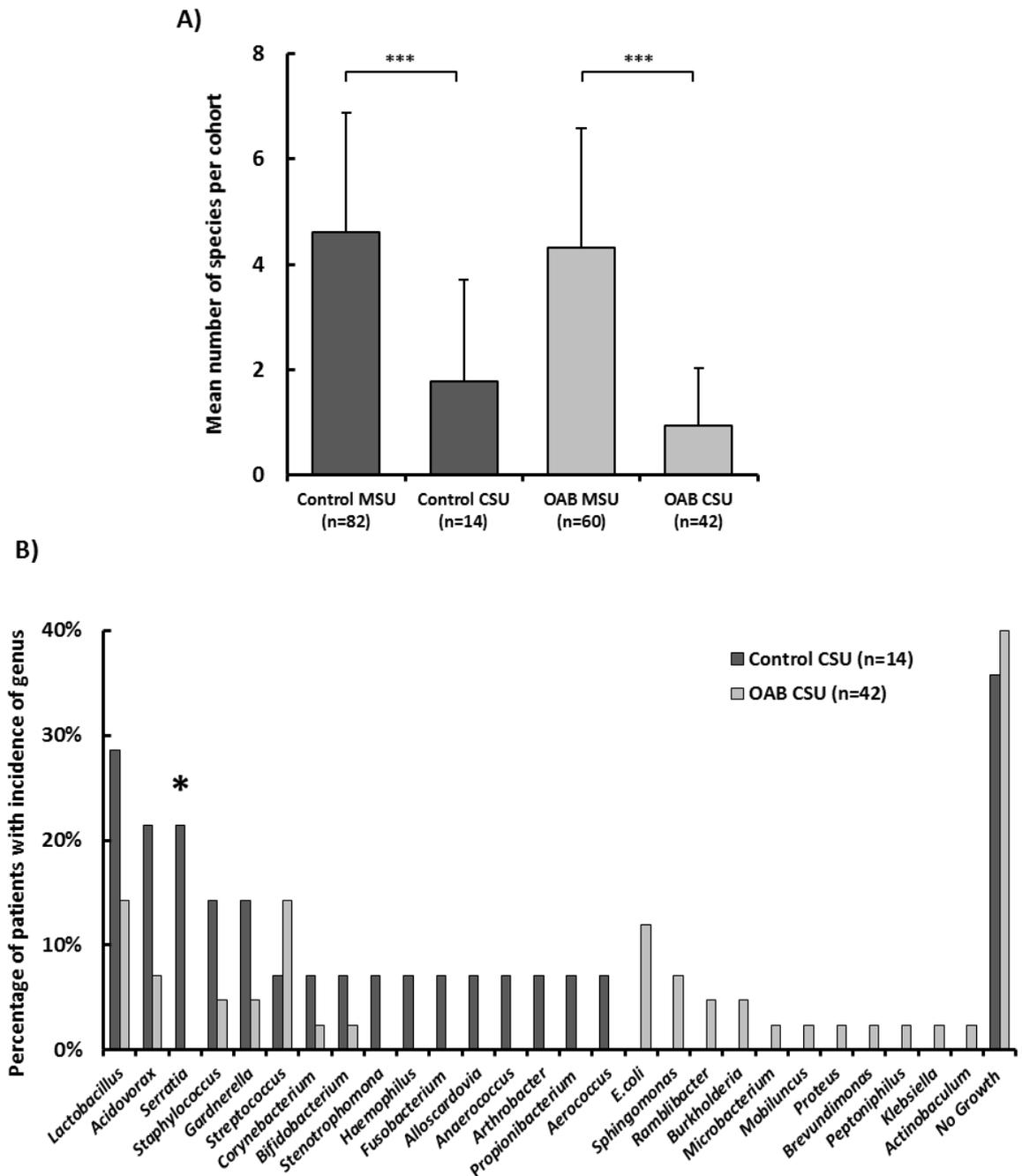
Figure 4.6: Frequency and percentage incidence of bacteria cultured from OAB and Control CSU samples of urine. A) The mean number of species successfully identified from Control MSU samples (n=82) and OAB MSU samples (n=60) are 4.61 and 4.32 respectively. Significantly fewer species were identified per sample from Control CSU (n=14) and OAB CSU (n=42) at a mean of 1.79 and 0.95 species per patient respectively ($p<0.0001$***by T test). This indicates MSU samples are contaminated with perineal bacteria. **B)** A total of 85 isolates cultured from MSU samples grown from 14 Control CSU samples and 42 OAB CSU samples were successfully identified by sequencing of the 16s rRNA gene. Incidence of each species is expressed as a percentage of samples with genus from the total number of samples. Decreased incidence from controls to OAB can be seen with *Lactobacillus* from 28.6% to 14.3% ($p>0.05$), *Acidovorax* from 21.4% to 7.1% ($p>0.05$), *Serratia* from 21.4% to 0.0% ($p<0.01$*), *Staphylococcus* from 14.3% to 4.8% ($p>0.05$), and *Gardnerella* from 14.3% to 4.8% ($p>0.05$). Increased incidence from controls to OAB can be seen with *Streptococcus* from 7.1% to 14.3% ($p>0.05$), and *Escherichia coli* from 0.0% to 11.9% ($p>0.05$).

By collating data on the incidence of bacterial presence for all MSU, CSU, Control and OAB samples, it can be more easily compared. Figure 4.7 takes the 12 most commonly found species from this study and compares the incidence between the patient cohorts. *Staphylococcus* is found in high percentages (50% to 65.5%) in both control and OAB MSU samples but in low percentages in both control and OAB CSU samples (4.8% to 14.3%, p=0.00001***)(figure 4.7A). *Lactobacillus* is found in high percentages in MSU (54.88%) and CSU controls (28.6%) but in low percentages in MSU (18.3%) and CSU OAB (14.3%, p=0.00001***)(figure 4.7B). *Streptococcus* is found in high percentages (50% to 53.3%) in both control and OAB MSU samples but in low percentages in both control and OAB CSU samples (7.1% to 14.3%, p=0.000***)(figure 4.7C). *Corynebacterium* is found in relatively high percentages (33.3% to 36.6%44) in both control and OAB MSU samples but in low percentages in both control and OAB CSU samples (0% to 7.1%, p=0.0001***)(figure 4.7D). *Peptoniphilus* is found in relatively high percentages (20% to 26.8%) in both control and OAB MSU samples but in low percentages in both control and OAB CSU samples (0% to 2.4%, p=0.0427*)(figure 4.7E). *Enterobacter* is found in relatively high percentages (1316.6% to 20.7%) in both control and OAB MSU samples but is not present at all in control and OAB CSU samples (p=0.0265*, figure 4.7F). *Acidovorax* is not found in MSU samples but can be found in 21.4% of control CSU samples and 14.3% of OAB CSU samples (p=0.00016***, figure 4.7G). *Serratia* is found in few control and OAB MSU samples (3.7% to 10%), however it was found in 21.4% of control CSU samples and 0% OAB CSU samples (p=0.03316*, figure 4.7H). *E.coli* was found in few control MSU samples (11%) and no control CSU samples, however it was present in 30% OAB MSU samples and 11.9% OAB CSU samples (p=0.00797***, figure 4.7I).
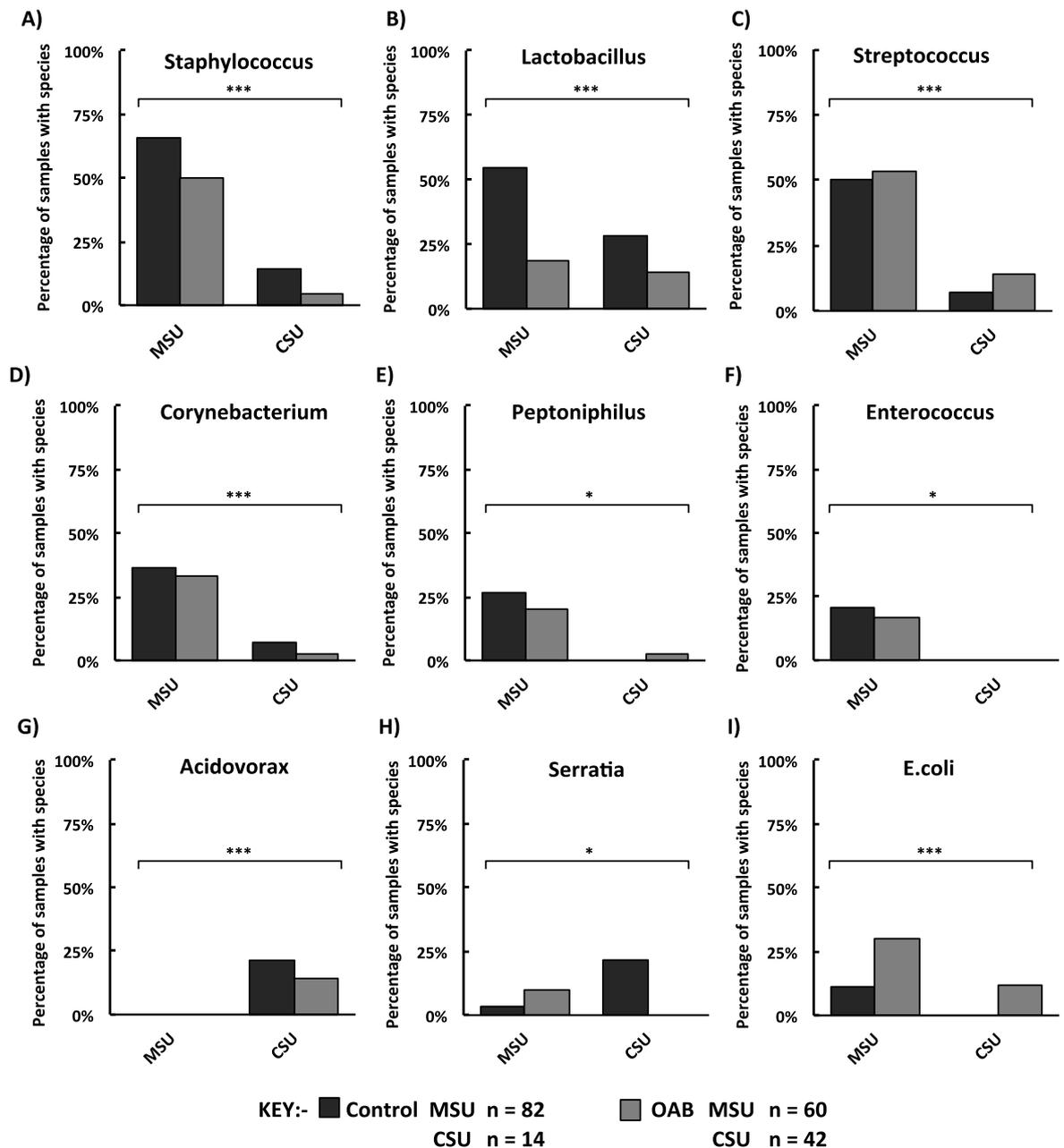
Figure 4.7: Incidence of bacterial presence in samples by patient cohort. A) *Staphylococcus* is found in high percentages (50% to 65.5%) in both control and OAB MSU samples but in low percentages in both control and OAB CSU samples (4.8% to 14.3%)(p=0.00001***). **B)** *Lactobacillus* is found in high percentages in MSU (54.88%) and CSU controls (28.6%) but in low percentages in MSU (18.3%) and CSU OAB (14.3%)(p=0.00001***). **C)** *Streptococcus* is found in high percentages (50% to 53.3%) in both control and OAB MSU samples but in low percentages in both control and OAB CSU samples (7.1% to 14.3%)(p=0.00044***). **D)** *Corynebacterium* is found in relatively high percentages (33.3% to 36.6%) in both control and OAB MSU samples but in low percentages in both control and OAB CSU samples (0% to 7.1%)(p=0.0001***). **E)** *Peptoniphilus* is found in relatively high percentages (20% to 26.8%) in both control and OAB MSU samples but in low percentages in both control and OAB CSU samples (0% to 2.4%)(p=0.0427*). **F)** *Enterobacter* is found in relatively high percentages (1316.6% to 20.7%) in both control and OAB MSU samples but is not present at all in control and OAB CSU samples (p=0.0265*). **G)** *Acidovorax* is not found in MSU samples but can be found in 21.4% of control CSU samples and 14.3% of OAB CSU samples (p=0.00016***). **H)** *Serratia* is found in few control and OAB MSU samples (3.7% to 10%), however it was found in 21.4% of control CSU samples and 0% OAB CSU samples (p=0.03316*). **I)** *E.coli* was found in few control MSU samples (11%) and no control CSU samples, however it was present in 30% OAB MSU samples and 11.9% OAB CSU samples (p=0.00797***). All P values calculated using a 2x4 Chi squared test.

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4.4.5 IDENTIFYING INTRACELLULAR BACTERIA

Intracellular bacteria were identified by deduction of CFU count post-wash from CFU count post Triton X-100 during the antibiotic protection assay. Total percentage of patients with intracellular bacteria identified from OAB MSU (n=31) was 58.1% compared with 56.7% from Control MSU (n=30, p>0.05 by chi squared test) (figure 4.8A).

The species of intracellular bacteria from each group are shown in figure 4.8 and show reduced incidence of *Staphylococcus* 36.7% in control to 23.3% in OAB, as well as reduced *Arthrobacter* (10.0% to 0.0%, p=0.236), and *Peptoniphilus* (10.0% to 0.0%, p=0.236). Increased incidence of *Streptococcus* (0.0% to 10.0%, p=0.236) and *Serratia* (0.0% to 6.7%, p=0.472) are also observed. Statistical significance on incidences of bacterial presence was calculated using chi-squared test for all species (figure 4.8B).
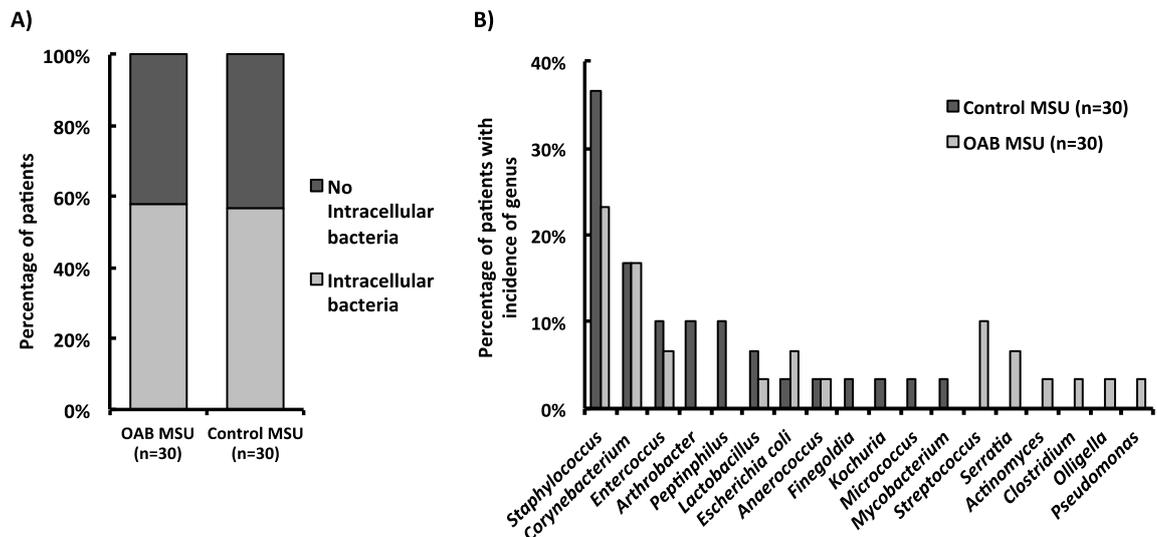
Figure 4.8: Frequency and percentage incidence of intracellular bacteria cultured from OAB and Control MSU samples of urine. A) The percentage of patients from Control MSU samples (n=30) and OAB MSU samples (n=30) from which intracellular bacteria were cultured are 58.1% and 56.7% respectively, which is not statistically significant (p>0.05). This indicates intracellular bacteria are not unique to OAB. **B)** A total of 60 isolates from 30 Control CSU samples and 30 OAB CSU samples were identified as intracellular by antibiotic protection assay followed by sequencing of the 16s rRNA gene. Decreased incidence from controls to OAB can be seen with *Staphylococcus* from 36.7% to 23.3% (p>0.05), *Arthrobacter* from 10.0% to 0.0% (p>0.05), and *Peptoniphilus* from 10.0% to 0.0% (p>0.05), Increased incidence from controls to OAB can be seen with *Streptococcus* from 0.0% to 10.0% (p>0.05), and *Serratia* from 0.0% to 6.7% (p>0.05).

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4.4.6 USING BLADDER BIOPSIES TO IDENTIFY INTRACELLULAR BACTERIA

Hultgren *et al* have previously identified intracellular bacteria from bladder biopsies taken from individuals with UTI (figure 4.9). By the EM images of bladder biopsies from individuals with OAB, it can be seen that many autophagosomes were observed (figure 4.10A). Furthermore, we found in one sample a particular blood vessel with an infiltrating neutrophil (figure 4.10B).
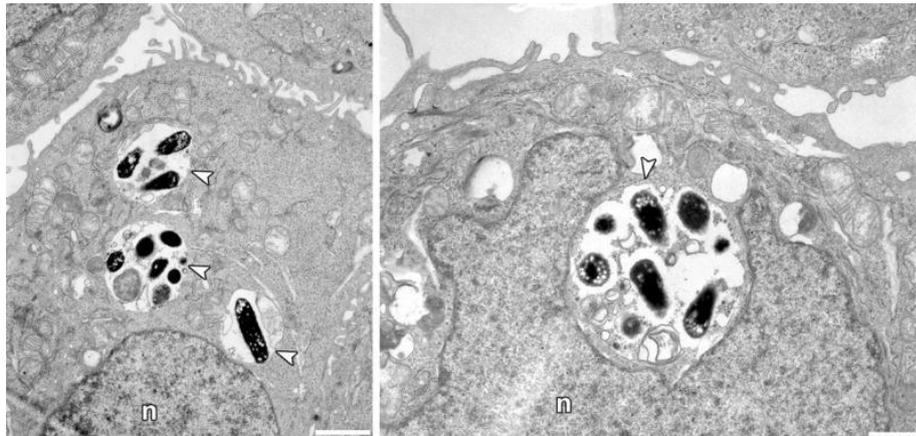
Figure 4.9: Example of transmission electron microscopy images of intracellular bacteria in bladder urothelial cells, adapted from Anderson *et al* (144)*.* Large white round biopods containing dark elongated intracellular bacteria identified by white arrows. Images taken of bladder urothelial cells infected with uropathogenic *Escherichia coli* (UPEC) by Anderson *et al*, 2003 **(144)**
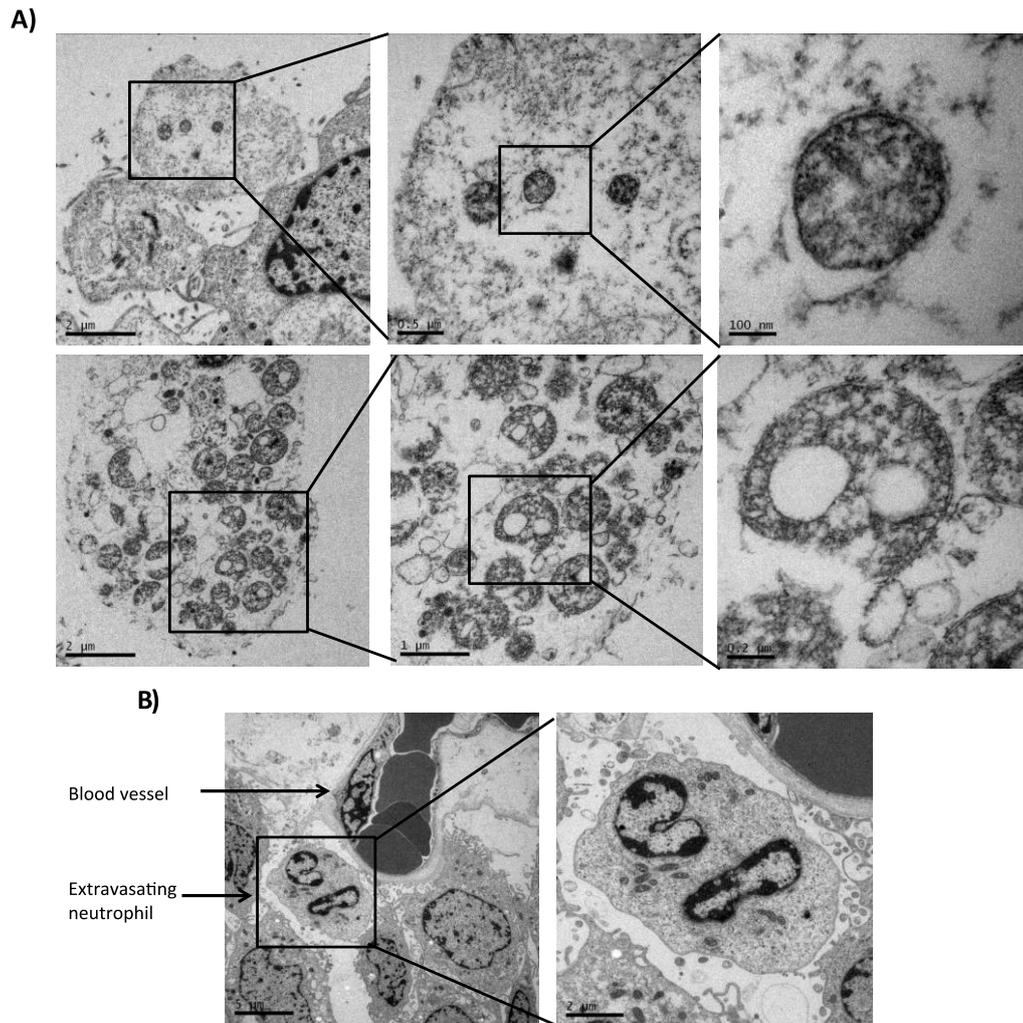
Figure 4.10: - Transmission electron microscopy (TEM) images of autophagosomes in bladder biopsies from patients with OAB. A) Autophagosomes are seen in all samples processed for TEM (n=6). Multivesicular bodies (MVBs) are probably late endosomes (autophagosomes) associated with autophagy. Programmed cell death and/or intracellular bacteria can cause autophagy. **B)** Extravasating neutrophil seen in 1 of 6 samples processed for TEM suggests possible infection.

Bladder biopsies were also used for culture, allowing potentially more insight into intracellular bacteria and those closely associated to the urothelium. Of 24 biopsies taken, only 5 cultured bacteria, which leave 79.2% of samples with no growth. From figure 4.7 it can be seen that the percentage of MSU samples with no growth from OAB is 40.5%. Of the biopsies where there was positive culture, species identified are *Staphylococcus* (n=2), *Peptoniphilus* (n=2), *Haemophilus* (n=1) and *Acidovorax* (n=1) (data not shown).

4.4.7 FACTORS AFFECTING THE BLADDER MICROBIOME

It has been recognised that factors such as age, hormones, and diet can affect other human microbiomes such as the gut. From the groups obtained, the mean age of OAB MSU and OAB CSU combined (n=111) is 57.8 years (SD=15.9 years), whereas the mean age of control MSU and CSU combined (n=91) is 39.1 years (SD=14.7 years) (p<0.0001*** by T test) (figure 4.11A). The mean BMI of the OAB MSU (n=39) cohort was significantly higher (29.1, SD=5.8) than that of the control MSU group (26.4, SD=5.3) (p=0.008**). This significance carries into the CSU cohorts also with the mean BMI of the OAB CSU cohort of 29.7 (SD=6.0) compared with 23.7 (SD=4.2) for controls (p=0.005**) (figure 4.11B).
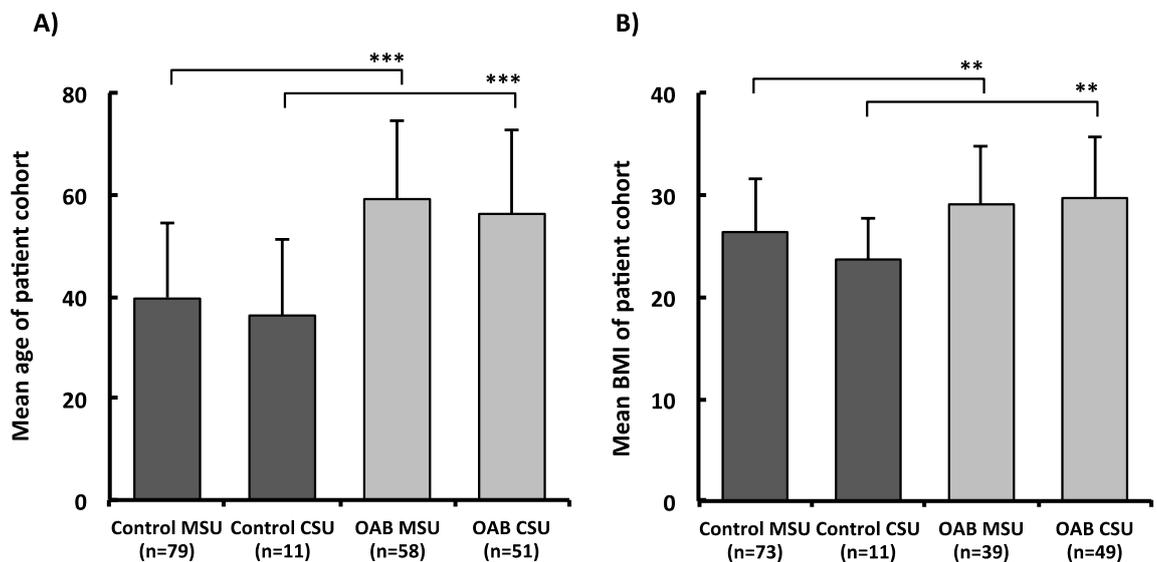
Figure 4.11: Mean AGE and BMI of OAB and control MSU and CSU samples of urine. A) The mean ages of both control MSU (39.6 years, n=79) and control CSU (36.3 years, n=11) differ significantly (P<0.0001***) from the ages of both OAB MSU (59.1 years, n=58) and OAB CSU (56.2 years, n=11) suggesting OAB is more prevalent in older women. **B)** The mean BMI of both control MSU (26.4, n=73) and control CSU (23.7, n=11) differ significantly (P<0.001**) from the BMI of both OAB MSU (29.1, n=39) and OAB CSU (29.7, n=49) suggesting individuals with OAB are generally heavier than controls. Statistical significance calculated using a Student's T-test.

By grouping patients by age for MSU samples, it is possible to identify trends with increasing age from controls that can be compared to OAB. Control MSU samples were split into three age groups for analysis; 20 to 44 (n=53), 45 to 64 (n=18), over 65 (n=5). *Staphylococcus* incidence remains relatively equivalent between OAB and controls across the age groups although incidence decreases in both cohorts with age (figure 4.12A). *Lactobacillus* incidence is much lower in OAB than controls in ages 20-64 years however no control patients over 65 years had Lactobacillus in their samples (figure 4.12B). *Streptococcus* incidence remains relatively equivalent between OAB and controls in ages 20-64 years, however in 65+ years, there are more OAB patients with *Streptococcus* than controls (figure 4.12C). *Corynebacterium* incidence remains relatively equivalent between OAB and controls across the age groups and ages (figure 4.12E). *Peptoniphilus* incidence remains relatively equivalent between OAB and controls in ages 20-64 years, however in 65+ years, there are more control patients with *Peptoniphilus* than OAB (figure 4.12E). *Fusobacterium* incidence remains relatively equivalent between OAB and controls in ages 20-64 years, however no control patients over 65 years had *Fusobacterium* in their samples (figure 4.12F). *Enterococcus* incidence remains relatively equivalent between OAB and controls across the age groups and ages (figure 4.12G). *Gardnerella* incidence increases with age in controls however is reduced in patients with OAB over 65 years (figure 4.12H). *Anaerococcus* incidence is reduced in patients with OAB with increased age, however control patients over 65 years have *Anaerococcus* (figure 4.12I).

From this it can be deduced that in OAB there are a few differences in trends with age; i) incidence of *Staphylococcus, Corynebacterium,* and *Enterococcus* remain relatively unaffected by diseases state or age., ii) post 65 years, incidence of *Lactobacillus, Streptococcus* and *Fusobacterium* increases in OAB but decreases in controls, iii) post 65 years, incidence of *Peptoniphilus, Gardnerella* and *Anaerococcus* decreases in OAB but increases in controls.

Statistics were not performed for this data due to the fact that the samples sizes for the older cohort of control and the younger cohort of OAB were very small and therefore rendered Chi squared tests invalid. However, trends were identified, which can inform of species of interest in age and the bladder microbiome.
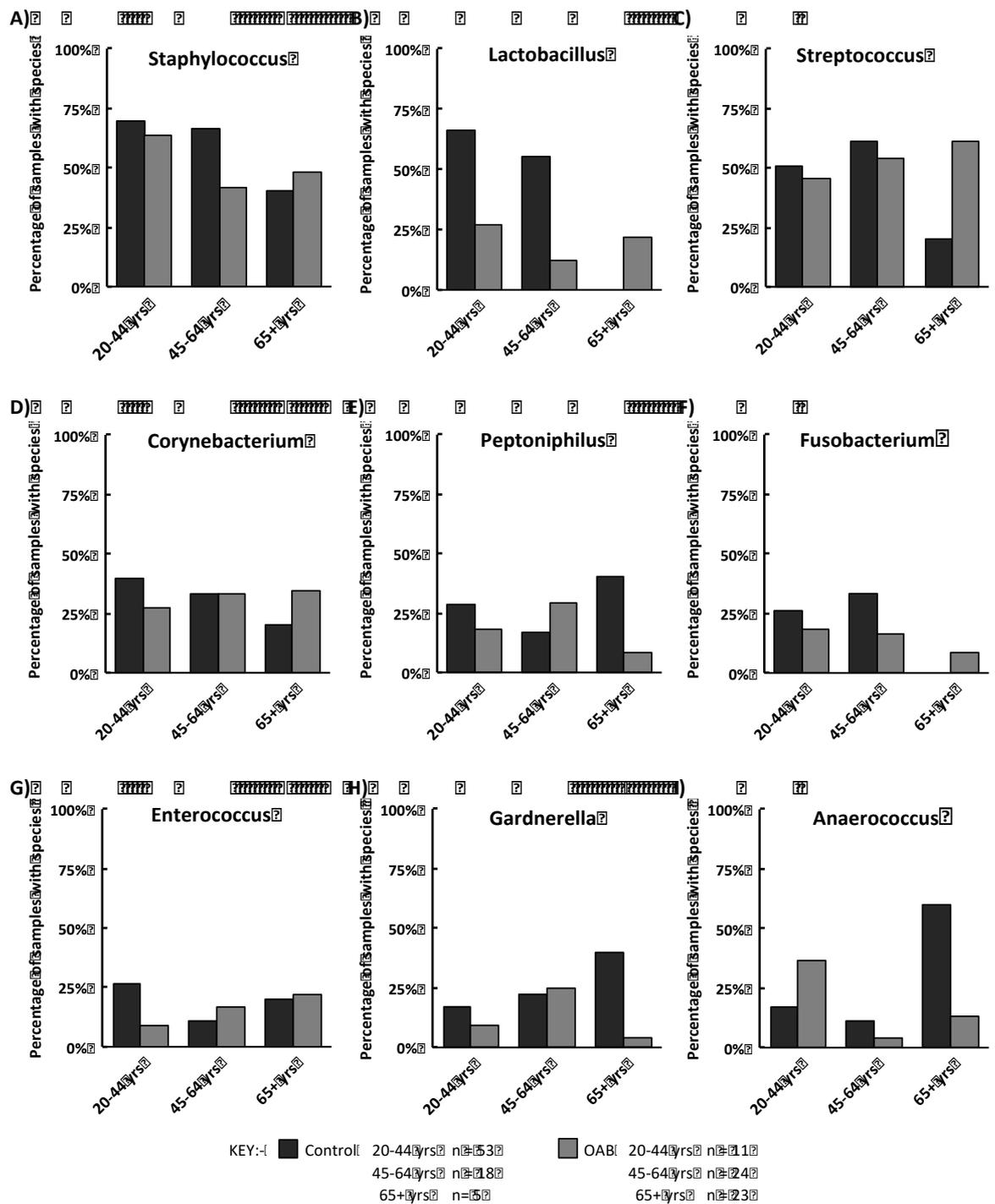
Figure 4.12: Percentage incidence of bacteria cultured from OAB and Control MSU samples of urine by age.
Control samples (n=82) are split into age groups of 20-44 (n=53), 45-64 (n=18) and 65+ (n=5). OAB MSU samples (n=60) are split into age groups of 20-44 (n=11), 45-64 (n=24) and 65+ (n=23). **A)** Incidence of *Staphylococcus* remains relatively unaffected by diseases state or age. **B)** Post 65 years, incidence of *Lactobacillus* increases in OAB but decreases in controls. **C)** Post 65 years, incidence of *Streptococcus* increases in OAB but decreases in controls. **D)** Incidence of *Corynebacterium* remains relatively unaffected by diseases state or age. **E)** Post 65 years, incidence of *Peptoniphilus* decreases in OAB but increases in controls. **F)** Post 65 years, incidence of *Fusobacterium* increases in OAB but decreases in controls. **G)** Incidence of *Enterococcus* remains relatively unaffected by diseases state or age. **H)** Post 65 years, incidence of *Gardnerella* decreases in OAB but increases in controls. **I)** Post 65 years, incidence of *Anaerococcus* decreases in OAB but increases in controls. Statistics were not performed for this data due to the fact that the samples sizes for the older cohort of control and the younger cohort of OAB were very small.

By comparing various factors of the bladder environment, it is possible to see if the bladder environment differs between OAB and controls. There is no significant difference between the pH of OAB (mean pH 6.0, n=62) or control groups (pH 6.2, n=68) (p=0.35) (figure 4.13A). The mean concentration of ATP/creatinine for controls was found to be 3.75×10^{-11} nM/mg/dL (SD=3.41×10^{-11} nM/mg/dL), slightly higher than that of controls at 2.92×10^{-11} nM/mg/dL (SD=2.13×10^{-11} nM/mg/dL), however not significantly so (p=0.19) (figure 4.13B). Contrary to expectations, IL-8 tends to be higher in controls (0.86pg/ml, SD=1.14pg/ml) than OAB (0.58pg/ml, SD=0.95pg/ml), however this is also not significant (p=0.23) (figure 4.13C). Statistical significance of means of cohorts was calculated using an unpaired two tailed t-test.
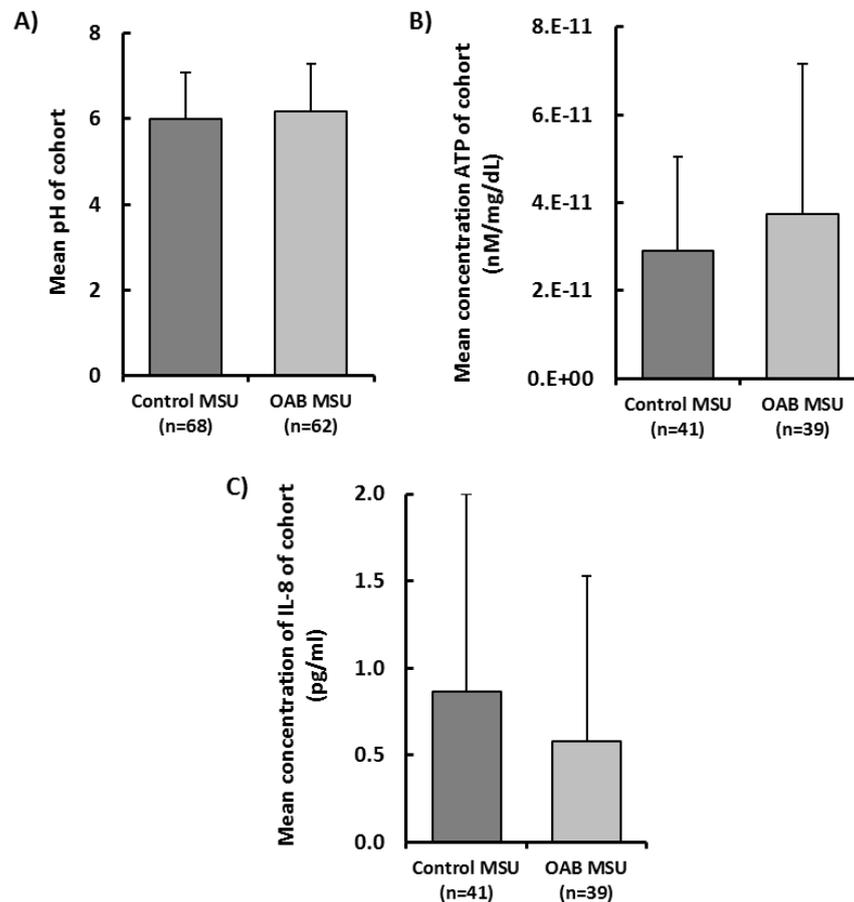
Figure 4.13: Mean pH, ATP and IL-8 concentration of OAB and Control MSU samples of urine. A) Mean pH of OAB MSU samples (pH 6.0, n=62) does not differ significantly from that of control MSU samples (pH 6.2, n=68) (p>0.05). **B)** Mean concentration of ATP/creatinine of OAB MSU samples (2.92×10^{-11} nM/mg/dL, n=41) does not differ significantly from that of control MSU samples (3.75×10^{-11} nM/mg/dL, n=39) (p>0.05). **C)** Mean concentration of IL-8/creatinine of OAB MSU samples (0.58pg/ml, n=41) does not differ significantly from that of control MSU samples (0.86pg/ml, n=39) (p>0.05).

4.4.8 GRAM NEGATIVE SPECIES

As opposed to looking at how factors may affect the microbiome, it is just as likely that the increased incidence of Gram negative bacteria in the microbiome could affect the bladder environment. Gram negative species are taken here as; *Bordetella, Campylobacter, Dialister, E.coli, Fusobacterium, Haemophilus, Klebsiella, Olligella, Prevotella, Proteus, Pseudomonas, Serratia, Shigella, Sneathea,* and *Viellonella.* Taken together there is a 73.4% incidence of Gram negative bacteria in OAB MSU (n=60) compared to 54.2% in control MSU (n=82) (p=0.027* with chi squared test) (figure 4.14).
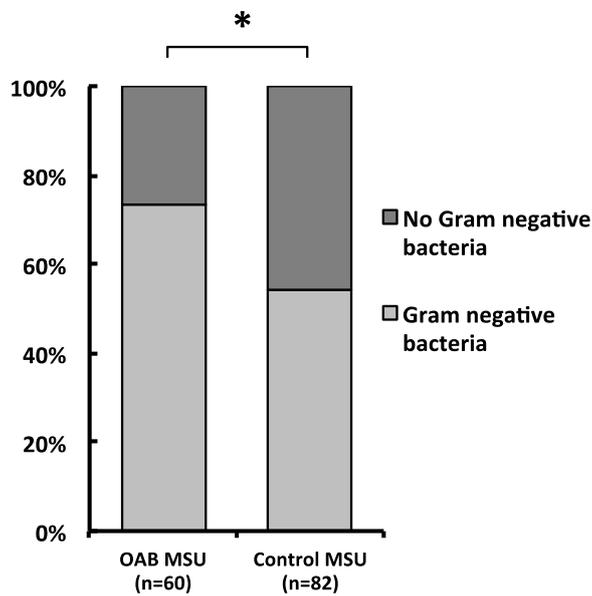
Figure 4.14: Frequency of Gram negative bacteria cultured from OAB and Control MSU samples of urine. Gram negative bacteria included are *Bordetella, Campylobacter, Dialister, E.coli, Fusobacterium, Haemophilus, Klebsiella, Olligella, Prevotella, Proteus, Pseudomonas, Serratia, Shigella, Sneathea,* and *Viellonella.* Incidence of Gram negative bacteria is significantly higher in OAB MSU (73.4%, n=60) than control MSU (54.2%, n=82) (p<0.05*).

Downstream signalling from LPS stimulation of PRRs are release of cytokines and increased ATP release, therefore by comparing Gram negative bacteria with the concentration of IL-8 and ATP it may be possible to determine if the presence of Gram negative bacteria is affecting the bladder environment. OAB MSU samples were separated into those with Gram negative species in their urine (n=28) and those without (n=11), and control samples also separated into those with Gram negative species (n=20) and those without (n=21). The mean concentration of IL-8 for OAB MSU with Gram negative species is 0.67 pg/mL, SD=1.08 pg/mL, slightly higher than that of OAB MSU samples without Gram negative species, 0.36 pg/mL, SD=0.44 pg/mL. This is in keeping with the theory that Gram negative species increases IL-8 concentration however is not statistically significant by a T test (P=0.37) (figure 4.15A). Interestingly however, the concentration of IL-8 is higher in controls without

Gram negative species (0.93 pg/ml, SD=1.42 pg/ml) than those with (0.80 pg/ml, SD=0.78 pg/ml) which is contradictory, however this is again not a statistically significant difference (figure 4.15A). The mean concentration of ATP for OAB MSU with Gram negative species is 3.41×10^{-11} nM/mg/dL, (SD=3.57×10^{-11} nM/mg/dL), slightly lower than that of OAB MSU samples without Gram negative species, 4.60×10^{-11} nM/mg/dL (SD=2.93×10^{-11} nM/mg/dL) (figure 4.14B), however is not statistically significant by a T test (P=0.37). The concentration of ATP is relatively similar in both groups of controls with a mean concentration of 2.77×10^{-11} nM/mg/dL (SD=2.05×10^{-11} nM/mg/dL) ATP in those with Gram negative species and 3.05×10^{-11} nM/mg/dL (SD=2.25×10^{-11} nM/mg/dL) ATP in those without, and the overall concentration ATP is lower in controls than OAB combined however this is not statistically significant by a T test (p=0.19) (figure 4.15B).
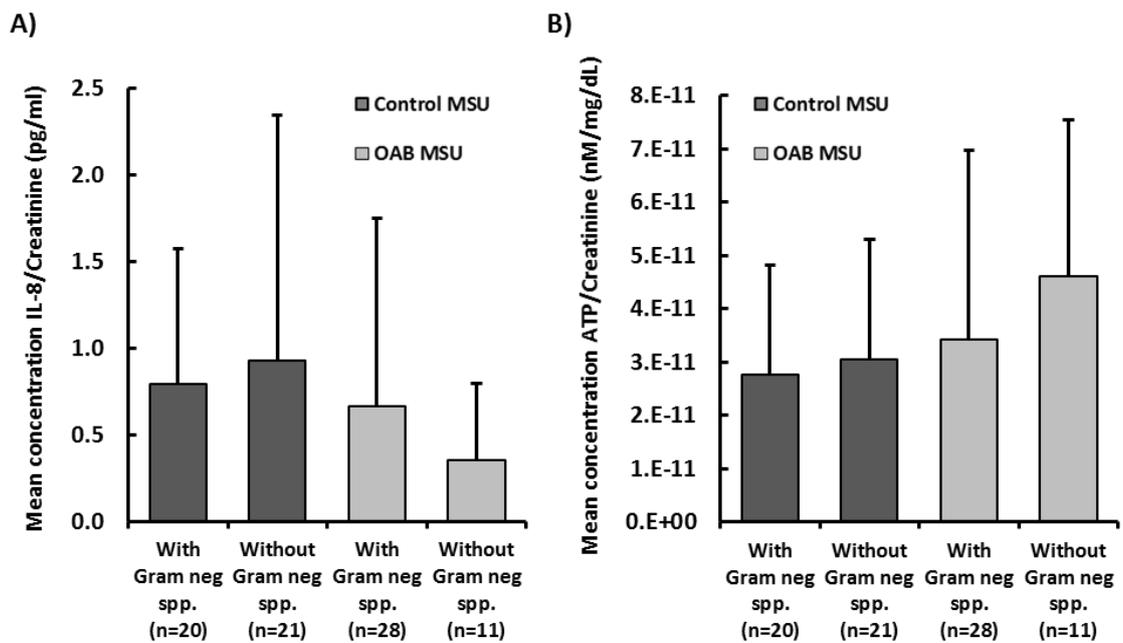
Figure 4.15: Mean concentration of Interleukin-8 and ATP for OAB and control cohorts with and without Gram negative species. On 41 control MSU and 39 OAB MSU samples, a luciferin/luciferase assay and IL-8 ELISA was performed on frozen urine samples. Results were divided by creatinine to standardise them. Of the 41 control MSU samples assayed, 20 cultured Gram negative species and 21 did not. Of the 39 OAB MSU assayed, 28 cultured Gram negative species and 11 did not. **A)** Mean concentration of IL-8 for OAB MSU with Gram negative species (n=28) is 0.67 pg/mL, SD=1.08 pg/mL, higher than that of OAB MSU samples without Gram negative species (n=11)(0.36 pg/mL, SD=0.44 pg/mL, P>0.05) however the opposite is observed in the Control cohort; mean concentration of IL-8 is higher for those without Gram negative species (n=21)(0.93 pg/ml, SD=1.42 pg/ml) than with Gram negative species (0.80 pg/ml, SD=0.78 pg/ml). **B)** Mean concentration of ATP for OAB MSU without Gram negative species (n=11) is 4.60×10^{-11} nM/mg/dL (SD=2.93×10^{-11} nM/mg/dL), higher than that of OAB MSU samples with Gram negative species (n=28) (3.41×10^{-11} nM/mg/dL, SD=3.57×10^{-11} nM/mg/dL) **(P>0.05)**. This is higher still in control cohort with mean ATP measured at 2.77×10^{-11} nM/mg/dL (SD=2.05×10^{-11} nM/mg/dL) in those with Gram negative bacteria (n=20) and even higher in the control group without Gram negative bacteria (n=21) (3.05×10^{-11} nM/mg/dL, SD=2.25×10^{-11} nM/mg/dL) (p>0.05).

4.4.9 REDUCED FREQUENCY OF *LACTOBACILLUS* IN OAB

From MSU (figure 4.5) and CSU data (figure 4.6B), it was observed that there was a significantly reduced incidence of *Lactobacillus* in OAB compared to controls By reanalysing the exact species of *Lactobacillus* found in MSU samples, it is possible to determine the exact species present in healthy controls which may be missing in those with OAB and may be protective to the bladder. From control MSU (n=82), 20.7% of individuals' samples grew *L.gasseri* and *L.psittaci*, whereas these frequencies are reduced to 11.7% and 8.3% respectively in the OAB cohort (n=60). 7.3% of controls grew *L.iners*, *L.crispatus* and *L.hominis* (figure 4.16), all of which are reduced in OAB. Across all species of *Lactobacillus*, there is a reduced incidence, except with *L.casei* and *L.plantarium,* however these exist in only 1.7% of OAB MSU. Of particular interest is the complete lack of *L.crispatus, L.hominis,* and *L.acidophilus* in OAB (figure 4.16). None of the differences are statistically significant by Chi squared test (p>0.05).
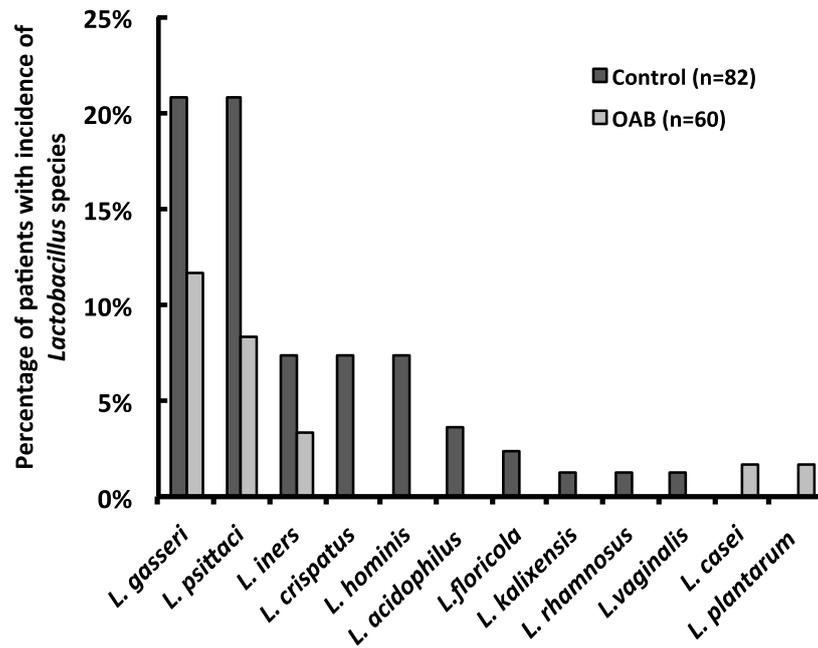
Figure 4.16: Percentage incidence of *Lactobacillus* species cultured from OAB and Control MSU samples of urine. A total of 75 *Lactobacillus* isolates cultured from MSU samples grown from 82 Control CSU samples and 60 OAB CSU samples were successfully identified by sequencing of the 16s rRNA gene. Incidence of each species is expressed as a percentage of samples with genus from the total number of samples. Decreased incidence from controls to OAB can be seen with all species of *Lactobacillus* particularly *L.gasseri* from 20.7% to 11.7% (p>0.05), *L.psittaci* from 20.7% to 8.3% (p>0.05), and *L.iners, L.crispatus* and *L.hominis* from 7.3% to 0.0% (p>0.05).

As mentioned, *Lactobacillus* has probiotic capabilities particularly against Gram negative species. By comparing incidence of *Lactobacillus* with Gram negative species it can be observed that in control MSU (n=82), there are fewer individuals with Gram negative species only, 24.4%, compared to OAB (n=60) at 60.7% (figure 4.17A). Furthermore, the percentage of patients with *Lactobacillus* only is 21.9% in controls, lower than that of OAB at only 8.2%.

Since *Lactobacillus* frequency is significantly reduced in OAB groups, and Gram negative species are significantly increased, these differences could affect observed pH or ATP levels in the bladder. The mean pH of individuals with *Lactobacillus* is pH 6.0 for OAB MSU (n=61) and pH 6.2 for control MSU (n=67), however the difference between the means is greater between individuals with OAB without *Lactobacillus* (pH 6.2) and controls without Lactobacillus (pH 5.8) (figure 4.17B). These results are not statistically significant by performing a one-way ANOVA dependent on both *Lactobacillus* status and OAB status (p>0.5) and was confirmed with a Mann-Whitney U (p=0.694) due to non-normal distribution of the pH demographics.
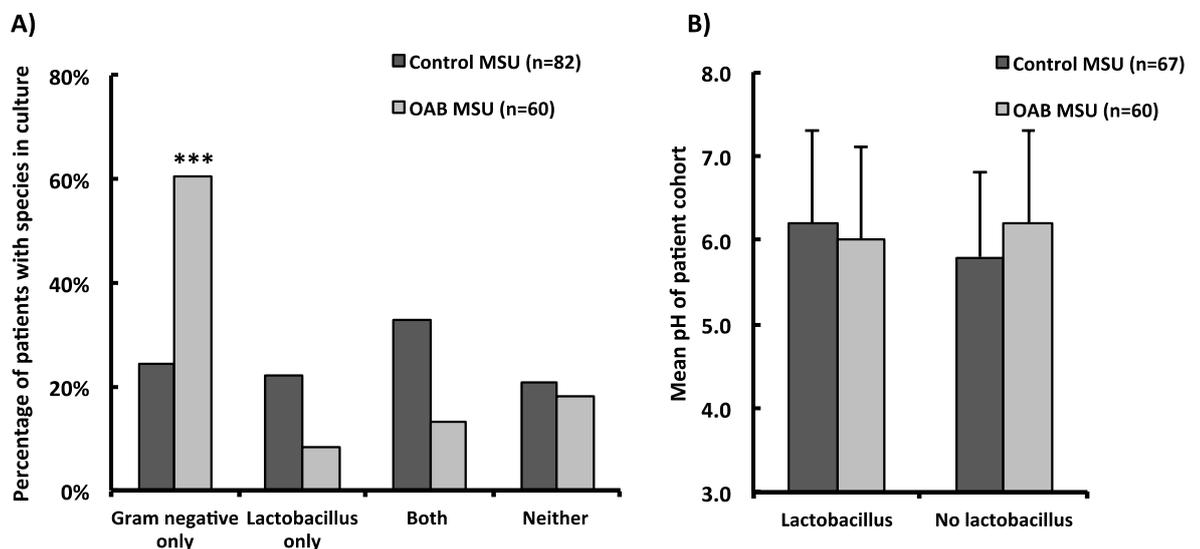
Figure 4.17: Gram negative bacteria incidence and pH of urine in combination with *Lactobacillus* incidence from OAB and Control MSU samples of urine. A) In controls (n=82) the percentage of individuals with Gram negative species only (24.4%) is relatively similar to that of those with *Lactobacillus* only (22.0%) with slightly more (33.0%) growing both. In OAB (n=60), 66.7% cultured Gram negative species only and only 8.2% grew *Lactobacillus* only, suggesting a lack of *Lactobacillus* may be allowing Gram negative species to grow in this cohort. **B)** The mean pH is slightly lower in those with *Lactobacillus* (pH 6.0) (n=11) than without *Lactobacillus* (pH 6.2) (n=49) in the OAB cohort (n=60), however not significantly by ANOVA (p>0.05). This is not seen in controls however (n=67), where pH is 6.2 in those with *Lactobacillus* (n=35) compared to pH 5.8 in those without (n=32).

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4.5 DISCUSSION

The aims of this chapter were to determine key bacterial species in OAB, to investigate alternative biomarkers for OAB, and to determine if there is an intracellular bacterial component to OAB. Following investigation, the main findings are: - i) low-level infections (10^3 CFU/ml as determined by university culture) does not correlate with symptoms of OAB, ii) incidence of emerging (*i.e.* recently hypothesised) Gram positive uropathogens (*e.g. Aerococcus, Arthrobacter, Corynebacterium* and *Gardnerella*) are not increased in patients with OAB, iii) there is an increase in the incidence of Gram negative species (*e.g. E.coli, Proteus* and *Serratia*) in patients with OAB, iv) intracellular bacteria are equally present in control and OAB samples, and as such likely a normal part of the bladder microbiome*,* v) alterations in the microbiome over time (*i.e.* with increasing age) may predispose women to OAB rather than any particular bacterial infection, vi) *Lactobacillus* is significantly reduced in patients with OAB and correlates with increased incidence of Gram negative uropathogens and may be developed into a possible biomarker for OAB.

4.5.1 GRAM-NEGATIVE UROPATHOGENS IN OAB

Gram negative species are the leading cause of UTI and of particular interest following the recent discovery of low-level bacteriuria in OAB suggesting UTI may be a cause of OAB. As expected, by reducing the threshold of infection to 10^3 CFU/ml, the percentage of patients with urinary infections increases two fold in both MSU and CSU samples. However, the percentage of control MSU samples with low-level infections is 35.0%, higher than in OAB MSU, which is 21.43%, suggesting that not all species at 10^3 CFU/ml or more may be detrimental as previously suggested (134-136). On the other hand it could be proposed that mere incidence is more relevant that colony count or that changes in an individual's microbiome over time predisposes OAB.

Our data has shown however that Gram negative species are found in increased incidence in OAB compared to controls. Of particular interest are those species which were increased significantly; *E.coli* and *Proteus.* The more recent investigations using ECUQ and culture-independent 16s rRNA sequencing have not noted these species to be of particular interest. This could be due to the rapid growth rates of *E.coli* and

Proteus, which swarm and outgrow other bacterial species and hence, supports the use of culture-independent methods for further investigation.

Looking at incidence of other Gram negative species of interest, it was seen that *Acidovorax*, was only found only in CSU samples which highlights the highly adhesive properties of the genus and it's ability to form biofilms in the bladder (figure 4.7G). *Enterococcus*, on the other hand, was not found in any CSU samples (figure 4.7F). This could suggest that once in the bladder, is much more intracellular, which has been supported by evidence showing this to be a highly intracellular species (152). *Serratia* was found to be highest in control MSU samples, which, considering the high pathogenicity and highly adhesive properties of this species, can only be explained as a data anomaly.

Our data were inconclusive in determining how Gram negative species may alter the environment since comparisons with Gram negative incidence and IL-8 and ATP concentration were not significantly different in either OAB MSU or control MSU but also IL-8 was higher in controls without Gram negative species. The concentration of ATP tended to be higher in OAB than in controls however this does not appear to be due to the presence of Gram negative bacteria. Taken together this contradicts the theory that Gram negative species leads to downstream pro-inflammatory cytokines and ATP release.

A possible continuation from this would be to perform an endotoxin assay to quantify the concentration of LPS in urine, which will hopefully show a more realistic correlation between LPS and cytokine production in OAB than has previously been shown using cells in tissue culture. Furthermore, there are no mentions in the literature of measuring LPS concentrations directly and this could provide insight into a possible biomarker for OAB for the future.

4.5.2 GRAM-POSITIVE UROPATHOGENS IN OAB

Figure 4.7 highlighted that many of the key species in the bladder are Gram positive. Furthermore, it showed that most of the more prevalent Gram positive species are found in much higher incidence in MSU samples than CSU samples, irrespective of disease state. This generally reflects the less-capable adherence and colonisation of Gram positive bacteria compared to Gram negative bacteria.

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From the introduction to this chapter, it was mentioned that species such as *Aerococcus, Arthrobacter, Corynebacterium* and *Gardnerella* are underreported Gram-positive uropathogens more prevalent in the elderly and that the advanced studies undertaken by Pearce *et al* have found these species to be of particular interest in OAB (138, 197). Our data has not found these species to be significant in either MSU or CSU samples. Unfortunately, due to the difficult accessibility to control CSU samples, the low numbers of patients in this cohort will significantly skew the data for CSU. Furthermore, our investigations have used restricted culture methods that may have missed out many incidences of these species.

4.5.3 INTRACELLULAR BACTERIA MAY NOT BE RELEVANT

Malone-Lee's 2013 study on the antibiotic protection assay was the first of its kind to look at culturing lysed urothelial cells and they identified much the same species as those found in the work of Hultgren on IBCs in UTI; *i.e. E.coli., E.faecalis,* and *P.mirabilis* (111, 137). As previously mentioned these are all Gram-negative bacteria with the ability to invade host cells and form IBCs. Much of the work in IBCs has, up until recently, focused mainly on *E.coli* however with the recent contribution from Malone-Lee revealing a more invasive capability from *E.faecalis*, our data was an interesting addition. We have shown that there are Gram positive species of bacteria capable of being intracellular in the bladder, notably, *Staphylococcus*, *Corynebacterium*, *Arthrobacter*, *Peptoniphilus* and *Streptococcus*, however no individual species stands out as significantly more predominant in OAB compared to controls. The observation of increased intracellular *Serratia* in OAB contradicts the reduced *Serratia* seen in CSU samples. This could be due to low numbers of Control CSU patients. Increased *Streptococcus* is consistent in both CSU and intracellular data suggesting this may be a potential species of interest in the bladder. Interestingly however, *Corynebacterium* and *Arthrobacter* have been noted to be underreported Gram-positive uropathogens, therefore this is exciting additional information for the pathogenicity of these species in UTI, particularly in reference to the previous chapter.

Hultgren refers in one of his publications to work done in 1985 on biopsies from patients with UTIs (242). The authors of this paper showed that half of patients (8 out of 16) who had negative urine cultures grew bacteria from bladder biopsies

indicating that urine culture may not reflect the true bacteriological status of the bladder. Interestingly, our research group has noted the opposite by using the antibiotic protection assay followed by 16s rRNA gene sequencing methods to identify intracellular bacteria cultured from biopsies taken from patients with idiopathic DO. Very few biopsies cultured bacteria when CSU samples taken from the same patients on the same day did grow bacteria. The biopsies were vigorously broken up with a combination of shaking and vortexing before spreading on chocolate agar. Furthermore, the application of triton X-100 at the lysing stage of the antibiotic protection assay should theoretically have released further intracellular bacteria however this was not the case. It can therefore be concluded that using biopsies for culture of IBC is not as effective as urine culture.

Hultgren *et al* have also previously identified IBC from bladder biopsies taken from individuals with UTI (figure 4.8). By taking EM images of bladder biopsies from individuals with OAB, we aimed to observe and image IBC present in OAB. Although no IBC were seen from the biopsies, there were many autophagosomes observed (figure 4.9A). Hultgren *et al* have described autophagosomes as being similar in morphology to IBCs but notably absent of bacteria (20). Interestingly, autophagosomes tend to occur when a cell is undergoing apoptosis triggered by activation of PRRs and is important in eliminating intracellular bacteria (21). Despite the fact intracellular bacteria have not been identified, the presence of autophagosomes in urothelial cells of OAB patients is in itself an exciting finding.

All species identified by the antibiotic protection assay have the capability to form IBCs suggesting our data are not wrong but rather that the prevalence of IBC in OAB and healthy controls is simply not that high or perhaps our methods are not as effective as others used. Alternatively, it suggests that intracellular bacteria may not be unique to OAB but may in fact also be part of the normal microbiome.

4.5.4 ALTERATIONS IN THE MICROBIOME OVER TIME

Our data has shown that a low-level infection can often be asymptomatic since 35% of the control MSU samples presented with an infection based on 10^3 CFU/ml. It could be said that only women who present with symptoms are likely to require microbiological urine culture however it does highlight concern for misdiagnosis. It

has also been shown that intracellular bacteria may be a normal part of the human bladder microbiome since no particular species stood out as being more prevalent in OAB compared to controls. Furthermore, the emerging uropathogens we would have expected to see in higher numbers in OAB were not significantly different to those of controls. Cumulatively, these data indicate that infection may not be a cause for OAB but it does not take away from the fact that bacteria may be important. As discussed in the General Introduction, the human microbiome is tolerated by the immune system and works in synergy to maintain health. Therefore, it could be hypothesised that changes in ones microbiome over time is a more likely cause for OAB than a hospital grade or low-level infection. This can be supported by the fact that incidence of OAB increases with age and BMI. Hormones changing with menopause, metabolites from our diet and stress are all factors that can alter a microbiome.

This thesis attempted to look at how age and the onset of menopause could be altering the microbiome. Although data on menopausal status was not available, the cohorts of patients were sub-categorised based on the average age of menopause onset (45 years). There are no obvious differences between the incidence of any species of interest between the age groups in either control or OAB however this could be due to the inaccuracy of the sub-grouping based on menopausal status since status was not known only assumed. Since the exact menopause status could not be determined, this restricts the value of the data, therefore no firm conclusions can be made regarding the changes in microbiome with age.

Another consideration for alterations in the bladder microbiome is BMI and diet. The food we eat and the breakdown products from digestion can act as 'prebiotics' for our microbiome. Prebiotics act as a nutrient source and support the growth of microorganisms particularly in the gut, therefore what we eat is key to our gut health. With the right nutrients, probiotic species tend to thrive, *e.g.* plant protein and unsaturated fat increase *Lactobacillus* and *Bifidobacterium* growth, whereas animal protein and saturated fat decrease these genera and increase risk of opportunistic infections and/or development of Crohn's disease, ulcerative colitis and inflammatory bowel disease (243). The onset of menopause reduces many hormones including oestrogen and leptin, both of which suppress appetite when reduced. Therefore,

investigating diet, in particular the consumption of high-fibre foods, in correlation with leptin levels and OAB incidence could highlight whether diet not only affects the gut microbiome but can extend to the bladder microbiome also (due to colonisation from rectum to urethra).

Unfortunately, the fine balance of the bladder microbiome is far from being well-understood but it can be assumed that as one species becomes compromised, another/numerous others take its place. However, since every individual microbiome and synergistic relationship with the immune system is unique, every patient would need to act as their own control. This is a much more difficult study to perform, since predicting OAB is impossible and many participants will be required in order to obtain a minimum number of women who will develop OAB. However, with the remarkable reduction in *Lactobacillus*, a known probiotic, this species may be key in microbiome alterations and susceptibility to OAB. Investigation is required to better understand the environment in which this species is best established and how it interacts with other species in the bladder.

4.5.5 CO-CULTURE OF *LACTOBACILLUS* WITH UROPATHOGENS

Our results have shown that the percentage of patient with OAB that grew *Lactobacillus* is significantly lower than that of controls, which is in keeping with findings from all other studies looking at the microbiome in OAB (137, 138, 197). From our results, this is the only genus of bacterium where there is a significantly reduced incidence in OAB. Furthermore, we have shown that incidence of *Lactobacillus* has a negative correlation with incidence of Gram negative species. This suggests *Lactobacilli* may have a protective, even probiotic potential in the bladder and may be key in alterations of the bladder microbiome, predisposing women to OAB. How this species grows and interacts with other species in the bladder requires further examination. Interestingly, in 2013, Abdulwahab *et al* assessed the ability of cultured *Lactobacillus,* taken from asymptomatic women, to inhibit growth of UPEC taken from women with UTI. Three strains of *Lactobacillus; L.acidophilus, L.fermentum* and *L.delburekii* were isolated as the most common species in asymptomatic women and were used in co-culture with UPEC. All three species were able to inhibit the growth of UPEC. Unfortunately, the species identified here do not

correlate with the most common *Lactobacillus* species normally found in premenopausal asymptomatic women as noted by Vasquez *et al* in Sweden and Antonio *et al* in the USA (203, 244). It is worth noting however, that this study was performed in and took sample swabs from women in Egypt. Differences in *Lactobacilli* colonization between these North African and Caucasian women has been investigated and supported by Fourney and Foster (245), supporting the notion that every microbiome is unique between individuals and cultures. In light of our results indicating a complete absence of *L.crispatu*s in OAB, it would be interesting to know the capability of *L.crispatus* to inhibit UPEC growth in culture as well as other known pathogens such as *Proteus mirabilis*, *Serratia* and *Clostridium*, which were all significantly higher in OAB from our data.

4.5.6 *LACTOBACILLUS* AS A PROPHYLACTIC AGAINST UROPATHOGENS

Lactobacilli are so called due to their capacity to ferment sugars into either lactic acid (homofermentative) or lactic acid or alcohol (heterofermentative), depending upon the species. Lactic acid reduces the pH of the surrounding environment and acts as an antibacterial by breaking down cell walls of pathogenic bacteria leading to leakage and cell death (246). Many *Lactobacilli* species also produce bacteriocins and hydrogen peroxide (H_2O_2). *L.crispatus* produces the highest levels of H_2O_2 of all the *Lactobacillus* species, seconded by *L.jensenii* (203, 204)*.* Interestingly, our data shows a complete lack of *L.crispatus* from the OAB cohort, which is also noted by Pearce *et al* (138).

Unfortunately, there have been no publications on the diversity of *Lactobacillus* species in the bladder, only the vagina; however, due to the proximity of the vagina to the urethra and the fact that urogenital colonisation is usually from the gut, it can be assumed the vaginal colonisation will be fairly representative of bladder colonisation in individuals. The four most common species identified in the vagina are *L.iners, L. crispatus, L.gasseri* and *L.jenesenii* followed by *L.acidophilus*, *L.gasseri*, *L.fermentum*, *L.oris*, *L.reuteri*, *L.ruminis*, and *L.vaginalis* (203, 244)*,* which is closely representative of the species found in our data, apart from the inclusion of *L.psittaci*. *Lactobacillus* has been widely researched as a probiotic in the gut, the oral cavity, the vagina and less so in the bladder however, using *Lactobacillus* as a prophylactic against UTI is not

a novel concept. Two studies of particular interest have generated optimistic results for this as a prospective prophylactic for UTI.

The first of these studies gave oral *L.rhamnosus* and *L.reuteri* or trimethoprim-sulfmethoxazole as prophylatics against UTI to 252 postmenopausal women and assessed incidence of UTI over 12 months in a randomised control trial. On average women in the antibiotic group had 7 UTI's per year prior to antibiotics. This was reduced to 2.9 with antibiotics. Similar figures were seen with *Lactobacillus* probiotics; 6.8 in the first year before treatment and 3.3 during. All data are significant suggesting oral *Lactobacillus* had a similar prophylactic effect on UTI compared to antibiotics (126).

Another group led by Stamm, has performed a phase 1 and phase 2 controlled trial, again looking at the role of *Lactobacillus* as a prospective prophylactic for UTI. These investigations have used *L.crispatus* as a vaginal suppository due to its ability to produce high levels of H_2O_2. A vaginal suppository would theoretically lead to higher rates of colonisation compared to oral probiotics due to the proximity of the urethra to the vagina, but also since oral probiotics can be significantly depleted by the gastrointestinal tract before reaching the anus. Results showed a reduction of roughly half incidence of UTI compared to placebo with minimal side effects (247, 248).

Taken together these studies and their results show that *Lactobacillus*, in particular *Lactobacillus crispatus*, can provide an effective prophylactic effect on UTI, in particular on UPEC. A randomised trial examining the effects of probiotics on OAB would be exciting since our results have clearly indicated reduced incidence of *Lactobacillus* in these patients. Furthermore, investigating the possible role of *Lactobacillus* metabolites on bladder function might highlight how a loss of this genus could lead to the symptoms of OAB.

4.5.7 POTENTIAL BIOMARKERS FOR OAB

Our data suggests that leukocyte esterase and nitrites are unreliable for identifying infection even at the higher threshold of 10^5 used by hospital pathology. Furthermore, there were many false positives in our data, which has been noted and explained by others (215). Therefore, dipstick analysis cannot be used to identify a possible UTI particularly if it is a low-level infection. By reducing the threshold of

bacteria to 10^3 the incidence of infection is not greatly increased, therefore a low-level infection is not likely to be the cause of OAB from our cohorts despite suggestions by other groups. Age does appear to be a predisposing factor for OAB as incidence increases rapidly with age, however our cohort has seen patients as young as 14 with idiopathic OAB therefore this cannot be used as an indicator for OAB. Our ATP and IL-8 results were not statistically different between OAB and controls despite others reporting ATP to be a potential biomarker for OAB. There were significantly higher number of patients presenting with positive culture for uropathogens *E.coli* and *Proteus,* therefore these individuals may be experiencing low-level rUTI which is leading to their symptoms. Unfortunately, despite being significant, the low numbers of individuals with these particular uropathogens does not qualify their incidence to be used a biomarker. The reduced incidence of *Lactobacillus* on the other hand could be used as a potential biomarker for OAB, particularly when combined with incidence of Gram negative species as 60% of patients with OAB grew Gram negative species with a lack of *Lactobacillus*. Further work is required to determine if this reduced incidence of *Lactobacillus* is found in other LUTS such as IC and rUTI before it can be considered as a biomarker. Also, due to the small, slow growing colony size of *Lactobacillus*, a *Lactobacillus* specific agar such as deMan Rogosa Sharp (MRS) could be used to promote this genus over others to easier determine its presence in urine samples.

4.5.8 IMPROVING OUTPUT OF 16S RRNA SEQUENCING

The percentage of non-concordant Gram stains from the total number of isolates was 14.47%. This is remarkably high and suggests that 16s rRNA sequencing may not be accurate. The original primers used (DG74 and RDR080) are taken from Griesen *et al*'s 1994 study on PCR primers for pathogenic bacteria and isolate the 1170-1540 region in *E.coli.* This publication quotes however "RDR080, and DG74 correspond to regions of the 16S rRNA gene which are highly conserved among divergent groups of eubacteria and therefore would be expected to amplify DNA from most pathogenic bacteria. The primer locations were chosen to be relatively specific for eubacterial genes;" but then later refers to these primers as Universal Primers (220). As mentioned, the location for the primers is 1170-1540bp in *E.coli,* which suggests this is in the V9/VT region for *E.coli* (249). V9 and V3 regions are most

variable however are not the most suitable to species ID. A study by Chakravorty *et al* identified V2 and V3 are most effective for identifying species to genus level however not for closely related *Enterobacteriacae.* They also indicate that using the V9 region does not provide complete data and do not include it in the remainder of their analysis (250). With more time, it would be advantageous to go back to isolates with a non-concordant Gram stain and re-perform PCR with primers designed for the V2 or V3 regions instead. Fortunately, despite the lack of evidence supporting the V9 region in 16s rRNA sequencing, the hypervariability of this region has allowed us to generate results very similar to those of almost all previous studies investigating the microbiome in OAB.

4.5.9 IMPROVING CULTURE METHODS

Since the start of this investigation, alternative methods have come to light that improve the culture of many uncultured species. The following chapter discusses how improved methods were developed in our laboratory for further studies investigating the microbiome in OAB by our team.

4.6 CONCLUSIONS

1. Hospital methods of dipstick should not be relied upon to diagnose infection and are even poorer at diagnosing low-level infection in OAB.

2. By reducing the threshold of CFU/ml to 10^3 more potential infections can be identified, however there are more low-level infections in controls than in patients with OAB suggesting low-level infection does not always correlate with incidence of OAB.

3. Intracellular bacteria appear to be a normal part of the bladder microbiome.

4. Individually, Gram negative species are found to be more predominant in CSU samples and Gram positive species are found to be more predominant in MSU samples.

5. Cumulatively, Gram negative bacteria are found in significantly higher frequency in OAB than controls.

6. *Lactobacillus* is found to be significantly lower in patients with OAB and this correlates with increased Gram negative bacteria indicating that OAB may be due to a lack of protective pro-biotic bacteria.

7. There do not appear to be any strikingly significant differences in the microbiome with increased age supporting the idea that patients act as their own controls as the microbiome changes.

8. Culture methods need to be expanded to include other conditions and alternative primers ought to be tested prior to further studies.

CHAPTER 5 – RESULTS 3:
IMPROVED METHODOLOGY FOR URINE CULTURE

5.1 INTRODUCTION

Since the start of this project, various published studies have highlighted potential weaknesses in performing sediment culture and growing bacteria in only basic conditions such as 5% CO_2 and anaerobic at a single temperature. Twelve most apposite studies have recently employed 2 different methodologies to investigate the non-cultivated species in urine samples of males, females, healthy individuals, and those with LUTSs (138, 189-196, 235, 236, 251). The first of these methods is culture of urine on a larger variety of agars in a variety of gas mixes and temperatures enhance quantitative urine culture (EQUC). The second method is culture-independent quantitative PCR whereby bacterial DNA is extracted directly from the urine samples followed by next generation sequencing and bioinformatics. One particular study has adopted both methods in order to compare them against each other concluding that even EQUC misses uncultured species and thus culture-independent methods are preferred (195). The details of each of these methods and the results from the studies using them shall be discussed in the following section, before going on to investigate how these can be adapted into our current methodology with in the capabilities of our laboratory facilities.

5.1.1 CURRENT METHODS

The UK Health Protection Agency suggests growth on cysteine lactose electrolyte deficient (CLED) and chromogenic agars for as the most efficient method for quantification of bacteria in urine (252). In CLED agar the presence of cysteine supports the growth of bacteria that require this amino acid; the presence of lactose and bromothymol blue act as an indicator for lactose and non-lactose fermenting microbes; and a lack of electrolytes prevents the swarming growth of *Proteus* species which can contaminate other colonies (252). Chromogenic agars contain various substrates which can identify particular species by the colour of the colonies. For example; primary UTI chromogenic agar contains ß-galactose, ß-glucose and tryptophan which turns enterococci blue, *E.coli* pink and *Proteus* brown based on the

presence of ß-galactosidase, ß-glucosidase and tryptophan deaminase activity, respectively (252). The UK Health Protection Agency references Kass's $\geq 10^5$ CFU/ml rule as indicative of an infection and anything less than this as contamination (252). This basic method misses out approximately 47% of possible urinary genera in females (n=70 genera) and 63% in males (n=46 genera) highlighted by Lewis *et al* (2013) who identified bacteria from MSU samples by culture-independent quantitative PCR methods (236).

The methods for culturing urine described in the General Methodology (Section 2.3) were adapted from Khasriya *et al*'s 2013 study (137). Their method was to perform sediment culture by plating 5 µl spun urine on blood agar and fastidious anaerobic agar and growing in 5% CO_2 and anaerobic conditions, respectively, at a single temperature of 37ºC. Previous work in our laboratory (unpublished data) determined that blood agar and chocolate grew similar cultures, however chocolate agar can support growth of *Haemophilus* species which is a known uropathogen (253). Therefore, chocolate agar was chosen as a preferred agar for the clinical studies at the start of this thesis (Chapters 3-4). Subsequent clinical studies by our group required us to update our methods in order to keep up with recent advances in urine culture. The aim was therefore to adapt our urine culture methods to potentially include culture-independent molecular PCR and/or EQUC.

5.1.2 CULTURE-INDEPENDENT MOLECULAR PCR

This method removes the need to culture bacteria and therefore eliminates the risk of missing non-cultivated species. 16s rRNA sequencing is still performed in order to identify species, however the DNA is extracted directly from the bacteria in the urine samples and the more advanced technique of next generation sequencing (NGS) is used rather than Sanger sequencing. This requires enzymatic lysis of bacteria with lysozyme followed by mechanical breakdown with lysing matrix B tubes. The DNA is then extracted from the lysate with a phenol chloroform alcohol extraction and ethanol precipitation. Upon obtaining bacterial DNA, a PCR reaction is prepared with primers barcoded for multiplexing (performing multiple PCR reactions at once). After the PCR has run, the amplicons are pooled and sent for NGS. NGS is a high-output method of sequencing which can read thousands of different DNA sequences per sample. This can allow a pool or library of different sequences to be created per

sample/multiple samples. The use of barcoded primers, allows for demultiplexing whereby each sequence is separated into individual FASTA files for each barcode, which requires specialised software. From these FASTA files a taxa can be allocated and from this the number of any particular taxa in each sample based on the number of amplicons related to that taxa.

One study which has adapted/utilised these methods identified 45 bladder species of which 20 could not be routinely cultivated (193). And a later, wider study, found 94 bladder genera, of which 63 could not be routinely cultivated (236) suggesting culture-independant methods are much more sensitive than culture-dependant methods. However, later studies with EQUC have shown that many of these are cultivatable with inclusion of extended culture (138, 195), and thus cheaper options may still be employed to identify the majority of species found in the urinary microbiome. Furthermore, these methods are complex and would require much practice before perfecting in the lab. Fortunately, NGS services are available which can perform the DNA extraction, prepare the DNA for NGS, and perform the NGS for you, as well as delivering a bioinformatics report detailing the results of your reads as a further option (*e.g.* Eurofins Genomics and Source BioScience).

5.1.3 ENHANCED QUANTITATIVE URINE CULTURE

Hilt *et al* (2014) published the first data using EQUC as a method for isolating various non-cultured bacteria from urine (n=260 isolates) (195). They inoculated 100 µl of unspun urine onto blood agar plates (BAP), chocolate agar, and colistin and nalidixic acid agar (CNA) plates and incubated them for 48 hours at 35°C in 5% CO_2. They also inoculated 100 µl of unspun urine onto BAP for incubation in aerobic conditions at two temperatures, 30°C and 35°C, for 48 hours each. And finally they inoculated 100 µl of unspun urine onto fastidious anaerobic agar (FAA) for culture in anaerobic and campygas (5% O_2, 10% CO_2, 85% N) conditions, both at 35°C for 48 hours. This generated a total of 5 different conditions summarised in the table below (table 5.1). Interestingly, Hilt *et al* used matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) to identify species instead of 16s rRNA gene sequencing. This newly emerging technique for identifying species is cheaper and faster than 16s rRNA sequencing.

Agar	Conditions	Temperature	Time
BAP/Chocolate/CNA	5% CO_2	35°C	48 hours
BAP	Aerobic	35°C	48 hours
BAP	Aerobic	30°C	48 hours
FAA	Anaerobic	35°C	48 hours
FAA	Campygas	35°C	48 hours

Table 5.1 Types of agars and conditions used in by Hilt *et al* (195) for culturing urine by the enhanced quantitative urine culture method (EQUC). For example, urine was cultured on blood agar plates (BAP), chocolate plates and colistin and nalidixic acid agar (CNA) in 5% CO_2 at 35°C for 48 hours.

Hilt's tabulated results indicate which species grew in each condition and can therefore be used to identify species missed from other studies. The original methodology for this thesis (Chapter 3-4) was adapted from Khasriya *et al's* study, which also listed all species identified by their methodology. From the published data of both these studies, missed cultured species from Khasriya *et al,* and therefore this study, can be extrapolated; of 93 individual species identified by EQUC, 15 of them (16.1%) were cultured from conditions not included by Khasriya *et al*, that is, aerobic at 30°C and 35°C, and Campygas. Furthermore, Khasriya *et al* plated spun urine which contains concentrated urothelial cells. This method is essential if the initial plating is followed by an antibiotic protection assay to determine intracellular bacteria (detailed in section 2.3.2), but is not necessary for determining planktonic species only and can in fact generate data which is bias for species capable of attachment to urothelial cells. The differences between spun and unspun urine has been shown by the differences in reported species by Khasriya and Hilt. Furthermore, by plating only 5 µl, any species present at levels lower than 10^2 CFU/ml are at risk of being missed, therefore plating 100 µl is more effective for culturing low numbers of bacteria. In 2014, Pearce *et al*, recognised that even with EQUC some species are still missed, therefore they combined the methods of culture-independent sequencing of the 16s rRNA gene and EQUC and MALDI-TOF MS to obtain the most complete description of bladder bacterial species (138). They noted a high-level of overlap in species suggesting that both methods are effective, however EQUC was not able to identify many incidences of *Atopobium* and high-throughput sequencing missed *Trueperella*.

As mentioned, Hilt and Pearce used MALDI-TOF-MS to identify the species found in their studies. Although this new technique is effective at determining bacteria to a species level with up to 85% accuracy, it is dependent upon known species peptides being included in the spectral database (254). Much the same as when a BLAST search is performed, discovered peptides are matched to a database of known peptides from known species. The use of MALDI-TOF-MS as a method of identifying species has previously been compared with 16s rRNA in regards to vaginal *Lactobacilli* by Anderson *et al* in their 2014 publication (255). They collected 77 samples of vaginal *Lactobacilli* of 15 different species including *L.iners, L.crispatus, L. jensenii* and *L.gasseri.* It was discovered that MALDI-TOF-MS analysis was able to identify 97.4% of the samples in concordance with 16s rRNA, however in some cases MALDI-TOF-MS was only able to identify isolates to genus level. This publication confirmed that MALDI-TOF-MS relies on a developed spectral database and as a new method, this has yet to be extended and fully established. Therefore, 16s rRNA sequencing appears to remain the most effective method for identifying bacteria to species level, confirmed by the highly concordant results from MALDI-TOF-MS. The results from the previous chapter of this thesis has identified exciting results for *Lactobacillus*, however there could be some discrepancies about the accuracy of 16s rRNA sequencing based on the choice of primers used. As mentioned in the discussion of the previous chapter (Section 4.5.1), the location for the original primers is 1170-1540bp in *E.coli,* which suggests this is in the V9/VT region for *E.coli* (249). V9 and V3 regions are most variable however are not the most suitable to species ID. Therefore it was concluded that a more selective set of primers should be used to better identify *Lactobacilli* isolates to species level.

5.1.4 CURRENT RESEARCH QUESTIONS

It is clear that the basic culture methods advised by the UK Health Protection Agency and those performed by Khasriya *et al* are likely not culturing and identifying all species of bacteria in urine samples. EQUC can improve this by culturing in extra conditions such as aerobic and campygas, and at a lowered temperature of 30°C, however even then many species are missed. Culture-independent molecular PCR can remove the need to culture bacteria at all and with the use of next generation sequencing methods the results can still be quantifiable. We wanted to determine if

our method of culturing urine, which was adapted from Khasriya *et al*, could be improved and yet remain cost efficient and within the capabilities of our laboratory facilities. Since the methods for culture-independent PCR are complex and would require considerable comprehension to perfect, various NGS service suppliers were investigated to perform the full service on our behalf. Also, we wanted to confirm that the *Lactobacillus* species found in our study were reliably identified. Therefore, we chose to repeat the 16s rRNA sequencing on these isolates using the same primers as those used by Anderson *et al* for comparing 16s rRNA sequencing with MALDI-TOF-MS for vaginal *Lactobacillus* (255).

5.2 HYPOTHESIS AND AIMS

We hypothesise that the EQUC and culture-independent 16s rRNA sequencing will show more variation in species per patient sample than the basic method adapted from Khasriya *et al*.

In order to begin to test this hypothesis the following objectives will be to:-

1. Compare culture growth using the original method with EQUC to determine how many species could be being missed in our studies.
2. Determine the cost and efficiency of an external NGS service to reduce work-load.
3. Compare results from NGS service with original culture method and EQUC to determine the best method for future culturing of urine samples.

5.3 Methodology

5.3.1 Disclaimer

All work performed in this chapter was a joint effort by Miss Tash Curtiss and myself.

5.3.2 Selecting patients

Three control samples of catheter specimen urine were obtained from patients attending Sunderland Day Case Clinic at Medway Maritime Hospital, Gillingham, Kent for non-bladder surgeries such as hysterectomy, myomectomy and laparoscopy. All samples were collected under general anaesthetic in a sterile operating theatre prior to the scheduled operation. All patients scored 0 on the International Consultation on Incontinence Modular Questionnaire – Female Lower Urinary Tract Symptoms Short Form (ICIQ-FLUTS-SF) and gave written consent prior to surgery.

5.3.3 Sediment culture

5 ml of fresh urine was centrifuged in a 15 ml falcon tube at 800 RPM for 5 minutes. The sediment was resuspended in 100 μl 0.1M PBS and 5 μl was plated on two chocolate agar plates per patient sample (E&O Labs) with a sterile single-use plastic 5 μl inoculation loop (Better Equipped). One plate was placed in 5% CO2 at 37-^{o}C for 48 hours and one was placed in a 2.5 L anaerobic jar with anaeogen (Fisher) and anaerobic indicator, resazurin (Fisher) and kept at 37°C for 7 days. Anaerobic bacterial growth is slower than in bacterial growth in 5% CO_2 therefore the plates in anaerobic jars were left for longer to ensure growth was optimised. At the end of the respective growth periods, plates were checked for growth and all colonies were identified by their appearance, described and purity plated on chocolate agar. Purity plating was performed by inoculating individual colonies with a sterile inoculation loop (Better equipped) and streak plating on fresh chocolate plates. The purity plates were allowed to grow in 37°C for a further 48 hours/7 days in their respective environments. Once pure plates were obtained for each isolate, the isolates were Gram stained, and the remaining bacterial growth from the purity plates was frozen at -80°C in 1.5 mL Nunc® CryoTubes (Sigma) containing Brain Heart infusion broth (Sigma) containing 10% Glycerol (Sigma) to prevent cell lysis during freezing.

5.3.4 Enhanced Quantitative Urine Culture

Seven conditions were chosen for the EQUC for which 100 µl of unspun urine was plated onto each of the following; i) Columbia blood agar (CBA), ii) chocolate agar, and iii) colistin and nalidixic acid agar (CNA) each incubated for 48 hours at 37°C in 5% CO_2, iv) CBA for incubation in aerobic conditions at two temperatures, 30°C, and v) 37°C, for 48 hours each, and vi) fastidious anaerobic agar (FAA) for culture in anaerobic, and vii) campygas (5% O_2, 10% CO_2, 85% N) conditions, both at 37°C for 48 hours. A colony count was performed on each unique species and the number multiplied by 10x to calculate the CFU/ml. In order to compare EQUC to the original methodology used in chapter 3 and 4, urine was also cultured as discussed in *Section 2.1.3.2 Streak plating and antibiotic protection assay* by plating 5 µl of centrifuged urine on two chocolate plates and incubating for 48 hours at 37°C in 5% CO_2 and 37°C in anaerobic conditions. For these plates, the CFU/ml was calculated by multiplying the CFU count by 40x due to the centrifugation step concentrating the 5 ml of urine.

Purity plating was performed by inoculating individual colonies with a sterile inoculation loop (Better equipped) and streak plating on fresh chocolate plates. The purity plates were allowed to grow in in their respective environments. Once pure plates were obtained for each isolate, the isolates were Gram stained, and the remaining bacterial growth from the purity plates was frozen at -80°C in 1.5mL Nunc® CryoTubes (Sigma) containing Brain Heart infusion broth (Sigma) containing 10% Glycerol (Sigma) to prevent cell lysis during freezing.

5.3.5 16s rRNA gene sequencing

Isolates were identified by sequencing of the 16s rRNA gene as discussed in the General Methodology; samples were revived by regrowing on agar in their respective environments, lysed in H_2O at 94°C, the 16s rRNA gene isolated by PCR, gel electrophoresis to check for the correct band size, purification, nanodropping to determine the final concentration on DNA, and sequencing by Eurofins Mix2Seq.

Three methods for submitting samples for sequencing to Eurofins Genomics are available; Mix2Seq service, Value Read service and Service à la Carte. Mix2Seq costs £3.18* per read where primers and sample are submitted in the same vial.

Value read service is £4.75*/reaction where primer and template can be sent separately allowing repeated reactions if no read is generated due to technical issues. Service à la Carte costs £6.80* per reaction and offers a quality check prior to read, DNA concentration adjustment, a free one time repetition and heterozygote analysis. Due to the number of isolates which may be generated in the future it was necessary to choose the first option of the Mix2Seq service to keep costs down.

5.3.6 SELECTING A NEXT GENERATION SEQUENCING SERVICE

Three companies were contacted regarding their 16S metagenomics profiling services; Eurofins Genomics, Germany; Beckman Coulter Genomics, USA; and Source BioScience, UK. The easiest, most efficient and cheapest service was selected from these three.

Eurofins Genomics offer an NGS service called '16s rRNA microbiome profiling MiSeq' which offered a quality check of each sample, amplicon generation of the genes region of interest, pooling and normalization of amplicons, sequencing of the amplicon on an Illumina MiSeq with 2 paired-end reads of 300bp. This was followed by bioinformatics analysis provided on a disk or paper format. The cost of this service is £175*/sample with a minimum of 6 samples per submission. This cost would be reduced to £128*/sample for greater than 48 samples.

Beckman Coulter offer an NGS service called 'Metagenomic Sequencing'. This service was not able to provide DNA extraction or PCR services therefore this would have to be performed beforehand. Submitted PCR product would be cleaned up, quantified and pooled and then read on a Roche-NGS. Each sample in a pool needs a different primer with a MID tag on it so that it is possible to demultiplex the data later. The same primer sets can be used again in separate pools but the primer costs still needed to be factored into the project. The price per pool would be £2,257* and therefore the price per sample would be dependent on how many samples were submitted to the pool. Metagenomic analysis would be a further £36* per sample or £1,582* per project.

Source BioScience offer an NGS service called 'Metagenomic sequencing' which consisted of a quality check of each sample, amplicon generation of the gene's region of interest, pooling and normalization of amplicons, sequencing of the amplicon on an

Illumina MiSeq with 2 paired-end reads of 300bp. This was followed by bioinformatics analysis provided on a disk or paper format. The cost of this service is £350*/sample to perform the quality check and prepare for sequencing then £1900* to create a pool.

After communication with sales representatives from all the above companies it was agreed that Eurofins would be most efficient as this allowed simple sample submission (discussed next) with the lowest cost

5.3.7 NEXT GENERATION SEQUENCING SAMPLE SUBMISSION

As discussed, Eurofins Genomics was selected to run the test samples for metagenomics sequencing. Due to the cost reduction for larger sample submission (£128*/sample for 48+ samples), any future sample submission would have to be on frozen samples to allow for collection and storage until a large batch could be submitted at once. For this reason the samples were frozen prior to submission in order to reproduce future submission. Since a minimum of 6 samples was required for submission, 5 ml of each of the 3 CSU samples was filtered prior to freezing and 5 ml was frozen without filtering to determine the most effective method of capturing bacteria. Each CSU sample (5 ml) was frozen for 2 weeks at -20°C and 5 ml of each CSU sample was passed through a 0.22 μm Millipore filter to capture any bacteria, and then frozen for 2 weeks at -20°C. Within 14 days the samples and filters were wrapped in tin foil, placed in labelled plastic bags and sent to Eurofins in Germany via recorded DHL delivery.

*All prices correct as of November 2015

5.3.8 CREATING A *LACTOBACILLUS* PHYLOGENETIC TREE

All species which were initially identified as *Lactobacillus* from the OAB study (Chapter 4) were resuscitated in their original conditions and 16s rRNA gene sequencing performed on each as discussed in the General Methodology (*Section 2.1.3.5*). The PCR was set up with new primers taken from Anderson's study which confirmed 16s rRNA to be more accurate at differentiating between vaginal *Lactobacillus* species than MALDI-TOF-MS (255): - Forward 5'-

GAGAAGAACGTGCGTGAGAG-3' and Reverse 5'-ATGTGAAAGCCTTCGGCTTA-3'. Another PCR was set up with new primers taken from Herlemann *et al* who used DegePrime to design a broad-taxonomic-range primer pair that targets the bacterial V3-V4 region (256)- Forward 5'- CCTACGGGRSGCAGCAG-3' and Reverse 5'-GACTACHVGGGTATCTAATCC -3'.

From each set of primers, a phylogenetic tree was created from the sequencing results using CLC Genomics Viewer 7, which can be used to join species to each other by sequence similarity. The sequences were extracted as FASTA files and open in CLC Genomics Viewer 7. Failed sequences and any anomalies in sequencing were removed. Sequences were renamed with their respective BLAST result species identity. When aligning sequences there are three parameters which must be considered; i) the gap open cost which is the price for introducing gaps in an alignment where nucleotides are missing, ii) the gap extension cost which is the price for every extension past the initial gap, and iii) the end gap cost which is the price of gaps at the beginning or the end of the alignment. These sequences were aligned and the costs optimised to a gap open cost of 10.0 and gap extension cost of 10.0. By making these values the same and as high as possible, the best alignments were generated. The End gap cost was set to 'As any other' so that gaps at the ends of sequences are treated like gaps in any other place in the sequences. Once aligned, a phylogenetic tree was created using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) tree construction method, which assumes a constant rate of evolution, compared to the Neighbour Joining method which assumes varying rates of evolution. The Jukes-Cantor substitution method was selected for the nucleotide distance measure, which assumes equal base frequencies and equal substitution rates, compared to the Kimura 80 Method which assumes equal base frequencies but distinguishes between transitions and transversions. Once generated, the tree layout was changed to a phylogram.

5.4 RESULTS

5.4.1 EUROFINS 16S RRNA MICROBIOME PROFILING MISSES ISOLATES

Eurofins Genomics performed the bacterial DNA-isolation and qPCR on 16s rRNA genes with bacteria-specific primers was used for quantification. The results of the 16s rRNA genomic profiling were returned by email. Relating to the bacterial standards of known concentration, lower than required DNA-concentrations were detected; approximately 0.001 ng/µl DNA per sample compared to the required 10 ng/µl (Table 5.2). Eurofins tried to amplify the target-region, but no PCR-product could be detected. They also performed some supplementary tests in order to verify whether human DNA can be detected in the samples. Two of the urine samples contained low amounts of human DNA, suggesting that DNA was present but bacteria were not.

Eurofins discussed the results within their team and proposed two reasons for the low bacterial DNA concentrations; i) the samples were taken from clinically healthy persons, having neither a bladder infection, or a tumour, ii) the samples were not the first void of the day nor were they MSU samples. Both of these suggestions can be argued against since recent investigations have proved that urine is not sterile even in healthy persons. It was therefore not possible to obtain any results from the NGS sequencing and this method had to be dismissed as an option for analysing urine samples for bacterial DNA content.

Sample name	DNA concentration per sample [ng/µl]
Control CSU sample 1 Filter	0.0013275
Control CSU sample 2 Filter	0.00115
Control CSU sample 3 Filter	0.00116
Control CSU sample 1 Urine	0.00121
Control CSU sample 2 Urine	0.000963
Control CSU sample 3 Urine	0.001154

Table 5.2 Concentrations of DNA obtained from Eurofins Next Generation Sequencing service for three control CSU samples. Samples were either filtered through a 0.22 µm Millipore filter to capture any bacteria and frozen for 2 weeks at -20°C, or the frozen urine was sent unfiltered. Eurofins require a minimum of 10 ng/µl DNA, therefore for all samples, concentrations of bacterial DNA were too low for Eurofins to perform next generation sequencing. This method for identifying species of bacteria from urine was therefore rejected.

5.4.2 EQUC COMPARED TO SEDIMENT CULTURE

Control sample-1 did not grow any colonies across all conditions in both EQUC and sediment culture (data not shown). These were left for a further 48 hours which also did not result in growth suggesting very low level bacteria in the sample. Focusing therefore on control samples-2 and -3, and considering only the differences between plating 5 µl spun urine and 100 µl unspun urine with the original conditions of 5% CO_2 and anaerobic, there are many inconsistencies when comparing the CFU/ml of individual species. In the case of *Lactobacillus iners* growth in control sample-2, 1000 CFU/ml grew in CO_2 37°C when plating 5 µl spun urine however no colonies grew when plating 100 µl spun urine but were seen in the anaerobic conditions (figure 5.3A). Also in sample-2, *Alloscardovia omnicolens* grew at 80 CFU/ml when plated with 5 µl spun urine but only 10 CFU/ml with 100 µl unspun urine. A further discrepancy can be seen in sample-3 in the case of *Lactobacillus hominis* which was found to grow at 320 CFU/ml with 5 µl spun urine but did not grow when plating 100 µl unspun urine (figure 5.3B). Despite the discrepancies in CFU/ml on some bacteria, the 100 µl unspun urine was able to grow 2 extra bacterial species which were not seen in 5 µl unspun urine; *Anaerococcus lactolyticus* and *Finegoldia magna.* Also, sample-3 was shown to culture an extra 3 species with 100 µl unspun urine compared to 5 µl spun urine; *Actinobaculum massilae, Olligella ureolytica* and *Peptoniphilus timonensis*

When looking at the new conditions and agars introduced with EQUC since with sediment culture for control sample-2 a further 2 species were identified; *Paenibacillus wynii* and a species of unknown identity (table 5.3A). Similar was seen in control sample-3 whereby EQUC was able to culture a further 4 species of bacteria; *Actinomyces suimastitidis, Corynebacterium tuscaniense, Lactobacillus acidophilus* and *Lactobacillus ultunensis* (figure 5.3B).

A) Control sample 2

Species identified	CFU/ml from 5 µl spun urine		CFU/ml from 100 µl unspun urine		CFU/ml from EQUC (100 µl unspun urine)			
	Choc CO$_2$ 37°C	FAA Anaerobic 37°C	Choc CO$_2$ 37°C	FAA Anaerobic 37°C	CBA CO$_2$ 37°C	CNA CO$_2$ 37°C	FAA Campygas 37°C	CBA Aerobic 37°C
Actinomyces furicensis	-	-	80	70	-	-	-	-
Alloscardovia omnicolens	160	80	500	10	-	70	-	40
Anaerococcus lactolyticus	-	-	-	20	-	-	-	-
Finegoldia magna	-	-	-	10	-	-	-	-
Lactobacillus iners	-	1000	250	-	60	10	-	-
Paenibacillus wynii	-	-	-	-	-	-	-	10
Unknown species	-	-	-	-	-	-	100	-

B) Control sample 3

Species identified	Choc CO$_2$ 37°C	FAA Anaerobic 37°C	Choc CO$_2$ 37°C	FAA Anaerobic 37°C	CBA CO$_2$ 37°C	CNA CO$_2$ 37°C	FAA Campygas 37°C	CBA Aerobic 37°C
Actinobaculum massilae	-	-	-	50	-	-	-	-
Actinomyces suimastitidis	-	-	-	-	-	-	-	20
Aerococcus urinae	40	-	40	-	30	20	60	-
Corynebacterium tuscaniense	-	-	-	-	-	-	10	-
Fusobacterium nucleatum	-	40	-	250	-	-	-	-
Lactobacillus acidophilus	-	-	-	-	500	-	-	-
Lactobacillus hominis	-	320	-	-	-	-	-	-
Lactobacillus iners	-	1000	800	-	800	500	-	-
Lactobacillus johnsonii	1080	-	1000	1000	-	-	600	-
Lactobacillus psittaci	-	-	-	-	100	100	1000	-
Lactobacillus ultunensis	-	-	-	-	-	600	-	-
Olligella ureolytica	-	-	10	-	-	-	-	-
Peptoniphilus timonensis	-	-	-	150	-	-	-	-

Table 5.3 Species identified by sediment culture and expanded quantitative urine culture (EQUC). A) Results for control sample 2, **B)** results for control sample 3. Species identified in the first column were found to be present in the various conditions signified by CFU. Sediment culture included two conditions (5% CO$_2$ and anaerobic at 37°C) whereas EQUC included 2 extra conditions and an extra temperature (Campygas at 37°C, and aerobic at 37°C). Two extra types of agar were used: Columbia blood agar (CBA) and colistin and nalidixic acid agar (CNA). Fastidious anaerobic agar (FAA) was used for campygas and CBA was used for aerobic conditions. Blank cells signify species did not grow under that condition.

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By looking across the table of EQUC findings, it can be seen that CNA plates, Aerobic and Campygas conditions were able to select for previously uncultured species in the standard sediment culture used in chapters 3-4. Looking again at control sample-2, culture on CBA and CNA did not contribute any further species than culture on chocolate. In fact, chocolate grew more colonies across all species found in CO_2 conditions (table 5.3A). This was not the case with control sample 3 however (table 5.3B). From this sample, CNA was able to grow *Lactobacillus acidophilus* and *Lactobacillus psittaci*, and CBA was able to grow *Lactobacillus psittaci* and *Lactobacillus ultunensis,* whereas chocolate agar did not.

Campygas conditions cultured 1 unique species not found in any other conditions for control sample 2 (table 5.3A). This was not possible to identify due to sequencing failing twice for this isolate. Looking therefore at control sample-3 it was possible to see that Campygas conditions were able to isolate *Corynebacterium tuscanense* where this isolate did not grow in other conditions (table 5.3B).

Aerobic conditions at 37°C identified *Paenibacillus wynii* from control sample-2 where other conditions did not grow this species (table 5.3A). From control sample-3, this condition also identified a unique species, *Actinomyces suimastitidis* (table 5.3.B)*.* Growth in aerobic conditions at 30°C was not performed due to restricted access to incubators that could be altered for temperature and used for bacterial growth.

Looking at the exact CFU/ml from individual *Lactobacillus* species there are some more discrepancies across the conditions in EQUC. For example, for control sample-2, 250 CFU/ml of *Lactobacillus iners* were identified on chocolate agar however only 60 CFU/ml on CBA and 10 CFU/ml on CNA (table 5.3A). The same is the case for *Lactobacillus psittaci* from control sample-3, which grew with 100 CFU/ml on CBA and CNA but with 1000 CFU/ml in Campygas (table 5.3B). This could suggest either that 16s rRNA sequencing has not been accurate in identifying the exact species from each condition, or that certain *Lactobacillus* spp. grow better in other conditions. This further supports the need to use MALDI-TOF to determine the accuracy of 16s rRNA sequencing.

5.4.3 *LACTOBACILLUS* CONCORDANCE

A total of 45 isolates from this study which were previously identified as *Lactobacillus* were re-suspended for repeated PCR with two sets of PCR reactions, one with primer set 1 from Anderson *et al* (255) and one with primer set 2 from Herlemann *et al* (256) to make a total of 90 PCR reactions. Upon BLASTing the newly generated sequences, concordance with the original species identification was recorded. Where sequencing failed, these isolates were removed from the calculations. Percentage concordance was calculated as follows:

$$\left(\frac{Number\ of\ concordant\ species}{Total\ number\ of\ species}\right) \times 100$$

It was found that from 19 that were initially identified as *L.psittaci*, 0% were identified as this species with both sets of new primers (table 5.4). All (100%) of successful sequences identified these isolates as *L.jensenii* with the new primers (n=17). There is also a 0% concordance for *L.hominis* which was identified as *L.gasseri* with both sets of new primers (n=4). There is however a 100% concordance for isolates first identified as *L.iners* (n=7) and *L.crispatus* (n=2). There is 82% concordance of successful sequences for isolates first identified as *L.gasseri (n=8).* Therefore, an amended version of *figure 4.25: Percentage incidence of Lactobacillus species cultured from OAB and Control MSU samples of urine* found in chapter 4 for the *Lactobacillus spp.* in OAB versus asymptomatic controls based on these results has been created and new statistics performed using Fisher's exact test (figure 5.1).

The inclusion of a phylogenetic tree checks the evolutionary distances between species based on their genetic sequences. The tree created from the 33 *Lactobacillus* isolates indicates that all species identified as *L.jensenii* are closely related with little evolutionary difference between sequences (between approx. 0.03% and 0.18%). The same is the case for the other species.

	# concordant isolates			
Original species ID	**Primer set 1** (255)	**Primer set 2** (256)	**% concordance**	**Correct species ID**
Lactobacillus psittaci (n=19)	0	0	*0%*	*Lactobacillus jensenii* (100%)
Lactobacillus gasseri (n=10)	6	8	*81.7%*	*Lactobacillus gasseri* (81.7%)
Lactobacillus iners (n=7)	6	7	*100%*	*Lactobacillus iners* (100%)
Lactobacillus hominis (n=4)	0	0	*0%*	*Lactobacillus gasseri* (100%)
Lactobacillus crispatus (n=2)	1	1	*50%*	*Lactobacillus crispatus* (100%)

Table 5.4: Percentage concordance of *Lactobacillus* species identified by original primers and new primer subsets. The original primers used in Chapter 4 of this thesis identify *Lactobacillus* species as *L.psittaci* (n=19), *L.gasseri* (n=10), *L.iners* (n=7), *L.hominis* (n=4) and *L.crispatus* (n=2). Alternative primers derived from Anderson *et al* **(255)** and Herlemann *et al* **(256)** are used to compare species ID of the *Lactobacillus* isolates. The number of concordant isolates by species ID are listed beneath the alternative primer sets used and relative percentage concordance calculated next to them. In cases where there is a 0% concordance, the correct species is listed. *E.g.* 0% of isolates originally identified as *L.psittaci* are identified as *L.psittaci* with the new primers. 100% of these isolates are identified as *L.jensenii* instead.
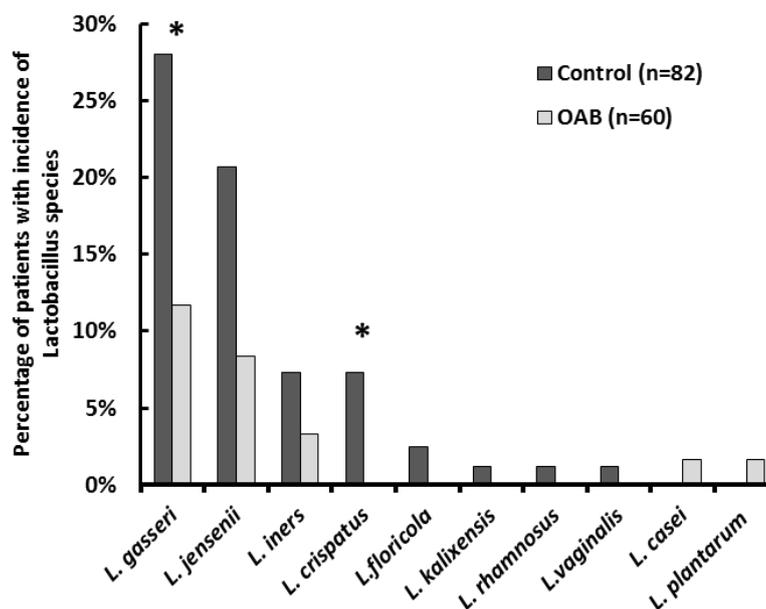
Figure 5.1: Percentage incidence of *Lactobacillus* species cultured from OAB and Control MSU samples of urine (amended version from *Section 4.4.9 Reduced incidence of Lactobacillus*). A total of 75 *Lactobacillus* isolates cultured from MSU samples grown from 82 Control CSU samples and 60 OAB CSU samples were successfully identified by sequencing of the 16s rRNA gene. Incidence of each species is expressed as a percentage of samples with genus from the total number of samples. Decreased incidence from controls to OAB can be seen with all species of *Lactobacillus* particularly *L.gasseri* from 20.7% to 11.7% (p=0.02*), *L.jensenii* from 20.7% to 8.3% (p>0.05), *L.iners,* from 7.3% to 3.3% (p>0.05) and *L.crispatus* 7.3% to 0.0% (p=0.04*). All P values were calculated using Fisher's exact test.
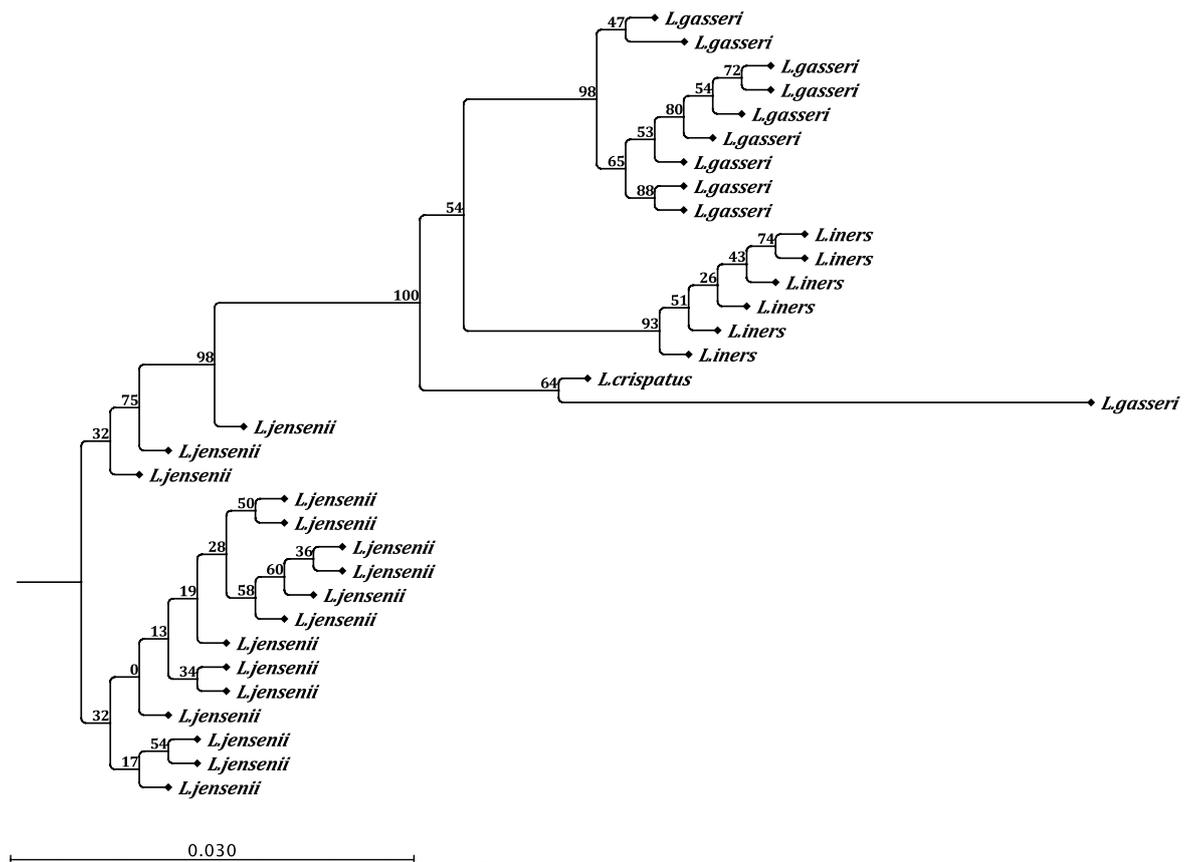
Figure 5.2: Phylogenetic tree of *Lactobacillus* species cultured from OAB and Control MSU samples of urine. An alignment was performed followed by the creation of a phylogenetic tree on sequences generated from 33 *Lactobacillus* isolates cultured from MSU samples obtained from asymptomatic individuals and those with OAB. This tree shows the evolutionary distances of *Lactobacillus* species from each other and confirms that all the sequences bar one are likely to be correct. Scale represents the number of differences between sequences (0.030 means 0.3% differences between two sequences. Junction values represent the % support for the nodes.

5.5 DISCUSSION

The aims of this chapter were to determine a cost-effective method to better investigate the bladder microbiome based on the capabilities of our laboratory. Following investigation, the main findings are: - i) Eurofins Genomics offers the most cost effective 16s metagenomics service compared to Source BioScience and Beckman Coulter Genomics, ii) Eurofins Genomics 16s metagenomics service was unable to detect bacterial DNA in the three CSU samples sent, iii) two of these three urine samples were not sterile, iv) inclusion of CNA agar, campygas and aerobic conditions increased the diversity of species found in the two non-sterile samples, v) centrifuging urine samples is bias for species closely associated to urothelial cells.

5.5.1 DISREGARDING EUROFINS 16S rRNA MICROBIOME PROFILING

Since the NGS service was not able to identify sufficient bacterial DNA in order to perform NGS, this option was discarded as a future improved methodology. A restriction with the methodology used for NGS was that CSU samples were sent. Although this type of sample was intentionally chosen since CSU samples provide a more accurate insight into the exact bacteria within the bladder (129), this has likely reduced the yield of bacterial DNA from extraction compared to using MSU samples. These results have shown that even species at 10^3 CFU/ml are not being captured by Eurofins method of DNA extraction (table 5.3A). Since it has been shown that 10^3 CFU/ml is sufficient to be considered low-level bacteriuria (134-136), if species are being missed at this level then even by sending MSU samples it could be predicted that key species would not be represented.

The quantity of DNA detected, even after amplification, was 10,000-fold lower than the required concentration in all 3 samples, however two samples grew bacteria even with the sediment culture method. This suggests that the Eurofins method for extracting DNA is not appropriate for CSU samples. The studies discussed in *Section 1.6 The bladder microbiome* which used culture-independent sequencing had the ability to adapt and amend methods to increase the yield of bacterial DNA from urine samples. Two principal methods were employed by the investigating teams using NGS to extract bacterial DNA from urine. The first method is more laborious, using centrifugation, enzymatic digestion with lysozyme, and physical fragmentation using beads, to break up cells in the first instance. This is then followed by DNA extraction using phenol chloroform isogamy and then alcohol precipitation to precipitate DNA (213, 236, 251). The second method uses Triton X-100 and lysozyme as well as vortexing to break up cells followed by DNA extraction using a Qiagen DNeasy DNA extraction kit, which uses a silica-based membrane containing chaotropic salt to remove water and contaminants from DNA (190-192, 194, 195). The latter method removes the need to use phenol extraction and alcohol precipitation, which can introduce human error. Eurofins Genomics used a method nwhich has been optimised for capturing bacteria from a wide range of starting materials including wastewater, marine water, food and faeces. Upon contacting Eurofins regarding the exact method used to extract DNA, they disclosed that filters were treated with lysis

buffer and the supernatant entered the extraction procedure using Maxwell 16 FFS nucleic acid extraction kit. This suggests that the inclusion of vortexing or bead beating would be necessary for better DNA extraction in our samples.

5.5.2 EQUC INCREASES DIVERSITY OF CULTURED SPECIES

EQUC has clearly shown that many species may have been missed from the sediment culture used in the previous two chapters; the number of species from both samples was more than two-fold greater using EQUC compared to sediment culture. The inclusion of Campygas cultured *Corynebacterium tuscaniense,* which did not grow in other conditions. Interestingly however, this species has been identified in many samples in the previous two chapters which did not employ the use of Campygas as a condition. It could indicate that this species prefers Campygas conditions, may have been missed in a number of samples in the previous chapters, and therefore actual incidence may in fact be higher than reported. Inclusion of aerobic conditions has cultured *Paenibacillus wynii* and *Actinomyces suimastitidis,* both of which are previously unreported in this thesis. Therefore, further investigations ought to include both of these conditions. The use of CNA and CBA was not effective in culturing extra species for one of the samples, however it was with the other sample whereby CBA and CNA were unique in culturing *Lactobacillus acidophilus* and *Lactobacillus ultunensis*, respectively.

 Due to restricted access to incubators, it was not possible to use $30^{\circ}C$ as a condition. It would have been interesting to incorporate this condition as it has been shown to increase diversity to include species such as *Actinomyces naeslundii, Gemella haemolysans, Gamella sanguinis,* and *Kocuria rhizophila*, all of which are previously unreported in this thesis (195).

 Despite EQUC showing increased diversity, there were multiple discrepancies in the CFU count between sediment culture and EQUC suggesting that centrifuging urine skews data and does not give a true representation of the species in the urine. As mentioned in Hilt *et al's* study; centrifuging urine increases the yield of bacteria which are attached to urothelial cells (195). Unless culture is to be followed with an antibiotic protection assay, the sediment culture method should not be used to quantify planktonic bacteria in urine samples.

This was a small study including only 3 samples, of which only 2 had positive culture. This is a very small sample size for comparing methodologies however it was adequate for our laboratory to determine the effectiveness of EQUC compared to NGS and sediment culture. Therefore, for future studies within our laboratory, the following conditions will be used; i) chocolate agar incubated for 48 hours at 37°C in 5% CO_2 and ii) 48 hours at 37°C in aerobic conditions, iii) fastidious anaerobic agar (FAA) for culture in anaerobic and iv) campygas (5% O_2, 10% CO_2, 85% N) conditions, both at 37°C for 48 hours.

5.5.3 SELECTING DIFFERENT PRIMERS

The results from the *Lactobacillus* concordance study showed that the original primers used in Chapters 3 and 4 were not identifying all *Lactobacillus* species correctly. Where *L.psittaci* was identified with the original primers, these isolates should have been *L.jensenii* as identified by the new primers. This is also the case with *L.hominis,* which should have been identified as *L.gasseri*. By amending isolates originally identified as *L.psittaci* and *L.hominis* to *L.jensenii* and *L.gasseri,* respectively, the *Lactobacillus* isolates from asymptomatic controls (figure 5.1) correlate better with findings from other studies showing that the most common *Lactobacillus* species are *L.iners, L. crispatus, L.gasseri* and *L.jenesenii,* where *L.hominis* is not included (203, 244)*.* This could be due to the fact that the original primers are designed to select for the V9 region of the 16s rRNA gene which is not the most suitable for species identification, but is the most highly variable region. Both sets of new primers appear to be more accurate since the species ID from both of these primers are concordant with each other and correlate more accurately with results from other studies. Since 'primer set 1' were *Lactobacillus* specific yet 'primer set 2' generated the same species identity but are genus wide, the latter primers were preferred for future 16s rRNA sequencing on isolates captured from EQUC.

5.5.4 A PHYLOGENETIC TREE CONFIRMS CORRECT IDENTIFICATION

The inclusion of a phylogenetic tree has further confirmed that the sequences generated are correct since the BLAST result for each isolate correlates with the groupings seen in the tree *e.g.* all the *L.jensenii, L gasseri* and *L.*iners are grouped together as expected, with the exception of only one outlier.

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5.6 CONCLUSIONS

1. Methods for detecting bacteria in urine adopted by Eurofins Genomics are not sensitive enough to detect very low CFU/ml and therefore this method is not best suited to future projects.

2. Inclusion of aerobic and campygas conditions and the use of fastidious anaerobic agar for anaerobic conditions should be included in the culture methods of future studies.

3. Spun urine creates bias for cell-associated bacteria and therefore unspun urine should be used for culture in future projects.

4. Alternative universal primers are better able to identify species to a species level than those used in Chapter 3 and 4.

5. Despite the use of less-efficient primers, our data from Chapter 3 and 4 still qualifies since these primers were able to identify bacteria to genus level.

CHAPTER 6 - RESULTS 4
THE ROLE OF PERICYTES IN BLADDER INFECTION

6.1 INTRODUCTION

6.1.1 PERICYTES AND MYOFIBROBLASTS

As has been discussed in the general introduction, pericytes are present in the lamina propria of the bladder, shown through immunohistochemistry staining with α-SMA, NG2, c-kit and PDGFRβ in mouse bladder (50). They have been shown to transdifferentiate into myofibroblasts (46), which contribute to collagen deposition, scarring and tissue remodelling, all of which have been observed in OAB (44). This chapter focuses on how a low level bladder infection could be leading to the symptoms of OAB by stimulation of pericytes. Pericytes have not only been shown to respond to vasoactive compounds, but also LPS and cytokines, which is discussed in more detail in the following section and provides a sound basis for our hypothesis.

6.1.2 ROLE OF PERICYTES IN INFECTION IN OTHER ORGANS

Pericytes have been shown to express TLR4 (257), the principal receptor for LPS, in both the lung and the brain and a number of studies have investigated the effects of LPS on pericytes and associated downstream signalling, cytokine production and contractile abilities of pericytes (54, 257-269).

6.1.2.1 PERICYTES RESPOND TO LPS BY RELEASING CYTOKINES AND COX-2

Stimulation of rat lung pericytes *in vitro* with 10-100ng/mL LPS leads to increased expression of the pro-inflammatory transcription factor NF-κB and IL-1α, IL-1β and TNF-α mRNA measured by reverse transcriptase PCR (RT-PCR) of pericyte cytoplasm and immunoblotting of supernatant (257, 259). A similar study on isolated TLR4^{+} brain pericytes measured 23 cytokines and chemokines by ELISA (263). Expression of the following cytokines and chemokines was increased post stimulation with LPS: - IL-3, IL-10, IL-13, MCP-1, GM-CSF and CXCL10, CCL20, CXCL1, CCL2 and CCL5 respectively (figure 6.1A). Another study has performed a similar experiment on brain pericytes with LPS and found that LPS increased pericyte production of IL-1α, TNF-α, IL-3, IL-9 and IL-13 (265). These two studies highlight the diversity of

downstream cytokines released from pericytes and the importance of pericytes during infection for upregulating chemokines necessary for migration of monocytes, neutrophils and other immune cells. Furthermore, it has been shown that downstream signalling from LPS to pro-inflammatory cytokines is TLR-4 and NF-κB dependant in brain pericytes (263).

6.1.2.2 LPS RELAXES PERICYTES AND INDUCES APOPTOSIS

Three very different studies have indicated that LPS has a relaxatory effect on lung pericytes (261, 264, 267). One of these studies investigated the contractile ability of isolated lung pericytes on collagen lattices in culture (261). After incubation with LPS the percentage contraction of these lattices was significantly reduced. This contractile reduction was not altered with the addition of various inhibitors of iNOS indicating that LPS relaxation of pericytes occurs in an iNOS independent mechanism (261). The second of these studies showed through patch clamping experiments that LPS leads to the opening of pericyte K^+ channels, which in turn could be causing relaxation in the pericyte cells (264). Finally, a recent study in 2015 by Zeng et al showed that by injecting mice with LPS and then staining sections of lung tissue with NG2 (pericytes) and isolectin 4 (vasculature), it was possible to quantify pericyte loss and pericyte/capillary coverage (267). Mice injected with LPS had significantly reduced pericyte density along microvessels in their lungs (267). LPS has also been shown to induce pericyte apoptosis by activating caspase-3 shown by increased p11 and p17, which may explain the reduced pericyte density in the previous study (268).

The resulting effect of LPS relaxing pericytes might increase vascular permeability to innate leukocytes migrating to the site of infection. One particularly interesting publication has highlighted that pericytes stimulated with LPS upregulate ICAM-1, a protein essential for innate leukocyte extravasation. Furthermore, this study indicates that not only do pericytes facilitate in extravasation by increasing vascular permeability, but they also instruct leukocyte migration and cell survival by providing essential signals (270).

6.1.2.3 PERICYTES RESPOND TO TNF-α AND IL-1β

Interestingly, two of the afore mentioned downstream cytokines, IL-1β and TNF-α, have also been investigated on their specific role on pericytes in both the lung and brain (262, 266, 269). One study has shown that TNF-α can induce further cytokine

production particularly MIP-1α, IL-6 and IL-1β (266), which can induce acute phase response, macrophage activation, pericyte relaxation and leukocytosis (figure 6.1B, table 6.1). Another study by Pieper et al showed that presence of TNF-α and IL-1β led to pericyte phagocytosis, and increased iNOS mRNA and COX-2 mRNA production in pericytes (269). iNOS leads to nitric oxide synthesis and COX-2 leads to vasodilation and activation of prostaglandins, both of which are essential in pro-inflammatory responses (figure 6.1A, table 6.1). Another exciting study by Kerkar et al, who in 2005 investigated the role the downstream cytokines IL-1β and TNF-α on lung pericyte function, showed that TNF-α stimulation of lung pericytes leads to capillary leak and influx of immune cells (262). IL-1β and TNF-α also induced an iNOS mRNA increase in turn leading to RONS production, increased expression on ICAM-1 (essential for immune cell infiltration), and increased production on IL-1β, IL-6, G-CSF and CCL5 (figure 6.2B, table 6.1). Taken together these studies suggest that pericytes respond in similar ways to IL-1β and TNF-α irrespective of the resident tissue.

To summarise, LPS and the downstream pro-inflammatory cytokines IL-1β and TNF-α can lead to the production of a variety of pro-inflammatory cytokines (262, 263, 265, 266) via activation and translocation of NF-κB to the nucleus (263), production of chemokines(54, 263, 266)leading to immune cell infiltration to the site of infection (54, 262), activation of COX-2 leading to vasodilation (269), as well as phagocytosis (269), apoptosis (268), and pericyte loss (267). Taken together these studies suggest that pericytes are instrumental to the development of localised inflammation but may be able to dissociate from endothelial cells and migrate elsewhere; potentially differentiating into myofibroblasts leading to fibrosis during chronic inflammation.

A)

LPS TLR-4

Pericytes become phagocytic/
pericyte loss

Nitric oxide

NF-κB

COX-2

Cytokines
IL-1α, TNFα, IL-6
IL-3, IL-10, IL-13,
MCP-1, GM-CSF

Chemokines
IL-8
CXCL1, CXCL10
CCL2, CCL20

B)

TNFα/
IL-1β

ICAM-1

COX2
mRNA

iNOS
mRNA → RONS

Cytokines
IL-1a, IL-6
RANTES, G-CSF
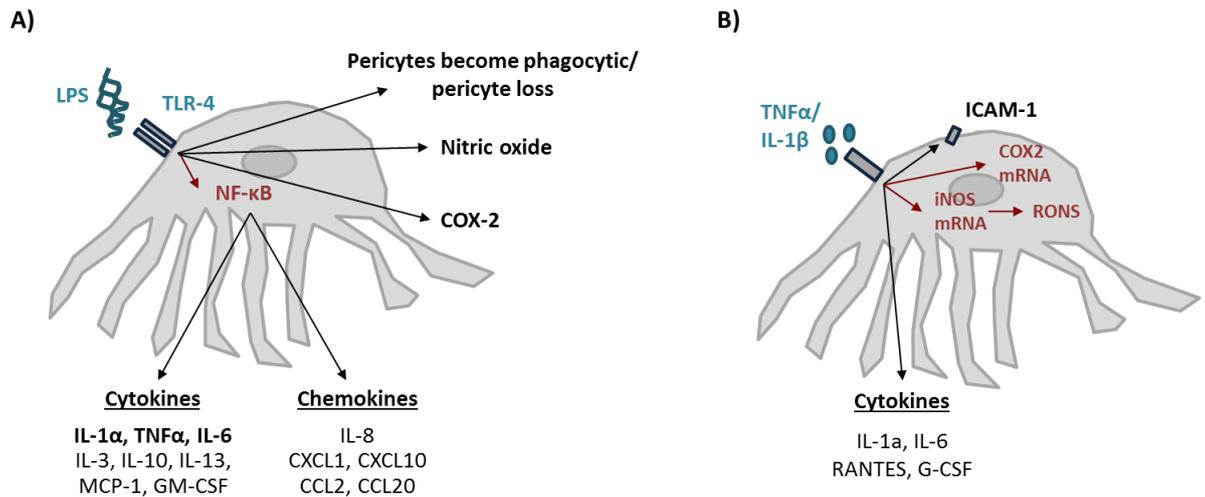
Figure 6.1: Schematic of downstream effects of LPS binding to TLR-4 on pericytes and TNFα and IL-1β stimulation of pericytes. A) LPS from Gram-negative bacteria binds to TLR-4 on pericytes and leads to nitric oxide and COX-2 production (263), as well upregulation of the transcription factor NF-κB (263) leading to production of a multitude of cytokines and chemokines, in particular IL-1α, TNFα, IL-6 and IL-8 (54, 263, 265). LPS stimulation also leads to pericytes becoming phagocytic (269) and pericyte loss (267). **B)** TNFα and IL-1β stimulation of pericytes acts in a positive feedback loop to produce more pro-inflammatory cytokines such as IL-1α and IL-6 (262, 266), and upregulates ICAM-1 facilitating extravasation of leukocytes (262). RONS and mRNA for iNOS and COX2 have also been identified in pericytes stimulated by TNFα and IL-1β (269).

Downstream	Function
Pro-inflammatory cytokines:- IL-6, TNF-α, IL-1α and IL-1β	Acute phase response (C-reactive protein, serum amyloid A, fibrinogen, mannose binding protein, complement). Macrophage activation. Pericyte relaxation. VEGF Leukocytosis (increased production on white blood cells) Prostaglandins → fever.
IL-8	Neutrophil chemotactic factor.
COX-2	Vasodilation COX-2 inhibitors Prostaglandin-E2, thromboxane A2.
iNOS	Nitric oxide synthesis.
IL-12 and IFN-γ	Activation of Type 1 T-helper cells → Adaptive immunity
MCP1	Monocyte chemotaxis. Macrophage activation. Myofibroblasts differentiation.

Table 6.1: Function of downstream compounds released by pericytes. Pro-inflammatory cytokines such as Il-6, IL-1β and TNFα lead to acute phase response, leukocytosis and pericyte relaxation, which, along with IL-8 and MCP-1's chemotactic properties and vasodilation caused by COX-2, will lead to influx of immune cells to the site of infection. Inducible nitric oxide synthase synthesises nitric oxide which acts as a defence mechanism against pathogens. COX-2 further acts to breakdown arachidonic acid into prostaglandins and thromboxane A2, which can stimulate smooth muscle contraction or dilation.

6.1.3 VASOACTIVE COMPOUNDS ACTING ON PERICYTES

As mentioned in the main introduction, pericytes are able to control blood flow by responding to various vasoactive compounds. These compounds may be endogenous to the body or a pharmaceutical drug. In order to form a better understanding of the role of pericytes in the bladder it is essential to determine which vasoactive compounds pericytes are able to respond to and to what extent by creating experiments where responses can be measured and quantified. The responsiveness of perictyes to various vasoactive agents such as angiotensin II, noradrenaline, endothelin, indomethacin, and ATP, and vasodilatory agents such as prostaglandin E2, NO, bradykinin, and carbachol has been described in the vasa recta of rat kidney by Crawford *et al* (52). Each of these compounds is endogenous in most tissues, although the mechanisms by which they lead to mediated change in vessel diameter in pericytes differs by the signalling pathways they induce. The principle technique used by Crawford *et al* in the kidney was using DIC imaging to acquire images of pericytes in live kidney slices and measuring capillary diameter changes at pericyte sites when tissue is superfused with vasoactive compounds. By adapting this technique it is possible to create a model to investigate the function of pericytes regulating blood flow in the bladder. This can then be expanded upon to investigate the role of IL-1β, TNFα and LPS in regulation of pericytes, which has not yet been explored in the bladder.

6.2 Aims and Hypothesis

We hypothesis that pericytes can react to pro-inflammatory components generated during an immune response to the presence of bacteria in the bladder. We also hypothesise that the pericytes will respond in a measurable contractile manner to control blood flow and, in chronic cases, pericyte loss will occur.

In order to begin to test these hypotheses the following objectives will be to investigate:-

1. Identify whether pericytes are in proximity to the vasculature in murine bladder urothelium by immunohistochemistry.
2. Use DIC imaging to investigate pericyte function by superfusing known vasoactive compounds over isolated murine bladder urothelium and visualising pericyte mediated changes in vessel diameter. .
3. Use the above model to investigate the acute effect of TNFα on murine bladder pericytes and vessel diameter.
4. Determine how prolonged exposure (4 hours) of bladder tissue to LPS, TNFα and IL-1β can affect pericyte density in the murine bladder.

6.3 Methodology

6.3.1 Disclaimer

All experiments were performed by myself.

6.3.2 Ethics

All culls were performed by trained personnel by cervical dislocation in accordance with the Animals Scientific Procedures Act of 1986.

6.3.3 Optimising dissection for DIC imaging

The methods for dissection in *Section 2.2.1 Tissue preparation* and set up of tissue in *Section 2.2.4 DIC imaging of live tissue for DIC imaging* are optimised. Various challenges were met when trying to obtain DIC images of live bladder urothelium. Initially, rat bladder was used (n=8, data not used) but due to the structure of the bladder in this species, the detrusor muscle could not be removed properly, and much

of the muscle tissue would be left behind. This meant the detrusor continually contracted under the objective making imaging impossible. Therefore, the tissue was pinned out with insect pins onto Sylgard® 184 (figure 6.2). Even in cases where an attempt was made to remove the detrusor (n=5), pinning out did not reduce the movement of the tissue making imaging impossible. Upon discussions with collaborators, it was learnt that mouse bladder is easier to dissect and the detrusor could be peeled off in one piece. This meant that pinning out was no longer required and only a harp was necessary to hold the tissue in place under the objective (figure 2.13A). Similar effects on the detrusor were observed with SNP at 100µM (n=1) and 10µM (n=1). In these experiments, the tissue was not pinned out. Therefore, mice were used for all experiments in this chapter as per the methods discussed in *Section 2.2 Bladder pericyte observation and functional experiments.*
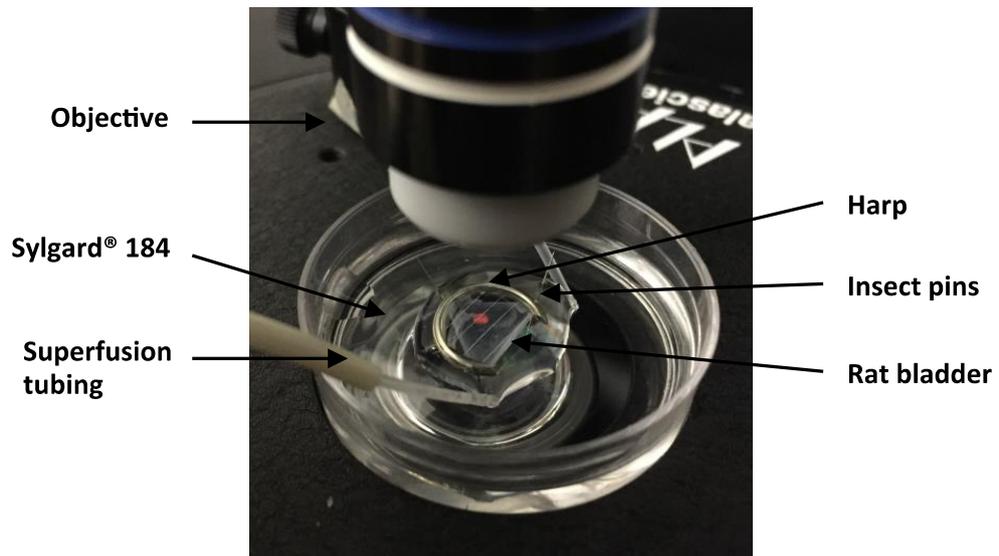
Figure 6.2: Image of DIC set up optimised for rat bladder imaging. Rat bladder detrusor was difficult to remove from the urothelium therefore the tissue contracted under the objective. In an attempt to combat this the rat bladder was pinned down with insect pins into Sylgard® 184 with a harp placed over the top. This set up was then placed on the microscope stage and superfused with physiological saline solution. This set up was not able to reduce rat bladder contraction sufficiently for imaging and murine bladder was used instead as it was easier to dissect.

6.3.4 CALCULATING PERICYTE DENSITY

Four mice were included in the experiment; urothelium was removed from the bladder, and cut into 3 or 4 strips (dependant on bladder size) and pre-incubated with LPS, TNFα, and IL-1β in PSS and a control of just PSS by leaving tissue strips in Nunc wells with the respective cytokines at the concentrations discussed in the General Methodology *Section 2.2.3 Immunohistochemistry NG2/IB4* for four hours prior to staining. This generated a total of n=4 controls, n=3 LPS, n=3 IL-1β and n=3 TNFα. Three images were taken per urothelium strip where staining was clearest. Distances between branch pericytes along vessels was measured in Image J. Pericyte density was calculated per strip and given as a mean distance between pericytes per field of view (390 μm^2), per mouse, per condition (control, LPS TNFα, or IL-1β). By comparing the pericyte number and density across individual mice it was possible to observe variability in pericyte density between conditions in each animal. By comparing across all mice it was possible to determine if differences in pericyte density between conditions were due to chance or were statistically relevant. Statistical significance was calculated using an unpaired student T-test.

6.4 RESULTS

6.4.1 OBSERVATION OF PERICYTES IN THE MURINE BLADDER

By staining murine urothelium with anti-IB4 (vasculature, green) and anti-NG2 (pericytes, red), it was possible to see where pericytes lay in the vasculature and determine average pericyte cell count per 100μm^2 and pericyte density at vessel branch points. Since C-fibres are also IB4 positive, vessels were identified due to their thick network structure and since SMC can also be NG2 positive, pericytes were identified due to their bulbous structure. It can be observed that pericytes tend to be densely populated in the vasculature and tend to be present at all branch points, where they are able to control directional blood flow (figure 6.3A and B). Vessel pericytes are more spaced out along the vessel (figure 6.3C-E).
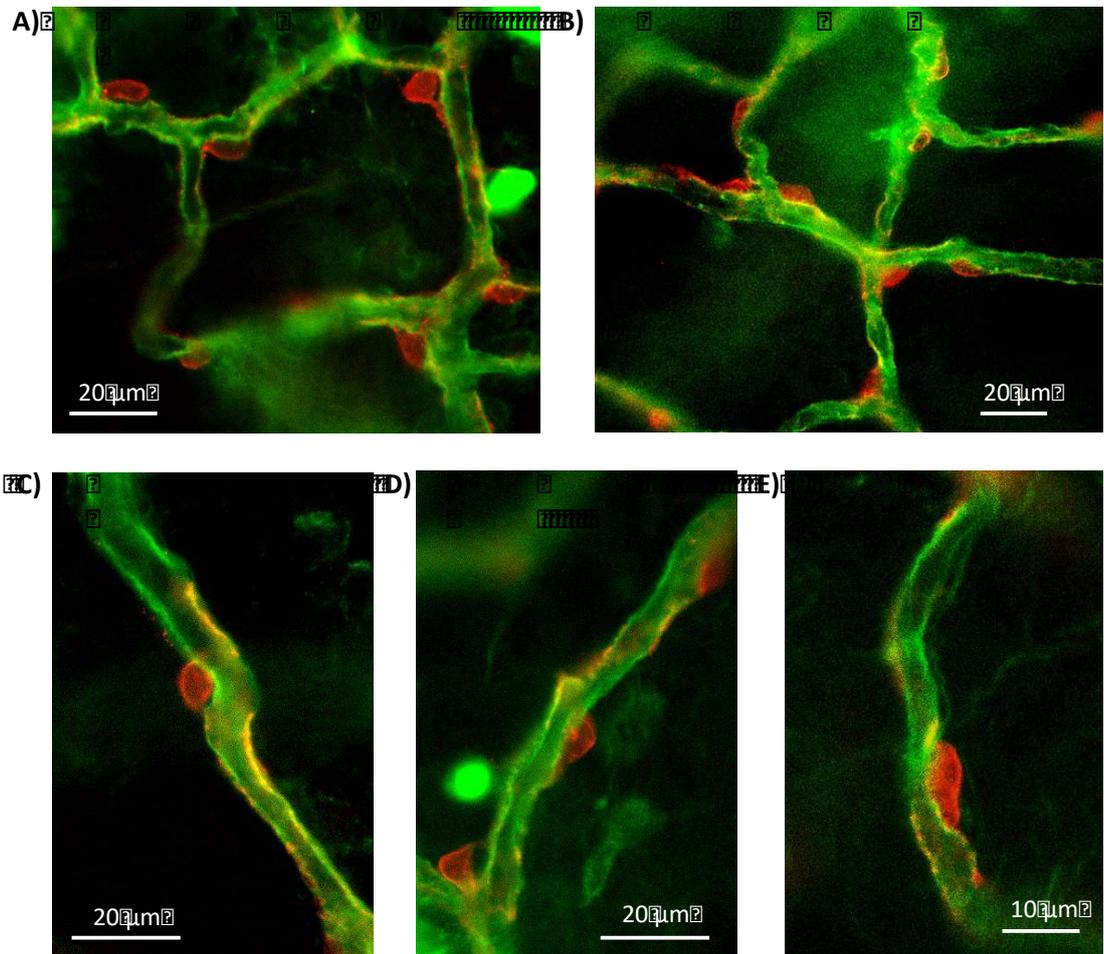
Figure 6.3: Isolectin B4 (IB4) and Neuroglial 2 (NG2) staining of mouse bladder urothelium. IB4 (conjugated with Alexa 488 green) staining for vasculature and anti-NG2 (probed with Alexa 555 conjugate 2° antibody, red) staining for perivascular pericytes in the bladder urothelium of adult male mice shows vast winding capillary network with many pericytes at both branch points and along branches. **A-B)** Network of microvasculature highly populated with pericytes at branch points. **C-E)** Branches of microvasculature are also populated with pericytes periodically spaced along the length.

6.4.2 CALCULATING PERICYTE DENSITY PRE- AND POST-INCUBATION WITH LPS, TNFα AND IL-1β

LPS acts as a potent activator of innate pro-inflammatory responses in many cells including pericytes. TNFα and IL-1β are examples of two cytokines released during these innate pro-inflammatory response which have been shown to act in a paracrine manner to further stimulate pericytes (figure 6.1B). By incubating bladder urothelium for 4 hours in either LPS, TNFα, or IL-1β in PSS prior to staining, it was possible to investigate pericyte responses to Gram negative bacteria and associated downstream cytokines which may be found in the bladder during an infection with a Gram negative species such as UPEC.

Mean number of branch pericytes per field of view (390 μm^2) in control (n=4) is 8.17 per field of view (390 μm^2) (figure 6.4). This is slightly lower in urothelium pre-incubated with LPS for 4 hours in PSS (7 pericytes per field of view (390 μm^2), p= 0.5013). The number of pericytes per field of view (390 μm^2) is significantly reduced when tissue is pre-incubated with TNFα to 5 cells (p=0.04*) and further reduced when pre-incubated with IL-1β to 3.78 cells (p= 0.007**) indicating that these cytokines could be leading to pericyte cell death (figure 6.4). All statistical differences were calculated using an unpaired student T-test.

From mouse 1, the strip incubated with IL-1β had a significantly greater mean distance between pericytes (5.9μm) compared to controls (3.03μm, p=0.006**), whereas only a slight increase is observed with the strips incubated in LPS (3.65μm, p=0.57) and TNFα (3.85μm, p=0.27) (figure 6.5A). From mouse 2, the strip incubated with LPS had a slightly greater mean distance between pericytes (3.98μm) compared to controls (3.52μm, p=0.47), whereas a decrease in mean distance is observed with the strip incubated with TNFα (p=0.23) (figure 6.5B). From mouse 3, mean distance between pericytes remained relatively similar across control (2.75μm), IL-1β (3.1μm), and TNFα (2.77μm)(all p>0.05) (figure 6.5C). From mouse 4, the strip incubated with IL-1β had a slightly greater mean distance between pericytes (3.91μm) compared to controls (3.10μm, p=0.30), and an even slighter increase in mean distance is observed with the strip incubated with LPS (3.56μm, p=0.23) (figure 6.5D).

Comparing all mice cumulatively, there is a significantly greater mean distance between pericytes in strips incubated in only PSS (3.02μm, n=4) and those incubated in IL-1β (3.76μm, n=3)(p=0.014*) and LPS (3.78μm, n=3)(p=0.031*). Mean distance between pericytes is not significantly greater in strips incubated with TNFα (3.06μm) but it is slightly greater than that of controls (p=0.91, figure 6.5E).
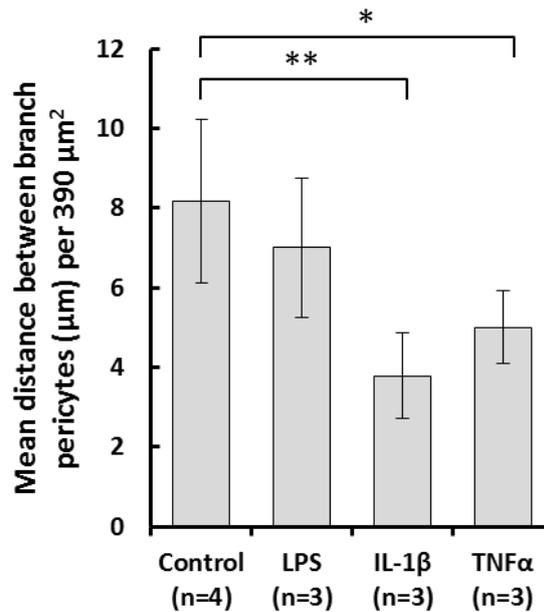
Figure 6.4: Mean number of branch pericytes per 390 μm² in mouse bladder urothelium post-incubation with LPS, IL-1β and TNFα. Mean number of branch pericytes in control (n=4) is 8.17 per field of view. This is lower but not significantly lower in urothelium pre-incubated with LPS for 4 hours in PSS (7 pericytes per field of view, p= 0.5013). The number of pericytes per field of view is significantly reduced when tissue is pre-incubated with TNFα to 5 cells (p=0.04*) and further reduced when pre-incubated with IL-1β to 3.78 cells (p= 0.007**) indicating that these cytokines could be leading to pericyte cell death. Statistical significance noted as * = p<0.05, ** p<0.01 calculated with an unpaired student T test.
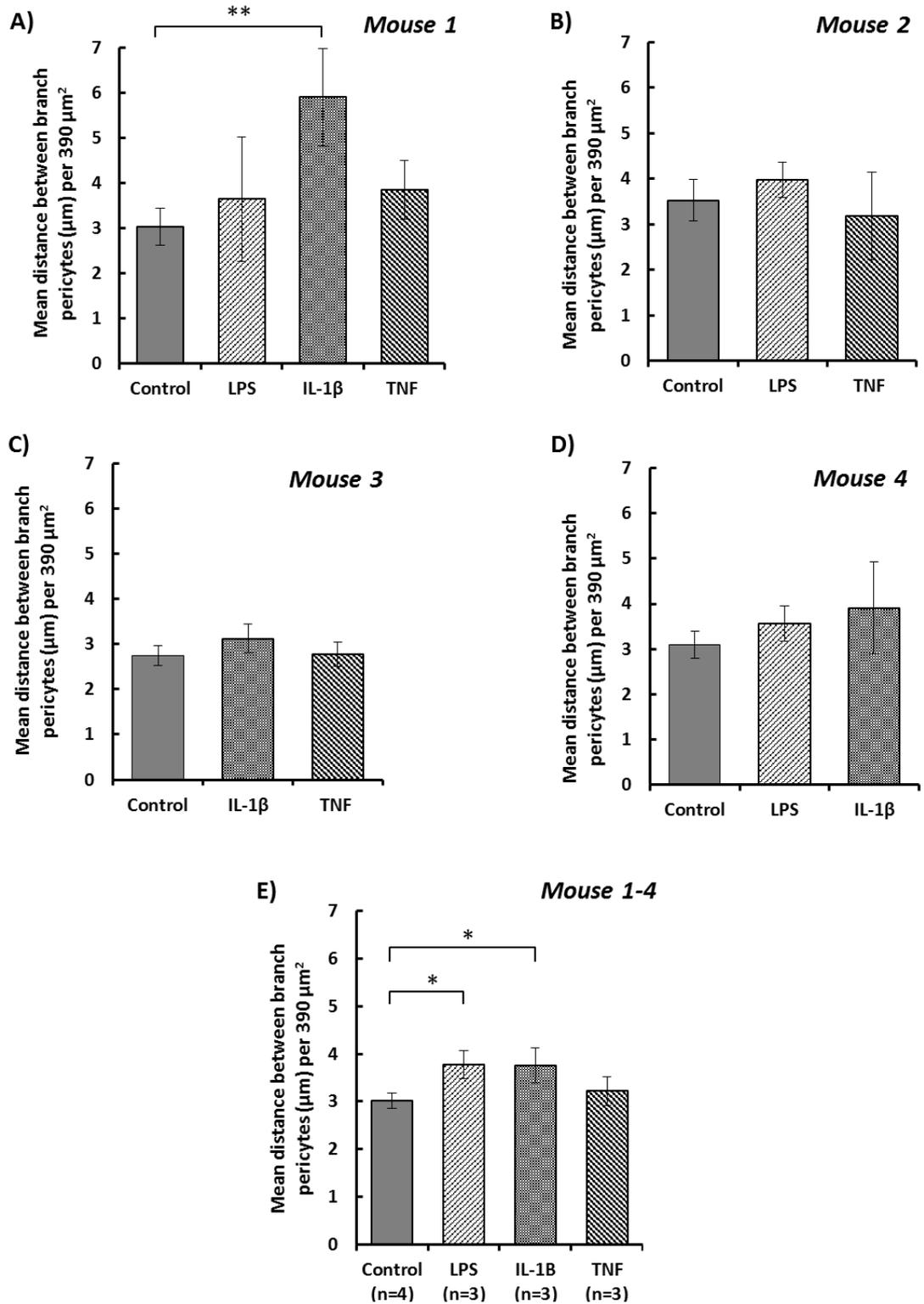
Figure 6.5: Mean distance between branch pericytes per 390 μm² in mouse bladder urothelium post-incubation with LPS, IL-1β and TNFα. Mean distance between branch pericytes in controls varies from 2.75 to 3.52μm with a mean on 3.02μm (n=4). When pre-incubated for 4 hours with LPS the mean distance increases slightly in all mice and significantly increases to 3.78μm when data is pooled (n=3, p=0.014*). When pre-incubated for 4 hours with IL-1β the mean distance increases slightly in two mice and increases significantly in one mouse from 3.02μm in control to 5.91μm in IL-1β. When data is pooled for all mice, mean distance increases significantly from control to IL-1β (3.76μm, n=3)(p=0.031*). Mean distance between pericytes pre-incubated with TNFα is not significant however trends to be slightly increased. Statistical significance noted as * = p<0.05, ** p<0.01 calculated with an unpaired student T test.

6.4.3 Pericyte functional responses to Angiotensin II

Angiotensin II is one of the most potent vasoconstrictor compounds found endogenously throughout tissues. It is a member of the renin-angiotensin system and is produced by cleavage of angiotensin I by angiotensin-converting enzyme. It plays a role in vasoconstriction and increased blood pressure throughout the body, and in the bladder it has been shown to increase smooth muscle cell growth and hyperplasia, as well as increased collagen deposition (271).

DIC imaging of live bladder urothelial preparation with addition of angiotensin II showed that at concentrations of 100 nM 10 nM and 1 nM, vessels constricted in response to the drug with varied response rates (table 6.2). Responses to angiotensin were highest (100%) with an intermediate concentration of angiotensin of 10 nM (n=3) (table 6.2) reducing to 50% with 100 nM (n=10) and 33.3% with 1 nm (n=6). Where there was a notable percentage response rate, pericyte sites were clearly visible as the areas of maximal constriction (figure 6.6E). In responsive tissues, higher concentrations of angiotensin II induced a more rapid constriction; on average within 76.6 seconds after the drug is added to the tissue (n=3) compared to 122.75 seconds with 10 nM (n=3) and 157.5 seconds with 1 nM (n=2) (table 6.2, figure 6.6A). Tissues responding to higher concentrations angiotensin II also reached maximal constriction faster, 98.3 seconds with 100 nM (n=5) compared to 218.25 seconds with 10 nM (n=3) and 395 seconds with 1 nM (n=2). The maximal constriction of the vessels was not concentration dependant. An overall maximal constriction of Δ78.15% of original vessel diameter was reached using 10 nM (p=0.05*) angiotensin II, however this was only Δ81.19% with 100 nM (p=0.004**) and Δ83.29% with 1 nM (P=0.03*) (figure 6.6D). These differences are considered significant by a paired Student T test.

Ang II concentration	Drug response rate	Time to induce constriction	Time to max constriction	% max constriction	Time to desensitisation	% of Δ diameter at end of video
100 nM	5/10 50%	76.6 s	98.3 s	81.19%	100 s	95.98%
10 nM	3/3 100%	122.75 s	218.25 s	78.15%	224.5 s	88.29%
1 nM	2/6 33.3%	157.5 s	395 s	83.29%	N/A	80.43%

Table 6.2: Drug response rate, the time taken for Angiotensin II to induce constriction, reach maximal constriction and become desensitised. Time to induce constriction post application of Ang II increases from a mean 76.6 seconds (n=5) with 100 nM Ang II to a mean 157.5 seconds with 1 nM (n=2). Time to maximal constriction also increases as concentration increases. In experiments performed with 100 nM Ang II the tissue desensitizes (vessel diameter returns to normal before drug is removed) in only 100 seconds post-application. As the concentration is lowered, the tissue desensitizes slower and with 1 nM does not desensitize. Overall maximal vessel constriction is obtained by application of 10 nM Ang II (78.15% original diameter)(n=3). % diameter of original diameter at the end of the video indicates that application of higher concentrations allows vessels to return to original diameter (95.98%), likely due to desensitisation, compared to lower concentrations such as 1 nM which remain at 80.43% constriction.

In all responding tissues at 100 nM and 10 nM, cells become desensitised to the drug allowing the vessel diameter to begin to return normal diameter before the drug is removed. (figure 6.6A-B). Time to desensitisation is measured from the point where constriction is reversed and the vessel begins to return to normal diameter. On average, desensitisation takes 100.0 seconds from when the drug is first added and 23.3 seconds since constriction begins with 100 nM angiotensin (n=3) (table 6.3, figure 6.6A). This time is slower with a lower concentration of 10 nM (224.5 seconds from when the drug is added). At the lower concentration of 10 nM, angiotensin II vessel constriction takes longer to respond (figure 6.6B), however constricts more than with 100 nm. When the drug is removed the vessel diameter does not return to normal as with 100 nM (figure 6.6B). Furthermore, since there is more constriction at pericyte sites, non-pericyte sites are also affected so that the difference between the constriction at pericyte sites and non-pericyte sites is less significant.
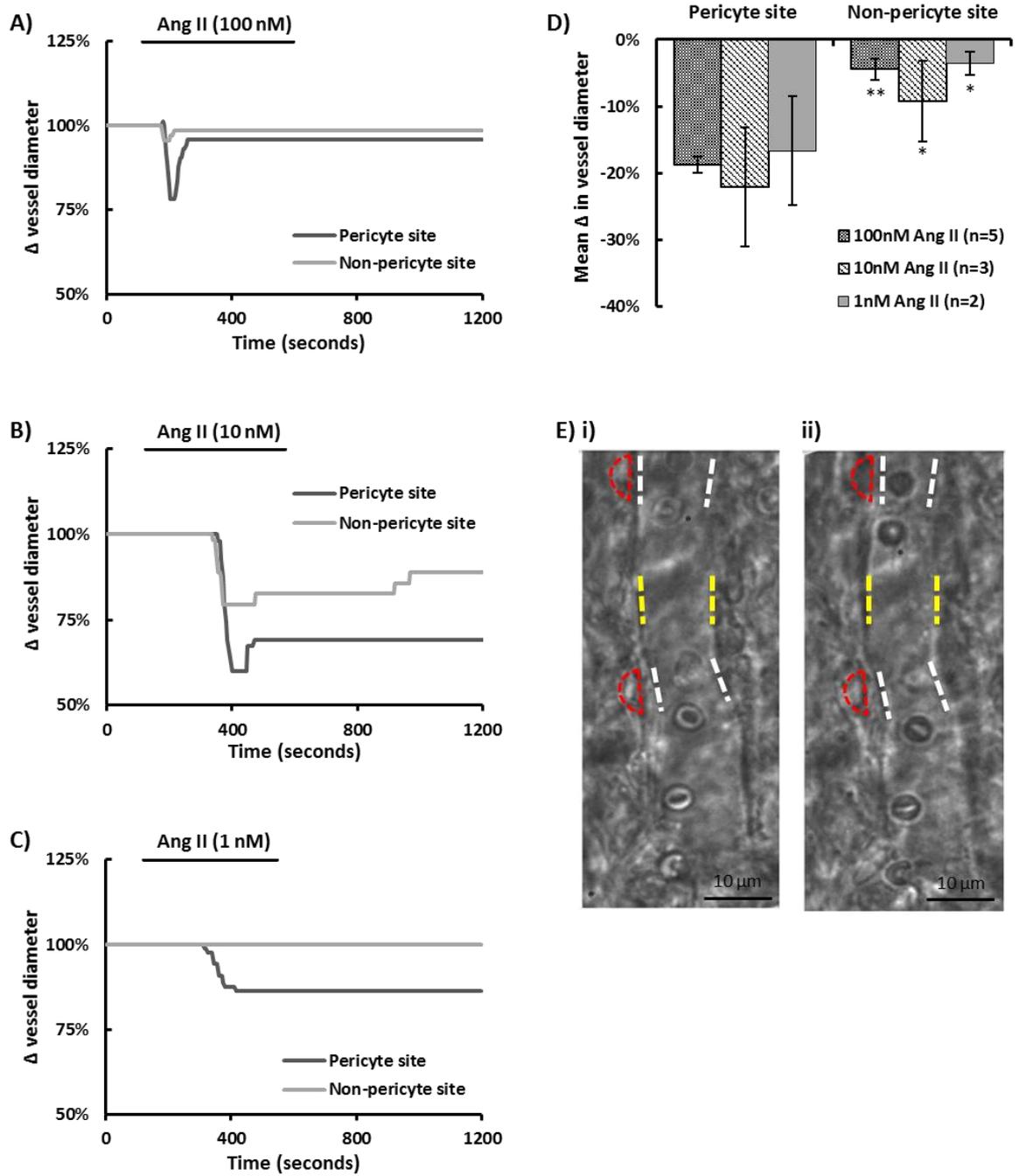
Figure 6.6: Effect of vasoactive compound Angiotensin II on diameter of bladder urothelium microvasculature. Superfusion of Angiotensin II (Ang II) over live mouse bladder urothelium. **A)** 100 nM Ang II induces a rapid response constricting the capillary which returns to baseline before Ang II is removed indicating drug desensitisation at higher concentration. **B)** 10 nM Ang II induces a delayed response constricting the capillary which reverses when Ang II is removed but does not return to baseline. **C)** 1 nM Ang II induces a delayed response constricting the capillary which stops constricting when Ang II is removed but does not reverse. **D)** Maximal constriction is obtained by application of 10 nM Ang II (-16.71%, n=3) (p=0.05*), however this concentration also affects non-pericyte sites more than other concentrations. The most significant change in diameter was with the higher concentration of 100 nM (p=0.004**). Statistical significance noted as * = p<0.05, ** p<0.01 calculated with a paired student T test. **E)** Example of DIC images of capillary i) pre- and ii) post- 10 nM Ang II addition. Pericytes are indicated by red dotted circles, white dotted lines indicate where maximal constriction/dilation occurs at pericyte sites, and yellow dotted lines indicate non-pericyte region of the same vessel.

6.4.4 PERICYTE FUNCTIONAL RESPONSES TO ATP

Not only is ATP an important signalling molecule in normal bladder function but it has also been shown by many studies to be increased in patients with overactive bladder (17, 18, 72, 272). ATP binds to P2 receptors on afferent nerves, myofibroblasts, smooth muscle cells, and urothelial cells with in the bladder and can induce constriction in pericytes (52), and is therefore of particular interest to this study. DIC imaging of live bladder urothelial preparation with addition of ATP showed that at concentrations of 1 mM and 0.1 mM, vessels constricted in response to the drug with response rates of 33.3% and 25%, respectively (table 6.4). Responses to ATP were highest (33.3%) with a higher concentration of ATP of 1 mM (n=9), reducing to 25.0% with 0.1 mM (n=8). Where tissues responded, pericyte sites were clearly visible as the areas of maximal constriction (figure 6.7D).

In responsive tissues, the higher concentration of ATP induced a more rapid constriction; on average within 73.3 seconds after 1 mM is added to the tissue (n=3) compared to 252 seconds with 0.1 mM (n=2)(table 6.4, figure 6.7A-B). Furthermore, in the higher concentration of 1 mM, constriction reaches a maximum faster; 685 seconds compared to 757 seconds with 0.1 mM ATP (table 6.4). (table 6.4, figure 6.7A-B). Tissues responding to the higher concentration of ATP also reached maximal constriction faster, 685 seconds with 1 mM (n=3) compared to 757 seconds with 0.1 mM (n=2). The maximal constriction of the vessels was concentration dependant. An overall maximal constriction of Δ82.28% of original vessel diameter was reached using 1 mM ATP (p=0.0006***), however this was slightly lower at Δ86.40% with 0.1 mM (p= 0.1294, figure 6.4C). Significance is determined by a paired Student T test.

In 4 of the 5 responding tissues at 1 mM (n=2) and 0.1 mM (n=2), pericytes continue to constrict once the ATP has been removed (figure 6.7B). For this reason, in these tissues the maximal constriction is the same as the % constriction at the end of the video. In the one remaining video, cells become desensitised to the drug (1 mM) allowing the vessel diameter to begin to return normal before the drug is removed (figure 6.7A). Since only one of five experiments shows desensitisation, the time to desensitisation is not be included for ATP.

ATP concentration	Drug response rate	Time to induce constriction	Time to max constriction	% max constriction	Time to desensitisation	% of Δ diameter at end of video
1 mM	3/9 33.3%	73.3 s	685 s	82.28%	N/A	85.41 %
0.1 mM	2/8 25.0%	252 s	757 s	86.41 %	N/A	86.41 %

Table 6.3: Drug response rate, the time taken for ATP to induce constriction and reach maximal constriction. Time to induce constriction post application of ATP increases from a mean 73.3 seconds (n=3) with 1 mM ATP to a mean 252 seconds with 0.1 mM (n=2). Time to maximal constriction also increases as concentration increases. In 2/3 experiments performed with 1 mM ATP and 2/2 with 0.1 mM ATP the tissue continues to constrict once the drug has been removed (vessel diameter returns to normal before drug is removed) in only 100 seconds post-application Overall maximal vessel constriction is obtained by application of 1 mM ATP (82.28% original diameter)(n=3). % diameter of original diameter at the end of the video indicates that at a lower concentration the maximal diameter remains the same until the end of the video even once the drug is removed.
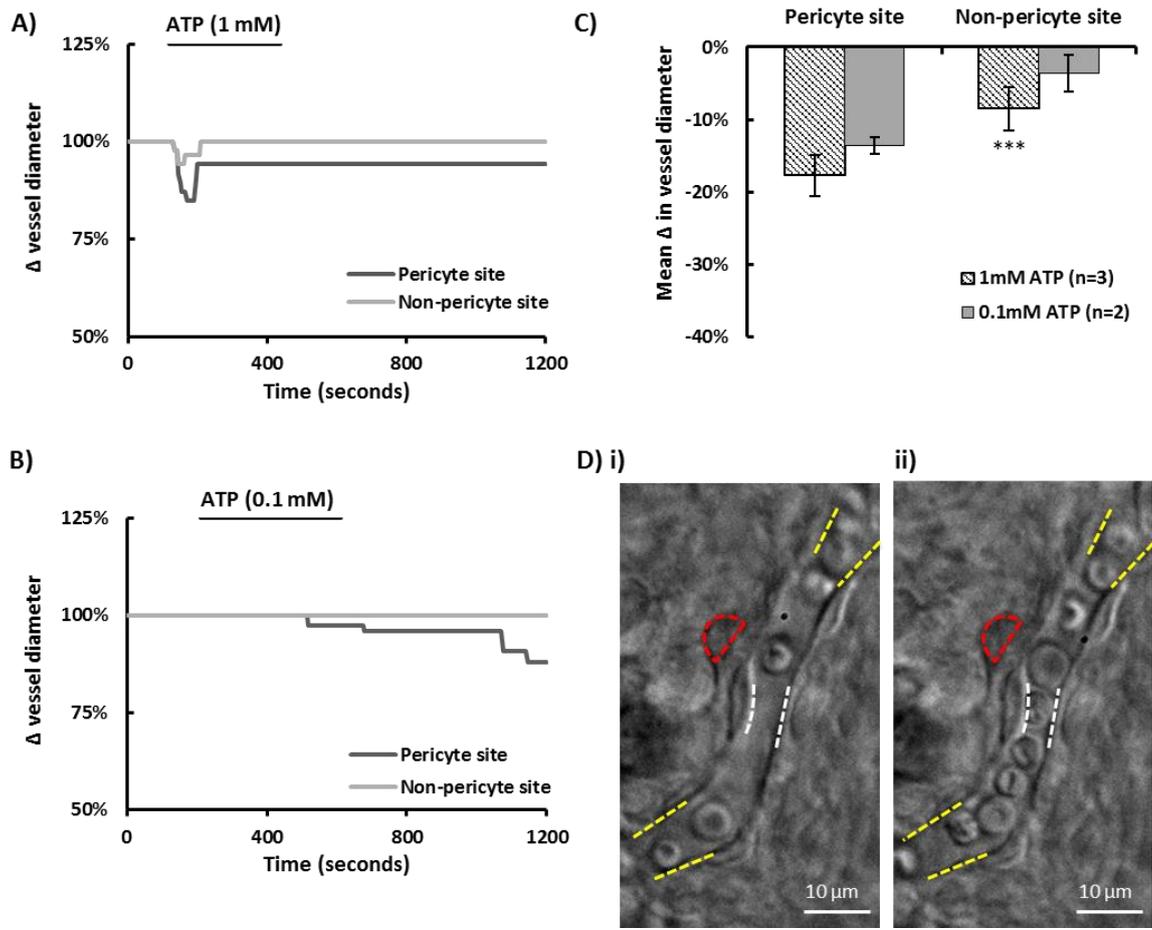
Figure 6.7: Effect of vasoactive compound ATP on diameter of bladder urothelium microvasculature. Superfusion of ATP over live mouse bladder urothelium. **A)** 1 mM ATP induces a rapid response constricting the capillary which begins returns to baseline before ATP is removed indicating drug desensitisation at this concentration. **B)** In 2/3 experiments performed with 1 mM ATP and 2/2 with 0.1 mM, ATP induces a delayed response constricting the capillary which does not stop constricting when ATP is removed and does not reverse. **C)** Maximal constriction is obtained by application of 1 mM ATP (-17.72%, n=3) (p=0.0006***), however this concentration also affects non-pericyte sites more than other concentrations. Statistical significance noted as *** = p<0.001 calculated with a paired student T test. **D)** Example of DIC images of capillary i) pre- and ii) post- 0.1 mM ATP addition. Pericytes are indicated by red dotted semi-circles, white dotted lines indicate where maximal constriction occurs (1200 frames) at pericyte sites, and yellow dotted lines indicate non-pericyte region of the same vessel.

6.4.5 PERICYTE FUNCTIONAL RESPONSES TO BRADYKININ

As well as using vasoconstrictor drugs to determine whether bladder pericytes constrict in same way as kidney ones, it is necessary to perform experiments using vasodilators. Bradykinin is a member of the kinin system and plays a role in blood pressure regulation by causing vasodilation and increasing vascular permeability (reviewed in (273)). Bradykinin also causes contraction of non-vascular smooth muscle in the gut and lung of most animals (274).

DIC experiments with bradykinin were challenging since this drug appeared to have contractile effects upon the detrusor muscle as well as the vessels. Despite peeling the urothelium away from the detrusor during dissection, small muscle fibres still remained attached to the lamina propria. Upon addition of bradykinin, the tissue appeared to be pulled towards and moved from focus making imaging impossible (figure 6.8). Initially, bradykinin was used at a concentration of 1µM (n=1), to which the smooth muscle responded very rapidly, contracting within 8 seconds (table 6.4). The concentration of bradykinin was reduced to 0.5µM (n=1), 100nM (n=1), 10nM (n=1) and then further to 1nM (n=2) but even at the reduced concentrations, the effect was the same, however the muscle took longer to respond as concentration reduced, with exception to the single responding experiment with 1nM which can be taken as an anomaly (table 6.4). Despite other functional experiments with angiotensin II, ATP and SNP not requiring pinning out on Sylgard® 184 (figure 6.2), this method was revised for bradykinin for 100nM (n=2), 10nM (n=1) and 1nM (n=1) but did not offer sufficient prevention of tissue movement in order to measure any changes in vessel diameter. In two of the three experiments using 1nM bradykinin there was no effect on the muscle fibres but also none on the vessel, suggesting the tissue may have died by being over stretched or that this concentration was insufficient to generate responses in the vessel.

Bradykinin concentration	Response rate	Average time to induce constriction post-drug
1 μM	1/1 (100%)	8 seconds
0.5 μM	1/1 (100%)	25 seconds
100 nM	3/3 (100%)	40 seconds
10 nM	2/2 (100%)	65 seconds
1 nM	1/3 (33%)	35 seconds

Table 6.4: Bradykinin response rate, the time taken for the drug to induce muscle constriction. Smooth muscle attached to the mouse urothelium contracted with the addition of bradykinin. With the highest dose used of 1 μM the average time to constriction was 8 seconds. As the dose was reduced the average time to constriction increased in a dose dependant manner. Even at concentrations of 10 nM the smooth muscle still contracted too much to maintain focus on the tissue. At the lowest dose used of 1 nM the response rate reduced to 33.3% (n=3), however in the two non-responding videos there was also no response in the vessels.
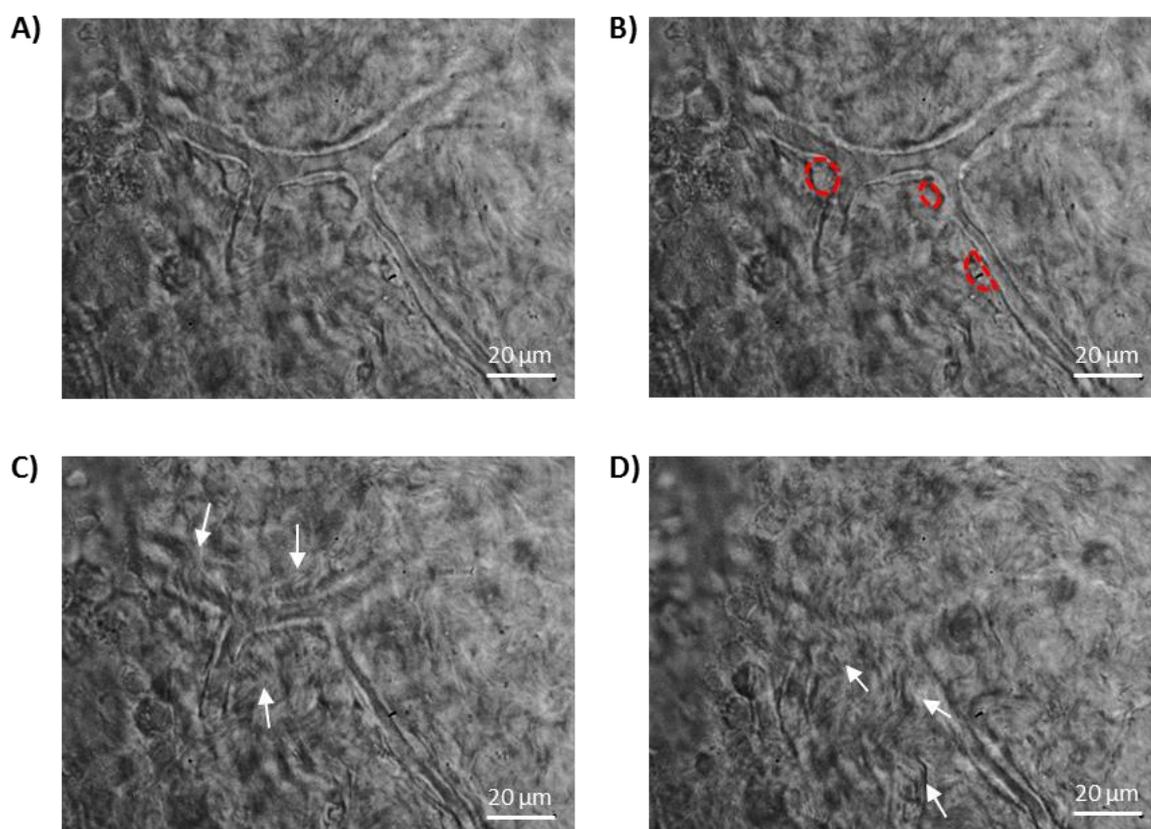
Figure 6.8: Image showing smooth muscle contraction upon addition of 1 nM Bradykinin. Singular images from DIC experiments pre and post drug application by superfusion. **A)** Snapshot of bladder urothelium at frame 1 pre-drug application. **B)** Snapshot of bladder urothelium at frame 1 pre-drug application with red dotted areas indicating pericyte location. **C)** Snapshot of bladder urothelium 45 seconds post-drug application. White arrows indicate direction of muscle contraction. **D)** Snapshot of bladder urothelium 60 seconds post-drug application. White arrows indicate direction of muscle contraction.

6.4.6 PERICYTE FUNCTIONAL RESPONSES TO NO

Nitric oxide signalling plays a valuable role in bladder function as a neurotransmitter stimulating myofibroblasts and afferent nerves during bladder filling, however it is unstable and only exists as a gas in standard conditions; therefore, it is necessary to use NO donors to experiment with this compound. Sodium nitroprusside (SNP) is one such NO donor. SNP also appeared to have contractile effects upon the detrusor muscle as well as the vessels, as was seen with bradykinin. Even at very small concentrations such as 10 μM SNP has a very strong affect on the smooth muscle in the bladder, which means that unless the entirety of the muscle is removed, the response of pericytes to SNP cannot be measured (figure 6.9). It can be seen in figure 6.9A and B that pericytes can be clearly observed (red dotted areas, figure 6.9B), which become harder to observe post SNP application (figure 6.9C-D) as the detrusor muscle contracts and the tissue moves out of focus (figure 6.9B), which cannot be regained by refocusing the microscope. According to the literature SNP relaxes smooth muscle (275), however the videos taken here appear to show contraction.

S-Nitroso-N-acetyl-DL-penicillamine (SNAP) is another example of an NO donor used in physiological experiments. Interestingly, SNAP, did not have the same muscle contractile effect on the detrusor muscle. A 0% response rate was observed on both vascular smooth muscle and detrusor smooth muscle when tissue was superfused with 100 μM SNAP (n=9, data not shown). Therefore, the structure of SNP or how this NO donor functions is likely leading to the constriction of smooth muscle in the bladder.
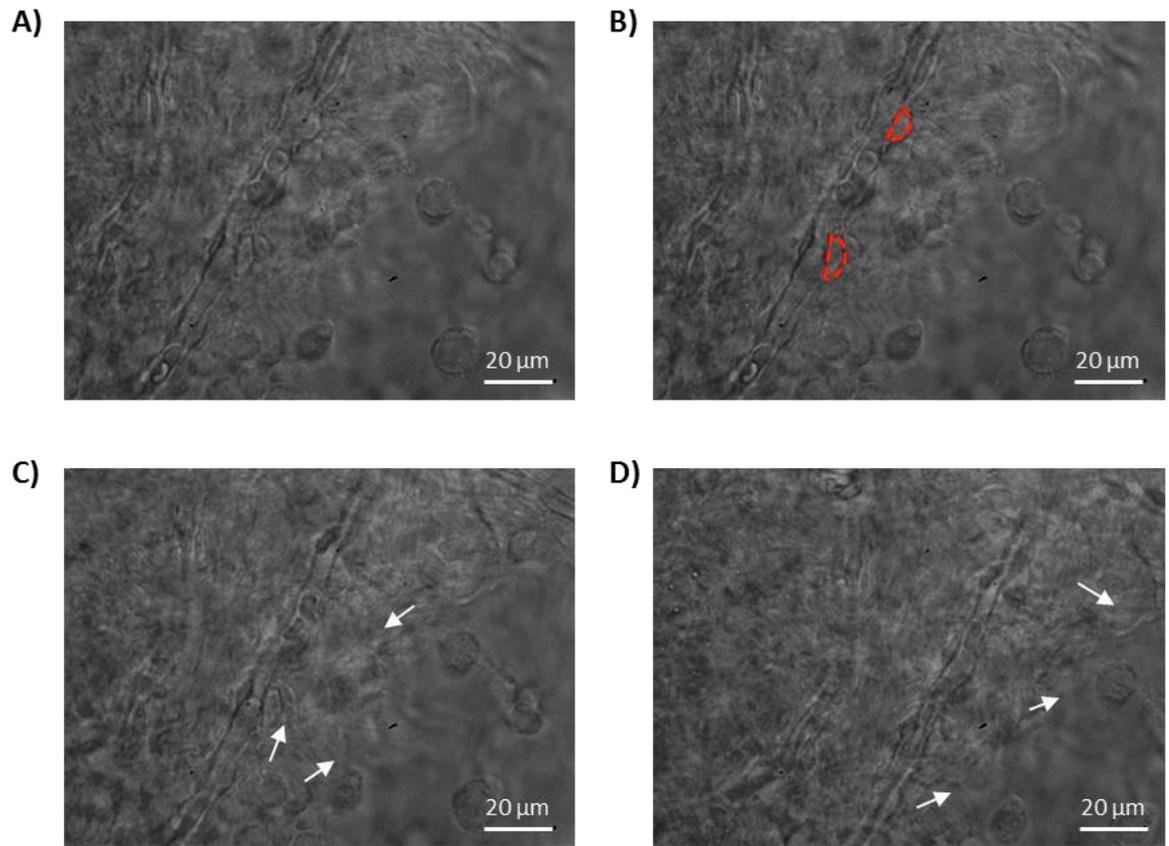
Figure 6.9: Images showing smooth muscle contraction upon addition of 10 µM SNP. Singular images from DIC experiments pre- and post-application of NO donor sodium nitropruside (SNP) by superfusion. **A)** Snapshot of bladder urothelium at frame 1 pre-drug application. **B)** Snapshot of bladder urothelium at frame 1 pre-drug application with red dotted areas indicating pericyte location. **C)** Snapshot of bladder urothelium 280 seconds post-drug application. White arrows indicate direction of muscle contraction. **D)** Snapshot of bladder urothelium 530 seconds post-drug application. White arrows indicate direction of muscle contraction.

6.4.7 PERICYTE FUNCTIONAL RESPONSES TO TNF-α

TNFα is a potent activator of pro-inflammatory responses and is produced in response to presence of pathogens. It has previously been shown to act on pericytes in other organs to induce production of COX2, RONS and other pro-inflammatory cytokines (262, 266, 269). Application of TNF-α to murine urothelium will hopefully give an indication to how pericytes respond to TNF-α acutely and in turn provide insight into pro-inflammatory cytokines' effect on blood flow during infection.

DIC imaging of live bladder urothelial preparation with addition of TNF-α showed that at a concentration of 10 ng/μL, vessels constricted at pericyte sites in response to the drug. TNF-α had a 100% success rate (n=3) with tissues responding on average 38.8 seconds after the drug is added to the tissue (n=3)(table 6.5, figure 6.10).

Responding tissues reached maximal constriction on average 115 seconds post-application and an overall maximal constriction of Δ90.55% of original vessel diameter was reached, which remained relatively the same even once the drug had been removed, until the end of the video (Δ91.05%, n=3) (p=0.0715, figure 6.10C). This difference is not considered significant by a paired Student T test.

TNF-α concentration	Drug response rate	Time to induce constriction	Time to max constriction	% max constriction	Time to desensitisation	% of Δ diameter at end of video
1 ng/μM	3/3 100%	38.3 s	115 s	90.55%	N/A	91.05%

Table 6.5: Drug response rate, the time taken for TNF-α to induce constriction and reach maximal constriction. Time to induce constriction post application of TNF-α is a mean 38.3 seconds (n=3). Time to maximal constriction is 115 seconds and the maximal constriction is 90.55% of the initial diameter. % diameter of original diameter at the end of the video indicates that maximal diameter remains similar (91.05%) until the end of the video even once the drug is removed.
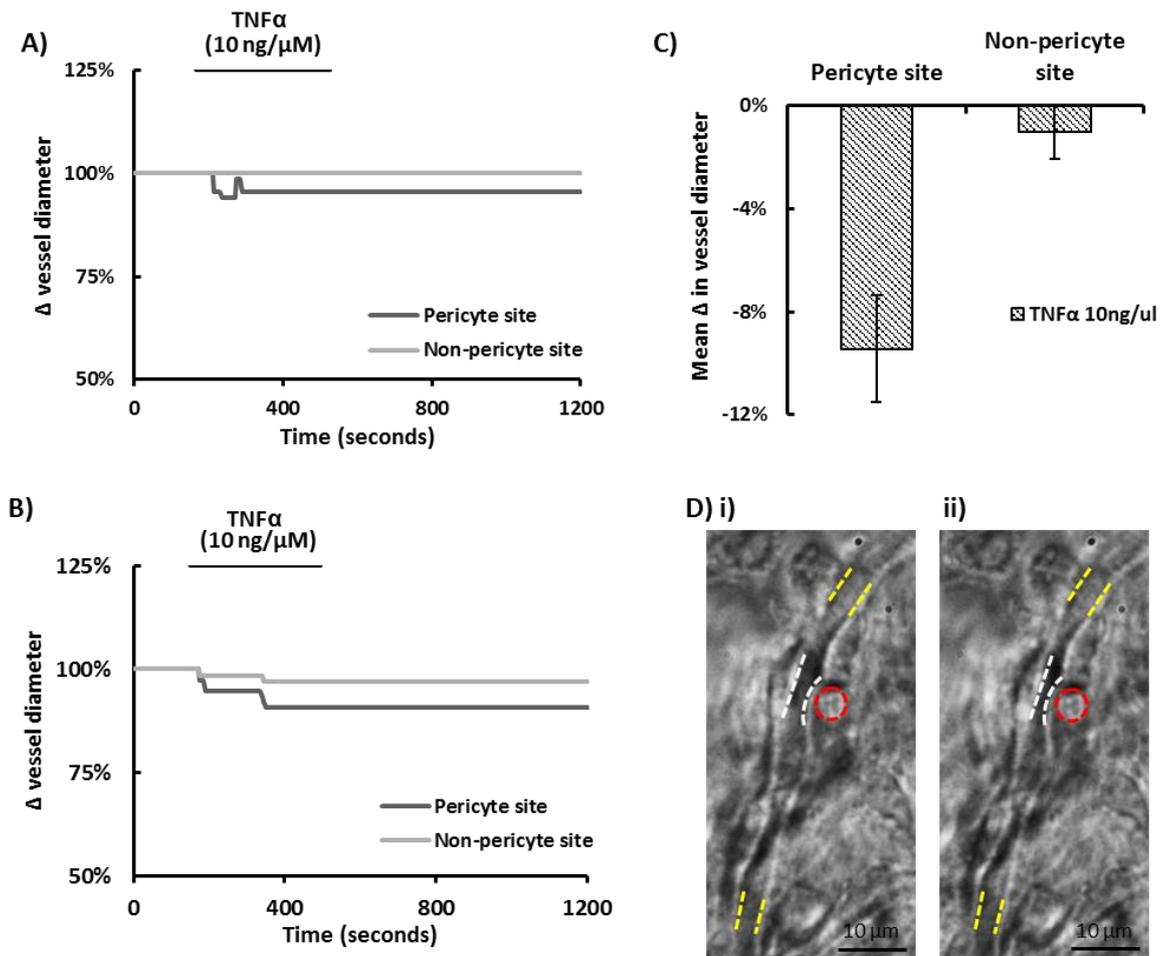
Figure 6.10: Effect of pro-inflammatory cytokine TNFα on diameter of bladder urothelium microvasculature. Superfusion of TNFα over live mouse bladder urothelium. **A)** 10 ng/µl TNFα induces a rapid response constricting the capillary which reverses slightly before TNFα is removed indicating a desensitisation of response at this concentration. **B)** 10 ng/µl TNFα induces a rapid response constricting the capillary which continues to contstrict even once TNFα is removed**. C)** Mean maximal constriction obtained by application of 10 ng/µM TNFα is -9.95% (n=3, p=0.07), however this concentration also affects non-pericyte sites. Statistical significance calculated with a paired student T test. **D)** Example of DIC images of capillary pre- and post- 10 ng/µl TNFα addition. Pericytes are indicated by red dotted semi-circles, white dotted lines indicate where maximal constriction occurs (1200 frames) at pericyte sites, and yellow dotted lines indicate non-pericyte region of the same vessel.

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6.4.8 MURINE BLADDER UROTHELIUM CELL VIABILITY

As relatively low response rates were observed in DIC experiments with Angiotensin II, ATP and SNAP, a tissue viability stain was performed to determine the % cell viability after times of 0 mins, 30 mins, 1 hour and 2 hours of tissue being in PSS. Since the staining with PI required incubating with PI in PSS for 15mins therefore the times were amended to 15 mins, 45 mins, 1h 15min and 2h 15mins. PI is taken up by cells with compromised cell membranes and is therefore used to stain for nuclei of dead cells. A counter stain of DAPI stains for DNA in all cells. Therefore the cell viability was calculated as follows: -

$$\% \text{ cell viability} = \left(\frac{(\text{Total DAPI}) - (\text{Total PI})}{\text{Total DAPI}} \right) x \ 100\%$$

Image J was used to manually count cells assisted by the application of a grid set to 30,000 pixels so only cells on the lines of two sides of a square are counted avoiding counting cells twice. Cells from the image taken under the 350nm filter (for DAPI, figure 6.11A) were counted first, followed by a manual cell count from the image aquired using a 536nm filter (for PI, figure 6.11B). Areas where blue live cells can be seen under the 350 nm filter (figure 6.11A) but are absent under the 536 nm filter are indicated by white dotted areas. Overlaying these two images into a composite image shows purple cells where cells are dead and blue cells were cells are alive (figure 6.11C). All urothelial strips were imaged in 3 different areas selected at random. Total manual cell count of both DAPI and PI are found in table 6.6, where it can be seen that even at 15mins incubation in PSS, all cells in one urothelial strip are dead and only 13.7% alive in the other. This viability is reduced to 0% and 8.62% (figure 6.11D) after 45 mins and then 0% for all strips after 1 h 15 mins (figure 6.11E) and 2 h 15 mins (figure 6.11F). These data suggest that tissue is dying rapidly in the PSS despite being a buffered solution being bubbled with 95% O_2/5% CO_2.

Tissue time in PSS	DAPI cell count (all cells)	PI cell count (dead cells)	% cell viability	Total % cell viability
15 mins (n=2)	191	191	0%	
	218	218	0%	0%
	230	230	0%	
	162	162	0%	
	218	179	17.9% (fig 5.C)	13.7%
	181	139	23.2%	
45 mins (n=2)	215	168	21.9% (fig 5.D)	
	190	190	0%	8.62%
	200	192	4.0%	
	216	216	0%	
	235	235	0%	0%
	213	213	0%	
1 h 15 mins (n=2)	128	128	0%	
	173	173	0%	0%
	217	217	0%	
	158	158	0%	
	188	188	0% (fig 5.E)	0%
	202	202	0%	
2 h 15 mins (n=1)	212	212	0%	
	154	154	0% (fig 5.F)	0%
	190	190	0%	

Table 6.6: Cell viability staining with DAPI and propidium iodide cell counts. Tissue left in physiological saline (PSS) for 15 mins (n=2), 45 mins (n=2), 1h 15 mins (n=2) and 2h mins (n=1) followed by staining with DAPI (blue) for all cells and propidium iodide (PI, red) for dead cells to determine cell viability. 3 images taken per piece of tissue, excited at 350nm (DAPI, blue) and 530nm (PI, red) and cell count performed manually in Image J for each image (see figure 6.11A and 6.11B). 15 min incubation (n=2) had 0% and 13.7% cell viability. 45 mins
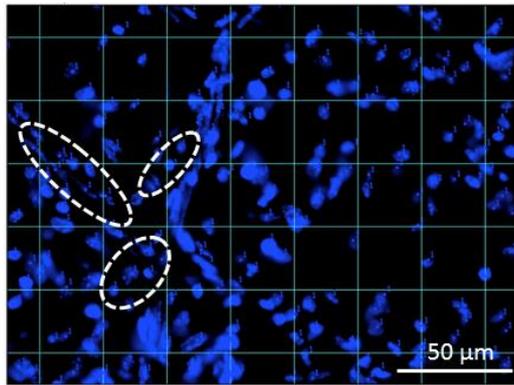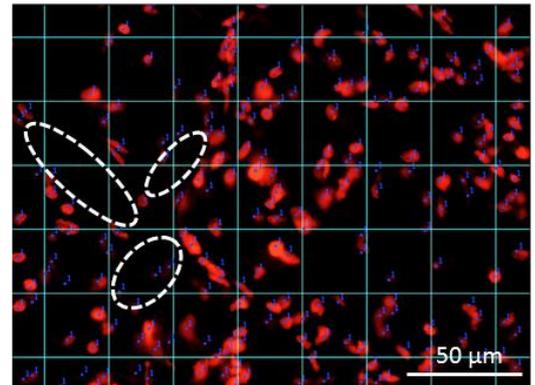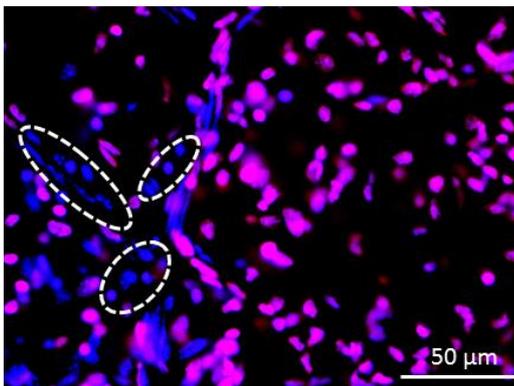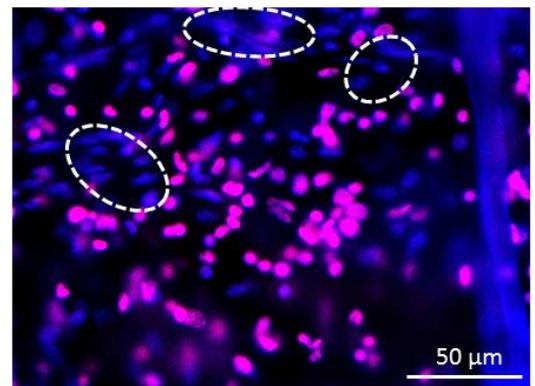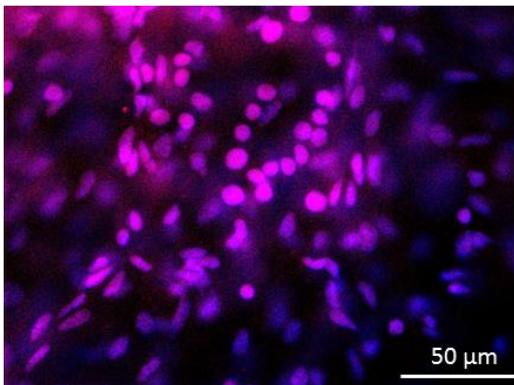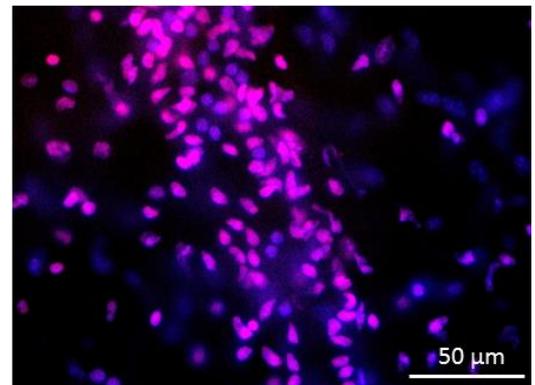
Figure 6.11: Cell viability staining of mouse bladder urothelium with DAPI and propidium iodide. Tissue left in physiological saline (PSS) for 15 mins (n=2), 45 mins (n=2), 1h 15 mins (n=2) and 2h mins (n=1) followed by staining with DAPI (blue) for all cells and propidium iodide (PI, red) for dead cells to determine cell viability. 3 images taken per piece of tissue, excited at 350nm (DAPI, blue) and 530nm (PI, red). **A)** an image of all cells labeled with DAPI at time point 15 mins indicates 218 total cells. **B)** an image of cells labeled with PI at time point 15 mins indicates 179 dead cells. **C)** an image of all cells labeled with DAPI and PI at time point 15 mins indicates 179 dead cells (=17.9% total cells). Total 6 images from n=2 15mins incubations gave total cell viability of 0% and 13.7%. **D)** an image of all cells labeled with DAPI and PI at time point 45 mins with 21.9% live cells (indicated by white dotted areas). Total 6 images from n=2 45 mins incubations gave total cell viability of 0% and 8.62%. **E)** an image of all cells labeled with DAPI and PI at time point 1h 15 mins with 0% live cells (indicated by white dotted areas). Total 6 images from n=2 1h 15mins incubations gave total cell viability of 0% and 0%. **F)** an image of all cells labeled with DAPI and PI at time point 2 h 15 mins with 0% live cells. Total 6 images from n=1 2h 15mins incubations gave total cell viability of 0%.

6.5 CONCLUSIONS

6.5.1 BLADDER PERICYTES RESPOND TO VASOACTIVE COMPOUNDS

By staining mouse bladder urothelium with anti-NG2 (pericytes, red) as well as IB4 (vasculature, green), it has been possible to show that pericytes are widely expressed throughout the bladder and are predominantly found at branch sites indicating that they are essential for controlling blood flow direction (figure 6.3). There are fewer pericytes along vessels than are branch pericytes (data not shown), and it is these pericytes that were most responsive to ATP, angiotensin II and TNF-α. This suggests that blood pressure is controlled along branches rather than at branch junctions within the bladder.

Data in this chapter indicate that in the bladder, angiotensin II functions directly at pericytes to induce constriction of blood vessels (figure 6.6). With higher concentrations of angiotensin the pericytes respond faster, both in terms of starting constriction and reaching a maximal constriction. At higher concentrations of angiotensin II the pericytes also become desensitised faster and the vessels begin to return to a relaxed state. With the lower concentration of angiotensin 1 nM, desensitisation does not occur and the vessels continue to constrict even once the drug has been removed. As mentioned in the introduction to this chapter, it has been shown that increased OAB has been linked to increased collagen deposition in the bladder (271). Since our data show that pericytes can respond acutely to angiotensin II and even continue to respond after this vasoactive compound has been removed, it opens up the question of whether pericytes can respond to angiotensin II by transdifferentiating into fibrosis producing cells. This requires further investigation outwith the time restraints of this project, however it could be an exciting line of research to support the theory that pericytes are a key cell in bladder fibrosis (46).

Data in this chapter also indicate that in the bladder, ATP functions directly at pericytes to induce constriction of blood vessels (figure 6.7). With higher concentrations of ATP the pericytes respond faster, both in terms of starting constriction and reaching a maximal constriction. At higher concentrations of 1 mM ATP the pericytes also become desensitised faster and the vessels begin to return to a relaxed state. With the lower concentration of 0.1 mM ATP, desensitisation does not

occur and the vessels continue to constrict even once the drug has been removed. As mentioned in *Section 1.5.6 Activator signalling and bacteriuria*, increased concentration of ATP in the urine correlates with OAB incidence and symptom score (75, 181-183). Furthermore, ATP is produced by both UPEC and by urothelial cells in response to UPEC infection in the bladder (75, 184, 185) (figure 6.12). Data from *Chapter 4 – Results 2: The Bladder Microbiome of Patients with Overactive Bladder* have shown that incidence of Gram negative bacteria is higher in patients with OAB than asymptomatic controls. Data in this chapter prove that pericytes respond acutely to ATP and continue to respond after this vasoactive compound has been removed, showing that ATP also acts upon this cell type in the bladder. What has not been deduced from this research is the type of receptors present on bladder pericytes. Different receptors have a different sensitivities/selectivity for range of nucleotides and nucleosides. By investigating the responses to each metabolite, one can deduce the receptors responsible for signalling in that cell type. The types of P2 receptors on pericytes has been investigated in other organs including the retina and the vasa recta of the kidney; the principal receptor on pericytes in the retina is the P2X7 receptor (276), and in the kidney, P2X1, P2X7 and P2Y$_4$ (277). Further investigation is required to determine the receptors responsible for pericyte-mediated vasoconstriction in the bladder. However, considering the two afore mentioned studies have both found P2X7 on pericytes, it could be theorised that this could also be present on bladder pericytes. This particular P2X receptor is known to require high levels of ATP in order to respond but continued exposure does not desensitise the receptor but instead causes a channel to pore formation (277), which is in keeping with our results. Additionally P2X7 has a role in immune response. Taken together these results support the theory that bacteriuria may lead to pericyte responses via induced neurotransmitter production, providing a pathophysiological explanation behind OAB (figure 6.12).

Unfortunately, the response rates for DIC imaging throughout this project were low and therefore n numbers for both angiotensin and ATP experiments were low. Furthermore, superfusion with bradykinin and SNP led to smooth muscle contraction, which meant it was not possible to investigate the effects of vasodilators on pericytes

in the bladder. The following section discusses this in more detail and suggests reasons why response rates may not have been as good as expected.

6.5.2 MODEL FOR INVESTIGATING BLADDER PERICYTE RESPONSES TO VASOACTIVE COMPOUNDS NEEDS IMPROVEMENT

In general the model of investigating pericyte function by DIC imaging has not been as sensitive as anticipated. Response rates to superfusion with low concentrations of ATP and angiotensin II were very low with only 25-33% of experiments having measurable responses. This meant that 9-12 mice were used to obtain n=3. These n numbers are relatively low; although they have provided provisional insight into the function of pericytes in response to these vasoactive compounds, these results can be improved upon.

One particular reason that may explain low response rates can be seen from the results of the cell-viability staining which indicated that much of the tissue is dead after only 30 minute. It is therefore essential to even more gentle during the dissection process and to use the tissue immediately. Due to the size of murine bladders, it is possible to obtain 3-4 pieces that are practically sized for staining and 2-3 pieces that are appropriate for DIC imaging. It could be suggested that by cutting the tissue smaller for staining experiments causes more damage than one single cut to half the bladder for DIC experiments and that one or two experiments per animal would be more effective although if less ethical.

An alternative reason which may explain low response rates to ATP is that small nucleotide polymorphisms in the P2X7 gene exist in C57BL/6 mice(278), which means that higher concentrations of ATP are required to evoke responses in these strains than in other strains or species. This includes the concentrations used in the rat model designed by Crawford *et al* from where the concentrations used in this thesis were obtained (52). With hindsight it would be worth increasing the concentration of ATP in the DIC imaging experiments to amend for this, or use rat bladder, which is harder to dissect but does also increase the number of experiments possible per animal.

Strangely, NO donors generally act as smooth muscle relaxants and have even been shown to act upon urethral muscles in a relaxatory manner (275). Both SNP and SNAP were used in the DIC experiments as NO donors, which would theoretically evoke vasodilation, however SNP induced smooth muscle contraction, which made it impossible to visualise and measure vessel diameter. This is believed to be due to the contractile nature of SNP rather than the donation of NO from this chemical. Switching to SNAP this effect was no longer a problem however the vessels were non-responsive. Alternatives to bradykinin and SNP could be used in order to determine the extent to which vasodilatory compounds increase capillary diameter and the concentrations at which they are most effective, including prostaglandin-E2, epinephrine and carbochol (52). SNAP may have provided this information however there were no responses from any of the experiments performed which may be due to the fact that this drug is extremely light sensitive. Furthermore, despite the fact that NO has been shown to be dilatory in renal pericytes, this does not suggest that an NO system exists within bladder pericytes. No published data has been found to prove or disprove this theory.

Bradykinin was also shown to induce smooth muscle contraction in DIC imaging experiments, however this result is less surprising. Bradykinin is known to cause smooth muscle contraction in the bronchia and the small intestine (279), and has been shown to induce contraction of the bladder detrusor in rats and guinea pigs (274). One particular study of interest has observed that by blocking bradykinin receptor 1 in rats with spinal injury induced OAB, the symptoms are reduced indicating that bradykinin may play a role in OAB (280). Interestingly, LPS has been shown to increase bradykinin 1 receptors in the mouse bladder, which could be an exciting lead for further investigations (281). During bacterial cystitis, spontaneous contraction is increased, as is the sudden need to urinate. This could be due to increased responses to bradykinin induced by presence of LPS, which could be investigated by staining for bradykinin 1 receptor in human bladder biopsies from UTIs, OAB and controls.

6.5.3 TNF CAUSES ACUTE PERICYTE-SITE CONSTRICTION

Data indicates that TNF-α functions directly on bladder pericytes to induce constriction of blood vessels and even once TNF-α has been removed, the vessels do

not return to normal diameter (figure 6.10). Despite the low response rates to vasoactive compounds investigated, and inconclusive and incomplete data for vasodilators, the data from TNF-α was exciting and promising since this is one of the cytokines that is a downstream compound of LPS stimulation (figure 6.12), is known to be released from pericytes (54, 263, 265), and acts in a paracrine and autocrine manner to further stimulate pericytes (262, 266). Our data have shown that TNF-α leads to acute vasoconstriction at pericyte sites, which at 10 ng/μM evokes a variety of responses; both desensitisation can occur, as well as extended contraction once the drug has been removed. This suggests that TNF-α has a very acute and a slightly prolonged effect on pericytes in the bladder as seen from the DIC experiments. TNF-α was also seen to affect pericytes in a chronic manner from IB4/NG2 staining, whereby prolonged exposure (4 hours) of bladder urothelium to TNF-α showed a significant reduction in the number of pericytes but not the distance between pericytes suggesting that this cytokine leads to rapid pericyte cell death. This suggests that a bladder infection, particularly with Gram negative species, could lead to downstream pericyte mortality and pericyte loss.

Due to the time restraints of this study it was not possible to investigate the role of other cytokines or LPS on the acute function of pericytes by DIC imaging. With hindsight it would have been exciting to include DIC imaging with LPS, IL-8, IL-1β, COX-2 and PGE-2 since these are notable cytokines and compounds produced downstream of LPS stimulation of pericytes (54, 263, 265) (figure 6.1). It could be hypothesised that LPS does not directly lead to pericyte vasodilation or vasoconstriction but rather leads to cytokine production and cell death/transformation in pericytes, and in turn, the downstream cytokines and PGE-2 would evoke vasodilation at pericyte sites.

6.5.4 LPS AND PRO-INFLAMMATORY CYTOKINES LEAD TO PERICYTE LOSS

Prolonged exposure (4hours) of bladder urothelium to TNF-α and IL-1β leads to a significant decrease in pericyte numbers, which suggests that cell death may be occurring (figure 6.4). The reason why this may only occur for TNF-α and IL-1β but not LPS is because these are downstream signalling molecules whereare LPS is a further upstream signalling molecule therefore this will take more time to have an effect.

The distance between pericytes is only significantly different when exposed to LPS or IL-1β (figure 6.5E), suggesting that these compounds are either transdifferentiating or migrating whereby pericytes are moving away from the capillaries. In this case it would be exciting to confirm that this is indeed the case. Unfortunately, myofibroblasts, which can be derived from pericytes, express many of the same antigens as pericytes, which means identifying them is very difficult (47). Tracking experiments can be designed to work around this and are discussed in the next section. To summarise, these data show that pro-inflammatory cytokines have a significant effect on the number of pericytes in the bladder, which during an infection, would reduce controlled blood flow and potentially lead to bladder ischemia and dysfunction as well as transdifferentiation into myofibroblasts (figure 6.12).
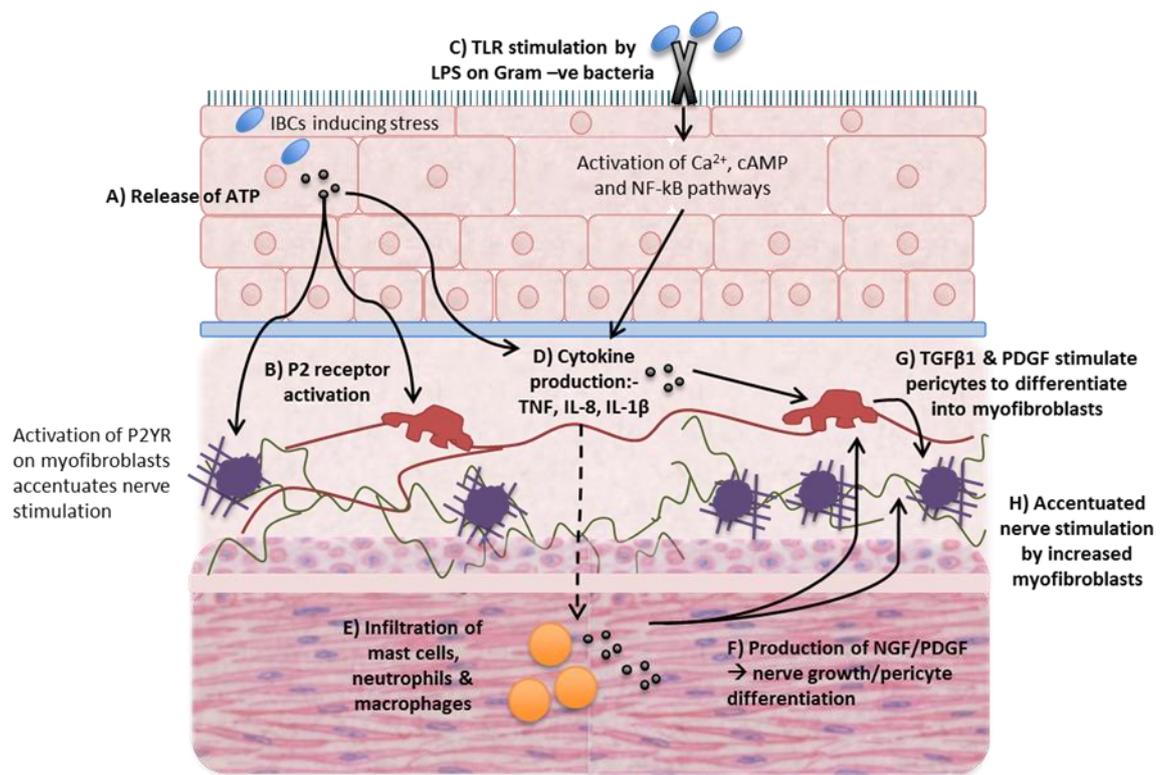
Figure 6.12: Schematic of how bacteria can alter the physiological function of the bladder. (By reviewing the schematic from the introduction, the points which have been proven in this thesis are discussed alongside areas for further research). A) ATP tends to be higher in OAB compared to controls however this is not significant (figure 4.22B) **B)** Pericytes do respond to ATP in an acute manner to constrict and reduce blood flow (figure 6.7). **C)** Gram negative bacteria were found to be higher in OAB compared to controls (figure 4.23) **D)** IL-8 production was measured and not found to be higher in OAB compared to controls (figure 4.22C) **E)** There is increased pyuria in OAB compared to controls (figure 4.3) **F)** No research was performed to support this, **G)** Cytokines and LPS were found to reduce the number and density of pericytes (figures 6.4 and 6.5) **H)** No research was performed to support this,

6.5.5 TRACKING PERICYTE TRANSDIFFERENTIATION IN THE BLADDER

A variety of recent studies have opened new doors to ways of fate tracking pericyte transdifferentiation into fibrotic cells such as myofibroblasts (33, 46, 61, 63, 276-279). Transdifferentiation studies in the bladder would be relatively easy compared to many of the other tissues used as it is a mucosal surface therefore stimulants can be applied to the tissue in the live animal and later observed in comparison to controls. Fate tracking experiments can be performed, as has been shown in a variety of other tissues, by employing the Cre-Lox recombinase method of cell tracking to stimulate pericytes *in vivo* and observe their destinations (33, 46, 61, 63, 282-285). This method takes two F_0 generation mice, one with a Cre gene inserted into the genome and the other with two LoxP genes inserted around a target gene of interest, followed by a marker *e.g.* green fluorescent protein. These two F_0 generation mice are bred and the result is an F_1 generation mouse with both the Cre and the LoxP genes. The constitutively expressed Cre protein binds to the LoxP genes and disrupts the target gene, instead transcribing the reporter, GFP. Using this method it is possible to target genes that are constitutively expressed in pericytes (*e.g.* NG2, PDGFRβ and αSMA in conjuction with collagen α1), and allows them to fluoresce enabling tracking.

Furthermore, studies have also highlighted that identification of the fibronexus of myofibroblasts by TEM can act as a unique way of recognising this cell type over others (table 1.1). By injecting stimulants, such as cytokines, bacteria or LPS, directly into the bladder of a live mouse and later counting the number of myofibroblasts by TEM compared to controls, it could be determined if these stimulants are increasing the number of myofibroblasts. Furthermore, it would be exciting to compare human bladder biopsies for numbers of myofibroblasts. Methods for performing TEM on human bladder biopsies have been described in the General Methodology section of this thesis. By comparing the numbers of myofibroblasts in healthy controls to those with rUTIs or OAB it could be determined if myofibroblasts may potentially be exacerbating symptoms in these disease states by leading to fibrosis and bladder wall thickening which has been observed in OAB.

Cumulatively, this chapter has highlighted that vasoactive compounds such as ATP and angiotensin II have direct effects on bladder pericytes causing constriction of

blood vessels which can reduce blood flow and in turn lead to ischemia. ATP has continually been shown to be increased in OAB and during bladder infection. The effects of bradykinin on smooth muscle are also interesting, as they have highlighted another possible avenue for which the causes of OAB could be investigated further since this compound appears to cause rapid detrusor contraction and has been associated to OAB.

Furthermore, this chapter has shown that LPS and the pro-inflammatory cytokines TNF-α and IL-1β have very similar effects on bladder pericytes as has been shown in other organs. TNF-α evokes acute pericyte constriction, which will inevitably reduce blood flow, but many other constituents of the inflammatory response could be investigated to determine if acute inflammation induces vasodilation over vasoconstriction. The effects of these compounds on pericyte density have also been investigated and highlights that the cytokines lead to pericyte loss, and LPS and IL-1β lead to reduced pericyte density. Fate mapping techniques or TEM could be used to investigate the fate of these pericytes in mouse models. Furthermore, TEM on human biopsies could determine if fibrosis caused by increased myofibroblasts presence is a potential cause of OAB.

6.6 CONCLUSIONS

1. Pericytes are found in close proximity to bladder capillaries and venules and can be found in high numbers around vessel branches.
2. ATP and Angiotensin II evoke bladder pericyte constriction of blood vessels.
3. Bradykinin and NO donor SNP cause smooth muscle contraction.
4. TNF-α evokes bladder pericytes constriction of blood vessels.
5. TNF-α and IL-1β lead to reduced pericyte numbers and LPS and IL-1β lead to reduced pericyte density.

CHAPTER 7 -
GENERAL DISCUSSION

The recent discovery of a bladder microbiome has led to the development of a spectrum of bacteria known to colonise the bladder, including various recognised and emerging uropathogens. With the development of more up to date techniques, many species previously undetected have also been identified, and thus the spectrum can be reliably referred to (197). Comparisons between the bladder microbiomes of asymptomatic individuals and bladder disease states has highlighted that alterations in the microbiome or low level infections may be a cause for certain syndromes including rUTI in post-renal transplant (213), OAB (138, 194, 195), and cystitis (286). Unfortunately, due to the high threshold CFU count for UTIs, any low level infections can go underreported (127), and therefore alternative biomarkers ought to be determined for both predicting rUTI and for OAB. Furthermore, despite some investigations stressing a role for bacteria, the exact pathophysiology has not been explained or hypothesised. Pericytes are a perivascular cell type that has been found in multiple organs including the bladder and are capable of responding to a variety of neurotransmitters (52), which are essential in bladder function, and can lead to changes in capillary diameter (52), and pericyte transdifferentiation into fibrosis producing myofibroblasts (46). Although the exact role of pericytes in the bladder has not been widely investigated, one study by Hashitani *et al* made the following concluding remark: "the pluripotency of pericytes may contribute to the remodelling of the bladder suburothelium that is commonly seen in an overactive bladder" (50). Bringing together the two fore mentioned hypotheses; i) that bacteriuria can lead to OAB, and ii) that pericyte transdiffentiation can lead to OAB, proposes the theory that bacteriuria may lead to pericyte transdifferentiation via induced neurotransmitter and cytokine production, providing a pathophysiological explanation behind this syndrome.

The aims of this thesis were to determine key bacterial species in rUTIs in RTRs and OAB, to investigate alternative biomarkers for rUTIs and OAB, and to determine whether bacteriuria affects pericytes, which may be a cause of fibrosis seen in patients with OAB. Following investigation, the 5 main findings are: - i) clue cells may

be used as an effective means to predict UTIs in RTRs since tests for WBCs such as leukocyte esterase and WBC counts do not always correlate with an infection, ii) three key species appear to be significantly reduced in RTRs at all time points, however these are not generally known to be affected by co-trimaxazole, iii) *Lactobacillus* is significantly reduced in patients with OAB and correlates with increased incidence of Gram negative uropathogens, iv) pericytes in the bladder respond to LPS and downstream pro-inflammatory cytokines both acutely and chronically. This supports the theory that bacteriuria may lead to pericyte transdifferentiation via induced neurotransmitter and cytokine production, providing a pathophysiological explanation behind this syndrome.

7.1 SUMMARY OF FINDINGS

Urinary tract infections are generally diagnosed by dipstick, WBC count and bacterial culture however recent publications and data from this thesis have recognised that neither of these means are effective. Malone-Lee *et al* published data suggesting that both dipstick and microscopic WBC count were not effective for detecting pyuria but rather that automated WBC count was the preferred method (215). Results from chapter 3 of this thesis support this in that automated WBC count is able to detect pyuria in patients with a UTI better than the other two methods. However, with approximately 70% of patients with a hospital grade infection have accompanying pyuria (figure 3.4), WBC count cannot always be used to diagnose an infection and with the threshold of CFU count set high at 10^4 CFU/ml, many infections could be being missed.

Chapter 3 of this thesis attempts to cumulate all clinical and biological data for RTRs over a number of time points post-transplant in order to determine if rUTIs can in some way be predicted or prevented before manifestation. Clue cells are a key medical sign of bacterial vaginosis (BV), which is caused by *Gardnerella vaginalis,* and their presence in vaginal swabs has been used as a means for diagnosing BV since their discovery in 1955 (287). This thesis is the first study to look at using clue cells as a biomarker for UTI in RTRs. Since these patients are particularly predisposed to recurrent infections, clue cells indicate a potential reservoir of intracellular bacteria, which could be used to predict a UTI. Results show that previous incidence of clue cell

observation could indicate a possible UTI in approximately 69% of cases, however this was based on low n numbers (n=13, figure 3.5A). Although investigations have not been able to confirm intracellular bacteria in these patients, it has highlighted that clue cells could be used as a prospective biomarker in order to prevent infections becoming too manifested in these immunocompromised patients. Further investigation is required to determine whether intracellular bacteria are important in rUTIs in RTRs. An antibiotic protection assay would highlight key species, the exact percentage of patients with rUTIs who have intracellular bacteria, and whether intracellular bacterial presence correlates with recurrence.

By culturing and sequencing every species from RTRs, it was possible to see the rich diversity of bacterial species in the urine, further affirming that urine is not sterile, but rather that the bladder has its own microbiome. Various recent studies have attempted to show the diversity of bacterial species in the bladder microbiome and compare that of healthy asymptomatic controls to those of disease states. Our data compares well to these studies, even those using more advanced methods. Interestingly, 3 key species were significantly reduced in RTRs at all time points indicating that a shift in the bladder microbiome may be a cause for concern in these patients. These species; *Actinomyces, Corynebacterium* and *Peptoniphilus* are not known to be affected by co-trimaxazole, therefore more investigation is required to determine how these species are reduced in RTRs.

The second results chapter of this thesis applied the same methodology of 16s rRNA sequencing to investigate the species of bacteria colonising the bladder of patients with OAB and comparing it to that of asymptomatic controls. It was observed that patients with OAB have significantly reduced *Lactobacillus* incidence suggesting that symptoms may be due to alterations in the microbiome rather than low-level infection as originally hypothesised. It was observed however that incidence of *Lactobacillus* correlated with increased incidence of Gram-negative uropathogens such as *Proteus* and *E.coli*. Although incidence of Gram-negative uropathogens is not significantly increased in OAB, these data suggest that a lack of *Lactobacillus* may have implications on other species of the bladder microbiome. *Lactobacillus* is a key

probiotic in both gut and vaginal health and could be implemented in bladder health also. Some studies have shown that *Lactobacillus* can be used as a prophylactic for UTI, however there have been no studies to date looking at this species as a prophylactic or treatment for OAB. It is also interesting to note that *Lactobacillus* was not reduced in RTRs therefore even though RTRs are vulnerable and susceptible individuals, RTRs cannot be used to study or predict rUTIs and microbiome changes in OAB. In other words, OAB is a unique syndrome, different from rUTIs.

The first results chapter of this thesis was not able to confirm intracellular bacteria from clue cell observation, therefore the methodology was adapted for the second chapter to include an antibiotic protection assay. This data was the first to describe the full spectrum of species that are capable of forming IBC in bladder urothelial cells. From the results, no particular species were any more prevalent in OAB than in asymptomatic controls indicating that intracellular bacteria may not be relevant in this particular bladder syndrome. Unfortunately, many CSU samples of urine were not rich enough in bacteria to have any growth on culture plates. Development of the methodology in the third results chapter included more conditions but also plated 100 μL instead of 5 μL. Using this method, various species were cultured which were previously missed, and therefore this method ought to be adapted for repeat on samples from both RTRs and patients with OAB.

The final results chapter of this thesis attempted to explain how an infection may lead to particular symptoms of OAB such as overactivity and fibrosis. Many studies have suggested that OAB may be caused by a low-level infection left unreported by hospital tests. However, explanations for the resulting symptoms of OAB have only been loosely hypothesised. One of the main indications of infection is the production of cytokines and inflammation. The pro-inflammatory cytokines most associated to UTI are TNF-α, IL-6 and IL-1β and have also been implemented in stimulating resident pericytes to increase vascular permeability, transdifferentiate and move away from their post along microvasculature. The final results chapter aimed to test the theory that bladder pericytes also respond to these cytokines and some neurotransmitters in an attempt to provide a pathophysiological explanation

behind the symptoms of OAB. By exposing pericytes to LPS and the downstream cytokines produced during an infection, it was possible to determine how pericytes respond both acutely and chronically. Exposure to TNF-α showed rapid constriction of pericytes, which *in vivo* would cause reduced blood flow and increased blood pressure. Exposure to TNF-α and LPS over 4 hours led to reduced density of pericytes indicating they may be moving apart from each other, moving away from the capillary or transdifferentiating. This confirms that bladder pericytes are able to respond to bacterial components as well as pro-inflammatory agents. Further investigation is required to determine if pericytes are indeed transdifferentiating by performing fate tracking studies *in loco* as discussed in *Section 6.5.5 - Tracking pericyte transdifferentiation in the bladder.* Other cytokines, complement factors *e.g.* C3a and C5a, and other bacterial components need to be investigated in gain a wider knowledge of influences leading to pericyte changes. Furthermore, a study based on infecting germ free mice with known uropathogens would be more informative of the effect of whole bacterium *in vivo* discussed in *Section 7.3.4 - The role of bladder pericytes in infection.*

7.2 METHODOLOGY ERRORS AND LIMITATIONS

Reflecting upon the methodology used for the RTR study, it is essential to include an antibiotic protection assay when culturing all samples in order to gain information on possible intracellular as well as planktonic bacteria. Although this was adapted for the OAB study, key information is missing from the renal study. Future studies should increase the volume of urine plated on agar in order to capture bacteria in very low numbers that would otherwise be missed if plating only 5 μL. Furthermore, inclusion of Campygen and aerobic conditions for culture will help to obtain the maximum diversity of cultureable species.

A number of isolates were not able to be resuscitated from cryogenic storage in order to perform the PCR. Upon resuscitation and regrowth, isolates were replated on chocolate agar plates sectioned into 8 in the same conditions as they were initially discovered (*i.e.* 5% CO_2 or anaerobic) which was not enough space to obtain an isolated colony. Any isolates that had contaminated or unsuccessful regrowth were replated using more of the cryopreserved broth and more of the chocolate plate. The

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initially colony description was referred to in order to identify the correct isolate in cases of contaminated regrowth. In some cases however, contamination was too great or regrowth was too low to perform a PCR. Therefore, not all isolates were identified and may skew the accuracy of the data obtained.

It was essential to compare the observed Gram stain/shape of the sequenced isolates with the true Gram stain/shape of the bacterium proposed by BLAST. The reason for this is that Gram staining is more accurate than sequencing; despite using a high-fidelity polymerase, which ensures insertion of the correct nucleotide during PCR, an error rate still exists. Since the program used for the PCR in this project was set at 37 cycles per reaction and the average size of a 16s rRNA gene is 320bp this works out as roughly 0.26% PCR products with an error. In addition, the protocol for PCR purification included 4 centrifugations at 13,000RPM which can damage the DNA, removing small numbers of nucleotides from the end of the DNA fragment. Furthermore, due to the nature of Sanger sequencing, further errors are possible. Therefore, the sequences generated cannot be wholly relied upon even if the Gram stains are concordant.

The identity of a number of isolates was not able to be confirmed by concordant Gram stains since the Gram stain was not established. Some species of bacteria are both Gram positive and negative depending on the stage of growth, notably *Gardnerella vaginalis* and *Arthrobacter.* Determining Gram positive or negative could be difficult in many other cases if many bacteria were dead in which case they would show as Gram negative when their true form was Gram positive. Furthermore, if the microscope slide had been too heavily overloaded with bacteria prior to staining, the crystal violet stain would remain in between the bacteria showing Gram positive when the true nature should have been Gram negative.

The Mix2Seq service used to sequence the isolates does not offer a re-run service. That is, if the sequencing was unsuccessful the first time, Eurofins will not perform a free re-run of the sample but will instead send the sequence as it has been generated. Therefore, in some cases, samples would have to be resent for sequencing, incurring extra time and cost.   In three cases, a successful sequence was generated however there was no significance found upon BLASTing. These could have been new or

uncultured species however due to the slow nature of the evolution of 16s rRNA gene, it is unlikely that a similar match would not be suggested by the BLAST search.

In three cases, a successful sequence was generated however there was no significance found upon BLASTing. These could have been new or uncultured species however due to the slow nature of the evolution of 16s rRNA gene, it is unlikely that a similar match would not be suggested by the BLAST search.

7.3 Application and scope for future studies

7.3.1 Intracellular bacteria in RTRs

As mentioned in the conclusions of the discussion of chapter 3, *Section 3.5.5 Methodology errors and limitations*, intracellular bacteria may be a reservoir for reinfection and recurrence in RTRs however the methods used were not able to confirm intracellular bacteria in these samples. Since the strongest correlation with rUTI was observation of clue cells, and clue cells may indicate intracellular bacteria, more work is required on this to clarify this potential biomarker. Future studies could be used to identify patients with clue cells and put them under stricter observation by increasing sample collection to weekly and monitor clue cells with the inclusion of an antibiotic protection assay to determine whether clue cells are a reservoir for infection in RTRs.

If the results of such a study show the clue cells are indeed a reservoir for infection in RTRs, then the stricter monitoring could be used to predict rUTIs and preventative mechanisms can be put in place. For example, the identification of clue cells in a urine sample of a RTR, could instigate prescription of more advanced broad-spectrum, prokaryote-penetrating antibiotics which will target intracellular bacterial reservoirs. A reduction in rUTI incidence will inevitably reduce graft rejection and decrease patient mortality.

7.3.2 *Lactobacillus* as a prophylactic

This thesis in conjunction with other publications has shown that in differences in the bladder microbiome between patients with OAB and asymptomatic controls is only consistently significant in respect to incidence of *Lactobacillus*. As

mentioned in *Section 4.5.7 Lactobacillus is a probiotic,* this species has prophylactic capabilities and has been used in the gut, vagina and more recently as a prophylactic for UTIs. As discussed in *Section 4.5.6 Lactobacillus as a prophylactic against uropathogens*, two studies of particular interest have generated optimistic results for use of *Lactobacillus* as a prospective prophylactic for UTI.

The first study performed a phase 1 and phase 2 controlled trial, again looking at the role of *Lactobacillus* as a prospective prophylactic for UTI. These investigations have used *L.crispatus* as a vaginal suppository due to its ability to produce high levels of H_2O_2. Results showed a reduction of roughly half incidence of UTI compared to placebo with minimal side effects (247, 248). The second study gave oral *L.rhamnosus* and *L.reuteri* or trimethoprim-sulfmethoxazole as prophylatics against UTI to 252 postmenopausal women and assessed incidence of UTI over 12 months in a randomised control trial and showed that *Lactobacillus* had a similar prophylactic effect on UTI compared to antibiotics (126). This study also measured the rate of development of antibiotic resistant UPEC over 12 months in both groups. After 12 months, 100% of UPEC isolated from UTIs in this study from the antibiotic group were resistant to trimethoprim and sulfmethoxazole compared to 0% in the *Lactobacillus* group. These data highlight just how rapidly antibiotic resistance can develop in the management of infection with antibiotics, which will inevitably lead to complicated rUTI over time. Given that the use of trimethoprim-sulfmethoxazole is used in renal transplant as a prophylactic for up to 6 months, if not more, this is a worrying indication as to the severity of the development of antibiotic resistance in these already vulnerable patients. Unfortunately, prophylactic *Lactobacillus* cannot be given as an alternative to co-trimoxazole as this antibiotic cocktail is specifically chosen due to its ability to prevent pneumonia, to which *Lactobacillus* cannot protect against. It could however be used in cooperation with immunosuppressants and antibiotics as a third protection for RTRs.

Taken together these studies and their results show that *Lactobacillus*, in particular *Lactobacillus crispatus*, can provide an effective prophylactic effect on UTI, in particular on UPEC. A randomised clinical trial examining the effects of probiotics on OAB would be exciting since our results have clearly indicated reduced incidence of *Lactobacillus* in these patients. Future studies could aim to monitor patient symptoms

in correlation with colonisation with *Lactobacillus* and perform various types of co-culture with isolated uropathogens and *Lactobacillus spp.* to determine how well *Lactobacillus* acts as a probiotic.

 If a clinical trial were to successfully show that *Lactobacillus* probiotics were helpful in suppressing symptoms of OAB then this could progress into Phase 2 clinical trial and even become a recommended treatment for OAB in some patients. Oral *Lactobacillus* therapy for women's health is already a manufactured product and therefore there would be no major requirement for drug development in order to progress this to clinical trial and potential treatment.

Furthermore, by investigating the possible role of *Lactobacillus* metabolites (*e.g.* hydrogen peroxide, lactic acid and bacteriocins) on bladder function this may highlight how a loss of this genus could lead to the symptoms of OAB. This could lead to research opportunities investigating alternative treatments for OAB which do not involve the use of live bacteria, reducing OAB symptoms as well as reducing the risk of infection and therefore the overall burden on the NHS.

7.3.3 THE ROLE OF BLADDER PERICYTES IN INFECTION

As discussed previously in the general discussion, murine infection studies and transdifferentiation studies are essential to confirm the hypothesis that chronic bacteriuria can lead to fibrosis of the bladder. Germ free mice are born and kept in sterile conditions so that they never develop a microbiome. By infecting germ free mice with a known uropathogen, it would be possible to say with confidence that any differences in physiology are the result of the infection. By comparing pericyte density or fibrosis in both of these mice, one could conclude the effects of bacteria on pericytes and their contribution to fibrosis. Transdifferentiation studies utilise target genes in pericytes to insert fluorescent tags so that they can be tracked as they differentiate and migrate. By using Cre-Lox-GFP Germ free mice it could be possible to induce a bladder infection in germ-free mice and track pericytes as they respond to infection.

Transdifferentiation studies cannot be performed in humans, however as discussed in section *6.5.5 Tracking pericyte transdifferentiation in the bladder*, the measurement of fibrosis pre- and post-infection and in controls and OAB patients can be assessed by

observing the fibronexus of bladder biopsies by TEM. If the number of pericytes is decreased and the number of myofibroblasts is increased, one could conclude that this was due to pericyte transdifferentiation. By determining whether pericyte transdifferentiatinon is a potential cause for OAB, treatments could involve anti-TNF-α therapy which could prevent potential increase in myofibroblasts and hence, reduce spontaneous contractions.

7.4 FUTURE ADVANCES

The main conclusion of this thesis is that bacteriuria may lead to pericyte transdifferentiation via induced neurotransmitter and cytokine production, providing a pathophysiological explanation behind OAB. There are other researching groups studying alternative but related concepts behind OAB and bacteriuria and the concluding findings of this thesis also support those theories.

This section attempts to tie the results of this thesis with other recent advances in current research in order to develop new ideas. To do this a literature search was performed for key researchers in the field and 'OAB in aging populations'. Two key theories emerged from this that were of much interest. The first is that of 'inflammaging' a term first coined in 2000 derived from inflammation and aging whereby the body deteriorates due to increased inflammatory responses and age (288). The second suggests that complicated UTIs lead to alterations in the immune system transcriptome and future infections are more likely to develop into cystitis due to COX-2 dependant inflammation (289). These two theories support each other and, with future investigation, they could be used to explain the cause of OAB in the absence of infections.

7.4.2 THE CONCEPT OF INFLAMMAGING

Inflammaging is an emerging field of research which suggests that as one ages the immune system undergoes various changes including immunosenescence (age-related deterioration of the adaptive immunity) and development of a chronic, low-level, systemic innate and pro-inflammatory state (288). This phenomenon increases susceptibility to diseases such as Alzheimer's, type II diabetes, atherosclerosis, arthritis and cancer. The main theory behind the concept of inflammaging is that there is a build-up overtime of non-resolved inflammation caused by persistent and

recurring infections and injury. This persistence can lead to increased stress, increased cytokines, oxidation-inflammation, DNA damage and autophagy, each of which will contribute to activation of one or a combination of signalling pathways including NF-κB, Notch, TGF-β, sirtuin, Ras, RIG-1 and TOR; the downstream effects of all of which are pro-inflammatory (reviewed by Xia *et al* (290)). Furthermore, this will alter the transcriptome, increasing the mRNA transcribed for pro-inflammatory genes. Recent investigations have focused on the signalling pathways to better understand the process of inflammaging but also to potentially develop targets for age-related diseases.

There is consistency in reporting a correlation between age and OAB, which data in Chapter 4 of this thesis supports. One particular study by Tyagi *et al* in 2013 hypothesised that OAB is a syndrome associated with inflammaging and thus measured the concentration of 8 cytokines and chemokines by MILLIPLEX of 140 individuals with OAB to determine if age-related biochemical changes correlates with OAB severity (291). They found a positive age-associated elevation of NGF (p=0.001) and MCP-1 (p=0.07). As discussed in the general introduction, increased expression of NGF has previously been associated to OAB, and furthermore, to increased severity of symptoms. They go on to hypothesise that NGF increases may be a homeostatic response to nerve senescence in the bladder and MCP-1 may be related to increased adipocytes in the detrusor leading to bladder stiffness (291).

A recent study investigating vascular remodelling in mouse gut via up-regulation of Angiopoietin-2 found that the source of Angiopoietin-2 up-regulation was TNF-α produced by M2-like macrophages (292). These macrophages release TNF-α in response to changes in the microbiota and inflammaging. The consequences of this increased TNF-α is angiotensin-II-dependant vascular remodelling by reduced pericytes, reduced VE-cadherin and increased permeability. Our data supports this in that we have shown that TNF-α can directly affect pericytes and lead to pericyte loss.

This is a stimulating and credible theory since many individuals with OAB do not have bacteriuria however many have a history of UTIs. Furthermore, in respect to the newly discovered bladder microbiome, it could be proposed that changes in this microbiome could also stimulate immune responses which lead to increased cytokine

production, leukocytosis and transcriptome alterations. The study by Tyagi *et al* has not included some of the more potent pro-inflammatory cytokines mentioned in this thesis including TNF-α and IL-1β, however it would be interesting to see whether these cytokines are increased with age and OAB incidence since it has previously been shown that these cytokines have a strong effect on pericytes as a consequence of microbiome changes.

Taking the data from these two studies, a potential research proposal could be to i) investigate if there is an age-associated elevation of TNF-α, IL-1β and Angiopoietin-2 in patients with OAB versus controls, ii) isolate macrophages from bladder biopsies taken from patients with OAB and characterise them to determine if there are any differences between their transcriptome/cytokine profiles, iii) incubate mouse bladder tissue with macrophages isolated from the bladder of patients with OAB to determine if there is a loss of pericytes as a consequence of TNF-α production, iv) a clinical trial to determine if anti-TNF-α can suppress the symptoms of OAB.

7.4.2 INFECTION HISTORY ALTERS SUSCEPTIBILITY TO RUTI

A unique and exciting study by Hultgren *et al*, who led the investigations into IBC in UTI discussed in the general introduction, was published near the end of this thesis. This new development in current research has revealed that mice previously infected with UPEC, are significantly more likely to develop chronic bacterial cystitis upon second exposure if the primary infection did not resolve spontaneously (resolved mice), but rather developed into cystitis (sensitised mice) (289) (figure 7.1). This work follows on from a publication by the same team of researchers who, in 2010, highlighted that development of chronic cystitis relied upon severe acute inflammatory responses during early infection (293).

Their recent publication further describes how prior UPEC infection results in differences in the way the urothelium remodels itself depending on whether the mouse was sensitised or resolved(289). Both mice had significantly smaller superficial cells post-infection however these were particularly small in sensitised mice and did not appear to be terminally differentiated (figure 7.1). Remodelling from a prior infection did however protect from colonisation and IBC formation when re-challenged with UPEC except in some sensitised mice.

To explain some of these differences they investigated the transcriptome of whole bladders from sensitised and resolved mice; over 800 genes were significantly differentially expressed for pathways relating to cell development, proliferation and inflammation suggesting severe chronic inflammation leads to alterations which can protect against future infections (figure 7.1).

As mentioned, a subset of sensitised mice were not protected from future infections but were highly sensitive to severe acute or recurrent chronic cystitis upon rechallenge. Hultgren's group investigated the expression of COX-2 in the bladders of both sensitised and resolved mice to explain how some sensitised bladders were not able to protect against rUTI despite significant remodelling. COX-2-dependent inflammation is leading to vasodilation, neutrophil influx and wounding. Findings indicated a significant reduction of COX-2 expression in resolved mice (figure 7.1).
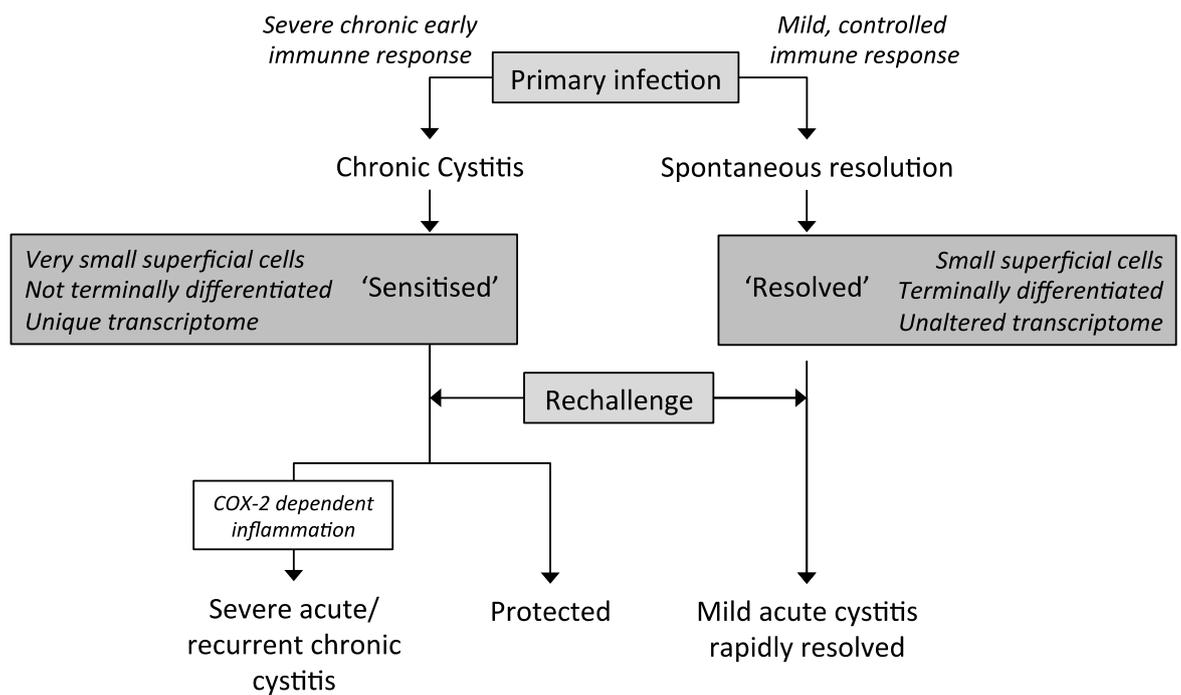
Figure 7.1 Flow diagram of resolution of secondary infection in mice dependent on resolution of primary infection. If a primary infection in mice is severe with early immune responses they will develop a chronic cystitis whereby the mice are 'Sensitised'. 'Sensitised' mice will have very small superficial cells which are not well differentiated. Furthermore, they will have an altered transcriptome ready for a secondary infection. Upon rechallenging, these mice will either be protected by the unique transcriptome or will develop a second severe chronic infection. Chronic infection in these mice is driven by COX-2 which was activated by the initial severe cystitis. If on the other hand a primary infection in mice is mild with spontaneous resolution they will be 'Resolved' and have an unaltered transcriptome and better superficial cell differentiation that 'Sensitised' mice. These mice will not activate COX-2 dependent inflammation and therefore will only develop a mild infection upon secondary infection. Diagram derived from results published by Hultgren *et al*, 2010 and 2016 **(289, 293)**.

Taken together, these findings suggest that severity of rUTIs are not only based on presence of bacteria or antibiotic resistance, but the means by which the immune system combats infection after infection. This also supports the theory of inflammaging whereby post infection, the transcriptome alters. Furthermore, it supports the theory that bacteriuria may lead to physiological changes induced by cytokine production providing a pathophysiological explanation for OAB.

This suggests that COX-2 inhibition could be a potential suppressor of the symptoms of OAB. There have been some studies performed in rats using COX-2 inhibitors, which have shown positive results in reducing frequency. Studies in humans have only looked at the efficacy of non-selective COX inhibitors in LUTS and cystitis and rUTI. Therefore, a clinical study to explore the use of COX-2 specific inhibitors in patients with OAB only warrants investigation.

A potential research proposal based on the above theory could be to i) investigate whether RTRs suffering with rUTIs have an altered transcriptome to determine whether these patients are more at risk, ii) investigate whether treatment with COX-2 inhibitors post-UTI could prevent RTRs from relapsing or developing severe rUTIs.

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APPENDIX

East Kent Hospitals University **NHS**
NHS Foundation Trust

Diagnosing urine infection in kidney transplant patients

Questionnaire Version 1.0

About Your Symptoms and their impact on quality of life (for use at visit 1)

Patient ID Sticker

Date ……../……../……..

SYMPTOMS	Please indicate whether you have had the following symptoms/problems in the past 24 hours and how severe they were: (*please circle one number for each symptom*)			
	Did not have	Mild	Moderate	Severe
Frequency of Urination (going to the toilet very often)	0	1	2	3
Pain or burning when passing urine	0	1	2	3
Not being able to empty your bladder completely/ passing only small amounts of urine	0	1	2	3
Pain or uncomfortable pressure in the lower abdomen / pelvis area caused by your urinary tract infection	0	1	2	3
Low back pain caused by your urinary tract infection	0	1	2	3
Blood in your urine	0	1	2	3

	If you have experienced these symptoms/problems in the past 24 hours, please indicate how bothersome they were: (*please circle one number for each symptom)*			
SYMPTOMS	Not at all	Not at all	Not at all	Not at all
Frequency of Urination (going to the toilet very often)	0	1	2	3
Pain or burning when passing urine	0	1	2	3
Not being able to empty your bladder completely/ passing only small amounts of urine	0	1	2	3
Pain or uncomfortable pressure in the lower abdomen / pelvis area caused by your urinary tract infection	0	1	2	3
Low back pain caused by your urinary tract infection	0	1	2	3
Blood in your urine	0	1	2	3

Please give an overall rating of the severity of your urinary tract infection symptoms as they are at the moment (*please circle the number of your answer)*

0   No symptoms at all

1   Mild

2   Moderate

3   Severe

MSU CULTURE INTERPRETATION

Leucocyte count
<50/mm^3
NOT cultured except

Leucocyte count
50-100/mm^3

< 10^4 orgs/ml
NSG

> 10^4 mixed growth* MXC

> 10^4 orgs/ml
Pure growth I/S

Leucocyte count
> 100/mm^3

Epithelial cells
+ or less

Epithelial cells
++ or more

< 10^4 orgs/ml
NSG RPTSI

> 10^4 orgs/ml
Pure growth

> 10^4 mixed growth **Equal amounts** of >2 types of coliforms/enterococci

< 10^4 orgs/ml
<10 cols

> 10^4 orgs/ml
Pure growth

> 10^4 mixed growth* **Equal amounts** of >2 types of coliforms/enterococci

Identify and report organism with
Sensitivities

Report MXR

Report NSGR
CCU

Identify and report organism with
Sensitivities

Report MXR CCU

> 10^4 orgs/ml
Pure growth

ONLY do Fastidious culture if leucs >100/mm^3 after medical advice and request

NSG
RPTSI

*Follow up **predominant organisms,** Identify and report with sensitivities

Insufficient urine to dilute Boric acid in specimen
Report as NSGR

UFAST
If female or male ≥ 65

UFASTM
If male

Glossery of Abbreviations

NSG	=	No significant growth
RPT		Please send a repeat sample if still indicated
SI		
NSG	=	No significant growth
SG	=	Significant growth (>10^4)
MX	=	Mixed growth of organisms
MXC	=	Mixed growth of organisms - probable contaminants
MXR	=	Mixed growth of organisms - Please send repeat
MXR	=	Mixed growth of organisms
CCU		Please send a clean catch urine
NSG	=	No significant growth - please repeat
RPT		Please send a repeat sample if still indicated
SI		Please send a clean catch urine
CCU		
MIN	=	Mixed growth including *
QS	=	Of doubtful significance in the absence of pus cells

Document number: MIC-WI-674
Author:: R Arkley
Approved by: R.Arkley

East Kent Hospitals University NHS
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CSU CULTURE INTERPRETATION

```
                                              ┌─────────────────────┐
                                              │   > 10⁴ orgs/ml     │
                                              │                     │
                                              │   >10 colonies      │
                                              └─────────────────────┘
```

$> 10^4$ orgs/ml

>10 colonies

$< 10^4$ orgs/ml

$<=10$ colonies

Pure growth

Mixed growth

Report

No Significant Growth

OR

Mixed growth with a predominant organism

Report as Mixed growth

Report identification – sensitivities suppressed

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Author:: R Arkley	NHS Foundation Trust		Date of issue: Jan 15
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□□□

DAY   MONTH   YEAR

Today's date

Urinary symptoms

Many people experience urinary symptoms some of the time. We are trying to find out how many people experience urinary symptoms, and how much they bother them. We would be grateful if you could answer the following questions, thinking about how you have been, on average, over the <u>PAST FOUR WEEKS</u>.

1. **Please write in your date of birth:**

□□□

DAY   MONTH   YEAR

2a. **During the night, how many times do you have to get up to urinate, on average?**

none	□	0
one	□	1
two	□	2
three	□	3
four or more	□	4

2b. **How much does this bother you?**
Please ring a number between 0 (not at all) and 10 (a great deal)

0 1 2 3 4 5 6 7 8 9 **10**
not at all   a great deal

3a. **Do you have a sudden need to rush to the toilet to urinate?**

never	□	0
occasionally	□	1
sometimes	□	2
most of the time	□	3
all of the time	□	4

3b. **How much does this bother you?**
Please ring a number between 0 (not at all) and 10 (a great deal)

0 1 2 3 4 5 6 7 8 9 **10**
not at all   a great deal

4a. **Do you have pain in your bladder?**

never	□	0
occasionally	□	1
sometimes	□	2
most of the time	□	3
all of the time	□	4

4b. **How much does this bother you?**
Please ring a number between 0 (not at all) and 10 (a great deal)

0 1 2 3 4 5 6 7 8 9 **10**
not at all   a great deal

5a. How often do you pass urine during the day?

1 to 6 times ☐	0
7 to 8 times ☐	1
9 to 10 times ☐	2
11 to 12 times ☐	3
13 or more times ☐	4

5b. How much does this bother you?
Please ring a number between 0 (not at all) and 10 (a great deal)

    0   1   2   3   4   5   6   7   8   9   **10**
not at all   a great deal

F score: sum scores 2a-5a ☐

6a. Is there a delay before you can start to urinate?

never ☐	0
occasionally ☐	1
sometimes ☐	2
most of the time ☐	3
all of the time ☐	4

6b. How much does this bother you?
Please ring a number between 0 (not at all) and 10 (a great deal)

    0   1   2   3   4   5   6   7   8   9   **10**
not at all   a great deal

7a. Do you have to strain to <u>urinate</u>?

never ☐	0
occasionally ☐	1
sometimes ☐	2
most of the time ☐	3
all of the time ☐	4

7b. How much does this bother you?
Please ring a number between 0 (not at all) and 10 (a great deal)

    0   1   2   3   4   5   6   7   8   9   **10**
not at all   a great deal

8a. **Do you stop and start more than once while you urinate?**

never	0
occasionally	1
sometimes	2
most of the time	3
all of the time	4

8b. **How much does this bother you?**
Please ring a number between 0 (not at all) and 10 (a great deal)

     0   1   2   3   4   5   6   7   8   9   **10**
not at all   a great deal

V score: sum scores 6a+7a+8a [ ]

9a. **Does urine leak before you can get to the toilet?**

never	0
occasionally	1
sometimes	2
most of the time	3
all of the time	4

9b. **How much does this bother you?**
Please ring a number between 0 (not at all) and 10 (a great deal)

     0   1   2   3   4   5   6   7   8   9   **10**
not at all   a great deal

10a. **How often do you leak urine?**

never	0
once or less per week	1
two to three times per week	2
once per day	3
several times per day	4

10b. **How much does this bother you?**
Please ring a number between 0 (not at all) and 10 (a great deal)

     0   1   2   3   4   5   6   7   8   9   **10**
not at all   a great deal

11a. Does urine leak when you are physically active, exert yourself, cough or sneeze?

never ☐ 0
occasionally ☐ 1
sometimes ☐ 2
most of the time ☐ 3
all of the time ☐ 4

11b. How much does this bother you?
Please ring a number between 0 (not at all) and 10 (a great deal)

0 1 2 3 4 5 6 7 8 9 **10**
not at all   a great deal

12a. Do you ever leak urine for no obvious reason and without feeling that you want to go?

never ☐ 0
occasionally ☐ 1
sometimes ☐ 2
most of the time ☐ 3
all of the time ☐ 4

12b. How much does this bother you?
Please ring a number between 0 (not at all) and 10 (a great deal)

0 1 2 3 4 5 6 7 8 9 **10**
not at all   a great deal

13a. Do you leak urine when you are asleep?

never ☐ 0
occasionally ☐ 1
sometimes ☐ 2
most of the time ☐ 3
all of the time ☐ 4

13b. How much does this bother you?
Please ring a number between 0 (not at all) and 10 (a great deal)

0 1 2 3 4 5 6 7 8 9 **10**
not at all   a great deal

I score: sum scores9a-13a ☐

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Thank you very much for answering these questions.

i

APPENDIX 1.4 PUBLICATIONS ARISING FROM THIS THESIS

ARTICLES

- Contreras-Sanz, A., **Krska, L.**, Balachandran. A, Curtiss, N., Khasriya, R., Kelley, S., Strutt, M., Gill, H., Taylor, K., Mansfield, K., Wu, C., Peppiatt-Wildman, C., Malone-Lee, J., Duckett, J., Wildman, S., (2016). Altered urothelial ATP signalling in major subset of human overactive bladder patients with pyuria. *AJP Renal Phys.* 311(4):F805-F81

- Curtiss, N., Balachandran, A., **Krska, L.**, Peppiatt-Wildman, C., Wildman, S., Duckett, J., (2017). A case controlled study examining the bladder microbiome in women with Overactive Bladder (OAB) and healthy controls. *Euro J Obstet Gynecol Reprod Biol,* 214:31-35

- Curtiss, N., Balachandran, A., **Krska, L.**, Peppiatt-Wildman, C., Wildman, S., Duckett, J., (2018). Age, menopausal status and the bladder microbiome. *Euro J Obstet Gynecol Reprod Biol,* 228:126-129

ABSTRACTS

- **Krska, L.,** Balachandran, A., Caballero, N., Curtiss, N., Peppiatt-Wildman, C., Strutt, M., Duckett, J., Wildman, S.P.W., (2016). Culture Of Urine Sediment Reveals Differences Between Patients With Overactive Bladder (OAB) And Controls: Are Changes In The Bladder Microbiome Responsible For Symptoms Of OAB?; *FASEB J.* 30, 1104.2

- Curtiss, N., **Krska, L.,** Balachandran, A., Duckett, J., Wildman, S.P.W., (2017); Age, Menopausal status and the bladder microbiome. *IUGA, Vancouver.*

AWARDS

- International Urogynecology Association annual meeting Vancouver Canada. Best basic science prize. (Credit to speaker Miss Natasha Curtiss)
  Curtiss NL, Balachandran A, **Krska L**, Wildman S, Duckett J "Age, menopausal status and the bladder microbiome".