The role of chronic and recurrent infection in the generation of overactive bladder symptoms in multiple sclerosis

Submitted by

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Declaration

I, Anthony Kupelian, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

This thesis provides original evidence that patients with multiple sclerosis may harbour undisclosed urinary infection that generates urothelial inflammation and overactive bladder symptoms. The studies within this thesis examine the performance of recommended diagnostic tests for urinary tract infection, and explore the bacterial ecology of urinary infection and its associated urothelial inflammatory response. The association between lower urinary tract inflammation, bacterial colonisation and the generation of overactive bladder symptoms is explored. A novel therapy with proposed immunomodulatory effects was tested as a candidate treatment for overactive bladder symptoms.

The urinary dipstick is the recommended diagnostic test for urinary infection in patients with multiple sclerosis. In patients with chronic lower urinary tract symptoms, the dipstick failed to identify culture-positive bacterial infection in more than half of cases. This failure is compounded by the poor performance of current culture-based diagnosis that employs erroneous quantitative diagnostic thresholds.

When a sensitive culture method was deployed and quantitative thresholds rejected, controlled data demonstrated that bacterial urinary infection was evident in sixty percent of patients. Quantitative and qualitative differences in the bacterial ecology of infecting isolates were evident amongst patients and controls. This was associated with increased urothelial inflammation amongst patients.

Prospective, controlled data demonstrated that escalating urothelial inflammation, characterised by the expression of pyuria, was associated with increased bacterial load in the lower urinary tract. Pyuria predicted the severity of overactive bladder symptoms and indices of bladder function.

Am immunomodulatory treatment for overactive bladder symptoms was tested as part of this work but no therapeutic effects were identified.

Dedication

This work is dedicated to the patients who made it possible.

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ABU	Asymptomatic bacteriuria
ACh	Acetylcholine
AE	Adverse event
AI	Autoinducer
AMP	Adenosine monophosphate
ANTT	Aseptic non-touch technique®
AR	Adverse reaction
ATP	Adenosine triphosphate
AUM	Apical asymmetric unit membrane
BBB	Blood-brain barrier
BMI	Body mass index
BTX-A	Onabotulinum toxin A
cAMP	Cyclic adenosine monophosphate
CI	Chief investigator
CNS	Central nervous system
CO	Carbon monoxide
CO_2	Carbon dioxide
CoNS	Coagulase-negative staphylococci
CRF	Case report form
CXCL-8	Interleukin-8
DAMP	Danger-associated molecular pattern
DDAVP	Desmopressin
DSD	Detrusor-sphincter dyssynergia
EBV	Epstein-Barr Virus
ELISA	Enzyme-linked immunosorbent assay
EAE	Experimental autoimmune encephalitis
FISH	Fluorescence in-situ hybridisation
GABA	Gamma-aminobutyric acid
GCP	Good Clinical Practice

GMP	Good Manufacturing Practice
HPF	High-power field
IBC	Intracellular bacterial community
ICH	International Conference on Harmonisation
IFN	Interferon
IL-6	Interleukin-6
ISC	Intermittent self-catheterisation
IQR	Interquartile range
LPS	Lipopolysaccharide
LUTD	Lower urinary tract dysfunction
LUTS	Lower urinary tract symptoms
MHC	Major histocompatability complex
MHRA	Medicines and Healthcare products Regulatory Agency
MCID	Minimal clinically important difference
MS	Multiple sclerosis
MSU	Midstream urine
NANC	Nonadrenergic, noncholinergic
NAT	Nucleic acid-based techniques
NF-κB	Nuclear factor kappa beta
NO	Nitric oxide
NRES	National research Ethics Service
OAB	Overactive bladder
PRR	Pattern recognition receptor
PAG	Periaqueductal grey
PG	Prostaglandin
PMC	Pontine Micturition Centre
PVR	Post-void residual bladder volume
QIR	Quiescent intracellular reservoir
QoL	Quality of life
RCF	Relative centrifugal force
RCT	Randomised, controlled trial
RNA	Ribonucleic acid

RPM	Revolutions per minute
SAE	Serious adverse event
SAR	Serious adverse reaction
RR-MS	Relapsing-remitting multiple sclerosis
SCI	Spinal cord injury
SOP	Standard operating procedure
SP-MS	Secondary progressive multiple sclerosis
SPA	Suprapubic aspiration
SUI	Stress urinary incontinence
SUSAR	Suspected unexpected serious adverse reaction
TLR4	Toll-like receptor 4
TCA	Tricyclic antidepressant
TCR	T cell receptor
UDS	Urodynamic studies
UK	United Kingdom
UTI	Urinary tract infection
UUI	Urgency urinary incontinence
UPEC	Uropathogenic E. coli
UP	Uroplakin
VBNC	Viable but not culturable

1 Introduction

1.1 Multiple sclerosis: An overview

Multiple sclerosis (MS) is a common neurological disorder, the hallmark of which is demyelination of the central nervous system (CNS). The core clinical manifestations of MS are evidence of neurological lesions, disseminated temporally and spatially in the CNS. The disease may present with diverse, insidious symptoms and follow a variable course, although some patterns can be usefully defined. Whilst the precise aetiology of MS remains elusive, a number of genetic and environmental factors have been implicated in its pathogenesis, although our understanding of how the disease is initiated remains limited.

1.1.1 Epidemiology of multiple sclerosis

Current population estimates suggest that 100 000 individuals in the UK are affected by MS and that figure might be 2.5 million globally (1). Mean age at diagnosis is 30 years, establishing MS as the leading cause of non-traumatic neurological disability in younger adults in the developed world (2). Whilst the disease most commonly presents in young adulthood, children, adolescents, and the elderly may occasionally be affected (3).

Ethnicity appears to have the strongest influence on disease development, and it is likely that such effects have a genetic basis. African American and Japanese populations demonstrate a much lower prevalence of the disease when compared to Caucasian populations, and the disease is almost completely absent amongst the Chinese (4). Significant familial clustering is a feature of MS, with the risk of developing the disease closely related to the degree of kinship (5, 6). These data suggest that a maternal parent-of-origin effect contributes significantly to familial aggregation of MS, although the mechanism by which this occurs remains unknown (7).

The influence of gender is widely recognised, and a female preponderance is reported by all epidemiological studies that examine the natural history of the disease. The majority of these data report twice as many affected females than males (8), although these estimates are subject to some variation (9-11). Genomic studies have consistently failed to support the existence of any x-linked, disease-associated genes (12).

Human leucocyte antigen (HLA) haplotypes appear to exert effects on disease development but this relationship is complex. There is significant geographic variation in HLA haplotypes associated with MS, and these genes may confer susceptibility or protection, and demonstrate interaction effects (13). In addition, distinct HLA haplotypes might influence the course and clinical features of MS. The biological effects of these polymorphisms remain relatively weak. Genomic association studies have uncovered other candidate genes that influence cytokines and inflammatory mediators, with modest effects on disease development and progression (14, 15).

Although genetic susceptibility may explain the familial clustering of MS, and the reduction in risk with increasing genetic distance, it cannot account for the geographic variations in disease prevalence, and changes in risk associated with migration. Whilst epidemiological studies implicate environmental factors as an important determinant of risk, direct evidence relating to specific agents is lacking with notable exceptions.

Within temperate regions, the prevalence of MS increases with higher latitude. With the exception of ethnicity, latitude has the largest influence on MS risk with sunlight exposure and vitamin D status proposed as a likely mechanism for this finding (16-19). Despite these observations, there remains no evidence that vitamin D supplementation reduces the risk of disease development (20, 21).

Migration studies have suggested that the influence of environmental factors in the development of MS may be dependent on the timing of exposure. First-generation

migrants appear to have a risk of disease development closer to that of their home country, with subsequent generations demonstrating a risk closer to that of the country of migration in which they were born (22, 23). Other work points to a critical window of exposure in childhood and adolescence in which environmental factors might exert their influence on the development of MS (24, 25).

Whilst multiple bacterial and viral agents have been implicated with the development of MS, the evidence associating Epstein–Barr virus (EBV) infection with MS is perhaps the strongest. Epstein–Barr virus infection is almost ubiquitous amongst patients with a diagnosis of MS. Although it is reported in approximately 94% of age-matched controls, seropositivity for EBV confers a twenty-fold increase in the risk of the disease (26). Thus, MS is rare in adults who have not been infected with EBV. Whilst EBV infection alone confers an elevated risk of MS, the development of infectious mononucleosis, and heightened anti-EBV antibody responses are associated with an even higher risk of disease development (27). The relationship between high EBV antibodies and the onset on neurological symptoms in MS appears to be temporal (28).

Infection with human herpes virus 6 (HHV-6) is another agent that has been implicated in the development of MS. Although seropositivity to the virus is also highly prevalent in both MS patients and controls, there are notable differences in the expression of viral antigen and antibody production in the CNS of affected patients when compared to healthy subjects (29). These data hint that it is not simply infection that mediates the development of MS, but the host immune response to the infecting pathogen.

Although EBV infection can explain many of the features of MS epidemiology, by itself the link between EBV and MS cannot account for the decline in risk amongst migrants from high to low MS prevalence areas. This decline implies that in low-risk areas, infectious strains might have less propensity to cause MS, or more likely that other infectious or non-infectious factors modify the host response to this initial infection.

1.1.2 Pathogenesis of multiple sclerosis

Multiple sclerosis has long been considered an autoimmune condition, mediated by the entry of peripherally activated autoreactive T lymphocytes into the CNS. These lymphocytes then react to specific antigen, initiating an inflammatory process that results in demyelination and the generation of symptoms. There is evidence that the relapsing forms of the disease, characterised by episodes of neurological deficit and recovery, demonstrate differences in underlying pathophysiology to the progressive disease forms.

Current opinion maintains that demyelination, mediated directly by T lymphocytes, and as a result of macrophage activity, is a central process in the pathogenesis of MS, irrespective of disease stage or subtype. This pattern of injury is further shaped by antibody-mediated and hypoxic damage. Whilst this process defines the relapsing-remitting forms of MS, new focal demyelination is much less common in progressive disease. Progressive variants of MS are characterised by enlargement of existing areas of demyelination and a generalised pattern of axonal damage and degeneration (30).

Early in the course of relapsing-remitting disease, complete recovery can be observed, even from marked neurological defects. Data from animal studies using a model of experimental autoimmune encephalitis (EAE) have proposed three mechanisms by which recovery might be achieved. Symptoms might be mediated by inflammation in the absence of demyelination, and in these inflamed but structurally normal axons, the resolution of inflammation might be associated with restoration of normal function (31). In demyelinated axons, adaptive changes including changes in the expression of ion channels and neuroplasticity may occur, and remyelination might also contribute to neurological recovery (32, 33).

In contrast to relapsing disease, progressive forms of MS are associated with a systematic and irreversible neurological deterioration. Magnetic resonance imaging

(MRI) and histopathological studies have demonstrated diffuse neuroaxonal loss in these patients, with little evidence of acute, focal inflammatory lesions in the CNS (34, 35). This manifests most often as spinal cord atrophy (36).

Axonal loss is thought to be the leading cause of irreversible neurological damage in progressive MS. Existing data suggest that generalised axonal loss may occur as a consequence of the inflammatory response within the CNS, with nitric oxide and glutamate implicated as mediators in this interaction (37-39). The eventual failure of damaged but functioning axons, culminating in persistent conduction block, may also contribute to the permanent loss of function in progressive disease. Axonal repair in MS is variable throughout the disease course and the adaptive changes proposed to moderate functional improvement after relapse might leave axonal function fragile (40). Wallerian degeneration secondary to neuronal apoptosis has also been proposed as a potential contributory factor (41). Some authors have hypothesised that a primary neurodegenerative process might precede the inflammatory response which characterises MS (42, 43). The notion that MS might be a primary neurodegenerative disease would neatly account for the diffuse pattern of neurological injury seen in its progressive disease variants, but there is no direct evidence to support this hypothesis.

The development of autoimmunity in MS is likely to be generated by similar mechanisms to those demonstrated in other autoimmune disease. During lymphocyte maturation, T cells demonstrating T cell receptor (TCR) with high avidity for self-antigen are usually deleted in the thymus. Nonetheless, it is known that some autoreactive T cells are able to escape destruction and settle in lymphoid tissue. In most circumstances, these autoreactive cells remain quiescent, as they are not excessively stimulated by self-antigen and subject to suppression by regulatory T cells. However, these cells could be activated against self-antigen under specific circumstances.

Whilst each TCR is unique, it can be activated by a spectrum of specific peptides that are structurally related. This feature of the TCR is known as poly-specificity (44).

Thus, a vast number of pathogens might share a peptide sequence or structural similarities with self-antigen and any such pathogen might be able to activate autoreactive T cells. The homology of microbial and self-antigen leading to T cell activation and host damage is termed 'molecular mimicry'. This process has been implicated in the initiation and propagation of autoimmune disease. Lymphocytes bearing dual TCRs have also been identified, permitting individual cells to harbour receptors that could respond to foreign and self-antigen (45).

Another mechanism by which previously quiescent autoreactive T cells might initiate autoimmune damage is 'bystander activation' (46). This term describes a process whereby autoreactive T cells residing in lymphoid organs can be activated by pro-inflammatory cytokines produced in response to acute infection. Such infections need not be a specific agent, rather any pathogen that can induce an inflammatory cytokine response in the host.

Some investigators have suggested that in a susceptible host, auto-reactivity might be primed by molecular mimicry or bystander activation, and autoimmune disease triggered by subsequent infection (47). The diversity of microbial antigens able to activate a specific TCR might explain why no single agent has ever been convincingly implicated as the cause of MS.

The available experimental data suggest that a model describing a single priming event and a further infective challenge leading to autoimmune disease may be an oversimplification. It may be that the priming and challenge phases of such a pathway could be multiple, separated temporally and spatially within the host. Other factors may moderate this process, including host characteristics, the nature and location of subsequent infective episodes, and their associated cytokine response (48).

1.1.3 Clinical features of multiple sclerosis

The diagnosis of MS hinges on the identification of neurological deficits disseminated in time and space. Previously, CNS lesions were defined solely on clinical grounds, although current diagnostic guidance advocates the use of MRI, evoked potentials and the examination of cerebrospinal fluid to complement clinical assessment (49). Whilst a full description of the diagnostic methods employed in the investigation of MS is beyond the scope of this work, these investigations are used to seek evidence of the dissemination of lesions in time and space, identify inflammation in the CNS and exclude competing diagnoses.

Whilst MS can follow a variable course, four patterns of disease are usefully described (50). These disease groups are helpful as they foster mutual understanding between clinicians and researchers, particularly when defining study populations in clinical trials (**Table 1**). Relapsing-remitting disease is most common at presentation, although around 20% of patients are diagnosed with progressive disease from the outset. Of those diagnosed with relapsing remitting disease, around 50% will enter a progressive phase of the condition within 10-15 years of diagnosis (51-53). Progressive disease heralds a stepwise decline in neurological function and is associated with the majority of neurological disability in MS.

The symptoms and signs of MS are diverse and particular patterns of neurological involvement can be seen in specific geographical locations and amongst some ethnic groups. Large population-based studies have helped to characterise the prevalence and impact of specific symptoms in MS and the results of a large, contemporary analysis undertaken in the UK are summarised in **Table 2** (54).

Table 1 *Classification of MS disease patterns (adapted from Lublin 1996).*

Relapsing remitting MS (RR-MS) *Characterised by 'clearly defined relapses with full recovery or with sequelae and residual deficit upon recovery; periods between relapses are characterised with a lack of progression'.*
Secondary progressive MS (SP-MS) *Characterised by an 'initial relapsing-remitting disease course followed by progression with or without occasional relapses, minor remissions, and plateaus'.*
Primary progressive MS (PP-MS) *Characterised by 'disease progression from the onset with occasional plateaus and temporary minor improvements allowed'.*
Progressive relapsing MS (PR-MS) *Characterised by 'progressive disease from onset, with clear acute relapses, with or without full recovery; periods between relapses are characterised by continuing disease progression'.*

Whilst these data are not stratified by disease status, they provide a pragmatic description of the disease experience reported by patients and the perceived impact of individual symptoms on health-related quality of life (QoL). Whilst fatigue is cited as the most prevalent and intrusive of these symptoms, it should be noted that bladder problems, more correctly termed lower urinary tract symptoms (LUTS), are extremely common. Lower urinary tract symptoms have a significant impact on those patients are affected. Whilst prevalence estimates of around 75% are often cited, this figure may be conservative, particularly in patients with a longstanding diagnosis (54-56).

Table 2 *Symptoms reported by 2265 UK patients with MS (adapted from Hemmet et al. 2004).*

Symptomatic problem	**Proportion affected (%)**	**Impact 'moderate' or 'high'**
Fatigue	96%	88%
Balance and dizziness problems	92%	74%
Loss of mobility	91%	79%
Sensory problems	88%	54%
Bladder problems	87%	70%
Loss of memory and concentration	87%	52%
Spasticity	82%	54%
Vision problems	82%	41%
Pain	81%	50%
Bowel problems	74%	45%
Sexual problems	70%	42%
Tremor	68%	30%
Speech and swallowing problems	68%	26%

1.2 Lower urinary tract dysfunction in multiple sclerosis

1.2.1 Structure and function of the lower urinary tract

The lower urinary tract comprises the bladder and urethra, functioning as a unit, which cyclically accommodate the storage and expulsion of urine. This cyclical activity is known as the 'micturition cycle' and the expulsion phase is termed 'voiding'. The filling phase of the bladder is known as the 'storage' phase. Regulation of normal lower urinary tract function requires intact and coordinated neurological control mechanisms, moderated by voluntary inputs. Much of our understanding of these mechanisms has been derived from animal models that demonstrate marked interspecies differences in lower urinary tract physiology. Caution should be exercised when extrapolating these data to humans.

1.2.1.1 Lower urinary tract structure

In males and females, the urinary bladder is located in the anterior pelvis. The bladder base, known as the trigone, is small and varies little in size as the organ fills. The trigone is bounded by two ureteric orifices laterally and the internal urethral meatus anteriorly. The inferolateral surfaces of the bladder are closely related to the walls of the pelvis and vary minimally in size as the bladder expands. The superior surface is known as the dome of the bladder, and it is this region that demonstrates greatest variation and expansion as the bladder distends (57).

The bladder wall is commonly described as having three layers. These include a mucosal layer, a muscular coat, and a serosal layer. The mucosa is further subdivided into three components: the urothelium, which is direct contact with the bladder urine; the basement membrane, which is a single layer of cells beneath the urothelium; and the lamina propria, which lies between the basement membrane and muscular wall of the bladder. In addition to its barrier function, it is now clear

that the mucosa plays a central role in the control of lower urinary tract function (58).

The muscular wall of the bladder, the detrusor, is comprised of smooth muscle, further sub-divided into smaller bundles called fascicles. These muscle units run in all directions in the detrusor, their orientation and activation influencing how the bladder responds to filling and contraction (59). The superior and superolateral surfaces of the bladder are in direct contact with the abdominopelvic cavity. Here, the detrusor is covered by a reflection of the pelvic peritoneum called the serosa. The inferior and inferolateral surfaces of the bladder are in direct contact with adjacent pelvic organs with a layer of connective tissue called adventitia at the interface.

The urethral mucosa is contiguous with the urothelium although it is composed of non-keratinizing, squamous epithelium in common with the vulva and lower vagina (57). Beneath this lies a submucosal layer, comprising a rich vascular plexus of arteriovenous anastomoses and multiple suburethral glands opening into the urethral lumen. Blood flow through the submucosal plexus is thought to enhance urethral closure, augmented by mucus production from the submucosal glandular tissue (60).

Whilst the male and female urethrae demonstrate marked anatomical differences, functionally they are similar. Continence is achieved by muscular sphincter mechanisms, consisting of both smooth and striated muscle types. The relative contribution of these muscular components to resting urethral tone and continence during bladder filling in humans is unresolved. The internal urethral sphincter is composed of smooth muscle, continuous with that of the detrusor and trigone (61). Animal studies have demonstrated that the smooth muscle of the urethra is arranged in circular and longitudinal orientations, enveloping the urethra in a horseshoe configuration. These muscular layers are thought to constrict and shorten the urethra, respectively (62). Tonic contraction of the internal urethral sphincter,

mediated by the autonomic nervous system, maintains continence under normal conditions.

The external urethral sphincter contains striated muscle and is under voluntary control. Anatomically, it comprises three elements, including a true circumferential component that surrounds the urethra, the compressor urethral muscle and the urethrovaginal sphincter (63). The external sphincter is composed of mainly slow-twitch muscle fibres, and is suited to prolonged periods of increased tone. Voluntary activation of the external sphincter and reflex contraction in response to increased abdominal pressure raise urethral pressure to maintain continence when intravesical pressures rise (64).

1.2.1.2 Innervation of the lower urinary tract

The innervation of the lower urinary tract includes parasympathetic, sympathetic, and somatic divisions (65). In humans, the parasympathetic nerves to the lower urinary tract, known as the pelvic splanchnic nerves, originate from sacral segments S2-S4. These preganglionic axons then descend and converge on ganglia in the pelvic plexus and bladder wall. The sympathetic supply arises from the thoracic and lumbar segments T11-L2 and preganglionic fibres synapse with ganglia in the sympathetic chain, superior hypogastric plexus and pelvic plexus. The pelvic plexus is therefore a mixed autonomic plexus containing nerves from both divisions. Branches of the sacral segments S2-S4 travel in the pudendal nerve and supply the striated urethral sphincter, in addition to the levator ani muscles of the pelvic floor.

1.2.1.3 Efferent neural control of the lower urinary tract

The lower urinary tract has only two functional states, storage and elimination, and the proposed neural pathways that moderate these activities appear to operate in a switch-like fashion. This contrasts with the tonic effects of the autonomic nervous system on other organ systems. The storage phase is dominated by sympathetic

activity from the thoracolumbar sympathetic plexus. Postganglionic nerves release noradrenaline, which stimulates adrenergic receptors in the lower urinary tract. This activation contracts urethral smooth muscle and maintains detrusor relaxation, ensuring continence and low-pressure bladder filling. Contraction of the striated muscle of the urethra and levator ani contribute to the sphincter mechanism, innervated by sacral somatic fibres carried in the pudendal nerve. Sympathetic nerves interact with parasympathetic ganglia, exerting an inhibitory influence on parasympathetic outflow.

The voiding phase is mediated by dominant parasympathetic discharge from nerves originating in the sacral nerve roots. Parasympathetic activity induces detrusor contraction, relaxation of urethral smooth muscle and bladder emptying. In the bladder, parasympathetic transmission appears to be mediated primarily by the release of acetylcholine (ACh) acting on muscarinic receptors (65).

In addition to parasympathetic cholinergic stimulation, 'nonadrenergic, noncholinergic' (NANC) transmission also generates detrusor contractions. The NANC neurotransmitters/modulators include the purine adenosine triphosphate (ATP), and the gases nitric oxide (NO), and carbon monoxide (CO). Lower urinary tract neurotransmission may vary in pathological states. Human studies imply that purinergic signalling makes almost no contribution to contraction generation in healthy bladders (66) but has a far greater role in detrusor activation when pathology is present (67-75). In the urethra, a variety of neurotransmitters have been implicated in parasympathetic transmission, including ACh and NANC agents, but human data are lacking (65).

1.2.1.4 Afferent neural pathways from the lower urinary tract

Studies in animals have demonstrated that afferent nerves from the lower urinary tract may be carried to the CNS by both divisions of the autonomic nervous system. Afferent nerves comprise myelinated A-delta (AΔ) fibres and unmyelinated C fibres.

A-delta fibres are found mainly, but not exclusively in the smooth muscle of the detrusor. C afferents are demonstrated throughout the human bladder, and interact directly with the urothelium, lamina propria, and the detrusor muscle (76).

Animal studies have demonstrated that both fibre types transmit information on bladder volume changes in response to the stretch-mediated activation of mechanoreceptors. In the rat, C afferents respond to slow distension but not bladder contractions, suggesting that volume-related change in response to bladder filling is transmitted via C fibres (77, 78). In cats, AΔ fibres are responsible for volume-dependent sensory outflow, and most C afferents remain quiescent under normal circumstances and are termed 'silent' afferents. When the feline urothelium is exposed to cold, or chemical irritation, C afferents demonstrate spontaneous discharge when the bladder is empty, and increased firing during bladder distension (79, 80). A definitive description of how these systems are organised in humans remains elusive.

Afferent C fibres are widely distributed in the human bladder and in vivo studies support the hypothesis that C fibre upregulation is a feature of some pathologic states. Increased C afferent recruitment and excitability has been implicated in the generation of LUTS in patients with spinal injury, bladder pain syndromes and the overactive bladder (81-83). In addition, C fibres respond to a wide array of stimuli which are able to moderate afferent signal transduction. These include the neurotoxins capsaicin, resiniferatoxin and botulinum toxin, and other agents including tachykinins, neurotrophic factors, prostaglandins (PG), ATP and NO (65, 81, 83-87). 'Silent' C afferents, which cannot be stimulated under normal conditions, may also be sensitised leading to sensory activation. The upregulation of C afferent activity might be mediated by changes in peripheral afferent organisation or central effects within the CNS (88, 89).

1.2.1.5 The urothelium

Traditionally, the urothelium was considered as a passive barrier at the luminal interface but it is now recognised as a participant in the regulation of lower urinary tract function. New insights have uncovered specialised sensory functions, and a capacity for complex interactions with other cells in the lower urinary tract. The urothelium expresses nicotinic, muscarinic, adrenergic, purinergic (P2X and P2Y), capsaicin (TRPV 1), and tachykinin (NK-2) receptors. It is also able to secrete and respond to molecules (eg. ACh, ATP and NO) that are known to modulate local neuronal activity and influence sensory/motor function. Changes in urinary pH, ion concentration, osmolality and chemical stimuli may also influence afferent sensory transmission, mediated by the release of local signalling molecules.

The capacity of the urothelium to moderate lower urinary tract function is now widely accepted. ATP instilled into the rat bladder induces detrusor contractions and abolition of NO secretion in the urothelium has a similar effect. Conversely, P2X receptor knockout mice have a hypoactive bladder. Studies of human and animal models of inflammatory cystitis have demonstrated greater urothelial ATP release in response to stretch, and P2X receptor up regulation (67, 69, 70, 72, 73, 75, 90, 91). Similar findings have also been reported in human detrusor from patients with overactive bladder symptoms (74, 92, 93). Other work has suggested that reduced extracellular ATP hydrolysis may occur in those with bladder pathology (94). Purinergic activation of bladder afferents in the lamina propria by urothelial-derived ATP has also been demonstrated (95).

1.2.1.6 The lamina propria

Whilst the urothelium has been the subject of much recent investigation, the functional role of the lamina propria, which lies beneath the urothelial basement membrane and the detrusor muscle, remains poorly understood. The lamina propria comprises an extracellular matrix containing myofibroblasts, also known as

interstitial cells, fibroblasts, adipocytes, and afferent/efferent nerves. It also demonstrates a rich lymphatic and vascular supply, and elastin and smooth muscle fibres (96, 97).

The extracellular matrix contains coiled collagen fibres that have been implicated in the regulation of bladder compliance, and elastin fibres that might facilitate recoil to augment detrusor contraction during bladder emptying (98). Myofibroblasts have long been suspected to play a role in signal integration in the bladder, although a full account of how this is mediated is yet to be described (99). Human data have suggested increased expression of myofibroblasts in pathologic states, and structural changes affecting the intercellular pathways that mediate cell-to-cell communication (100, 101).

1.2.1.7 Central nervous system regulation of the lower urinary tract

Animal models of induced brain injury provided the first evidence of how neural control of micturition might be organised. The findings indicated that a region in the brainstem could be responsible for coordination of lower urinary tract functioning and higher cortical centres appeared to have an inhibitory effect on micturition (102). It was hypothesised that a spinobulbospinal pathway carried afferent signals from the lower urinary tract to the brainstem, which then modulated efferent activity sent back to the bladder and urethra. This brainstem centre was named the Pontine Micturition Centre (PMC), a switch-like neural relay, actuated by critical afferent activity generated by tension receptors in the bladder (102). The PMC is deemed to switch between storage and voiding phases by inverting autonomic output to the lower urinary tract. The changes in autonomic tone that mediate the micturition cycle are highly coordinated and damage to any part of the spinobulbospinal pathways might be expected to generate symptoms.

Conscious control of micturition originates from the cerebral cortex, and cortical inputs have an inhibitory effect on the initiation of micturition. The periaqueductal

grey (PAG) is the major anatomic and functional interchange between the forebrain and brainstem and appears to integrate a host of behavioural responses, including those involved in micturition (103). It has been proposed that the PAG functions as an interface between bladder afferent input and forebrain modulatory influences controlling micturition (104).

1.2.1.8 Spinal reflexes and micturition

Spinal reflexes can mediate automatic micturition triggered by increasing bladder distension, dissociated from higher regulatory inputs. Such reflex voiding is usually absent in adult species with an intact CNS, although the re-establishment of reflex bladder emptying has been demonstrated in neonates and adults with spinal cord injury (SCI) (88, 105). In the feline model, C afferents are normally quiescent, but the recrudescence of spinal reflex voiding is associated with C afferent sensitisation (79, 80). Clinical data from human studies supports C afferent upregulation as a key mediator in the re-establishment of reflex voiding (83, 106).

1.2.1.9 Neurotransmitters in CNS micturition pathways

Animal studies have demonstrated a vast number of neurotransmitters that mediate excitatory, inhibitory or mixed effects on pathways controlling micturition in the CNS. Excitatory transmitters include glutamate, tachykinins, NO and ATP (107-114). Amino acids, such as gamma-aminobutyric acid (GABA) and opioid peptides mediate inhibitory effects on central micturition control (115). A number of neurotransmitters with complex, mixed effects have been identified which include dopamine, monoamines, noradrenaline, ACh and a variety of neuropeptides (116-119)

1.2.2 Damage to neural control mechanisms in multiple sclerosis

Spinal cord damage is cited as the main cause of lower urinary tract dysfunction (LUTD) in MS (104). The disruption of spinal connections between the brainstem and sacral nerves is thought to impair normal neural control of micturition. The clinical manifestations of such damage include disorders of bladder storage, problems emptying the bladder, or a combination of these symptoms. Absent bladder sensation and bladder atony are less commonly reported (55). In addition to the loss of higher neural control mechanisms, the emergence of automatic voiding reflexes may also contribute to symptoms. These reflexes are thought to be triggered at the spinal level, mediated by bladder filling, when higher neurological control mechanisms are no longer active (120).

Spinal cord damage has also been associated with organisation changes to neural afferent and efferent signalling. Human data has demonstrated enhanced C fibre neurotransmission in MS patients with spinal damage (89, 105, 121). These C afferents may be activated by many stimuli, including NANC agents. Studies of purinergic signalling in the spinal cord-injured rat have revealed a ten-fold increase in basal urothelial-derived ATP release and similar heightened secretion under stimulation. This evidence suggests that NANC mechanisms play a much greater role in the regulation of lower urinary tract function after spinal damage.

In addition to nervous system disruption, animal models of spinal cord transection have demonstrated rapid alterations in urothelial cell functioning after spinal injury. In a rat model, SCI was associated with epithelial dysfunction which was apparent by two hours and did not resolve for up to two weeks; epithelial damage manifest as loss of umbrella cells and increased urothelial permeability (122). Whether such changes may affect humans is not known.

1.2.3 Storage symptoms

The normal bladder acts as a quiescent reservoir for urine between voids. Bladder emptying is initiated under conscious control in response to sensory and cognitive inputs. Whilst increasing awareness of bladder filling indicates the impending need to empty the bladder, other factors such as habituation, opportunism and the fear of leakage play a significant role (123).

Storage symptoms include urinary urgency, increased daytime frequency, nocturia and urinary incontinence. Urinary incontinence is subdivided further into urgency urinary incontinence and stress urinary incontinence (124). Urgency, increased daytime frequency, urgency urinary incontinence and nocturia are the principal storage symptoms experienced by MS patients (55). This symptom complex is commonly described as the overactive bladder (OAB) syndrome, although urinary urgency is regarded as the defining symptom of this condition (125). Storage symptoms are believed to be mediated by loss of regulatory control, in particular inhibitory influences, and the emergence of spinal reflex voiding. Changes in the organisation of sensory processing and urothelial function have also been implicated in symptom generation.

1.2.4 Voiding symptoms

Voiding symptoms include hesitancy, slow stream, intermittent stream, straining, and terminal dribble. Post-micturition symptoms, although often defined in their own group, commonly accompany voiding symptoms. They include the sensation of incomplete emptying and post micturition dribble.

Voiding symptoms associated with MS are attributed to detrusor-sphincter dyssynergia (DSD) which describes involuntary contractions of the urethral sphincter during voiding (126). In the normally functioning lower urinary tract, there is a reciprocal relationship between contraction of the bladder detrusor muscle and

urethral relaxation. This coordinated activity, attributed to regulation in the brainstem, might be undermined or abolished as a result of damage to the spinal nerves connecting the lower urinary tract to the brain. Sensations of incomplete emptying are widely attributed to the retention of a residual urine volume after passing urine.

1.2.5 Recommended investigations and management

The management of neurogenic LUTD is influenced by the assumed underlying neuropathology. Serious renal complications may arise when the lower urinary tract is rendered a high-pressure system, as a result of persistently elevated bladder pressures and outlet resistance. This leads to severe renal outflow obstruction, glomerular damage and a progressive deterioration in renal function if untreated. Whilst pressure-mediated damage is relatively common in those affected by traumatic spinal cord injury or neural tube defects such as spina bifida (127, 128), patients with MS do not appear to have an elevated risk of renal complications.

Ten studies, including 1460 patients with MS, have reported the prevalence of upper tract complications to be 0.9-5.0% (129-138). Four additional studies were identified with higher reported rates of upper tract complications, although these estimates include non-obstructive radiological abnormalities of questionable clinical significance (139-142). No consistent disease-specific variables, including disease severity or duration since diagnosis, could predict the development of upper tract abnormalities. The majority of data relating to upper tract complications in MS are based on ultrasound identification of renal outflow tract dilatation mostly without estimates of renal function. Whether these structural abnormalities, many of them mild, would have any effect on renal function long-term is not known.

In the largest reported series, Krhut and colleagues (2008) reported the creatinine clearance of 92 patients with MS and demonstrated that only 3% (*n*=3) had a creatinine clearance of <90ml min 1.73 m^2 (134). Half of the patients in this study

had progressive MS and their mean age was 44 years. Only 4% (*n*=4) of patients demonstrated radiological abnormalities that comprised two cases of non-obstructive renal calculi and two cases of mild hydronephrosis.

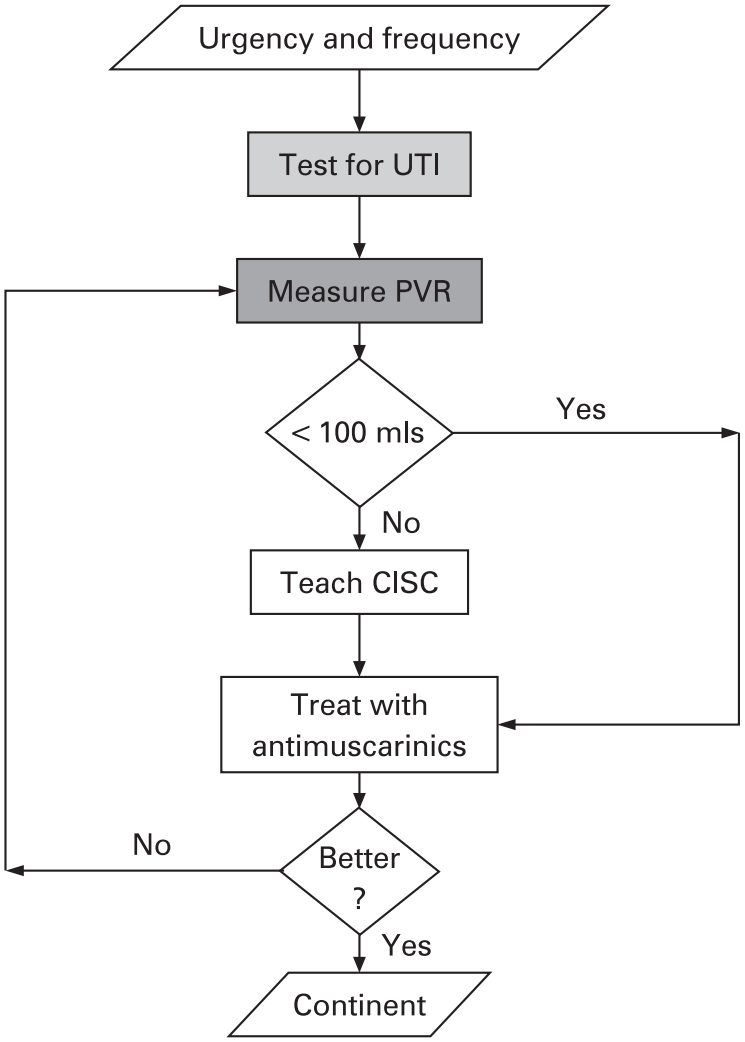
The risk of CKD increases with age, and amongst patients and population-based European data suggest that amongst adults aged 45-64, the prevalence of CKD is 3-20% (143). An autopsy study of 59,000 cadavers reported a hydronephrosis rate of 3.1% (144). Based on these data, the risk of upper tract complications in patients with MS is comparable to that of the normal population. The management of LUTS in patients with MS ought to differ from other neurological conditions where the risk of upper tract damage is significant (145).

Urodynamic studies (UDS) are often recommended in the initial assessment of bladder dysfunction associated with MS in order to identify those at risk of upper tract abnormalities, and improve therapeutic outcomes in treated patients with LUTS. Despite claims of efficacy, there is no evidence to support the utility of UDS in predicting upper tract complications or improving treatment outcome for patients (126, 129, 133-136, 146-148). The utility of UDS in the management of non-neurogenic LUTD has also been convincingly challenged, and in most circumstances, urodynamic variables have no diagnostic or prognostic value in the management of patients with LUTS (149-151).

The management of LUTS in MS was the subject of a recently published UK consensus document (104). The guidance acknowledges the low risk of renal complications associated with MS and the lack of evidence supporting the utility of UDS in the initial assessment of LUTS. The document offers a simple management algorithm for the initial assessment of MS patients who present with urinary symptoms that is summarised in **Figure 1**.

After screening for urinary tract infection using dipstick urinalysis, a measurement of post-void residual volume (PVR) is recommended. Voiding problems are

Figure 1 *Management algorithm for patients with MS presenting with LUTS (adapted from Fowler et al. 2011).*



CISC: Clean intermittent self-catheterisation; PVR: post-void residual volume; UTI: urinary tract infection

commonplace, and impaired bladder emptying is thought to aggravate storage symptoms by reducing the functional capacity of the bladder. The consensus document advocates the use of urethral intermittent self-catheterisation (ISC) if the patient has a PVR of >100 ml. The expert panel consulting on this guidance considered the introduction of ISC to be of pivotal importance, although they noted the absence of any formal evidence base for its use.

Intermittent self-catheterisation is recommended to achieve complete bladder emptying, circumventing any voiding problem, and increasing the functional capacity of the bladder. Another proposed benefit of ISC is the prevention of urinary tract infection (UTI), widely perceived to be a result of incomplete bladder emptying and consequent impaired bacterial clearance from the bladder. The detection of UTI is recognised as a critical component in the management of patients with MS, as infection may exacerbate LUTS, and has been implicated in functional deterioration and disease relapse (152-156).

Agents recommended for the treatment of storage symptoms include anticholinergic medications, which form the mainstay of therapy in non-neurogenic LUTD, and intradetrusor onabotulinum toxin A (BTX-A) injections; both of these treatments attenuate cholinergic and NANC transmission which is thought to be their primary mode of action. Desmopressin (DDAVP), a synthetic vasopressin analogue that mediates a temporary reduction in renal urine production, is also commonly prescribed for nocturia.

The use of anticholinergic medication for OAB symptoms in MS is largely based on extrapolation from their use in non-neurogenic LUTS (157). Data examining the efficacy of anticholinergic agents in MS patients is limited and comprises uncontrolled comparisons of different anticholinergic agents (158). Nonetheless, anticholinergic medication is offered as first line therapy for MS patients with OAB symptoms. Evidence from randomised-controlled trials demonstrates that BTX-A is associated with a significant reduction in urgency and urgency incontinence episodes when compared to placebo in patients with MS (159). Onabotulinum toxin A

treatment has been associated with high rates of ISC use following its administration in patients with MS, although the introduction of ISC is clearly dependent on the criteria used to trigger its initiation. The efficacy of DDAVP in reducing nocturia and nocturnal enuresis is supported by randomised studies (160), although long-term data are lacking.

For those patients refractory to these measures, long-term suprapubic catheterisation is an option if ISC is not possible as a result of disability. Radical surgery is rarely recommended since the advent of BTX-A as an effective treatment for OAB symptoms, although bladder augmentation and urinary diversion procedures are still undertaken for some patients. Sacral neuromodulation has also been successfully used to treat LUTS in MS although data are few (161, 162).

1.2.6 Consequences of lower urinary tract dysfunction

There is little doubt that LUTS have a disproportionate impact on QoL for patients and their carers living with MS. Data from large, symptom-based surveys consistently cite LUTS as a key determinant of QoL, with an impact comparable to that of loss of mobility (54). LUTS may also interact with other disease-related symptoms: fatigue may be confounded by disrupted sleep due to nocturia, and incontinence the result of poor mobility and dexterity during episodes of urgency.

1.3 Urinary tract infection in multiple sclerosis

1.3.1 Epidemiology

Whilst widely recognised as a cause of significant morbidity, epidemiological data relating to UTI in MS are sparse. A large population-based study of 221 patients with MS has estimated that the annual incidence of UTI is around 30% (55). These data were gleaned from a patient-reported, retrospective survey with twice as many female respondents than males. By comparison, a study of 2000 healthy adult

women demonstrated an annual incidence of urinary infection of approximately 10% (163). The incidence of UTI amongst healthy male subjects is around 3% (164) so these data may overestimate the difference in incidence of urinary infection between healthy adults and patients with MS.

Whilst risk factors for UTI in patients with MS have not been studied, the apparent increased incidence of infection has been attributed to poor bladder emptying and catheter use. Whilst a strong association between catheter use and consequent UTI is grounded in clinical data (165) the assumed association of infection with elevated residual urine volumes is not supported by the literature.

In a comprehensive review, Hampson and associates reviewed 342 studies reporting on the prevalence of UTI in patients demonstrating PVR of <100 ml and >100 ml and found no difference in the prevalence of infection between the groups (166). There are no available data to guide ISC use in patients with MS and LUTS, and its introduction in patients with potentially asymptomatic residual volumes above 100 ml could easily be associated with harm. Recent studies have highlighted the risk of infection associated with catheter use: chronic and recurrent, symptomatic, culture-proven UTI in almost 50% of patients with neurogenic LUTD using ISC, and 70% of patients using an indwelling catheter (165, 167, 168).

There are no studies that determine the impact of ISC in patients with MS and LUTS when it is initiated at a fixed PVR of 100 ml. The UK consensus document states that 'it seems highly improbable that a placebo controlled trial of its effectiveness will ever be undertaken as the non-treatment of patients with a raised post micturition residual volume in a placebo arm would now be considered unethical' (104). This statement assumes that the use of ISC is not associated with adverse events and that the non-treatment of post-void volumes that exceed 100 ml is likely to cause harm. There is no evidence to support either of these suggestions.

Multiple sclerosis is not associated with an elevated risk of renal deterioration and there is no evidence that supports a universal relationship between elevated PVR

measurements and UTI. In this context, ISC ought to be initiated only when there is clinical suspicion that an elevated residual bladder volume is generating or exacerbating symptoms. Patients who are unable to empty their bladders and those who experience difficult bladder emptying associated with distressing bladder distension symptoms would obviously benefit from intermittent catheter drainage.

In patients who maintain a large residual volume, functional bladder capacity is reduced and this might be associated with increased urinary frequency. A reduced functional capacity is also perceived to hasten the onset of urinary urgency. When other therapeutic interventions have been exhausted, it would appear reasonable to trial ISC in patients who report intractable OAB symptoms and assess its effects on the severity of LUTS and other patient-reported outcomes. The notion that ISC will be beneficial for all patients with OAB symptoms, based solely on a PVR threshold of 100 ml, and before other interventions have been instituted, is not grounded in evidence.

The question of what constitutes a 'normal' PVR remains unclear. There is no consensus in the scientific literature and PVR volumes vary dependent on the characteristics of the population under scrutiny. Volumes ranging from 50 ml to 200 ml are cited in consultation documents but these recommendations are based on opinion rather than scientific evidence (169). A PVR measurement of <100 ml is often cited as normal and this figure is drawn from a study of 96 ambulatory women without urinary symptoms (170). The results of such an analysis cannot be applied to other populations and provide no information about the relationship between PVR measurements and symptoms, or the impact of interventions such as ISC.

1.3.2 The impact of urinary tract infection

Whilst UTI is generally considered a self-limiting illness for most healthy individuals, the consequences of UTI for those affected by MS may be considerable, and not only confined to the lower urinary tract. Whilst the association of acute infection and

deteriorating lower urinary tract function is widely acknowledged, the systemic effects of infection on the disease process in MS are recognised by patients and professionals.

Approximately 30% of relapses are associated with systemic infection (152-154, 156, 171). Some of these data suggest that relapse which is driven by infection may result in more pronounced and sustained neurological deficits than non-infective disease exacerbations (156, 171). The risk of relapse appears to rise from just before the onset of the illness, and remains elevated for a few weeks after resolution (152, 154, 156, 171)

Whilst early studies linked viral and respiratory infections with relapse, more recent work has demonstrated that bacterial and viral infections are associated with an increased risk of relapse. Functional deterioration associated with infection may also occur in the absence of a clearly defined relapse. This is particularly true for febrile illness, which is thought to disrupt axonal function by inducing temporary conduction block. These episodes, termed pseudo-relapses, are typically transient and tend to resolve over the same period as the infectious illness (172).

Whilst urinary infection has been linked to relapse, the evidence implicates febrile UTI specifically in this interaction (171). Fever and chills were requisite symptoms in this work, and it remains unclear whether urinary infection without fever mediates similar risk (156). Whilst more data are awaited, clinical experience and case series would suggest that this is the case (173). Urinary tract infection was reported as the most common secondary diagnosis amongst 5834 acute hospital admissions with MS (174) so the condition is highly prevalent. Anecdotally, MS patients often describe a significant deterioration in function associated with UTI, even in in the absence of fever.

Although systemic infection has been associated with increased disease activity on MRI (171), some data has questioned whether neurological damage may occur in the absence of detectable inflammatory activity on CNS imaging (156). This process has

long been considered a pivotal event in the pathogenesis of neurological damage in MS, allowing the entry of autoreactive T-cells in to the CNS. It has been suggested that damage to the CNS could be mediated through mechanisms independent of BBB dysfunction and inflammatory cell migration, although this conclusion has been not been corroborated by other clinical studies.

Experimental data has suggested a possible mechanism for this phenomenon, formulated through experimental studies using a murine EAE model (175-177). It has been postulated that infection remote to the CNS generates pro-inflammatory cytokines that cross the BBB and activate CNS macrophages known as microglia. These microglia, already primed as a result of local CNS disease, shift to an aggressive pro-inflammatory state and mediate tissue damage through the production of inflammatory mediators. This is independent of BBB dysfunction or the influx of T-cells into the CNS.

In these studies, microglial activation in the CNS was associated the production of pro-inflammatory cytokines including NO, which has been specifically implicated in axonal loss in MS. Only 60% of the animals exposed to the systemic infective challenge developed a well-defined clinical relapse. Nonetheless, histological and immunohistochemical studies clearly demonstrated evidence of tissue damage within the CNS, often without clinical evidence of relapse, or in the face of very subtle symptoms (177). It is noteworthy that progressive variants of MS are characterised by a continuous deterioration in neurological function, mostly without discrete relapses, and in the absence of MRI evidence of new inflammatory lesions in the CNS. These data hint that infection remote to the CNS could plausibly drive disease progression although more data are awaited. Whilst EAE is often used as an experimental model in the study of demyelinating disease, in common with other animal models, its limitations should be recognised (178).

1.3.3 Recommended investigations and management

Recently published UK guidance on bladder management in MS recommends the use of dipstick urinalysis to exclude infection in patients with new onset LUTS (104). Whilst the role of microbiological culture is not specifically explored, the guidance does include recommendations on the management of asymptomatic bacteriuria (ABU). ABU refers to the isolation of significant bacteria in a urine culture, in the absence of symptoms indicative of infection. The treatment of ABU is not currently recommended except in pregnancy, and prior to urologic procedures (179). In patients with MS using ISC, or an indwelling urinary catheter, the presence of bacteriuria, indicated by standard urinalysis methods, is frequently encountered. Current guidance does not advocate treatment in the absence of indicative symptoms and the diagnosis of UTI in these circumstances is therefore 'clinical rather than microbiological'.

The UK consensus document of bladder management in MS recommends 'prompt treatment of UTI to minimise the risk of neurological deterioration'. Our ability to make symptom-based diagnoses in this context is important. Whilst ABU is defined as significant bacteriuria in the absence of infective symptoms, there are no data which inform us of which urinary symptoms indicate infection. Acute frequency and dysuria are widely cited as the classical symptoms of lower urinary tract infection but there is no evidence to refute the importance of other LUTS as indicators of UTI. Uncertainty relating to which symptoms signify infection, especially in those patients who have multiple, pre-existing LUTS makes the 'clinical' diagnosis of UTI extremely difficult.

1.4 Urinary tract infection: A review of diagnostic methods

Since it was first conceived, quantitative microbiological assessment has remained the most used diagnostic test for urinary infection. Microscopic pyuria, its key surrogate, is also widely applied in the clinical setting although dipstick urinalysis has

all but replaced direct microscopic assessment. Despite the universal deployment of these tests, the available evidence points to systematic problems in their development and application to patients, especially those who present without acute symptoms.

1.4.1 Diagnostic thresholds and spectrum bias

Dichotomous diagnostic thresholds are used extensively in clinical medicine, defining a test as 'positive/negative', or a disease 'present/absent'. From a biological perspective, disease is often expressed across a continuum, ranging from the first pathologic changes to advanced states. The partitioning of disease into a dichotomy is artificial and not demonstrated in nature (180). The prevalence of a disease in a population is a function of the sensitivity of the criteria used to identify it. The diagnosis of diabetes mellitus is based on an assessment of glucose tolerance, a continuous trait, yet a dichotomous threshold is used to diagnose the presence or absence of diabetes. The use of glucose tolerance was conceived to assess the risk of microvascular complications from diabetes, not define a disease as present or absent (181, 182). The tests we use to diagnose urine infection are affected by similar problems.

Perhaps the most pervasive influence on the performance of diagnostic testing is spectrum bias (183). Tests properties are affected by the spectrum of disease under scrutiny. Test validation is commonly achieved by case-control studies that overestimate accuracy because comparisons are limited to patients with advanced disease versus healthy control subjects. Patients with mild or moderate disease may be overlooked by the test.

1.4.2 Microbiological culture

Although not the first to explore the utility of quantitative bacterial counts in the diagnosis of UTI (184, 185), Kass (1957) was credited with the development of

definitive diagnostic criteria (186). His culture threshold of $\geq 10^5$ colony-forming units (cfu) ml^{-1} has exerted a dominant influence on the diagnosis of UTI for the last 50 years and remains the popular reference standard in work evaluating surrogate markers of UTI.

Whilst Kass' contribution to microbiological practice was undeniably significant, the application of his findings to the wider population of symptomatic patients may be inappropriate. His original study compared 74 women with pyelonephritis and 337 asymptomatic control subjects. The patients with pyelonephritis were hospitalised with marked systemic upset, loin pain, fever, and pus in the urine. It is clear that these groups are not representative of most patients presenting with UTI, occupying opposite ends of the clinical spectrum of infection. This quantitative bacterial threshold is only applicable to hospitalised patients with pyelonephritis.
In order to characterise the bacterial ecology of patients with clinical presentations other than pyelonephritis, investigators turned to acute cystitis, an infective syndrome confined to the lower urinary tract. The condition is classically characterised by the abrupt onset of LUTS and pain attributed to the lower urinary tract. This work produced compelling evidence that much lower bacterial counts in the urine are associated with clinically significant disease (187-192). Treatment was associated with the resolution of symptoms, bacteriuria and pyuria, whilst bacterial persistence and continued symptoms were noted when the infection was left untreated (187, 190). The findings demonstrated that the use of a $\geq 10^5$ cfu ml^{-1} bacteriological threshold erroneously classified almost half of the study subjects with genuine coliform UTI as having no disease. The treatment of patients with symptoms of acute cystitis but bacterial counts of $<10^5$ cfu ml^{-1} has also been subject to an RCT which demonstrated that patients responded to antibiotics but not to placebo (193). Unfortunately, this work is largely overlooked.

These factors expose the susceptibility of Kass' original work to spectrum bias, and question the applicability of his diagnostic threshold to infective syndromes other than pyelonephritis. In common with other test boundaries, the use of a dichotomous threshold to define the presence/absence of disease is also artificial

and distorts our understanding of pathophysiology. Kass also assumed dominant pathogenicity from coliform organisms, and considered mixed bacterial growth as likely contamination. The scientific community is becoming aware of the pathogenic significance of polymicrobial infection in human disease, and the bladder has been specifically implicated in this debate (194-196).

1.4.3 Surrogate markers (1): Microscopic pyuria

Whilst the identification of urinary leucocytes using light microscopy was first described in 1893, Dukes (1927) is credited with the development of modern cytometric urinalysis techniques. In work predating quantitative microbiological methods, he described the assessment of fresh urine, using a cell counting chamber, to quantify the inflammatory exudate in healthy patients without LUTS. His study of 300 midstream urine (MSU) samples from asymptomatic controls produced estimates for normal mean leucocyte counts of 1.6 wbc μl^{-1} and 5.4 wbc μl^{-1} for males and females respectively (197). Despite significant dispersion around these mean estimates (range 0-50 wbc μl^{-1}), he proposed a threshold of <10 wbc μl^{-1} as the upper limit of normal pyuria excretion. This was a convenient watershed, and in common with other early work, was obviously not subject to any statistical assessment. Microscopic pyuria ≥10 wbc μl^{-1} was subsequently adopted as our key surrogate marker of urinary infection.

There are problems with this analysis that may not have been apparent to early pioneers in this field. Most importantly, there were no attempts made to attenuate the effects of spectrum bias and no symptomatic groups were included in the analysis; pyuria expression in healthy controls cannot be used in isolation to construct diagnostic boundaries in the diseased. It is also apparent from the data that the distribution of pyuria is positively skewed and using the arithmetic mean as a measure of central tendency would be expected to produce inflated estimates of these values. The analysis was conducted on voided urine samples, which without

careful attention to handling may have been subject to contamination, particularly in females.

The development of quantitative microbiological techniques provided an opportunity to validate the performance of microscopic pyuria using new culture-based diagnosis. Several groups studied women with acute cystitis using positive reference cultures with a threshold of $\geq 10^5$ cfu ml^{-1} as diagnostic of infection. The threshold of ≥10 wbc μl^{-1} appeared to be a sensitive marker of UTI, (198-200). This finding is not unexpected, as acutely symptomatic women with marked bacteriuria would be most likely to demonstrate a significant urothelial inflammatory signal. Of particular interest was the finding that almost half of those studied with acute symptoms of cystitis were culture negative, but demonstrated leucocyte excretion of ≥10 wbc μl^{-1} (199). Even though the authors acknowledged the potential influence of a strict culture threshold on the prevalence of UTI, they concluded that these women were suffering from a non-infective urethritis causing a pyuria, rather than cystitis. Some of their contemporaries found similar perplexing results, demonstrating 'significant' pyuria and 'negative' culture, and attributed their findings to 'nervous tension' amongst patients (201). These data expose pyuria excretion ≥10 wbc μl^{-1} as common finding amongst women with acute cystitis symptoms, and cast doubt on the microbiological definition of UTI long before Kass' work was re-evaluated.

It is difficult to estimate the sensitivity of pyuria ≥10 wbc μl^{-1} in the diagnosis of UTI. The majority of available studies in adults are subject to poor reporting of clinical symptoms in their study subjects and employ disparate methods of analysis. The available data demonstrate sensitivities in the region of 80% with reported specificities of 90%. Nonetheless, these estimates only apply to patients who present with acute symptoms and a positive urine culture employing a $\geq 10^5$ cfu ml^{-1} threshold (202-204). The literature points to lower bacterial counts being clinically significant in patients with acute cystitis and it is likely that the sensitivity of microscopic pyuria would be blunted if a lower culture threshold were used as the reference standard.

1.4.4 Surrogate markers (2): Urinary dipstick testing

The development of the urinary dipstick began in an effort to limit reliance on laboratory urine diagnostics that were expensive, and time consuming. Whilst modern reagent strips are able to provide information on a variety of physical and biochemical variables, leucocyte esterase (an enzymic leucocyte product) and urinary nitrite (a nitrate reduction product of some uropathogenic bacteria) are the principal determinates of infection on dipstick testing. The drive to establish 'point-of-care' testing for UTI was initiated to circumvent direct microscopic examination, reduce the need for microbiological culture, and permit early treatment if indicated.

Whilst there is widespread enthusiasm for the use of dipstick testing in the detection/exclusion of UTI, evidence from individual studies and metanalyses of diagnostic accuracy cast serious doubt on their clinical utility (205-208). Most of the literature in this area is affected by inadequate reporting of presenting symptoms and inconsistent methods. Reported sensitivity estimates for leucocyte esterase cluster around 60%, with a commensurate specificity of around 70%. The sensitivity of nitrite is approximately 50%, with reported specificities around 90% (205-208). Again, these estimates relate to patients with acute symptoms and use a positive reference culture of $\geq 10^5$ cfu ml^{-1} as indicative of infection. The use of leucocyte esterase and nitrite as a disjunctive diagnostic pair confers marginal improvement but this is insufficient to redeem the dipstick as a reliable test.

The vast majority of the scientific literature scrutinise the performance of urinary dipstick testing in patients with acute symptoms of cystitis. Nonetheless, there have been a few attempts to determine the accuracy of leucocyte esterase and nitrite as surrogate markers of infection in patients without acute symptoms. Buchsbaum (2004) screened CSU samples from 265 women presenting with symptoms of incontinence using a reference culture threshold of $>10^4$ cfu ml^{-1} and specifying a pure growth of one organism to indicate UTI (209). Twelve percent of women demonstrated bacterial growth above this level but positive dipstick testing, defined

as leucocyte esterase ≥ 'trace' or positive nitrite, yielded a sensitivity of only 29%. Raza-Khan (2006) used similar methods and reported the sensitivity of dipstick testing to be 35% amongst a sample of 143 unselected women presenting with symptoms of pelvic organ prolapse or incontinence (210). Hessdoerfer (2011) screened 2252 women presenting to a urologic service with symptoms of OAB using MSU sampling and a positive culture threshold of $\geq 10^3$ cfu ml^{-1} (211). Thirty percent of patients demonstrated a positive bacterial culture, and the sensitivity of dipstick testing was 44%.

The poor performance of dipstick urinalysis is not entirely unexpected. The microscopic and microbiological reference standards employed in the development of these reagent strips appear to be inappropriate in most clinical settings and the calibration of the reagent pads may contribute to this. The leucocyte esterase assay is manufactured to show a 'trace/spurious' result at the equivalent of 15 wbc μl^{-1} which is commonly misconstrued as 'clinically insignificant'. A 'positive' result represents the detection of 70 wbc μl^{-1} which is a significant urothelial inflammatory response likely to be demonstrated only in the presence of pronounced inflammation. The specificity of the test would thereby be affected.

1.5 Urinary tract infection: Pathophysiology

Over the last decade, our understanding of the pathogenesis of UTI has been challenged. The concept of UTI being confined to the luminal surface of the lower urinary tract has been replaced by a more complex host-pathogen interaction, mediated by entry of bacteria into the intracellular space. Intracellular colonisation appears to confer protection from host defences and provide a sheltered environment for bacterial expansion. Some bacteria appear capable of forming intracellular bacterial communities that may persist over long periods and be a source of recurrent infection.

1.5.1 Bacterial uropathogens

Bacterial pathogens in the lower urinary tract are opportunists. Opportunistic pathogens might rise from commensal bacteria, such as gut symbionts, or colonise the host from an environmental source. There is much published data on the perceived importance of different bacterial genera/species in the pathogenesis of UTI. These data report significantly different findings dependent on the sample population that is influenced by demographic characteristics and clinical setting. Whilst the effects of a fixed, dichotomous microbiological threshold on the diagnosis of UTI is now being recognised, its influence on pathogen prevalence studies in UTI might easily be overlooked. The use of these thresholds in epidemiological studies, and the dismissal of polymicrobial cultures as likely contamination, may have perverted our understanding of the ecology of urinary infection.

Much of the data exploring bacterial ecology of urinary tract infection is derived from the study of acute cystitis. The largest of these studies, the multinational ECO·SENS project, provides some of the strongest ecological evidence (212). It sampled only community patients with acute cystitis and reported all recognised pathogens exhibiting growth $\geq 10^3$ cfu ml^{-1} including polymicrobial growth of up to two isolates.

In common with the findings of other studies, *Escherichia coli* was by far the most prevalent isolate, found in approximately 75% of samples. Other *Enterobacteriaceae*, including *Proteus mirabilis*, *Klebsiella spp.*, *Enterobacter spp.*, and *Citrobacter spp.*, were demonstrated in just under 15% of samples. *Staphylococcus saprophyticus* was isolated in 5% of samples with other staphylococci and enterococci cultured in the remaining 5%.

S. saprophyticus and Group B streptococci are known to colonise the female gastrointestinal and urogenital tract and are isolated more frequently from women. In this patient group, *S. saprophyticus* probably represents the second most common

uropathogen, whilst Group B streptococci may be associated with UTI in pregnancy. By contrast, *Proteus spp.* and *Pseudomonas spp.* are often associated with hospital acquired UTI (213).

1.5.2 Bacterial adhesion, invasion and intracellular growth

The evolution of disease hinges on the interaction between pathogen and host. Bacteria must be able to attach and adhere to host tissues, and specific carbohydrate adhesion mechanisms have been implicated in this process. Much of the data relating to the pathogen-host interaction in UTI has been generated from the study of uropathogenic *E. coli* (UPEC) in the murine model, although similar processes have since been observed in human UTI (214, 215).

The urinary tract is lined by terminal urothelial cells, known as 'umbrella cells', which protect the underlying tissues of the lower urinary tract from the toxic effects of urine. This barrier function is mediated by the production of an apical asymmetric unit membrane (AUM) consisting of membrane-bound proteins called uroplakins (UP). Uropathogenic E. coli possess Type 1 pili, hair-like structures with selective adhesive properties, which cover the external surface of the cell. Adhesion appears to be mediated by FimH, a terminal pili component, which binds to UP on the surface of urothelial cells of the lower urinary tract.

During bladder filling and contraction, there are dynamic changes in the surface area of the bladder. These changes are accommodated by the internalisation and egress of UP plaques in vesicles. UPEC exploit this process by pili-mediated attachment to UP and are transported into the cytoplasm of urothelial cells. Whilst the majority of these bacteria appear to be returned to the cytoplasm during bladder filling, some are able to remain inside the urothelial cell and thrive; the mechanisms underlying this process remain unknown.

In the murine model, bacterial adhesion and invasion are followed by the establishment of an 'intracellular bacterial community' (IBC) which is described in

three phases: (1) Early: loose aggregations of UPEC rapidly divide inside the cytoplasm; (2) Middle: bacteria adopt a coccoid morphology and pack together in a 'pod' formation (3) Late: bacteria at the periphery of the pod regain their motility and leave the cell to infect adjacent cells. These events represent the acute infection cycle, allowing sheltered intracellular bacterial expansion to fuel the infection of new cells.

1.5.3 Host defences and immune evasion by uropathogens

Studies of the immune response in the bladder have centred on Toll-like receptor 4 (TLR4), activated by bacterial lipopolysaccharide (LPS). TLR4 activation leads to the expression of cytokines including interleukin-6 (IL-6) and chemokine ligand 8 (CXCL-8), via the nuclear factor kappa beta (NF-κB) signalling pathway (216-218). IL-6 is central to the acute phase response, and CXCL-8 is an important mediator of neutrophil chemotaxis. Human studies have suggested an association between UTI susceptibility and TLR4 expression (219, 220). Evidence from murine studies has also suggested that TLR4 receptor activation can mediate cytoskeletal changes that render urothelial cells less receptive to invading bacteria.

Data from murine models and human tissue culture systems have demonstrated that UPEC express mechanisms that may subvert the innate immune response. These include suppression of TLR4 receptor activation, down-regulation of pro-inflammatory signalling, and inhibition of neutrophil migration and adhesion (221). In the late stage of IBC development, some UPEC also demonstrate marked morphological plasticity, forming large filamentous bacteria that are able to resist phagocytosis (214).

1.5.4 Bacterial persistence in the lower urinary tract

Urothelial cell exfoliation is an important component of the innate immune response in UTI. Infected superficial urothelial cells and associated intracellular bacteria are

ejected into the urine (222, 223). Whilst this process would appear to favour the resolution of acute infection, normal maturation in the urothelium may take months, and the rapid cell turnover exposes naive transitional cells to luminal interface. Uropathogenic E.coli are able to invade and occupy these cells, but their intracellular activity contrasts that observed during the initial invasion of mature urothelial umbrella cells. The bacteria exhibit limited growth and are confined in a localised lysosomal compartment, forming a 'quiescent intracellular reservoir' (QIR). The restriction of bacterial expansion and release has been attributed to lysosomal encapsulation in actin fibres. As the naive urothelial cells mature, the formation of AUM is associated with a reduction in actin expression, which permits the release of bacteria to establish a fresh cycle of infection (224). Studies of recurrent UTI in women have isolated identical UPEC strains as in recurrent infections in the same patient. Whilst these data implicate QIR formation in the pathogenesis of recurrent UTI, direct evidence is lacking (225). The formation of QIRs appears to confer protection from immune and antibiotic attack (226). Whether these bacteria are truly quiescent, or active and suppressing host responses, is unknown. These questions are relevant to the generation of chronic symptoms, and not only the recurrence of acute, infective episodes.

A biofilm is defined as a community of microorganisms contained within a self-generated polymeric matrix, adherent to a surface. Bacteria secrete extracellular polymeric substances (EPS), which include polysaccharides, proteins, glycoproteins, and glycolipids, forming a protective matrix that confers local regulation of the environment (227). Whilst much attention has been focused on UPEC exploiting an intracellular niche in the lower urinary tract, data relating to extracellular biofilm formation on the mucosal surface is scarce.

Biofilm formation is demonstrated by a vast array of bacteria in many environmental habitats. The bacteria within these biofilms are protected from environmental stressors, and in common with QIRs, extracellular biofilm formation fosters significant resistance to antibacterial agents (226, 228). Research has focused mainly on biofilm formation associated with foreign bodies in the lower urinary tract, such

as catheters (229). This is presumably because these devices provide an easily accessible model for study. Whilst uropathogens demonstrate adhesive organelles which allow urothelial cell attachment, this may not be associated with the generation of EPS, the hallmark of biofilm production.

Whilst the literature remains silent on the production of extracellular mucosal-associated biofilms associated with the urothelium, the prostate gland in the male has been the subject of study. Bacteria may infect the prostate gland by ascending infection from the urethra, or by reflux of infected urine through the prostatic ducts causing a bacterial prostitis (230). These infecting bacteria are able to form biofilms that adhere to the epithelial cells of the duct system within the prostate (231-233). The resilience of bacterial communities within biofilms in the prostate may account for difficulties achieving bacteriologic eradication in patients with infection.

1.5.5 Anaerobic bacteria

Headington (1960) reported the prevalence of anaerobes amongst 15250 MSU samples submitted for culture from inpatients and outpatients. Only 195 (1.3%) of the samples grew anaerobic organisms, predominantly *Lactobacillus spp.* and *Clostridium spp.* (234). An analysis confined to a subgroup of 54 symptomatic patients demonstrated that a dominant aerobic uropathogen was present in almost all of these samples.

Banon (1998) examined 19429 MSU samples and demonstrated positive anaerobic cultures in only 6 (0.03%) patients (235). In this series, positive cultures were defined as a pure growth of at least 10^8 cfu ml^{-1} which likely accounts for the lower prevalence of infection in this work. All of the patients with positive cultures presented with acute symptoms and pyuria, implicating anaerobic bacteria in the generation of acute urinary symptoms. Other small case series and individual reports have convincingly linked anaerobic organisms and acute urinary infection in adults and children (236-238).

Some investigators have examined the urethral flora of female patients with recurrent UTI in order to examine the role of anaerobes in symptomatic infection. One controlled study (*n*=60) failed to identify any difference in the prevalence of anaerobic urethral carriage amongst young women with recurrent UTI and matched controls (239). Another smaller study provided evidence of urethral anaerobic colonisation in the asymptomatic, but not in patients with acute symptoms who expressed recognised aerobic uropathogens (240).

Anaerobic bacteria are clearly capable of mediating acute symptomatic urinary infection, although how commonly this occurs remains unclear. Anaerobic culture methods are rarely employed in routine clinical practice and there is a need for further work in this area. Based on the available data, the prevalence of positive anaerobic urine cultures appears to be very low, even when fixed bacterial thresholds are rejected.

1.5.6 The role of atypical organisms

Atypical bacteria including *Mycoplasma spp.*, *Ureaplasma spp.* and *Chlamydia spp.* are often cited as aetiological agents in UTI. Nonetheless, there are surprisingly few controlled data that examine the role of these microbes in acute infection (202, 241). Whilst these organisms have been isolated from patients who demonstrate acute symptoms, these data suggest that concurrent infection with other uropathogens drives symptom generation in the vast majority of patients.

Some investigators have sought to identify atypical organisms amongst patients with chronic LUTS. Humburg (2012) demonstrated a significantly greater prevalence of *Mycoplasma spp.* and *Ureaplasma spp.* amongst symptomatic patients than asymptomatic controls (30% vs. 15%), although no data relating to the isolation of other uropathogens were presented (242). Lee (2010) reported atypical organisms in 40% of urine samples provided by women with LUTS (243). These patients (*n*=29) were treated with a short course of azithromycin or doxycycline and 80% of patients

were ‘satisfied’ with their treatment at four weeks. Whilst two validated measures of symptom improvement were reported, the statistical methods employed precluded meaningful inferences being drawn from the data. No long-term follow-up was undertaken and the results of standard cultures were not available. Any potential therapeutic effects of antibiotic treatment could have been mediated through the eradication of other recognised uropathogens. Similar findings were also reported in another study that employed almost identical methodology (244).

Baka (2009) enrolled 81 women with chronic LUTS who were screened for atypical organisms using urethral, vaginal and cervical swabs (245). Urine samples were also cultured to exclude concurrent UTI although details of the quantitative threshold employed to indicate infection were withheld. Patients who screened positive for *Mycoplasma spp.* and *Ureaplasma spp.* were treated with azithromycin or doxycycline. Significant improvements in urinary symptoms and lower urinary tract pain were demonstrated at four weeks but change was based on a three-point likert scale rather than a validated outcome measure. Normality was assumed without evidence that it was tested for. The use of a fixed culture threshold to identify concurrent infection with aerobic uropathogens may have dismissed other bacteria that could have been the target of treatment.

Only one other retrospective study has described the treatment of patients with LUTS associated with atypical bacterial infection (246). A small sample of women with atypical bacteria isolated from the urine were treated with norfloxacin for three months and just over half of patients reported an improvement, although this was gleaned retrospectively from the notes and not subject to any validated outcome measure. Many samples yielded concurrent growth of other bacterial isolates and the perceived therapeutic effects of antibiotic treatment could have been mediated through the eradication of these microbes. In addition, a fixed culture threshold of 10^5 was employed to determine significant bacterial growth for uropathogens. This could have allowed infection with common aerobic bacteria at levels less than 10^5 cfu ml^{-1} to go undetected.

1.6 Chronic lower urinary tract symptoms: A reappraisal

Pyelonephritis and cystitis are well-recognised clinical manifestations of UTI. The former is characterised by acute pain and tenderness, and systemic illness including fever. Many patients will also describe lower urinary tract involvement, as pathogens may reach the upper tracts by an ascending mechanism. By contrast, acute cystitis is confined to the lower urinary tract, and is associated with the abrupt onset of symptoms that classically include acute urinary frequency, urgency, and pain. Whilst acute cystitis may be a short-lived and self-limiting illness, antibiotic therapy is advocated. Treatment with antibiotic therapy, compared to placebo, is significantly more likely to abolish symptoms and effect clinical cure, achieve microbiological eradication following treatment, protect against reinfection or relapse, and reduce the risk of upper tract involvement (247). Pyelonephritis is a more serious condition, commonly requiring hospitalisation and intravenous antibiotic therapy. However, both conditions are perceived to resolve clinically within a relatively short timeframe.

Whilst the infective origin of acute cystitis is widely acknowledged, the role of infection in the generation of LUTS that do not manifest acutely is unknown. Current microbiological and inflammatory indicators of infection have not been validated for use in patients without acute symptoms, and the influence of infection in such patients remains undetermined. Routine urinalysis and culture may have unwittingly dismissed significant infective pathology in patients with chronic symptoms by falling foul of accepted diagnostic constructs.

Confidence amongst clinicians in these tests may have been enhanced through confirmation bias, induced by the association of positive urinalysis in the presence of acute symptoms. These influences may be more pervasive in conditions such as MS where the onset or deterioration of LUTS might be easily attributed to evolving neuropathology, supported by negative routine urinalysis and culture that could be misleading.

Recent work has examined the nature of the urothelial inflammatory response and bacterial ecology in patients with non-neurogenic LUTD who present with non-acute symptoms. These studies have focused on OAB symptoms, as the pathogenesis of SUI is associated with impaired sphincteric function and the failure of normal urethral support (248). Surgical interventions have proved successful in treating this condition.

The study of patients with OAB symptoms has demonstrated increased inflammatory activity and bacterial colonisation not seen in asymptomatic control subjects. This evidence implicates bacterial infection and associated urothelial inflammation in the generation of chronic LUTS. In this context, the contribution of infection to the generation of urinary symptoms in MS needs to be reassessed, employing more sensitive indicators of urothelial inflammation and lower urinary tract infection.

1.6.1 Urothelial inflammation (1): Pyuria

Peripheral recruitment of leucocytes in response to inflammation, regulated by chemokines, has been widely studied (249). Microscopic pyuria is an established marker of urothelial inflammation, the result of urothelial infiltration by leucocytes that subsequently escape into the urine. Preliminary clinical observations in patients with OAB symptoms demonstrated that microscopic pyuria was common, and often not associated with positive routine urine culture.

On the strength of these findings, a prospective, cross-sectional study of patients with OAB symptoms was undertaken. MSU samples were subjected to immediate microscopic assessment and routine microbiological culture to determine the urothelial inflammatory response and associated microbiological ecology. Between 2004 and 2009, 785 patients (F=719; M=68; mean age=54) with OAB symptoms were recruited of which 452 (58%) manifest pyuria ≥10 wbc μl^{-1}. Of these 452 patients, only 53 (12%) demonstrated a positive microbiological culture defined as the growth of a single recognised uropathogen at $\geq 10^5$ cfu ml^{-1}. Forty patients (9%) produced

polymicrobial cultures (250). 'Culture negative pyuria', defined as the expression of ≥10 wbc μl^{-1} without routine culture evidence of infection, was demonstrated in 51% of the patient sample.

Whilst these exploratory findings were uncontrolled, this study provided the first evidence of urothelial inflammation in patients with OAB, which in the majority was not associated with a positive culture. There were 47 patients with MS in this sample of which 32 (68%) presented with a significant pyuria ≥10 wbc μl^{-1} and only six (19%) demonstrated a positive routine culture. In the wake of these data, corroborative evidence of urothelial inflammation was sought from other sources.

1.6.2 Urothelial inflammation (2): Cytokines

Following preliminary studies that demonstrated cytological evidence of an inflammatory exudate in the urine of patients with OAB symptoms, controlled studies were completed using an alternative marker of inflammation. Urinary IL-6 expression has been quantified in studies of human UTI in patients with different infective presentations and has proven to be a reliable indicator of urothelial inflammation (251-258).

A comparative, prospective, cross-sectional study of IL-6 expression in patients with OAB symptoms was undertaken to compliment early data documenting cytological evidence of urinary inflammation. Paired urine samples were subject to immediate microscopic assessment, and frozen for later IL-6 quantification using a high-sensitivity, enzyme-linked immunosorbent assay (ELISA). Patients provided CSU samples for analysis, whilst asymptomatic control subjects submitted carefully collected MSU samples.

One hundred and seventy-two patients (F=157; M=15; mean age 57; *sd*=19) and 20 control subjects (F=9; M=11; mean age=34; *sd*=11) were included in the analysis. Ninety-nine patients (58%) demonstrated pyuria ≥10 wbc μl^{-1} which was not evident in controls. Urinary IL-6 expression was significantly greater in patients with OAB

compared to asymptomatic controls (*F*=9; *p*=0.003) (259, 260). Amongst patients, pyuria was associated with higher IL-6 levels, although patients without pyuria still demonstrated greater IL-6 expression than asymptomatic control subjects (*F*=3.2; *df*=2; *p*=0.045).

1.6.3 Urothelial inflammation (3): Histology

The finding of elevated pro-inflammatory cytokine release in symptomatic patients, in the absence of contemporary cytological evidence of urothelial inflammation, prompted further evaluation. A controlled, histological evaluation of bladder tissue from patients with OAB symptoms and asymptomatic control subjects was completed using tissue harvested by cystoscopic biopsy. Urothelial inflammation was defined as the presence of all of the following histological features: oedema; mixed inflammatory cell infiltrate; urothelial hyperplasia; and evidence of urothelial exfoliation.

Sixty-seven patients (F=52; M=15; mean age= 55; *sd*=15) provided biopsy specimens; three tissue samples were insufficient for histological analysis. Sixty-one patients with OAB symptoms were included in the analysis, all of whom had no microbiological culture evidence of infection. Whilst fifty of these patients did not demonstrate pyuria, 11 patients were known to have a longstanding microscopic pyuria but no culture evidence of bacterial infection. Recruitment of asymptomatic control subjects was difficult and only three subjects consented to inclusion; all were undergoing cystoscopy for a previous episode of haematuria, and had clear urine and a negative bacterial culture at the time of examination. All groups were age-matched. Forty-five (90%) OAB patients without pyuria and ten (91%) OAB patients with pyuria manifested all of the urothelial features of chronic cystitis. Whilst no features of inflammation were identified in any of the three control samples ($p<0.001$), the small number of control biopsies limits the strength of the findings (261).

1.6.4 Urothelial inflammation (4): Physiology

Purinergic signaling in the lower urinary tract has been explored in both animals and humans and these data demonstrate evidence of enhanced purinergic neurotransmission in some pathological states. Data from controlled human studies is limited by access to biopsy material but physiological studies of patients with neurogenic LUTD, bladder outlet obstruction and interstitial cystitis have demonstrated increased purinergic receptor expression and augmented release of ATP in response to stretch (67-75). Purinergic receptor up-regulation has also been demonstrated in *E.coli*-infected and cytokine-stimulated human urothelial cell lines (262). One small study, including just five patients with OAB symptoms, also implicated purinergic upregulation in the generation of symptoms (74). These findings have been replicated more recently, and on a larger scale (92, 93). In this context, urothelial inflammation could contribute to the generation of LUTS as a result of enhanced urothelial purinergic signalling and afferent/efferent activation. These data prompted a larger controlled study of basal and stretch-evoked urothelial ATP release in patients with OAB symptoms.

Cystoscopic bladder biopsies were obtained from patients with OAB symptoms and asymptomatic control subjects. Detrusor muscle was removed from the biopsy specimens. Basal and stretch-evoked ATP release from urothelium was quantified using a luciferin-luciferase assay and purinergic P2 receptor mRNA levels were explored using quantitative real-time polymerase chain reaction (PCR) analysis; P2 receptor expression was investigated in snap-frozen sliced tissue using immunohistochemical methods.

Nineteen patients with OAB symptoms and nine asymptomatic controls were recruited. Ten OAB patients demonstrated pyuria but all were culture negative. Basal ATP release was 50-fold greater from the urothelium of OAB patients who manifest a pyuria when compared to both non-pyuric OAB patients and asymptomatic controls ($p<0.01$). By contrast, the concentration of ATP released following stretch was

similar in all three groups. PCR and immunohistochemistry revealed distinct P2X receptor subtype expression in all three groups (92).

1.6.5 Urothelial immune response

Urothelial cell analysis has been confined to cytological assessment in suspected urinary tract malignancy. Outside of such assessment, their presence was thought to represent contamination. Animal studies have identified urothelial cell exfoliation as an important component of the innate immune response in UTI (222, 223). Increased urothelial cell expression in the urine ought to be a feature of the OAB syndrome if infection and inflammation plays a role in the generation of symptoms. A controlled, prospective cross-sectional study was undertaken to determine urothelial cell expression and leucocyte excretion in OAB patients and asymptomatic controls.

Female patients provided CSU samples to circumvent genital contamination and male patients and controls submitted carefully collected MSU samples. Urine was subjected to cytocentrifugation that produced a thin-layer, concentrated cellular deposit from a fixed volume of urine. Ninety-five patients with OAB (F=93; M=2; mean age=59; *sd*=18) and 21 controls (F=7; M=14; mean age=31; *sd*=11) were included in the analysis (263). Patient urine expressed significantly greater urothelial cell numbers when compared to controls, indicative of pronounced cell turnover (mean difference=22.2; t=4.19; df=114; p<0.001). Increased inflammatory cell expression was also seen in patients when compared to controls (mean difference=8.0; t=2.32; df=114; p=0.02). Other data has demonstrated that the exfoliation of urothelial cells is proportional to the inflammatory response in the urinary tract, characterised by microscopic pyuria (264).

1.6.6 Bacterial infection (1): Culture

Traditional culture-based methods of bacterial isolation in the urinary tract are subject to the effects of rigid diagnostic thresholds and spectrum bias. Few

investigators have discarded the ≥10^5 cfu ml^{-1} diagnostic threshold, and they have only reported data relating to acute cystitis (187-189, 191, 192). Until very recently, the bacterial ecology associated with chronic LUTS had not been examined.
Initial efforts to explore the bacterial ecology of patients with chronic LUTS employed variable microbiological criteria to diagnose infection. These criteria included a ≥10^5 cfu ml^{-1} threshold derived from the study of patients with pyelonephritis (186), and a ≥10^2 cfu ml^{-1} threshold, conceived from exploration of acute cystitis (187-189, 191, 192). Data from 198 CSU samples provided by patients with OAB demonstrated that a ≥10^2 cfu ml^{-1} threshold unearthed significant infection that went undetected when a ≥10^5 cfu ml^{-1} threshold was employed. The lower threshold yielded a 30% positive culture rate compared to a 12% positive culture rate using ≥10^5 cfu ml^{-1} threshold (X^2=100; *df*=1; *p*<0.001) (265). Another study has scrutinised the prevalence of UTI in OAB patients using a culture threshold of ≥10^3 cfu ml^{-1} and reported positive cultures in 28% of patients (211).

Microscopic analysis of the urinary sediment is a common diagnostic investigation undertaken in many laboratories. Concentrated cellular deposits produced by centrifugation are examined for the presence of cells, crystals, casts and bacteria that may indicate urinary tract disease or systemic disorders. In a departure from conventional methods, researchers in this unit decided to explore the use of centrifuged urinary sediments as an inoculum in bacterial culture studies. Early work showed that culture of the spun urinary sediment was able to demonstrate differences in the quality and quantity of urinary tract bacteria generated from OAB patients and asymptomatic control subjects (266). The technique has since been refined and more recent data shows clearly that the method cultivates a greater magnitude and variety of microbes than standard microbiological methods (267-269). This sediment culture was recently deployed in a study of 165 patients with LUTS (F=154; M=11; mean age=55; *sd*=16) of whom 69% demonstrated OAB symptoms (270). Ninety-eight percent of patients cultivated bacteria with mean growth of 10^2 cfu ml^{-1}.

The assumption that acute cystitis and chronic LUTS share identical bacterial ecology and should be subject to the same diagnostic criteria ignores the influence of spectrum bias (183). The data described above show that when diagnostic thresholds are dismissed, the prevalence of bacterial infection amongst symptomatic patients is high (266, 269). The dismissal of infecting microbes on quantitative criteria is a misplaced attempt to classify disease in binary terms (180).

1.6.7 Bacterial infection (2): Cytology

Whilst exfoliation of urothelial cells has been described as part of the innate response to infection (222, 223), closer examination of shed urothelial cells supported an infective aetiology in patients with OAB symptoms. Urothelial attachment and invasion of bacteria are key steps in the pathogenesis of UTI. A chance observation whilst examining centrifuged urinary sediments identified bacteria associated with exfoliated urothelial cells. These findings prompted a controlled, prospective, cross-sectional study to determine the proportion of shed urothelial cells exhibiting associated bacteria in patients with OAB symptoms.

Patients with OAB symptoms provided CSU samples, and asymptomatic control subjects provided carefully collected MSU samples. Urine was subject to microscopic pyuria quantification and routine bacterial culture employing a $\geq 10^5$ cfu ml^{-1} diagnostic threshold. Cytocentrifugation produced thin-layer, concentrated cellular deposits that were gram stained and the proportion of cells demonstrating associated bacteria enumerated. Forty-one patients (F=32; M=9; mean age=61; *sd*=17) and 23 controls (F=10; M=13; mean age=30; *sd*=13) were included in the analysis. Patient samples exhibited a greater proportion of urothelial cells with associated bacteria (32%) compared to control samples (8%) (X^2=21; *df*=2; p<0.001) (271). These results have been replicated in more recent work, using a novel immunofluorescence technique (272).

1.6.8 Bacterial infection (3): Antibiotic treatment

Whilst the efficacy of short-term antimicrobial treatment in acute UTI has been proven, the utility of similar agents in the treatment of chronic LUTS remains unknown. In this unit, emerging data prompted the empirical treatment of patients with significant microscopic pyuria and OAB symptoms with antimicrobial therapy. This approach appeared to confer symptomatic improvement with a commensurate reduction in pyuria, but only if protracted treatment regimens were maintained over many months. Early withdrawal of antibiotic therapy appeared to be associated with a return of symptoms and pyuria.

These preliminary observations prompted an observational cohort study of 440 patients (F=380; M=60; mean age=54; *sd*=18) divided into three groups: Group 1 - patients with OAB symptoms and pyuria at presentation (*n*=147); Group 2 - patients with OAB and no pyuria at any point (*n*=212); and Group 3 - patients with OAB, without pyuria at first consultation, who manifest pyuria during follow-up (*n*=81). All patients were treated with anticholinergic medication and bladder retraining. Urinary antibiotics, primarily nitrofurantoin and cefalexin, were introduced when pyuria was first detected. Group demographics were matched. Symptoms were monitored with validated questionnaires.

Whilst there were significant improvements in all symptoms and in all groups over the treatment period (F=59; p<0.001), the principal findings relate to treatment responses in Group 1 and Group 3. Group 3, prescribed antibiotics late, took longer to recover, and experienced a significant improvement in symptoms associated with the introduction of antibiotics (F=8; p<0.001). All groups demonstrated similar improvement by the end of treatment but Group 3, who manifest pyuria late, demonstrated accelerated resolution of symptoms after the introduction of an antimicrobial (273).

Whilst the role of common aerobic uropathogens in the generation of chronic LUTS has been overlooked, some investigators have targeted atypical organisms (243-246). The decision to pursue fastidious organisms rather than recognised aerobic bacteria could have been fostered by potentially erroneous negative cultures in the symptomatic. Microbiological diagnosis using standard quantitative thresholds could easily have dismissed aerobic uropathogens in patients with LUTS, leaving clinicians and researchers searching for alternative infective agents. Nonetheless, there are no reliable data that confirm the efficacy of antibiotic treatment in patients with LUTS and atypical bacterial infection (1.5.6).

1.7 Novel treatments for lower urinary tract symptoms

In the context of evidence implicating lower urinary tract infection and urothelial inflammation in the aetiology of chronic LUTS, antimicrobial therapy or manipulation of the immune response could represent novel treatments. Whilst antimicrobial therapy might seem a more likely candidate treatment, mechanisms mediating bacterial persistence make eradication of uropathogenic bacteria from the urinary tract difficult. Antibiotic therapy also applies a selection pressure to polymicrobial communities, and this may foster the emergence of less susceptible or resistant bacterial strains. Immune modulation, provided it does not compromise host defence, might present an attractive alternative treatment strategy.

1.7.1 Therapeutics: Immune modulation

In the development phase of this research proposal, the opportunity to test a potential modulator of the immune response on lower urinary tract function presented itself. The prospect of conducting a clinical trial to test the efficacy of this agent was grasped because of its particular relevance to lower urinary tract function in MS. AIMSPRO, a polyclonal hyperimmune serum product, contains high titres of antibodies against major histocompatibility complex (MHC) Class II proteins, which are key antigen presenting molecules in humans.

Antibodies to MHC Class II proteins may have the potential to mediate anti-inflammatory effects. In addition to anti-MHC Class II antibody activity, AIMSPRO contains other molecules known to regulate the hypothalamo-pituitary-adrenal (HPA) axis and moderate the inflammatory response (274). Based on these data, AIMSPRO was presented as a candidate drug for use in inflammatory disease including MS.

AIMSPRO has been made available to patients in the UK with inflammatory and autoimmune conditions through its registration as a 'special' with the MHRA. 'Specials' are unlicenced agents that may be prescribed on a named patient basis when clinical need cannot be met by licensed medicinal products. The designation of AIMSPRO as a 'Special' is based on anti-inflammatory effects described in pre-clinical studies, and data from small, uncontrolled series in humans (275).

One of the key areas of symptom improvement, gleaned from the open-label use of AIMSPRO in MS patients, was a perceived improvement in OAB symptoms. If bacterial infection and urothelial inflammation are implicated in symptom generation, an agent that could safely modulate immune function and the inflammatory response in the lower urinary tract would be welcomed.

1.8 Hypotheses and aims

Scientific evidence challenges the performance of diagnostic tests used to detect UTI. These inadequacies may have distorted our understanding of the role of urinary infection in the generation of LUTS. Emerging data have provided evidence of bacterial infection, urothelial inflammation, and immune activation in patients with OAB symptoms, undisclosed by routine testing. Observational data also points to evidence of an antibiotic treatment effect in patients with OAB, although further data are awaited.

Whilst compelling, control data in much of this work were gathered from younger subjects, a greater proportion of whom were men (259, 263, 271). The literature is strewn with reports associating advancing age, and to a lesser extent female sex, with the presence of bacteriuria and pyuria, apparently in the absence of infective symptoms (179, 276, 277). Epidemiological studies also demonstrate a higher prevalence of OAB symptoms in older patients, in the absence of infection identified on routine testing (278-280). Whilst these conclusions could easily be misplaced as a result of the inadequacies of routine urinalysis, and assumptions about the nature of infective symptoms, the lack of adequately matched control data in the studies supporting an infective, inflammatory aetiology for OAB, leave the work vulnerable to criticism. The need to match patients and control subjects for key demographic characteristics in future studies must be a priority.

The current clinical evidence of an antibiotic treatment effect is indirect (273). Prospective data scrutinising the interaction between symptoms, bacterial ecology, and the urothelial inflammatory response would strengthen the argument for a causal relationship between these variables. If such a relationship were supported, then an RCT of antibiotic treatment in patients with OAB symptoms would be justified.

1.8.1 Hypothesis to be tested

Bacterial infection of the lower urinary tract goes undetected by routine diagnostic testing, and contributes to the generation of lower urinary tract symptoms in patients with multiple sclerosis.

1.8.2 Study aims

(1) To determine the diagnostic performance of routine urinalysis methods used to detect urinary tract infection in patients with chronic lower urinary tract symptoms.

(2) To determine the prevalence of urinary tract infection and inflammation in patients with multiple sclerosis and overactive bladder symptoms, employing sensitive bacteriological methods and measures of the urothelial inflammatory response.

(3) To determine the relationship between bacterial infection, urothelial inflammation, manifest by pyuria and elevated local pro-inflammatory cytokines, and lower urinary tract symptoms in patients with multiple sclerosis and overactive bladder symptoms.

(4) To test the effects an immunomodulatory agent on lower urinary tract functioning in patients with multiple sclerosis and overactive bladder symptoms, without evidence of urinary tract infection on routine testing.

2 Methods

2.1 Study design

Study design is described in detail in the relevant chapters.

2.2 Ethical review, study recruitment and consent

2.2.1 Ethical review

All of the studies included in this work were subject to approval by the National Research Ethics Service (NRES) and where appropriate, the Medicines and Healthcare products Regulatory Agency (MHRA). Details of individual approvals are provided in the relevant chapters.

2.2.2 Recruitment

The recruitment of eligible MS patients required a number of complementary approaches. Contacts were established with MS clinical nurse specialists (CNS) throughout Greater London, and meetings conducted to explain the premise and scope of the work. The research programme was also discussed with consultant neurologists and continence advisors locally. Ethically approved publicity material was displayed in all major London units specialising in MS care. Local patient support groups were approached and presentations given at patient meetings and fundraising events. Approved recruitment advertisements were also placed in local and national patient publications.

Patients with non-neurogenic LUTD who contributed to the studies were recruited from the urological clinic at the Department of Medicine, UCL Archway Campus. Control subjects were recruited from the staff of University College London and her affiliates.

Only adult patients aged ≥18 years of age were eligible for study inclusion.

2.2.3 Patient information and consent

Informed consent and the provision of study-specific information were conducted in accordance with Good Clinical Practice (GCP) guidance. Written informed consent was taken prior to the initiation of any study-related procedures. The persistence of consent for the study was checked at each visit. Before the participants gave written consent, they were provided with written patient information, which described the risks and potential benefits of participation. Study information for participants, and consent forms, were approved by the relevant research ethics committee, in common with all other study-specific documents.

After reading the information documents the subjects were able to discuss the content with the researcher. Any questions posed during this interaction were answered. The subjects were encouraged to discuss the study with their friends and family. Patients considering participation in a clinical trial of an investigational medicinal product (IMP) were also encouraged to discuss the study with the primary care physician. Study subjects were advised that they were entitled to withdraw their consent for study participation at any time, without having to provide a reason, and this would not affect their clinical care. A copy of the informed consent form was provided to all participants for their records

The principal investigator was responsible for ensuring that written informed consent was obtained prior to study inclusion, and that subjects included were eligible, according to the inclusion and exclusion criteria described in the protocol. Study subjects were made aware that data would be anonymised, but the study sponsor, delegated monitoring staff, and representatives of regulatory authorities were authorised to inspect their medical records.

2.3 Data management and monitoring

2.3.1 Data storage and protection

All study data were securely stored in the Department of Medicine, UCL Archway Campus, unless otherwise stated. Storage was in accordance with GCP guidance for data management in clinical research. Source data, case report forms (CRF) and biological samples were identified only by study number and patient initials. Patient identifiable data and demographics were recorded on an NHS database, which was protected by encryption.

2.3.2 Data monitoring

Data monitoring plans for the individual studies are described in the relevant chapters.

2.4 Statistical methods

Statistical analysis was conducted using IBM® SPSS® Statistics 22 (IBM, New York, USA). Where appropriate, sample size computations were calculated using IBM® SPSS® SamplePower (IBM, New York, USA). Study-specific statistical methods are described in detail in the relevant sections of this work.

2.5 Adverse event reporting

2.5.1 Adverse events and reactions

In all of the studies undertaken during this research programme, an adverse event (AE) was defined as follows (281):

– *'Any untoward medical occurrence, including laboratory, considered to be related to the study'.*

In a clinical trial of an IMP, an adverse reaction (AR) was defined as follows (281):

– *'Any untoward and unintended response in a subject to an investigational medicinal product which is related to any dose administered to that subject'.*

2.5.2 Reporting and management of adverse events and reactions

All adverse events and reactions were recorded in the source documents and CRF, irrespective of the perceived relationship to any study intervention or the administration of an IMP. A report summarising all AEs/ARs was submitted on an annual basis to the following recipients: (1) NRES; (2) The study Sponsor; (3) The host organisation research and development office; (4) Any contracted independent medical monitor or pharmacovigilance organisation; (5) MHRA (when a clinical trial of an IMP was being undertaken). The management of AEs was the responsibility of the Chief Investigator but was conducted in accordance with the instructions of the Director of Research and Development.

2.5.3 Serious adverse events and reactions

A serious adverse event (SAE) was defined as an AE associated with one or more of the following outcomes (281):

– Death.
– Life-threatening illness.
– Hospitalisation or prolongation of an existing hospital stay.
– Persistent or significant disability/incapacity.
– A congenital anomaly or birth defect.

The term life threatening refers to an event in which the patient was at risk of death at the time of the event. It does not refer to an event that hypothetically might have caused death if it were more severe. Important events that did not meet the criteria above could be designated an SAE if they put the patient at risk of harm, and required medical or surgical interventions to prevent one of the outcomes listed above.

A serious adverse reaction (SAR) was defined as an AR associated with one or more of the following outcomes (281):

- Death.
- Life-threatening illness.
- Hospitalisation or prolongation of an existing hospital stay.
- Persistent or significant disability/incapacity.
- A congenital anomaly or birth defect.

An SAR was classified as unexpected if the nature and severity of the reaction was is not consistent with the information about the medicinal product in question set out as follows:

- In the case of a product with a marketing authorisation, in the summary of product characteristics (SPC) for that product.

- In the case of any other investigational medicinal product, in the investigator's brochure relating to the trial in question.

Any unexpected SAR was termed a suspected unexpected serious adverse reaction (SUSAR).

2.5.4 Causation

For all AEs/ARs, irrespective of severity, a causation assessment was undertaken. The following data relating to each event were recorded:

- Nature of the event.
- Date and time of onset and resolution (if resolved).
- Severity.
- Causation.
- Outcome.

Severity was defined using the scale described in **Table 3**. In clinical trials employing an IMP, causation was assessed using the framework described in **Table 4**. All SAEs and SUSARs were reported in accordance with timelines specified by the Sponsor, and those stipulated by the MHRA for trials of an IMP. All SAEs and SUSARs were reported to the Sponsor as soon as they were identified by the study site, and within one business day unless otherwise specified. Agencies providing data monitoring or pharmacovigilance services were also notified within this timeframe. The MHRA were notified of any such events within seven days if the outcome was life threatening or fatal, or within 15 days in all other cases. Medical and scientific judgment was exercised in deciding whether expedited reporting was appropriate in other situations.

2.5.5 Reporting of serious adverse events and reactions

All SAEs and SUSARs were reported in accordance with timelines specified by the Sponsor, and those stipulated by the MHRA for trials of an IMP. All SAEs and SUSARs were reported to the Sponsor as soon as they were identified by the study site, and within one business day unless otherwise specified. Agencies providing data monitoring or pharmacovigilance services were also notified within this timeframe. The MHRA were notified of any such events within seven days if the outcome was

life threatening or fatal, or within 15 days in all other cases. Medical and scientific judgment was exercised in deciding whether expedited reporting was appropriate in other situations.

2.6 Urine sampling

2.6.1 Midstream urine sampling

Samples were obtained by the midstream clean-catch and verbal and written instructions were provided (282). Subjects were required to cleanse their hands and genital area with antibacterial wipes prior to sample collection. Female subjects were instructed to hold the outer edges of labia apart and cleanse the genial region from front to back. Uncircumcised males were asked to retract the foreskin and cleanse the glans.

Subjects began urinating into the toilet or urinal. After urine flow for a few seconds, a sterile container was placed into the stream and approximately 100 ml of urine was collected without interruption of flow. The container was then removed from the stream and the urine decanted into three 30 ml sterile universal specimen tubes.

2.6.2 Catheter urine sampling

Cleansing of the genital area with an antibacterial wipe was undertaken prior to sampling as described above. A self-lubricating small latex-free catheter size 12 French gauge (Lofric™) was passed under aseptic conditions through the external urethral meatus into the bladder until urine flow was evident. Approximately 100 ml of urine was collected into a sterile container, and the catheter was then removed. The urine was then decanted into three 30 ml sterile universal specimen tubes.

Table 3 *Severity grading for study-related adverse outcomes.*

Severity grading for study-related adverse outcomes	
Mild	Discomfort noted, but no disruption to normal daily activities
Moderate	Discomfort sufficient to reduce or affect normal daily activities.
Severe	Inability to perform normal daily activities

Table 4 *Framework for assigning causation in clinical trials of an IMP.*

Causation and relationship to study intervention	
Definite	There can be no doubt
Probably	It follows a reasonable temporal sequence from administration of the drug*
	It cannot be reasonably explained by the known characteristics of the patient's clinical state, environmental or toxic factors, or other modes of therapy administered to the patient*
	It disappears or decreases on cessation or reduction in dose*
	It follows a known pattern of response to the suspected drug or intervention.
	It reappears upon re-challenge
Possibly	It follows a reasonable temporal sequence from administration of the drug
	It may have been produced by the patient's clinical state, environmental or toxic factors, or other modes of therapy administered to the patient.
	It follows a known pattern of response to the suspected drug
Unlikely	It does not follow a reasonable temporal sequence from administration of the drug†
	It may readily have been produced by the patient's clinical state, environmental or toxic factors, or other modes of therapy administered to the patient†
	It does not follow a known pattern of response to the suspected drug
	It does not reappear or worsen when the drug is re-administered
Not related	There is no evidence of a causal relationship

**These criteria are essential for probable causation to be assumed; †These criteria are essential for unlikely causation to be assumed.*

2.7 Serum sampling

Serum samples were collected by venepuncture using the BD Vacutainer® system (Becton Dickinson, Oxford, UK), employing a standard aseptic non-touch technique® (ANTT) (283). Samples for routine testing were submitted directly to the NHS laboratory in individual sample tubes selected according to the planned analyses.

Samples for exploratory analyses were subject to centrifugation and frozen storage. Five millilitres of venous blood was drawn in a BD Vacutainer® SST tube, which contains spray-coated silica and a polymer gel to promote serum separation. The sample was spun in a Denley BR401 centrifuge (R_{MAX}=140mm) at 2000 revolutions per minute (RPM) producing a relative centrifugal force (RCF) of 100 *g*. This separated the serum component from the red cell component, and the buffy layer, containing leucocytes. Without disturbing the cellular component, four 0.5 ml aliquots of separated serum were carefully transferred, using a sterile Pasteur pipette, into individual 1.5 ml freezing vials. These tubes were frozen immediately at the point of collection on dry ice before transfer to a freezer unit at -80°C.

2.8 Storage of biological samples

All biological samples were stored securely in the Department of Medicine, UCL Archway Campus, unless otherwise stated. Biological samples were identified only by study number and patient initials. Individual storage conditions for biological samples are described the relevant sections. Freezer units were fitted with alarms and subject to 24-hour temperature monitoring. Samples were kept in frozen storage at -80°C unless otherwise specified. Refrigeration units were temperature monitored only and maintained at 4°C.

2.9 Cytological assessment

2.9.1 Inflammation: Microscopic pyuria

Assessments were undertaken immediately after sample collection. A disposable Pasteur pipette (Sigma-Aldrich, Gillingham, UK) was used to load a Neubauer haemocytometer with a drop of fresh urine. This preparation was examined using an Olympus CX41 light microscope (x200) (Olympus, Southend-on-Sea, UK). The leucocyte count was enumerated using standard methods, and the results presented as a mean value, calculated from the assessment of two chamber counts.

2.9.2 Immune activation: Urothelial cell exfoliation

Assessments were undertaken immediately after sample collection. A disposable Pasteur pipette was used to load a Neubauer haemocytometer with a drop of fresh urine. The urothelial cell count was determined using the same methods as those outlined for the enumeration of urinary leucocytes. Results were presented as a mean value, calculated from the assessment of two chamber counts.

2.9.3 Bacterial colonisation: Urothelial clue cell analysis

Processing was undertaken within one hour of sample collection and samples were refrigerated at 4°C until assessment. Following mixing of the sample by inversion, 80 µl of urine was transferred into a cuvette assembly for centrifugation. The assembly comprised a single channel cuvette and retainer, a Shandon filter card (Fisher Scientific, Loughborough, UK), and a Superfrost Ultra Plus glass microscope slide (Thermo Scientific, Basingstoke, UK). The cuvette assembly containing the sample was spun at 75 *g* for five minutes in a Shandon Cytospin™ 2 cytocentrifuge (Thermo Scientific, Basingstoke, UK). The cellular and particulate components of the sample formed a visible deposit on the slide approximately 5 mm in diameter and excess liquid was absorbed by the filter card.

The deposit was circumscribed with an ImmEdge hydrophobic barrier pen (Vector Laboratories, Peterborough, UK) and 100 µl of 16% formaldehyde (Fisher Scientific, Loughborough, UK) was added for 15 minutes at room temperature (≈20^{o}C) as a fixative. The formaldehyde was then aspirated, and the preparation washed three times with 1% PBS (Sigma-Aldrich, Gillingham, UK) at 5 minute intervals.

The cellular deposit was stained using wheat germ agglutinin (WGA) conjugated to Alexa Fluor 488 (Invitrogen, Paisley, UK) to label the cell membrane. A stock solution of WGA (1 mg ml^{-1}) was prepared by dissolving 5 mg of Alexa Fluor 488 WGA conjugate into 5 ml of sterile 1% PBS. The stock solution was stable at -20°C for one month if protected from light. A working solution of Alexa Fluor 488 WGA conjugate (5 µg ml^{-1}) was produced by further dilution with Hank's balanced salt solution (HBSS) minus phenol red (Invitrogen, Paisley, UK). Prior to incubation with the sample deposit, this solution was briefly centrifuged to eliminate protein aggregation; approximately 100 µl of this supernatant was then added to the deposit. After incubation for 15 minutes at room temperature, the solution was aspirated and the deposit washed twice at 5-minute intervals with HBSS.

DNA in the deposit was counterstained with DAPI (4'', 6-diamidino-2-phenylindole) (Sigma-Aldrich, Gillingham, UK). Staining was achieved by the addition of 100 µl of DAPI (1 µg ml^{-1}) to the deposit which was incubated at room temperature for 15 minutes. This working solution was produced by the dilution of a stock solution (1 mg ml^{-1}) with 1% PBS; stock and working solutions were stable at -20°C until the expiry date of the reagent whilst protected from light. After incubation, the DAPI solution was aspirated and the deposit washed twice in 1% PBS. After staining, the deposit was immediately mounted with FluorSave reagent (Calbiochem, Darmstadt, Germany). A coverslip was fixed in place with clear nail varnish and the slide allowed to cure for at least one hour before examination. All slides were stored at 4°C in a light-protected environment.

Alexa Fluor 488 excites at a wavelength of 495nm and emits at 519nm staining cell membranes that appear green under fluorescence. DAPI excites at a wavelength of

360nm and emits at 460nm giving mammalian nuclei and bacteria a blue appearance under fluorescence. DAPI is capable of penetrating cellular membranes, and intracellular/extracellular pathogens can be labeled without the need for cell permeabilisation.

Samples were examined to identify urothelial cell expression and the proportion of urothelial cells exhibiting associated bacteria. Microscopic examination was undertaken using an Olympus CX41 upright epi-fluorescence microscope at the Department of Medicine, UCL Archway Campus, and a Leica DM4000B upright epi-fluorescence microscope (Leica Microsystems, Milton Keynes, UK) at the Royal Veterinary College Imaging Suit, Camden. Images were processed using ImageJ 1.44P and Axiovision Rel. 4.8 software (Carl Zeiss, Cambridge, UK).

2.10 Urothelial cytokine response

2.10.1 Interleukin-6

Samples for IL-6 measurement were frozen for later analysis. Aliquots of urine were transferred into individual 1.5 ml freezing vials within ten minutes of collection, (Sigma-Aldrich, Gillingham, UK) and stored at -80°C in the Department of Medicine, UCL Archway Campus (unless otherwise stated). Serum samples for cytokine analysis were collected by venepuncture and frozen at the point of collection (2.7). Analyses were undertaken at the Raine Institute, Division of Medicine, University College London. Frozen samples were transported to these laboratories in insulated boxes containing dry ice, and defrosted in an ice bath on site prior to testing.

The Quantikine® High Sensitivity ELISA Human IL-6 Immunoassay was used to quantify IL-6 expression in serum and urine (R&D Systems, Abingdon, UK). Standard solutions were prepared using the supplied IL-6 standard (10 pg ml^{-1}) and calibrator diluent. Six serial dilutions of the IL-6 standard were prepared, producing solutions between 5 pg ml^{-1} and 0.156 pg ml^{-1}; the stock IL-6 standard and calibrator diluent

were used as 10 pg ml^{-1} and zero standards respectively. Frozen samples for analysis were concurrently thawed and mixed thoroughly using a vortex mixer for five seconds at 3200 rpm (Scientific Industries, New York, USA).

Sixteen wells in the supplied microplate were filled 100 µl of the eight IL-6 standard solutions in duplicate, with the remaining wells accommodating 100 µl of each test sample. The plate was then incubated at room temperature on an orbital microplate shaker (0.12" orbit) set at 500 ± 50 rpm for two hours. The well contents were then emptied and the wells washed six times using the supplied wash solution.

After washing, 200µl of IL-6 conjugate was added to each well, and the plate incubated for a further two hours on the orbital shaker as described above. The well contents were again emptied and washed six times. Fifty microlitres of the supplied substrate solution was then added to each well, and the plate incubated on the benchtop at room temperature for one hour. Without washing, 50 µl of amplifier solution was added to each well, and the plate incubated for a further 30 minutes on the benchtop. Fifty microlitres of the supplied stop solution was then added to each of the wells and analysis undertaken within 30 minutes. Interleukin-6 concentration was determined using an Opsys MR fluorescence microplate reader (DYNEX Technologies, Worthing, UK), set at 490 nm.

2.11 Urinary purine expression

2.11.1 Adenosine Triphosphate

Urinary ATP measurement was undertaken on fresh urine samples on-site. Urinary ATP expression was quantified using a Lumitester PD-20 hand-held detector device in conjunction with a single-use LuciPac Pen employing a bioluminescence assay (Kikkoman Biochemifa, Toyko, Japan). Prior to testing, a LuciPac Pen (containing the reagents luciferase and pyruvate phosphate dikinase) was removed from refrigerated storage at 4°C and allowed to equilibrate to room temperature for 20

minutes prior to use. The swab component of the LuciPac pen was removed from the rest of the assembly and immersed in a freshly voided urine sample. Testing was undertaken within three minutes of sample production to avoid signal loss. The swab was immersed and agitated in the urine sample for five seconds before its removal. Following immersion, the swab was immediately replaced into the LuciPac Pen assembly and LuciPac Pen shaken vigorously for 10 seconds to allow the reagents to mix. The Lucipac Pen was then inserted into the Lumitester PD20 and measurement was initiated, taking care to maintain the Lumitester in an upright position. The result, expressed in relative light units (RLU), was produced after a 10 second analysis phase. The LuciPac Pen was then removed. The molar concentration of ATP was calculated using a standard curve generated from the analysis of known concentrations of ATP in solution.

2.12 Bacterial isolation

Existing data have demonstrated that anaerobic bacteria are isolated very infrequently in cultured urine, even when fixed thresholds are dismissed (234). A recent analysis of cultured urine from patents with LUTS demonstrated that anaerobic isolates were extremely rare (270). Although atypical organisms have been implicated in acute urinary infection, controlled studies have suggested that their role is small (284, 285).

Bacterial isolation in this study focused on aerobic bacteria. The isolation of these bacteria can be achieved simply, employing overnight culture techniques without complex culture media or conditions. This reflects standard microbiological practice in the UK.

2.12.1 Spun urinary sediment culture

All urinary sediment cultures were undertaken at the laboratories of the Department of Medicine, UCL Archway Campus. Cultures were processed within two hours of collection, and pending samples were refrigerated at 4°C.

2.12.1.1 Preparation and inoculation

Following mixing of the urine sample by inversion, five millilitres were transferred into a 15 ml sterile centrifuge tube. The sample was centrifuged using a Denley BR401 centrifuge (R_{MAX}=140mm) (Denley, Heckmondwike, UK) at 627 *g* for 5 minutes. The supernatant was carefully removed and discarded, leaving the undisturbed urinary sediment. The sediment was resuspended in 400 µl of 1% sterile PBS solution. The resuspended urinary sediment was then subject to four 1:10 serial dilutions to permit a fully quantitative bacterial count.

Serial dilutions were undertaken as follows: One-hundred microlitres of the sediment solution was transferred into a sterile Eppendorf 1.5 ml Microtube (Eppendorf, Stevenage, UK), and 900 µl of 1% sterile PBS was added. The tube was capped and inverted five times to mix. The new sediment solution was subject to a further dilution as described, and the whole procedure repeated twice more to produce five sediment suspensions, ranging in concentration from n to n^{-4}.

A chromogenic CPS3 agar plate (bioMérieux, Basingstoke, UK) was divided into five equal sectors radially, and 50 µl of each of the sediment suspensions plated and spread using a sterile cell spreader. Fifty microlitres of the stock 1% PBS solution used in the culture process was then transferred onto a Columbia Blood Agar (CBA) plate (Oxoid, Basingstoke, UK) to test for bacterial contamination. The culture plates were retained in an incubator for 24 hours at 37°C.

2.12.1.2 Bacterial identification: Colour and morphology

The CPS3 chromogenic medium generates distinct chromophores in the presence of specific bacterial genera/species. This permits colour-based identification of almost 90% of isolates to genus or species level on the primary culture plate, using the manufacturer's reference criteria (286). The use of chromogenic media, in conjunction with Gram staining and biochemical testing where indicated, accelerates bacterial identification and improves discrimination in polymicrobial cultures (287, 288). Chromogenic media have equivalent or superior sensitivity to Columbia Blood Agar (CBA), MacConkey's Agar (MCA) or Cysteine Lactose Electrolyte Deficient (CLED) media (286-289). Analytical Profile Index (API) testing was used to identify all organisms to species level.

2.12.1.3 Bacterial quantification

Each bacterial isolate was subject to a quantitative count. The number of colonies of each isolate was determined in each sector of the CPS3 plate, corresponding to one of five serial bacterial dilutions. The mean colony count from all sectors was calculated.

2.12.1.4 Bacterial sub-culture

Each bacterial isolate identified on chromogenic agar was sub-cultured prior to supplementary testing. A single colony of the bacteria was streaked on a CBA plate using a sterile 1µl inoculation loop (Sigma-Aldrich, Gillingham, UK), with care taken to avoid contamination. The inoculated CBA plate was incubated for 24 hours at 37°C and a pure growth of the cultured organism verified before isolate storage.

2.12.1.5 Bacterial identification: Gram staining

Gram staining was employed to determine the morphological characteristics and cell wall composition of isolates where further classification was required. Prior to staining, a bacterial smear was produced by applying a small deposit of bacteria to a Superfrost Ultra Plus glass microscope slide using a sterile 1µl inoculation loop. The bacteria were spread across the surface of the slide using the loop. Two drops of 1% PBS were then added to the slide using a sterile Pasteur pipette, and the bacterial deposit mixed with the PBS to produce a homogeneous suspension on the surface of the slide. The bacterial suspension was then heat-fixed using an electrothermal slide-drying bench at 70°C for approximately 15 minutes (Cole-Palmer, London, UK).

The surface of the fixed bacterial smear was flooded with Crystal Violet (Sigma-Aldrich, Gillingham, UK) and incubated for 30-60 seconds before washing with tap water. The preparation was then flooded with Gram's Iodine (Sigma-Aldrich, Gillingham, UK) and incubated for a further 30-60 seconds before washing with tap water. The slide was then flooded with acetone (Sigma-Aldrich, Gillingham, UK) but care was taken to wash within one to two seconds of its application. A Carbol-Fuchsin counterstain (Sigma-Aldrich, Gillingham, UK) was then applied and the preparation incubated for a further 30-60 seconds before washing with tap water. The slide was then dried using an electrothermal slide-drying bench at 70°C for approximately 15 minutes.

The preparation was examined using an Olympus CX41 light microscope (x400) employing an oil-immersion lens. The morphology and colour of the bacteria were recorded. Gram-positive bacteria appear purple and Gram-negative appear pink to red.

2.12.1.6 Bacterial identification: Rapid biochemical testing

Rapid reagent testing including indole, oxidase, coagulase and catalase testing were employed to supplement colour-based bacterial identification. The methods and indications for these tests are summarised below (**Table 5**).

For the indole test, two drops of the RapidID™ Spot Indole Reagent (Remel, Basingstoke, UK) were dispensed onto a piece of Whatman® (No. 1) filter paper (Sigma-Aldrich, Gillingham, UK). A viable bacterial inoculum was smeared over the saturated filter paper using a sterile 1µl inoculation loop. The preparation was observed for one to three minutes and the development of a specific colour indicated a positive test. The oxidase test was conducted using an identical method, but the BactiDrop™ Oxidase reagent (Remel, Basingstoke, UK) was used, and the incubation time confined to 10-30 seconds.

For the coagulase test, a drop of demineralized water was applied to a Superfrost Ultra Plus glass microscope slide. A loop of the test isolate was mixed with the water to produce a homogeneous suspension, and the suspension checked to ensure no spontaneous agglutination had occurred. A drop of Coagulase Plasma (Remel, Basingstoke, UK) was then added to the suspension and mixed gently using a sterile 1µl inoculation loop. The sample was observed for the immediate formation of a white, globular precipitate, indicating a positive result. The test results were read in the first few seconds, as false positive results can be observed associated with reaction times of more than ten seconds.

For the catalase test, a loop of the test isolate was smeared onto a Superfrost Ultra Plus glass microscope slide. Care was taken not to carry over any traces of blood agar as this can mediate a false-positive result. Using a sterile Pasteur pipette, one drop of 3% hydrogen peroxide was added to the isolate. A positive result was indicated by immediate effervescence.

Table 5 *Summary of rapid reagent testing for bacterial identification.*

Test	Target bacteria	Reaction	Positive test	Negative test
Indole	E.coli	Indole combines with dimethylaminocinnamaldehyde to form a blue-green compound	Blue colour	Pink colour
Oxidase	Pseudomonadaceae	Bacteria that produce the oxidase enzyme, in the presence of oxygen, cytochrome c, and phenylenediamine oxidase reagent, oxidise to form indophenol.	Purple colour	No colour reaction or delayed development
Coagulase	Coagulase-positive Staphylococci	The enzyme coagulase acts on a constituent of the rabbit plasma reagent to produce a thrombin-like substance	Immediate formation of white, globular precipitate	No reaction
Catalase	Staphylococcus and Micrococcus	Catalase expedites the breakdown of hydrogen peroxide into water and oxygen	Immediate effervescence	No reaction

2.12.1.7 Bacterial identification: API testing

API testing is frequently used in the diagnostic laboratory to permit accurate species-level identification. It is widely employed as a reference test for the validation of other identification systems. An API array consists of around 20 microtubes containing different reagents, each catalysing a distinct reaction. Colour changes associated with the individual tests are noted and the results entered into an online database (290). The database reports the likely species and provides a probability estimate of accuracy. Selection of the appropriate API test depends on suspected genera of the isolate based on chromogenic culture data, gram staining and additional biochemical 'spot tests'.

2.12.2 Routine microbiological culture

All routine microbiological cultures were undertaken in the Whittington Hospital and Royal Free Hospital microbiology laboratories. Thirty millilitres of urine in a sterile universal specimen tube was submitted for culture. Samples were processed immediately upon receipt, or after overnight refrigeration at 4°C. All analyses were undertaken by trained biomedical scientists.

2.12.2.1 Preparation and inoculation

One microlitre of the sample was transferred by sterile inoculation loop to a chromogenic CPS3 agar plate; inoculation was achieved by streaking the loop across the plate. The culture plate was then incubated aerobically for 24 hours at 37^{O}C.

2.12.2.2 Bacterial identification: Colour and morphology

Bacterial colonies were identified by colour and morphologic characteristics, as described previously (2.12.1.2).

2.12.2.3 Bacterial identification: 'Spot' biochemical testing

Rapid reagent testing ('spot testing') was employed to supplement colour-based bacterial identification as described previously (2.12.1.6).

2.12.2.4 Bacterial quantification

Routine NHS culture techniques are semi-quantitative and bacterial growth is estimated by visual assessment of colony density. A 'positive' culture is defined as the growth of a single recognised uropathogen at $\geq 10^5$ cfu ml^{-1}; polymicrobial growth

above this threshold is reported as 'mixed growth'. Any bacterial growth below 10^5 cfu ml^{-1} is reported as 'no significant growth'.

2.12.3 Storage of bacterial isolates for research

Bacterial isolates were indexed and stored in 2 ml cryopreservation vials (Thermo Scientific, Basingstoke, UK) at -80°C in the Department of Medicine, UCL Archway Campus.

2.13 Urine dipstick reagent testing

Dipstick testing was undertaken using an automated colourimetric system. Multistix® 8 SG reagent strips were paired with a Clinitek® Status analyser (Siemens, Munich, Germany). Leucocyte esterase was reported as: 'negative', 'trace', '1+', '2+' or '3+'. Nitrite was reported as 'negative' or 'positive'.

2.14 Lower urinary tract symptom measures

2.14.1 International Consultation on Incontinence Questionnaires

The measurement of symptoms was a key consideration in this work. Whilst there are many available instruments to measure the frequency and impact of LUTS, the International Consultation on Incontinence Questionnaires (ICIQ) were selected for use in this work (291). The ICIQ symptoms scores have been developed under the supervision of a board of international experts on incontinence and have been widely validated. The development of the ICIQ measures was initiated in an attempt to standardise the reporting of research findings in studies of lower urinary tract function, allowing ready comparison of outcomes between studies. The ICIQ-LUTS questionnaire was selected to quantify and characterise symptoms and the ICIQ-LUTSqol questionnaire used to assess bladder-related QoL.

2.14.2 Whittington Urgency Score

The severity of urinary urgency and its response to treatment was measured using an additional validated instrument. The Whittington Urgency Score is a simple ten-item scale that records the individual circumstances associated with urinary urgency described by the patient (292). Questionnaire responses are summed and the total score has a near linear relationship with urinary frequency and incontinence, which are independent measures of lower urinary tract symptom severity. The questionnaire has been fully validated and used successfully in RCTs exploring treatment efficacy in patients with OAB symptoms.

2.14.3 Whittington Pain score

Pain is an important presenting feature of acute infective urinary tract syndromes such as acute pyelonephritis and acute cystitis. An association between OAB symptoms and urinary tract pain has been described in clinical practice but no published evidence exists. In the context of preliminary evidence implicating urothelial inflammation in the generation of LUTS, a validated questionnaire measuring lower urinary tract pain symptoms was included as part of each patient assessment.

Validated instruments to measure pain associated with the lower urinary tract are few. The Whittington Pain Questionnaire, developed from a large symptom dataset provided by patients with interstitial cystitis (IC) was selected for use in these studies. It is a validated eight-item scale which records the most prevalent dysaesthetic/pain symptoms associated with the lower urinary tract (293).

2.14.4 Incontinence Quality of Life questionnaire

The Incontinence Quality of Life (I-QOL) questionnaire is an established measure of the impact of urinary incontinence on QoL. The questionnaire has been extensively

validated and translated into over 20 languages. It is a responsive measure of QoL in patients with neurogenic and non-neurogenic LUTS (294, 295). Whilst the development of ICIQ questionnaires was initiated to standardise the assessment of incontinence in clinical and research settings, the I-QOL is still widely used. It was employed to assess bladder-related QoL in this programme of research prior to the validation of the ICIQ questionnaires.

2.15 Lower urinary tract function

Lower urinary tract function was assessed using three-day bladder diaries. Each diary records data from three 24-hour periods, collected inside a one-week window. The patient is required to measure and document all volumes of urine passed in a 24-hour period and note any episodes of incontinence. Average voided volume, an estimate of functional bladder capacity, was calculated as an average of all recorded voids over the three-day diary period. Mean daily urinary frequency and incontinence episodes were calculated similarly.

Whilst bladder diary data can be collected for extended periods of up to fourteen days, the three-day diary has become a popular instrument to measure bladder function in most clinical and research settings. The process of completing a bladder diary may be arduous and ambulant adults report progressively increasing burden as diary periods are extended (296). Diary periods of more than three days have also been associated with poor compliance and incomplete data entry (297). Patients with MS frequently describe significant problems with fatigue, mobility and dexterity, and the burden of bladder diary completion might be expected to be greater in this group of patients. This could reduce compliance. One study has suggested that accuracy is compromised when bladder diary duration is reduced beyond four days (298), although the three-day diary has proven a reliable tool in the assessment of OAB symptoms (299).

2.16 Disability and function in MS

2.16.1 Short Form Health Survey

The Short Form Health Survey (SF-36) was administered as a generic measure of patient health. The RAND Corporation developed the questionnaire as part of the Medical Outcomes Study, conducted to explore variations in patient outcomes (300). The SF-36 comprises eight subscales comprising 36 questions that explore health and wellbeing, employing psychometric assessment of physical and mental health indicators.

The questionnaire does not target any specific demographic group or illness. The utility of the SF-36 in evaluating disease burden has been demonstrated in over 200 medical conditions (301). The measure has been cited in over 11000 publications and over 200 studies in MS have used the SF-36 as an outcome measure.

2.16.2 MS Impact Scale

The MS Impact Scale (MSIS-29) was used to measure the physical and psychological impact of MS on patients. It is fully validated and has been used extensively in the assessment of new therapies for the treatment of MS (302, 303). The MSIS-29 demonstrates greater responsiveness than any other disease-specific or general health measure in MS (303).

The questionnaire has two subscales that assess the influence of MS on physical and psychological wellbeing. Whilst the results from these two domains can be reported as a total summary score, they measure two related but distinct dimensions. The use of a summed score may conceal important but divergent changes in physical and psychological status.

2.16.3 MS Walking Scale

Impairment of mobility is a key determinant of QoL in MS (54). The MS Walking Scale (MSWS-12) is a validated, self-administered questionnaire that was used to measure the perceived impact of MS on walking ability (304). The MSWS-12 is the only measure designed specifically for the self-assessment of walking ability in MS, although other general measures include ambulation as a subscale. None of these patient-reported measures demonstrate greater responsiveness than the MSWS-12 (304).

2.16.4 Expanded Disability Status Scale

The Expanded Disability Status Scale (EDSS), an extension of the earlier Disability Status Scale (DSS), is a physician-reported measure of disability in MS (305). The EDSS is reported as a score of 0-10 (employing 0.5 unit increments) with higher scores indicating greater disability. In ambulant patients with lesser degrees of disability (scores 0-4.5) the EDSS is calculated by the neurological assessment of eight functional systems. Higher scores (5.0-10) are defined by impairment of ambulation.

Whilst the EDSS is ubiquitously deployed in the assessment of patients with MS, as a standard measure of disability it has significant drawbacks. It demonstrates significant inter- and intra-observer variability (306) and is an ordinal rather than linear variable. Thus, each increment on the EDSS rating is not associated with comparable changes in disability. Despite these problems, the EDSS is the only disability scale widely recognised by regulatory agencies, making its inclusion as an outcome measure in clinical trials widespread.

2.16.5 MS Functional Composite

The MS Functional Composite (MSFC) was devised in recognition of the shortcomings of existing outcome measures in MS research. The MSFC is a measure of three key clinical dimensions: (1) leg function and ambulation; (2) arm and hand function; and (3) cognitive function. The MSCF score is generated from the nine-hole peg test (9HPT) that evaluates upper limb dexterity, the timed 25-foot walk test (TWT) that measures lower limb function, and the three-second paced auditory serial addition test (PASAT3) that measures cognitive function (307). Whilst these three tests have different metrics, results can be standardised by calculating a *z*-score. The *z*-score is the number of standard deviations above or below the population mean at which the result lies. In this regard, performance is related to the characteristics of the reference population.

Whilst the MSFC has theoretical advantages over the EDSS, it does have its drawbacks. Whilst validated, it has only been used as the primary outcome measure in one clinical trial (308) (although it has been selected as a secondary outcome measure in many other studies). Unlike the EDSS, MSFC scores are not easily interpretable to clinicians and the measure has yet to gain wider acceptance as a primary tool to assess functional in MS. There have been recent calls to develop the MSFC into a measure that will ultimately become the key primary outcome measure for studies in MS but this work is ongoing (309).

2.16.6 Farnsworth-Munsell 100 Hue Colour Vision Test

The Farnsworth-Munsell 100 Hue Color Vision Test is used to test for color blindness (310). The test evaluates the ability of the patient to discriminate minute differences amongst 100 coloured tiles and arrange the tiles according to perceived hue. Four trays of tiles are provided with a fixed ‘anchor’ tile at the end of each tray. Patients had to arrange the loose tiles between these ‘anchor’ tiles in the four trays according to hue. Fifteen minutes were allowed for completion of the task. The test was administered binocularly, under controlled lighting conditions with D50 illumination.

The results were analysed using software provided with the testing trays (X-Rite, Poynton, UK). Patients using distance or reading spectacles during testing were required to use the same pair for all tests.

2.16.7 Logarithm of the Minimum Angle of Resolution (LogMAR) visual acuity testing

Logarithm of the Minimum Angle of Resolution (LogMAR) testing was undertaken to assess visual acuity (311). The LogMAR chart demonstrates five letters on each line, with descending letter size designated as Log^{10} of the visual acuity. The spacing between the individual letters on each line and between the lines become smaller in proportion to one another. This feature of the LogMAR chart reduces variation in contour interaction (the effects of letter crowding). The LogMAR chart is recommended as the instrument of choice for the assessment of visual acuity in the research setting. The test was administered to each eye, under controlled lighting conditions from a distance of 4 metres. Patients using spectacles during testing were required to use the same pair for all tests.

2.16.8 Threshold tracking

Threshold tracking is a neurophysiological test that evaluates peripheral nerve excitability (312). The technique is non-invasive and determines the electrical properties of the nerve membrane at the site of stimulation. Electrical stimulation was applied to the median nerve at the wrist and the resultant compound muscle action potential recorded using surface electrodes. Stimulus waveform and intensity were controlled by computer and delivered by a Digitimer DS5 isolated bipolar, constant-current stimulator (Digitimer, Welwyn Garden City, UK). Stimulus-response curves, strength-duration time constants, threshold electrotonus, current-threshold relationships and the recovery cycle were all measured. The patient's dominant hand was tested where possible. A carpal tunnel lesion was excluded prior to first testing in each patient.

3 Evaluating novel measures of urothelial inflammation and distress in the study of lower urinary tract symptoms

3.1 Background

The presence of uropathogenic bacteria in the lower urinary tract is not always associated with symptoms and asymptomatic bacteriuria is commonplace (179, 276, 277). The isolation of uropathogens from the lower urinary tract provides no evidence of an interaction between microbe and host, and the magnitude of bacterial growth alone cannot define the veracity of any pathogen-host exchange.

Prospective study of women with acute cystitis has demonstrated bacterial proliferation and escalating lower urinary tract inflammation that precedes the appearance of symptoms (258). Preliminary data suggest that patients with chronic LUTS manifest greater bacterial colonisation and urothelial inflammation than asymptomatic controls but prospective studies are lacking. If infection is associated with the generation of LUTS, assumptions about pathophysiology cannot be made based on observations made in acute UTI. Measures of urothelial inflammation and distress are essential in characterising the nature of any relationship.

Urinary tract infection stimulates the release of a variety of inflammatory mediators including platelet-derived growth factor (PDGF), tumour necrosis factor-α (TNF-α), IL-6 and CXCL-8 (313). Interleukin-6 is a multifunctional cytokine that modulates inflammatory, acute phase and immune responses. Interleukin-8 (CXCL-8) is a primarily chemotactic cytokine, recruiting neutrophils and other granulocytes towards a site of infection or inflammation.

Elevated IL-6 levels contribute to the pathogenesis of many inflammatory and autoimmune conditions and increased IL-6 expression has been demonstrated in prospective observational studies of human UTI (216, 251-258, 314). Human lower urinary tract inoculation studies have demonstrated rapid increases in IL-6 secretion within minutes of bacterial inoculation with E.coli (252).

There are few data on the cytokine profile of patients with other lower urinary tract syndromes such as OAB. One study reported significantly greater urinary IL-6 expression in patients with OAB compared to asymptomatic controls, although controls were significantly younger (F=9; p=0.003) (259). Amongst patients, pyuria was associated with higher IL-6 levels, although patients without pyuria still demonstrated greater IL-6 expression than asymptomatic control subjects (F=3.2; df=2; p=0.045). This finding suggests that in some patients urothelial inflammation may be present in the absence of inflammatory cell recruitment. Other published studies are small, inadequately controlled, and use microarray analysis to screen for large numbers of cytokines (315, 316). These data provide conflicting conclusions relating to the expression of urinary IL-6 in patients with OAB.

Immune cells and many other cell lines secrete IL-6. Data from animal and human studies implicate urothelial cells as the primary source of IL-6 after bacterial challenge (251, 252, 317). Collectively, these data promote IL-6 as a candidate cytokine for detecting immune activation in studies exploring the role of infection in the generation of LUTS.

Human data demonstrate enhanced purinergic neurotransmission in patients with lower urinary tract disease (67-75, 92, 93). Purine receptor up-regulation has also been demonstrated in E.coli-infected and cytokine-stimulated human urothelial cell lines (262). These data were generated from the physiological study of tissue biopsies and the expression of ATP in sampled urine from patients with lower urinary tract infection and inflammation remains unclear.

Adenosine triphosphate is present in high concentration in the intracellular space and under normal circumstances its concentration in the extracellular compartment is low. Adenosine triphosphate is a small solute and when released, it readily diffuses in the extracellular space. All cells demonstrate membrane-bound ecto-nucleotidases that rapidly hydrolyse extracellular purine nucleotides, although the products of hydrolysis are able to interact with a variety of purine receptors (318).

The release of ATP from injured host cells is widely acknowledged to serve as a 'danger' signal. Lytic secretion of ATP occurs in response to cell damage causing a loss of membrane integrity. Non-lytic secretion might occur in response to mechanical stretch, bacterial lipopolysaccharide (LPS), or be driven by ATP release locally (319-322). This can be mediated by exocytosis of ATP-containing vesicles or facilitated diffusion across trans-membrane proteins or channels. Other potentially potent sources of ATP in the lower urinary tract are bacteria. Whilst this could be related to lytic release from dead microbes, recent data demonstrates active ATP secretion from viable cells during growth (323, 324). The physiological function of secreted ATP in this context is not known.

Whilst existing data have implicated the urothelium as the primary source of host tissue IL-6 secretion in the lower urinary tract, the same is not true for ATP. Purines may be released from stimulated or damaged urothelial cells but innate immune cells may also contribute to total urinary ATP concentration. Adenosine triphosphate mediates its effects through autocrine and paracrine mechanisms, amplifying the response to chemotactic stimuli and cellular activation (325). Thus, the migration of leucocytes into the lower urinary tract may contribute to purine expression in human urine.

Adenosine triphosphate is known to interact with antigen presenting cells (APCs) in shaping the immune response. The effects of purine nucleotides on APCs depend upon the quality of purine release. Whilst this interaction is complex, it is clear that the effects of ATP are influenced by the presence of bacterial endotoxin or LPS (326). In their presence, ATP drives a potent pro-inflammatory cascade. It appears that this response requires the synergistic effects of ATP and bacterial antigen. Other data has suggested that ATP, released as a danger signal, may be capable of driving the inflammatory response directly (326-329). This relationship is not fully understood and there are no studies that explore this interaction in the bladder specifically.

Adenosine triphosphate is measured using a luciferin-luciferase bioluminescence assay. Biological samples are plated and buffered to approximately pH 7.8 to

optimise luciferase enzymic action. The luciferin and luciferase reagents are then added and bioluminescence measurements are made immediately using a benchtop luminometer. Luciferase catalyses the reaction between ATP and luciferin, producing adenyl-luciferin and inorganic phosphate. Adenyl-luciferin then undergoes oxidation to produce adenosine monophosphate (AMP), oxyluciferin, carbon dioxide (CO_2), and light that is quantified in RLU. Adenosine triphosphate concentration is calculated from RLU using a standard curve, constructed from the luminometric analysis of known concentrations of ATP in solution.

Buffering the samples prior to measurement is time consuming but the effect of delays in analysis on the recovery of ATP is unknown. In vivo, ecto-nucleotidases on cell membranes mediate the rapid hydrolysis of ATP (330). It is possible that similar enzymic degradation could happen in urine. The effects of freezing urine samples prior to analysis is also worthy of consideration, as a freeze-thaw cycle prior to analysis might damage cell membrane integrity liberating ATP. This might be of particular concern in samples with marked cellularity.

In the catering and food industries, and the environmental sciences, the measurement of ATP is used as a surrogate marker of bacterial contamination/activity. Hand-held luminometers for the measurement of ATP are available and employ single-use swab systems to sample biological materials and provide information on ATP concentration (331). Kikkoman Biochemifa (Tokyo, Japan) produces a hand-held luminometer, initially developed for use in Kikkoman manufacturing plants. The PD20 lumitester™ is used to identify bacterial contamination in manufactured foodstuffs and monitor equipment and hand cleanliness. Industry data and independent scrutiny have demonstrated a linear relationship between bacterial quantities and light generated by the luciferase assay (331, 332).

Hand-held luminometers have not been used in health sciences, but one study has evaluated the use of the PD20 lumitester™ in the quantification of ATP in biological solutions (333). This work demonstrated that the device reacted with linear

sensitivity to ATP concentrations in the micromolar to femtomolar range. Existing data show that urinary ATP levels are within this range (334). The LuciPac Pen™, used in conjunction with the luminometer, contains pyruvate orthophosphate kinase which converts AMP into ATP. Adenosine monophosphate is the product of ATP breakdown, so the addition of this enzyme should attenuate signal loss as a result of ATP degradation after sampling. The sensitivity of the PD20 luminometer™ to other purine nucleotides was also assessed using standard solutions, which confirmed that the device was only responsive to ATP and AMP (333). Based on these data, the PD20 luminometer™ offers an alternative to conventional benchtop bioluminescence assays in biomedical research.

By allowing the immediate quantification of ATP in biological samples, the PD20 lumitester circumvents the possible effects of storage on ATP measurement. Cell lysis liberating intracellular ATP, or bacterial growth might otherwise influence urinary ATP levels. Immediate testing precludes the requirement for frozen storage prior to analysis which could also be associated with cell lysis.

Whilst Adenosine triphosphate is reported to be stable in neutral solution, hydrolysis is accelerated at extremes of pH. A mean urinary pH value of 5.5 is often cited, although the pH of urine is known to vary considerably (range 4.5-8.5) (335). The LuciPac Pen contains a compartmentalised reagent mixture, with lyophilised powder and liquid components separated by an aluminium foil membrane. These mix when the sampling swab is inserted and perforates the membrane. The reagent mixture is buffered, maintaining a narrow pH range (336).

Purinergic upregulation has been demonstrated experimentally in tissues harvested from patients with selected lower urinary tract syndromes (67-75, 92, 259, 262). The expression of ATP in sampled urine from similar patient groups has not been explored. Only one study has reported a correlation between urinary ATP expression and pyuria excretion in patients with LUTS (334). The utility of ATP as a surrogate marker of bacterial infection and urothelial distress will be explored in this programme of study.

There are limited data that scrutinise the measurement of cytokines and purine nucleotides in human urine. Specifically, the effects of specimen processing and storage have not been determined. These factors are important for any clinical application. Cytokine stability in human urine has been examined in urothelial cell lines and human UTI (258, 337). The first of these studies examined the stability of CXCL-8 in only three urine samples stored at different temperatures, concluding that CXCL-8 did not decay during a 24-hour period (337). The second study explored the stability of a panel of cytokines, including IL-6 and CXCL-8, when stored in a proprietary urine preservative tube (258). Whilst these data were not explicitly reported in the paper, the commentary described 'no significant alteration' in cytokine levels after preservation and storage at 4°C or 20°C.

Little is known about the relationship between serum and urine IL-6 levels, and whether increased serum IL-6 concentrations can influence urinary IL-6 expression. Several studies have explored serum and CSF IL-6 levels in MS producing contradictory results, although the published data do not demonstrate increased serum IL-6 levels in patients compared to matched controls (338-343). The available evidence suggests that elevated levels of IL-6 are expressed in the CNS of patients with MS rather than the serum.

Human data examining the relationship between serum and urine IL-6 levels are limited to very small case series although this work has not demonstrated a correlation between serum and urinary IL-6 expression (252, 317). This relationship has never been formally tested in MS patients and without these data, the source of urinary cytokine release cannot be assumed to be local.

Whilst some cytokines are sensitive to freeze-thaw effects, IL-6 appears to be stable despite repeated freeze-thaw cycles (344). When stored at -80°C, IL-6 levels in cell-free serum, produced by cytocentrifugation, remain stable for up to two years before significant decay is recorded (344). Whether urinary cytokines demonstrate similar behaviour when stored is not known and the influence of frozen storage on

purine expression in urine has not been reported. The effect of centrifugation on the recovery of urinary cytokines and purines is also unclear.

3.2 Study overview

Laboratory experiments were conducted to evaluate the effects of urinary storage and processing on IL-6 and ATP recovery. The influence of chemical preservation, frozen storage conditions and cytocentrifugation were all subject to scrutiny. The relationship between serum and urine IL-6 levels in MS patients was also investigated in paired serum and urine. The optimal incubation period for samples subject to luminometric analysis was also examined, as this has not been described for purine recovery in urine using a hand-held luminometer.

The studies were conducted at the Department of Medicine, UCL Archway Campus, the Department of Neurophysiology, Royal Free Hospital, and the Raine Institute, UCL, London. Ethical approval for these studies was granted by the Whittington and Moorfields Research Ethics Committee (Ref: 07/H0704/74) and the NRES Committee London - Queen's Square (Ref: 07/H0716/69).

3.3 Safety considerations

There were no safety considerations for patients providing MSU samples only. Patients providing paired serum and urine samples for analysis were at risk of discomfort and bruising associated with venepuncture. The risks and benefits of study participation were provided in study-specific patient information materials and discussed before informed consent was provided.

3.4 Study objectives

The objectives of the study were to determine the following:

- The optimum test incubation period before luminometric measurement in the assessment of urinary ATP using the handheld PD20 lumitester.

- The influence of storage and chemical preservation on urinary IL-6 and ATP concentrations in stored urine.
- The influence of frozen storage temperature on urinary IL-6 and ATP recovery.
- The influence of centrifugation on urinary IL-6 and ATP recovery.
- The relationship between serum and urine IL-6 in patients with MS and LUTS.

3.5 Study population

3.5.1 Recruitment of participants

Recruitment of study participants was conducted as outlined previously (2.2.2).

3.5.2 Consent and eligibility

All patients provided written, informed consent prior to any study related procedures, and eligibility was checked before inclusion (2.2.3).

3.5.3 Inclusion and exclusion criteria

Adult patients with non-neurogenic LUTS provided samples for the laboratory analyses examining the effects of urinary storage and processing on IL-6 and ATP recovery. Study participation was not restricted by any other inclusion or exclusion criteria, as there was no expectation that these experiments would be influenced by any clinical or demographic patient factors.

MS patients enrolled in a clinical trial provided paired urine and serum samples for IL-6 quantification. Details of inclusion and exclusion criteria relating to this study are summarised elsewhere (7.5).

3.6 Study design

Urine and serum samples were subject to laboratory experiments that explored the study objectives. In the studies concerning urine analyses only, eligible patients were required to provide an MSU sample for analysis. These patients were recruited from a clinic specialising in the treatment of LUTS and submitted only one sample. A subset of patients participating in an RCT provided paired MSU and serum samples for analysis.

3.6.1 Blinding

In all comparative analyses, the researcher was blinded to the results of any previous assessments to attenuate bias.

3.7 Clinical and laboratory assessments

3.7.1 Biological samples

MSU samples were collecting using a clean-catch method (2.6.1). Serum samples were collected using a standard aseptic non-touch technique® (2.7). Where frozen storage was required prior to analysis, the samples were securely stored at -80°C in the Rheumatology Department, Royal Free Hospital, and the Department of Medicine, UCL Archway Campus (2.8).

3.7.2 Incubation and luminometric measurement of ATP in urine

Repeated luminometric measurements were made on the same LuciPac Pen in order to determine the optimum test incubation period before luminometric measurement. Fresh midstream urine was collected from each subject in a 30 ml sterile universal specimen tube (2.6.1). The PD20 lumitester was used to quantify

ATP concentration immediately after collection (2.11.1). Whilst the collected volumes varied, the minimum volume for study inclusion was 10 ml, in order to allow complete LuciPac Pen swab immersion prior to luminometric analysis.

After the first measurement was taken, the LuciPac Pen was removed from the lumitester but not discarded. The LuciPac Pen was replaced in the lumitester and the measurement retaken 30 seconds after the initial measurement was made. This process was repeated at 30-second intervals for a total of five minutes.

3.7.3 Storage and preservation of urinary IL-6 and ATP

The influence of storage and chemical preservation on urinary IL-6 and ATP concentrations were assessed by serial measurement in preserved and unpreserved urine samples. A minimum of 20 ml of midstream urine was collected from each subject in a 30 ml sterile universal specimen tube (2.6.1). Ten millilitres was stored in a plain 15 ml sterile universal specimen tube without preservative. The remaining 10ml sample was added to a BD Vacutainer® urine preservative tube (Becton Dickinson, Oxford, UK), containing a proprietary preservative compound (boric acid, formic acid, and sodium borate). The plain and preserved tubes were then gently inverted three times and ATP was quantified in each tube using the PD20 lumitester and LuciPac Pen (2.11.1). Two 0.5 ml aliquots of urine were then decanted from each tube using a sterile Pasteur pipette, transferred into individual 1.5 ml freezing vials, and stored at -80°C to allow later IL-6 measurement (2.10.1). This process was repeated at 12, 24, 48, and 168 hours. Both the preserved and unpreserved urine were stored at room temperature (≈20°C).

3.7.4 Frozen storage and recovery of IL-6 and ATP from urine

The influence of frozen storage temperature on urinary IL-6 and ATP recovery was assessed in paired samples frozen at -20°C and -80°C. A minimum of 20 ml of midstream urine was collected from each subject in a 30 ml sterile universal

specimen tube (2.6.1). Each sample was then divided as follows: (1) Two 5 ml aliquots were decanted into plain 10 ml sterile universal specimen tubes without preservative to allow later ATP measurement; (2) Two 0.5 ml aliquots of urine were transferred, using a sterile Pasteur pipette, into individual 1.5 ml freezing vials for later IL-6 quantification. The two specimen tubes prepared in (1) were stored at either -20°C or -80°C. Similarly, the freezing vials prepared in (2) were stored at either -20°C or -80°C. The samples were frozen for four weeks prior to ATP and IL-6 measurement. Frozen samples were defrosted fully in an ice bath before ATP and IL-6 quantification was undertaken (2.10.1;2.11.1).

3.7.5 Centrifugation effects of IL-6 and ATP recovery from urine

The influence of centrifugation on urinary IL-6 and ATP recovery was assessed in paired urine samples. A minimum of 20 ml of midstream urine was collected from each subject in a 30 ml sterile universal specimen tube (2.6.1). This urine was then divided into two 10 ml samples and decanted into plain 10 ml sterile universal specimen tubes. One tube was centrifuged prior to analysis, whilst the other was analysed without centrifugation.

The sample for analysis without centrifugation was processed as follows: (1) ATP was immediately quantified using the PD20 lumitester and LuciPac Pen (2.11.1); (2) Two 0.5 ml aliquots of urine were transferred, using a sterile Pasteur pipette, into individual 1.5 ml freezing vials for later IL-6 quantification (2.10.1).

The sample for analysis after centrifugation was spun in a Denley BR401 centrifuge (R_{MAX}=140mm) at 2000 revolutions per minute (RPM) producing a relative centrifugal force (RCF) of 100 *g*. This protocol has been shown to optimise cell sedimentation in urine whilst minimising cell loss overall (345). Following centrifugation, the tube was removed without disturbing any sediment and processed as follows: (1) Five millilitres of urinary supernatant were carefully transferred into a plain 10 ml sterile universal specimen tube using a graduated pipette and ATP quantified immediately

using luminometry (2.11.1); (2) Two 0.5 ml aliquots of urine were transferred, using a sterile Pasteur pipette, into individual 1.5 ml freezing vials for later IL-6 quantification (2.10.1).

3.7.6 Serum-urine IL-6 levels in patients with MS

The relationship between serum and urine IL-6 in patients with MS and LUTS was explored using paired samples collected from patients during an RCT. Serum samples were collected using a standard aseptic non-touch technique® as previously described (2.7). Four 0.5 ml aliquots of cell-free serum were transferred, using a sterile Pasteur pipette, into individual freezing vials for later analysis. Immediately after serum collection, patients provided a MSU sample for analysis. A minimum of 10 ml of fresh midstream urine was collected from each subject in a 30 ml sterile universal specimen tube (2.6.1). Four 0.5 ml aliquots of freshly voided urine were transferred, using a sterile Pasteur pipette, into individual 1.5 ml freezing vials for later IL-6 quantification. These tubes were immediately frozen at the point of collection on dry ice.

3.8 Data management

3.8.1 Data protection

The storage and protection of study-specific data was in accordance with GCP guidance for data management in clinical research (2.3.1). All study documents were kept in a locked cabinet in the Department of Medicine, UCL Archway Campus and the Royal Free Hospital, London.

3.8.2 Data monitoring

The studies that were conducted at the Department of Medicine, UCL Archway Campus, were subject to regular internal audit. The CRFs, source data records, and

study documents, were monitored in accordance with the Sponsor's standard operating procedure (SOP) for data monitoring in clinical studies. Data monitoring was conducted by designated research nurses, under the supervision of Mrs Elizabeth Denver, the Senior Clinical Research Nurse in the department. Studies at the Royal Free Hospital, London, were subject to independent external monitoring and audit conducted by PSR Group (Hoofdorp, Netherlands) (7.12)

3.9 Statistical methods and analysis

3.9.1 Sample size calculation

No existing data were available to permit sample size calculations for any of the analyses in this work.

3.9.2 Statistical methods

The results were reported and summarised using standard descriptive statistics. The data were assessed for normality using graphical methods, employing visual assessment of frequency distributions and Q-Q plots. Data that were not normally distributed were subject to transformation, allowing the use of parametric analysis methods if the transformation achieved a normal distribution. There were no missing data points.

Repeated measures and factorial repeated-measures analysis of variance (ANOVA) were employed to explore the influence of storage and processing on variables across time. The assumption of sphericity in ANOVA was assessed using Mauchly's test, and degrees of freedom were corrected using appropriate estimates of sphericity. Bland-Altman analysis was used to assess agreement between the results of different urinary processing and storage techniques (346). Linear regression analysis was used to assess the relationship between serum and urine cytokine levels.

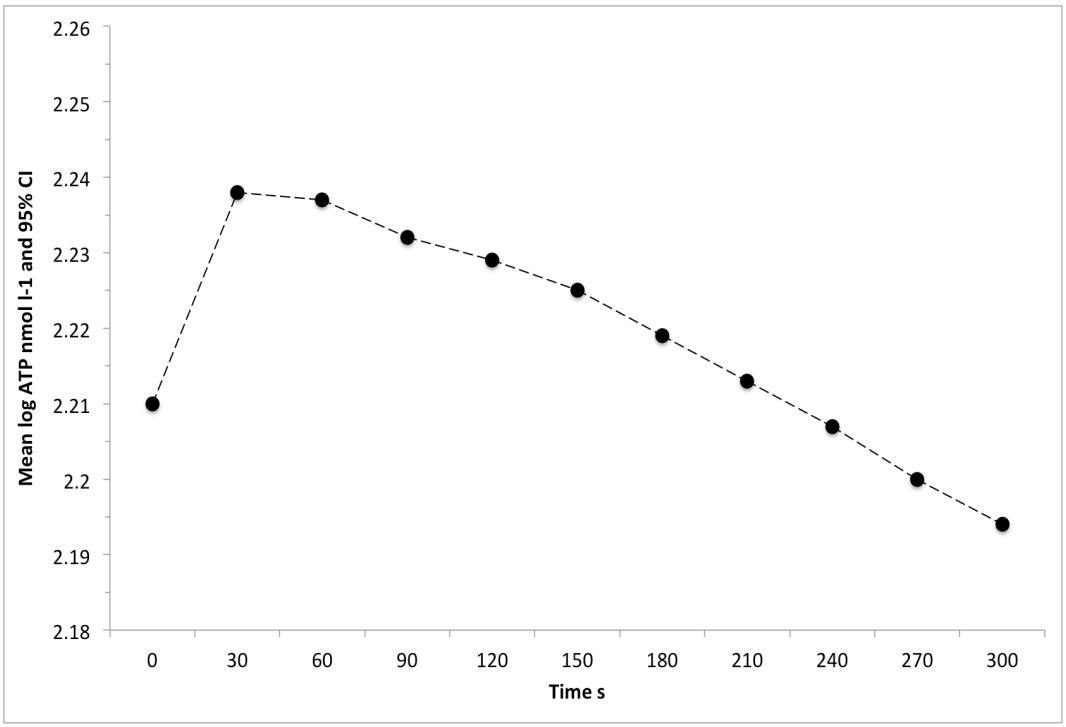
3.10 Results

3.10.1 Luminometry is best conducted after 30-60 seconds incubation

Fifty patients provided MSU samples for analysis. A factorial repeated-measures ANOVA was conducted to assess the effects of incubation time on urinary ATP measurements using the PD20 luminometer.

Mauchly's test indicated that the assumption of sphericity had been violated for the effect of incubation time on measured ATP (X^2=1760.20; p<0.001). The degrees of freedom were corrected with the Greenhouse-Geisser estimate of sphericity (ε=0.173). Adenosine triphosphate recovery was significantly influenced by duration of incubation (F=26.136; df=1.73; p<0.001). Peak bioluminescence was achieved after approximately 30-60 seconds of incubation prior to measurement using the PD20 luminometer (**Figure 2**).

Figure 2 *The influence of incubation duration and ATP recovery using the PD20 luminometer.*



3.10.2 Urinary preservation may influence ATP recovery from urine

Twenty patients provided MSU samples for analysis. A factorial repeated-measures ANOVA was conducted to assess the effects of storage duration and chemical preservation on the proportion of urinary IL-6 and ATP recovered.

Mauchly's test indicated that the assumption of sphericity had been violated for the main effect of storage duration on the recovery of IL-6 (X^2=44.95; p<0.001) and the interaction between storage duration and the use of a preservative (X^2=51.33; p<0.001). The degrees of freedom were corrected with the Greenhouse-Geisser estimate of sphericity (ε=0.58 for storage duration; ε=0.39 for the interaction between storage duration and the use of a preservative). The mean proportion of IL-6 recovered from urine was not influenced by the duration of storage (F=1.42; df=2.32; p=0.23) or the addition of a preservative (F=0.09; df=1; p=0.77). There was no significant interaction between duration of storage and whether a preservative was used (F=0.63; df=1.56; p=0.50).

When the dataset for ATP was analysed, Mauchly's test indicated that the assumption of sphericity had been violated for the main effect of storage duration on the recovery of ATP (X^2=204.69; p<0.001) and the interaction between storage duration and the use of a preservative (X^2=213.55; p<0.001). The degrees of freedom were corrected with the Greenhouse-Geisser estimate of sphericity (ε=0.26 for storage duration; ε=0.26 for the interaction between storage duration and the use of a preservative). The mean proportion of ATP recovered from urine was not influenced by the duration of storage (F=0.97; df=1.04; p=0.34) or the addition of a preservative (F=1.43; df=1; p=0.25). There was no significant interaction between duration of storage and whether a preservative was used (F=1.38; df=1.05; p=0.26).

Mean recovery of IL-6 (**Figure 3** and **Figure 4**) and ATP (**Figure 5** and **Figure 6**) is presented graphically below. The most striking feature of these data is the effect of preservation on the 95% confidence interval (CI) of the mean proportion of ATP recovered, which is a function of the standard deviation of the study sample. Urine preservation appears to confer significant effects on the variability of this measure in comparison to urine stored without a preservative agent. These effects are less pronounced with respect to the proportionate recovery of IL-6.

Figure 3 *Mean proportion of IL-6 recovered from urine stored at room temperature for up to seven days.*

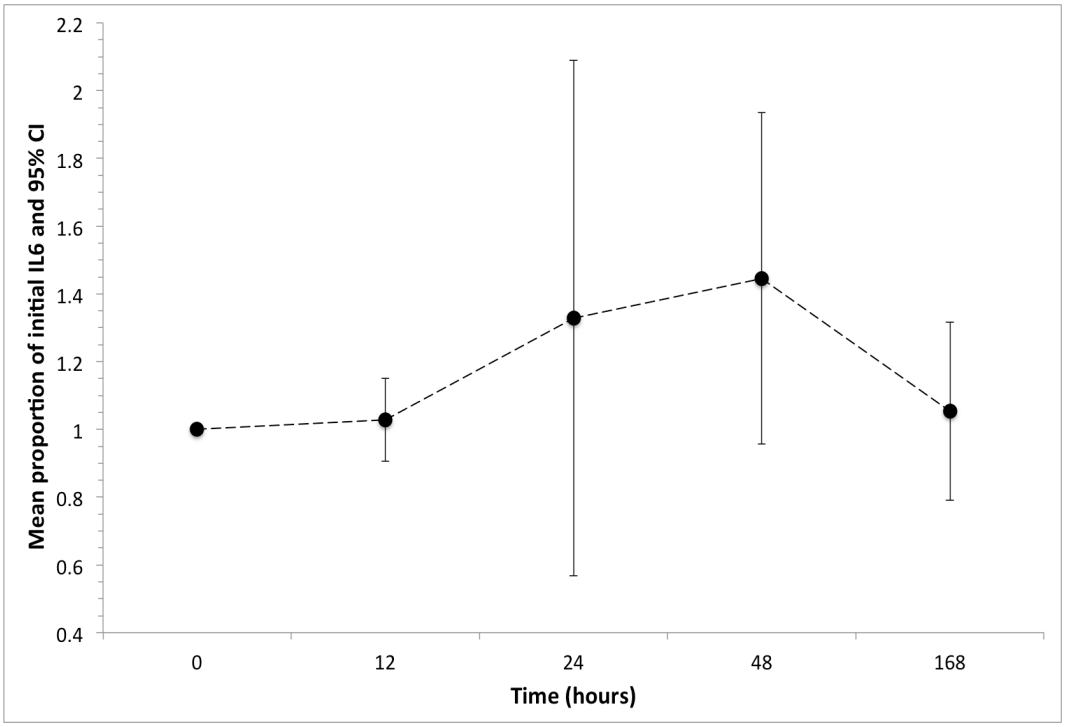


Figure 4 *Mean proportion of IL-6 recovered from preserved urine stored at room temperature for up to seven days.*

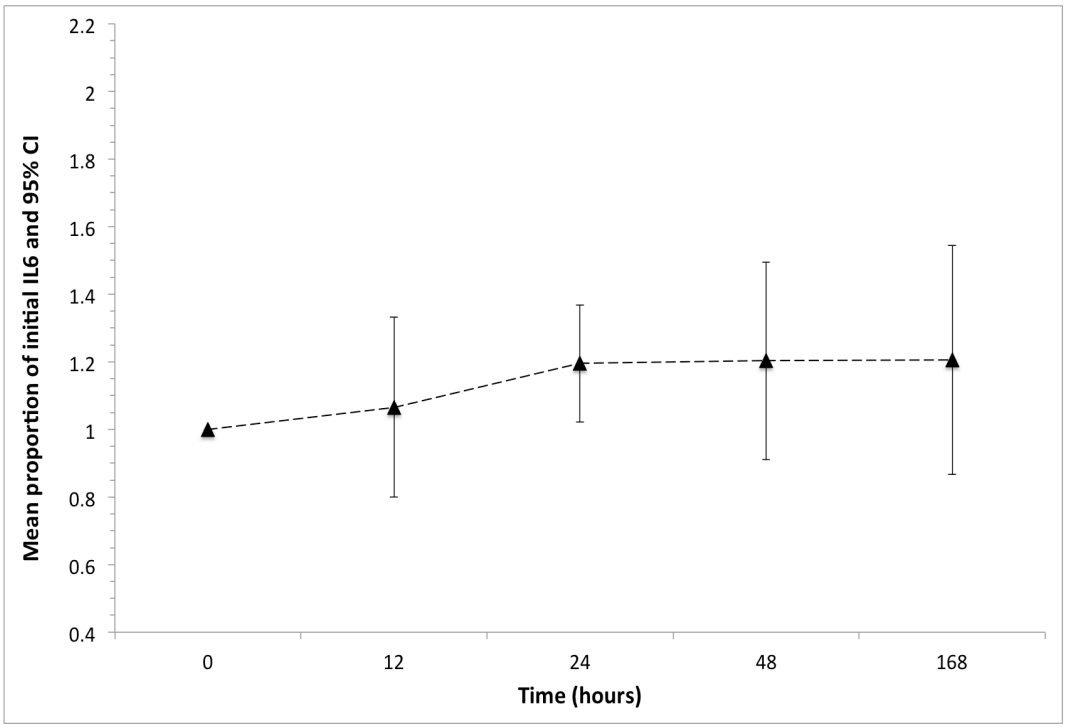


Figure 5 *Mean proportion of ATP recovered from unpreserved urine stored at room temperature for up to seven days.*

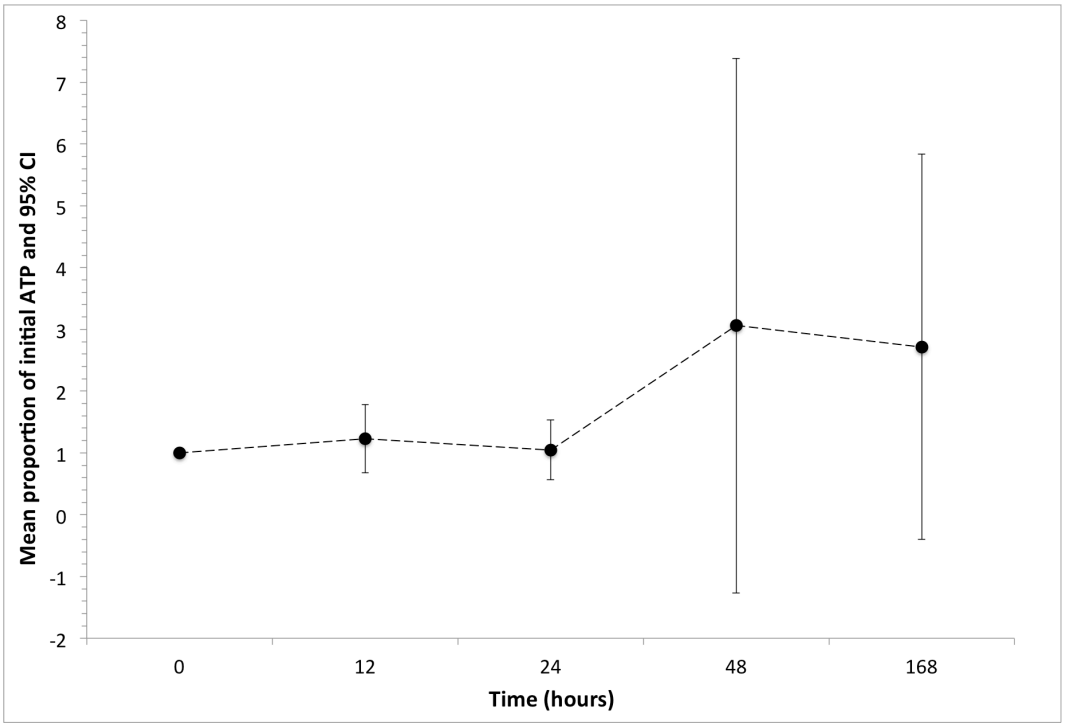
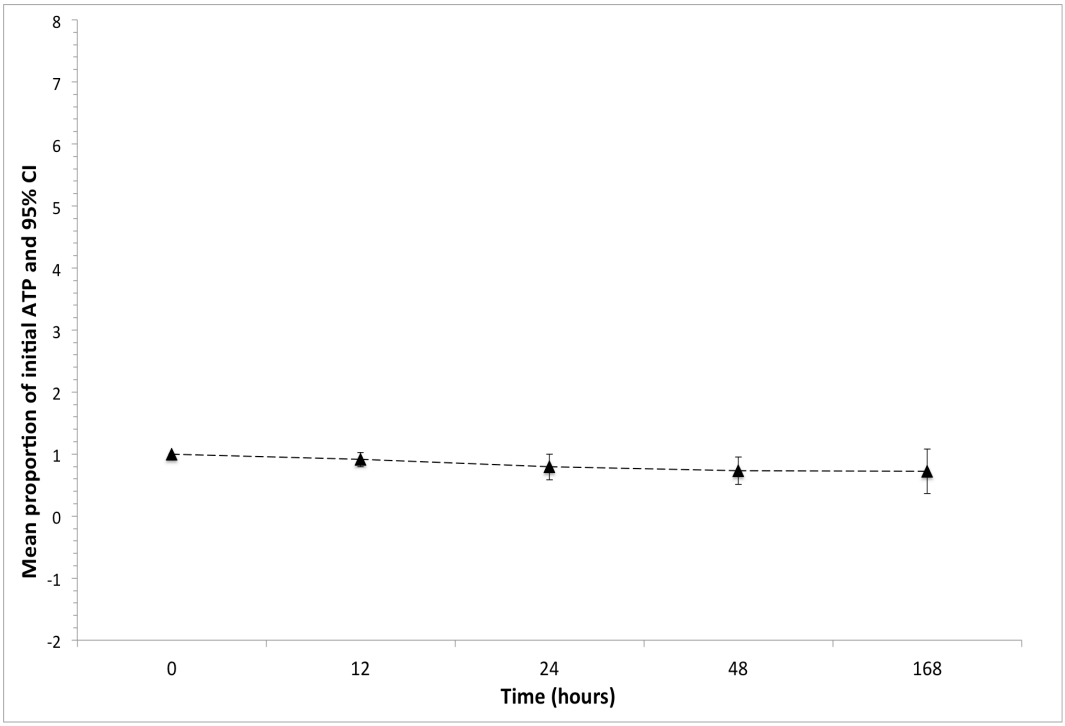


Figure 6 *Mean proportion of ATP recovered from preserved urine stored at room temperature for up to seven days.*



3.10.3 Frozen storage and recovery of IL-6 and ATP from urine

Forty patients provided MSU samples for analysis. A Bland-Altman analysis was conducted to assess agreement in urinary IL-6 and ATP recovery following storage at -20°C and -80°C.

The Bland-Altman plots for IL-6 and ATP are presented in **Figure 7** and **Figure 8**. The central reference line represents the mean difference in measurements, with the upper and lower lines indicating the 95% limits of agreement. Provided differences as large as those described by these limits of agreement would not be clinically important, the different storage conditions could be used interchangeably without affecting the recovery of either molecule. In both analyses, differences in the concentration of IL-6 and ATP as large as those described by the 95% limits of agreement would not have a significant influence on the interpretation of study data.

3.10.4 Centrifugation effects on IL-6 and ATP recovery from urine

Forty patients provided MSU samples for analysis. A Bland-Altman analysis was conducted to assess agreement in urinary IL-6 and ATP recovery in spun and unspun urine samples.

The Bland-Altman plots for IL-6 and ATP are presented in **Figure 9** and **Figure 10**. In both analyses, differences in the recovered IL-6 and ATP as large as those described by the 95% limits of agreement would not have a clinically meaningful impact on the data.

Figure 7 *Bland-Altman plot demonstrating satisfactory agreement between ATP recovery from frozen urine whether stored -20℃ and -80℃.*

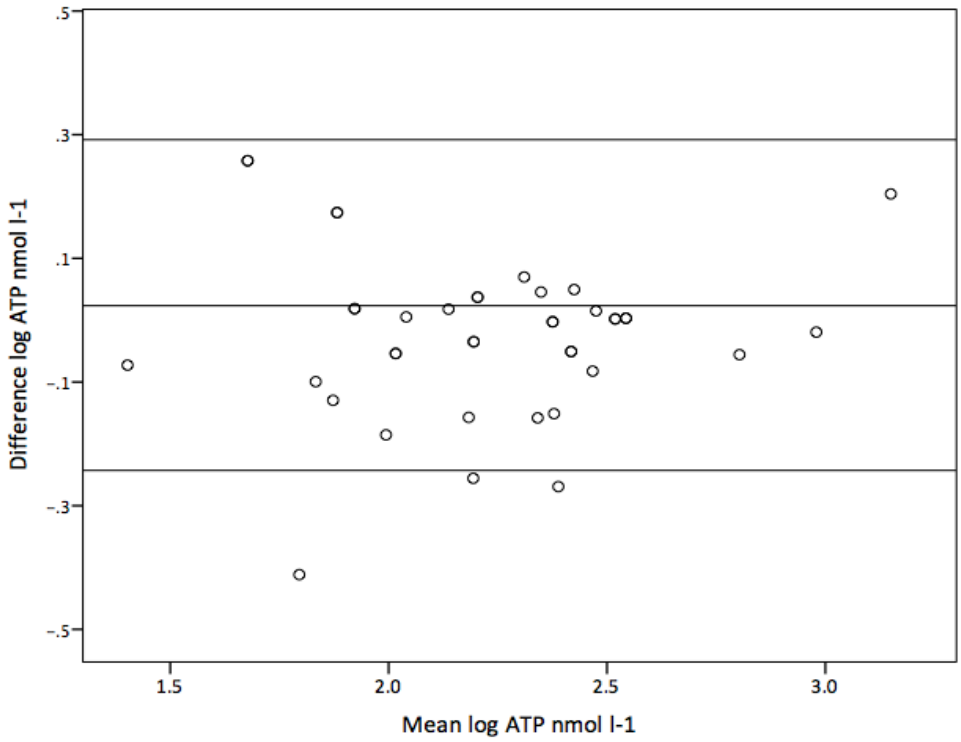


Figure 8 *Bland-Altman plot demonstrating satisfactory agreement between IL-6 recovery from frozen urine whether stored -20℃ and -80℃.*

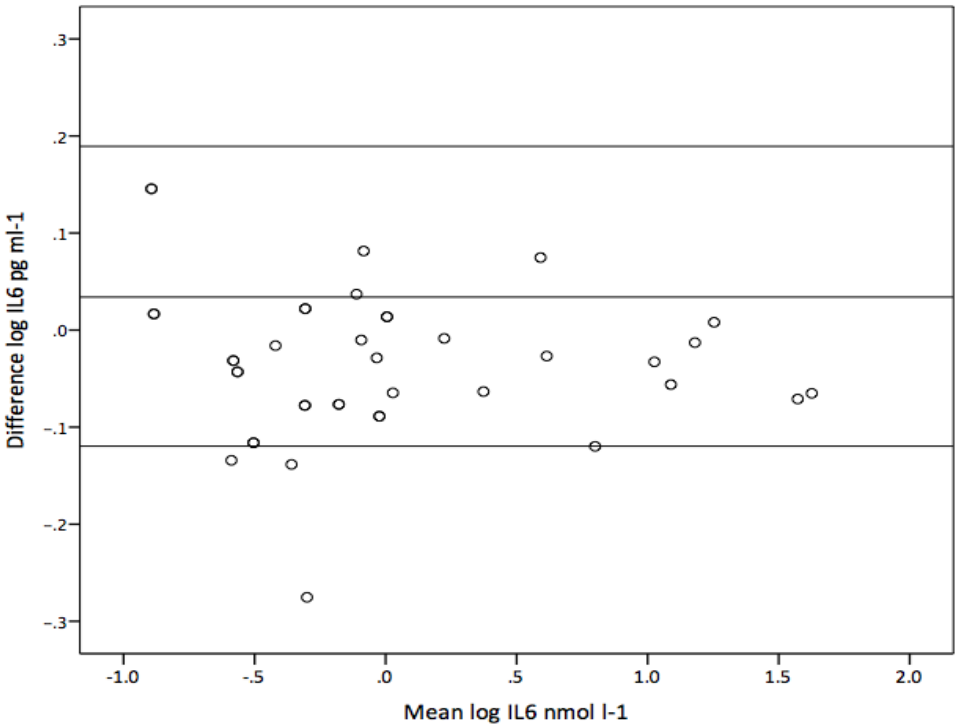


Figure 9 *Bland-Altman plot demonstrating satisfactory agreement between IL-6 recovery from fresh urine whether spun or unspun.*

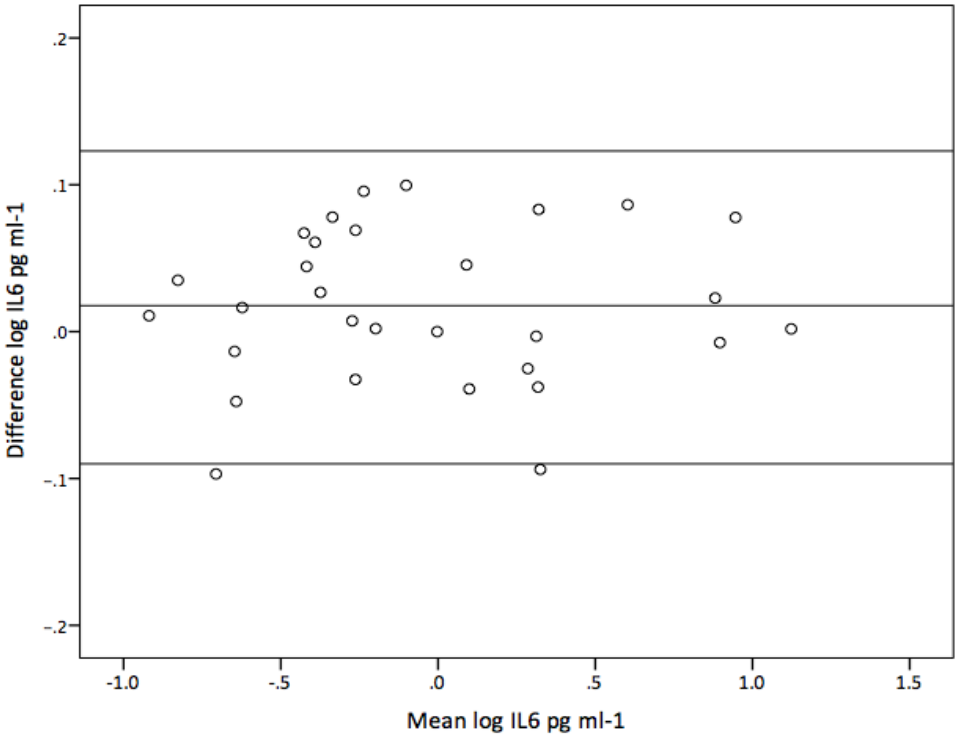
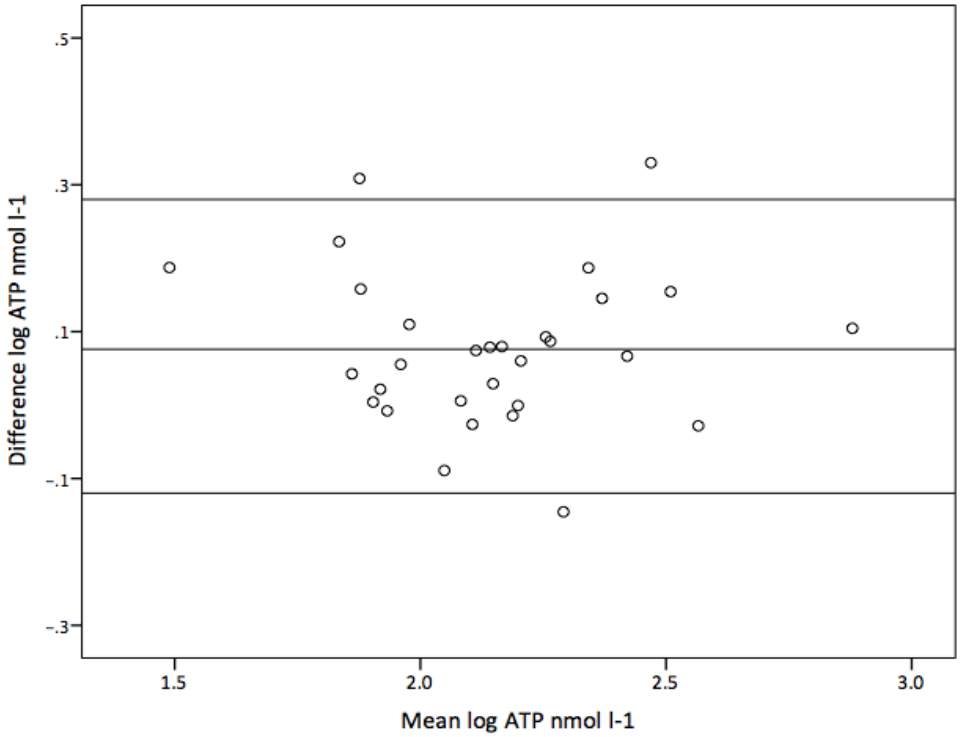


Figure 10 *Bland-Altman plot demonstrating satisfactory agreement between ATP recovery from fresh urine whether spun or unspun.*

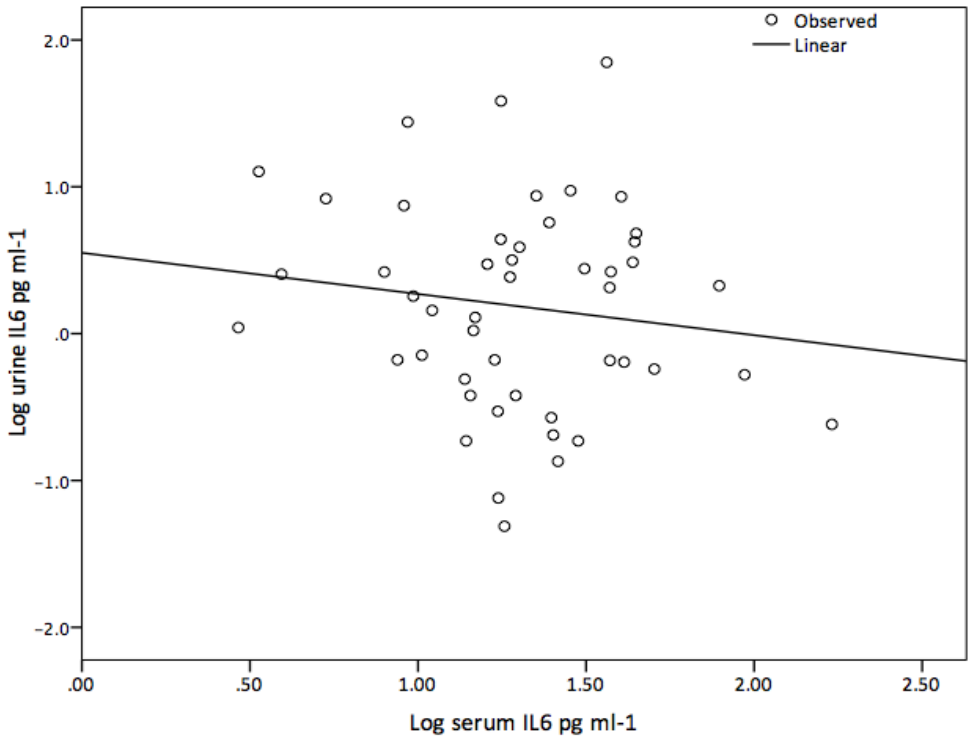


3.10.5 Serum IL-6 levels do not influence urinary measurements

Fifty paired urine serum and urine samples were submitted for analysis. Linear regression was used to explore the relationship between serum and urine IL-6 measurements.

A linear regression model was fitted to the log serum IL-6 data, with log urinary IL-6 designated as the dependent variable. Changes in serum IL-6 levels had no effect on urinary levels. In the regression model, serum IL-6 levels accounted for only 2% of the variation in measured urinary Il-6 (R^2=0.02; *F*=0.93; *df*=1; *p*=0.34). The relationship is demonstrated graphically in **Figure 11**.

Figure 11 *Regression plot demonstrating the relationship between serum and urinary IL-6.*



3.11 Discussion

Existing data suggest that the PD20 lumitester and LuciPac Pen is a suitable device for the measurement of ATP in human urine. It demonstrates linear sensitivity to ATP across a range of values and human urinary ATP levels fall well within these limits. The LuciPac Pen contains pyruvate orthophosphate kinase which regenerates ATP from its hydrolysis products and ameliorates potential signal loss after sampling. Maximal ATP recovery from human urine is achieved after the LuciPac Pen is incubated for only 30-60 seconds after sampling. Bioluminescence measurements are then processed by the luminometer in only 10 seconds. The simplicity of the PD20 luminometer and the speed at which measurements can be made make the unit ideal for use in biomedical research.

Statistical analysis demonstrates that neither storage nor preservation have any significant effect on the recovery of IL-6 and ATP. Nonetheless, visual scrutiny of the results demonstrates that preservation does influence the dispersion of these data, particularly with respect to the recovery of ATP. The conclusion that preservation does not have any effects on ATP recovery is driven by the wide confidence intervals generated for ATP recovery in unpreserved samples from 48 hours onwards. The mean estimates of ATP recovery are all contained within these limits and statistical analysis demonstrates that preservation has no significant effect.

When both ATP decay plots are compared, a difference is observed. In the unpreserved samples, proportionate ATP recovery rises from 48 hours onwards and this is associated with significant widening of the confidence intervals. This is a consequence of greater dispersion of the data around the mean estimates for ATP recovery. By contrast, the decay plot for the preserved samples demonstrates very little variation in mean ATP recovery and the confidence intervals are extremely narrow. Such effects are much less pronounced when the decay plots for IL-6 recovery are scrutinised. The effect of preservation on the variability of ATP in stored urine could be mediated through a number of mechanisms.

Bacterial growth is impeded by chemical preservation and boric acid is known to exert bacteriostatic effects in urine (347-352). Whilst lytic production of ATP was long thought to be the main mechanism by which ATP is released by bacteria, recent evidence has challenged this view (323, 324). In this context, the inhibition of bacterial growth by the addition of a preservative would be expected to arrest non-lytic ATP production.

The marked cellularity of the samples could have influenced ATP recovery by other means. Whilst IL-6 is generated as a result of gene expression in response to infection or trauma, ATP levels are consistently high in the intracellular compartment. As a consequence, cell damage or degradation is known to liberate significant quantities of ATP that act as a danger signal. By contrast, the disruption of cell membrane integrity would not be expected to have a comparable effect on the production of IL-6 as its secretion is tightly controlled by transcription. It is possible that cytoplasmic levels of IL-6 could be elevated if production of the cytokine had been initiated and IL-6 was being processed ready for release.

If preservation were able to restrain cell lysis then the effects of continued lytic production of ATP would be attenuated. The precise mechanism by which boric acid confers cell preservation remains unclear but could include buffering and osmotic effects. Plasma membranes are sensitive to excursions in acid-base status and boric acid is reported to provide favourable osmotic conditions for animal cells at concentrations recommended for preservation (350). The effects of cell lysis on the generation of lytic ATP and other cytokines could be explored by repeating the experiments in cell-deficient samples produced by cytocentrifugation.

The effects of chemical preservation on the stability of ATP in solution may have influenced ATP recovery. Whilst ATP is stable in buffered solution, it is sensitive to changes in pH, and undergoes accelerated hydrolysis in particularly acidic or alkaline environments (353). Whilst sample pH was not recorded during the study, differences in urinary pH may have influenced the breakdown of ATP. The buffering capacity of boric acid could be important in retarding this process.

In vivo, ecto-nucleotidases that are ubiquitously expressed in the plasma membrane rapidly break down extracellular ATP. How active these enzymes are in experimental conditions such as those described here remains unknown. Enzymic activity might also be influenced by environmental conditions such as pH and the function of these enzymes in urine could be affected by local factors.

Whilst enhanced purinergic transmission has been demonstrated in urothelial cell biopsies from patients with lower urinary tract disorders, bacteria and innate immune cells may also contribute to ATP expression in the urine. Purine recovery is a measure of biomass and not solely an indicator of urothelial distress. In this context, elevated urinary ATP levels could indicate urothelial distress, immune activation or bacterial colonisation of the lower urinary tract.

The temperature at which urine samples are frozen does not appear to confer clinically important differences in IL-6 and ATP salvage (when stored for up to four weeks prior to analysis and tested immediately after defrosting). The influence of long-term urine storage at -20°C and -80°C on cytokine recovery remains unclear. Some data examining the stability of IL-6 in frozen human serum at -80°C demonstrates little decay over the first two years, although no comparable data exist for urinary cytokines (14).

The use of cytocentrifugation had no significant effect on IL-6 and ATP recovery when urine samples were immediately tested. Cytocentrifugation can be safely omitted in order to reduce processing time. One study demonstrated that the physiological stress of centrifugation is able to up-regulate the expression of cytokine mRNA in human epithelial lung carcinoma cells (354), although these findings have not been corroborated in urine samples.

The implications of these results are of interest to the researcher. These data suggest that the storage of urine samples for later analysis in a standard -20°C freezer unit appears to be acceptable for at least four weeks. This might allow study subjects to store urine samples collected at home for subsequent analysis, reducing

reliance on visits to central laboratories or study sites. For patients with disabilities, or those living remote to study centres, self-storage of samples with appropriate temperature monitoring could allow the participation of subjects who might be otherwise unable to satisfy the visit requirements of more demanding protocols.

Published data have not demonstrated increased serum levels of IL-6 in patients with MS (338-343). Nonetheless, the possibility of elevated systemic levels of IL-6 influencing local concentrations in the lower urinary tract could be a significant source of bias. The comparative data exploring the concentration of IL-6 in paired serum and urine samples demonstrated no correlation between these measures. The strength of these data illustrate that local production of this cytokine accounts for almost all of the IL-6 recovered in sampled urine. These results support the use of urinary IL-6 as an appropriate measure of lower urinary tract inflammation in patients with MS and LUTS.

4 The performance of pyuria as a surrogate marker of infection in patients with chronic lower urinary tract symptoms

4.1 Background

Hottinger (1893) was the first to describe microscopic methods to quantify urinary cellular content (355), although Addis (1926) is often credited with this discovery (356). Following the publication of his methods, the technique of enumerating the cellular components of urine became eponymously named the 'Addis count'. Addis sampled urine from 74 overnight collections taken from male medical students, and following centrifugation to concentrate the sediment, employed microscopic examination to count the cells. Total cell numbers in each collection were calculated by extrapolation from the counts of 10ml samples that were subject to centrifugation and assessment. Addis enumerated urinary casts and erythrocytes, but did not differentiate between leucocytes and epithelial cells in his original report.

Dukes (1928) is credited with the development of modern cytometric urinalysis techniques and was the first to propose 'normal' limits for urinary leucocyte expression in healthy adults (197). Dukes assessed urine immediately after collection, and used a haemocytometer to quantify leucocyte numbers in 300 MSU samples from asymptomatic subjects. He rejected the analysis of urine collections and the use of centrifugation on the grounds that cell loss and deformation was likely to have significant and unpredictable effects (357). He proposed a threshold of <10 wbc μl^{-1} as the upper limit of normal pyuria excretion, based on mean estimates of leucocyte expression of 1.6 wbc μl^{-1} in males, and 5.4 wbc μl^{-1} in females.

Dukes' attempt to define a threshold for normal leucocyte excretion was not subject to any statistical analysis. He also reported significant dispersion around these mean estimates, with the range of counts cited as 0-50 wbc μl^{-1}. In this context, the distribution of cell counts must have been positively skewed, rather than normally distributed. The use of the mean as a measure of central tendency would be expected to generate inflated estimates for average counts.

Despite Dukes' criticism of centrifugation and the effects of urinary storage, Addis' methods or a modification were used by the majority of investigators for three decades (358-365). It was not the advent of quantitative microbiological diagnosis that interest in Dukes' methods enjoyed a resurgence. The enthusiasm for culture-based diagnosis prompted validation studies to examine the performance of pyuria as a surrogate marker of infection against positive reference cultures employing a $\geq 10^5$ cfu ml^{-1} threshold (186).

Stansfeld (1961) examined the urine of a large series of unselected paediatric patients. He demonstrated that 95% of patients with a positive culture at $\geq 10^5$ cfu ml^{-1} threshold demonstrated ≥10 wbc μl^{-1} (198). Mond (1965) looked at symptomatic and asymptomatic adults, and for those without symptoms of cystitis, pyuria <10 wbc μl^{-1} correctly identified 95% of those with a negative culture (199). In patients symptomatic of cystitis who had a positive culture employing a threshold of $\geq 10^5$ cfu ml^{-1}, all patients had ≥10 wbc μl^{-1} in the urine (199). The studies used MSU sampling methods used in ordinary clinical practice and seemingly corroborated microscopic pyuria ≥10 wbc μl^{-1} as a reliable surrogate of 'culture-proven' infection.

Whilst these findings appear to support Dukes' original conclusion that healthy adults should express pyuria <10 wbc μl^{-1}, the emergence of contemporary data exploring the microbiology of cystitis casts doubt on these findings. Critically, recent work has clearly demonstrated that symptomatic cystitis can be associated with much lower bacterial counts than those originally suggested by Kass (187-192).

Mond also reported the microscopic analysis of samples from acutely symptomatic adult patients whose cultures were reported as negative using a $\geq 10^5$ cfu ml^{-1} to define infection (199). In this group, classified as free of infection by standard microbiological methods, pyuria expression ≥10 wbc μl^{-1} was found in half of the symptomatic patients. Thus, a marked inflammatory signal was present in the urine of almost 50% of patients with acute symptoms. The authors concluded that these women were affected by a non-infective urethritis, leading to pyuria in the absence

of bacterial cystitis. This was despite all subjects providing a carefully collected MSU, using a clean-catch method and local antiseptic cleansing prior to urine collection.

These findings cast doubt on the veracity of Kass' original culture threshold long before it was examined again in more recent work (187-192). The apparent paradox of significant pyuria in patients with 'negative' bacterial culture was noted by investigators at the time, and the influence of a strict culture threshold on the findings was considered (198, 199). Nonetheless, the integrity of the $\geq 10^5$ cfu ml^{-1} threshold was not challenged. Such was the confidence in Kass' diagnostic criteria that significant pyuria in patients with 'negative' bacterial culture was even attributed to psychological disturbance (201).

The urinary dipstick was originally conceived to provide rapid diagnosis of infection in symptomatic patients, reducing reliance on microscopy and culture. The dipstick relies on the detection of leucocyte esterase (an enzymic leucocyte product) and urinary nitrite (a nitrate reduction product of some uropathogenic bacteria) to identify UTI. Leucocyte esterase is a measure of urothelial inflammation, and the enzyme is liberated from granulocytes including neutrophils, basophils and eosinophils, by secretion or lytic release. Conversely, nitrite is produced in the presence of urea-splitting bacteria and is a bacterial product. Thus, leucocyte esterase confers information about the host response to infection whilst nitrite is an indirect measure of bacterial colonisation.

Leucocyte esterase catalyses the breakdown of indoxylcarbonic acid ester to produce indoxyl. Indoxyl then reacts with a diazonium salt to produce a purple colour. The nitrite test is based on the reaction of urinary nitrite with sulphanilamide to form a coloured diazonium salt which then reacts with hydroxybenzoquinolone to produce a characteristic pink colour (366). Whilst *E. coli* and other *Enterobacteriaceae* are able to reduce nitrate to nitrite, gram-positive bacteria including *S. saprophyticus*, Group B Streptococci, and Enterococci do not have this capability.

Published data relating to the performance of microscopic analysis and dipstick testing in the diagnosis of UTI demonstrate significant variation. Much of the available evidence in adults fails to define the population under scrutiny, particularly with respect to the presenting symptoms, and employs different microbiological culture standards. This makes comparison difficult and such factors can mediate significant effects on test performance.

The sensitivity of a diagnostic test is defined as the proportion of individuals with a condition that are correctly identified by the test. Conversely, specificity relates to the proportion of individuals without the condition who are correctly identified. The sensitivity and specificity of a given diagnostic test are widely perceived to be fixed. The positive predictive value (PPV) describes the proportion of those with a positive test who have the disease, and the negative predictive value (NPV) reports the proportion of those with a negative test who do not have the disease. The PPV and NPV are modified by the underlying disease prevalence in the population under study. It is recognised that as the prevalence of the disease increases there will be a commensurate increase in the PPV and a fall in the NPV. The opposite would be observed if the disease prevalence fell. In the context of these statistical considerations, the characteristics of the sample have a significant impact on how a test performs.

A study sample comprising subjects with acute urinary frequency and dysuria would be expected to have a higher background prevalence of UTI than a group of asymptomatic individuals. Thus, the PPV of a diagnostic test would be greater in the group of symptomatic individuals when compared to subjects without symptoms. For this reason, a lack of clarity relating to the presenting symptoms in published research makes direct comparison of data difficult.

Whilst widely believed to be independent of sample characteristics, sensitivity and specificity can also vary as a result of sample selection (183, 367, 368). Subjects with more serious manifestations of the illness under investigation would be more likely to test positive than those individuals with less severe illness. Furthermore, changes

in sensitivity and specificity can occur even if the prevalence of the disease, defined by the reference standard, remains static (369). The influence of sample characteristics on diagnostic test performance is known as spectrum bias (183) and its influence in diagnostic studies assessing the performance of urinalysis has been specifically highlighted (370).

The impact of sample heterogeneity in much of the published work relating to surrogate markers of UTI has been overlooked. The influence of spectrum bias and potentially erroneous microbiological reference standards confounds this problem. Few studies have attempted to determine the accuracy of surrogate measures of UTI in patients with chronic LUTS. Two series including just over 200 women reported the performance of dipstick testing in women presenting with urinary incontinence. Employing reference cultures with diagnostic thresholds of 10^3-10^4 cfu ml^{-1}, sensitivity estimates of leucocyte esterase and/or nitrite in the detection of UTI were 29% and 35% respectively (209, 210). Another study examined the performance of dipstick testing in women with OAB symptoms who provided 633 positive MSU cultures using a $\geq 10^3$ cfu ml^{-1} threshold (211). One-third of patients demonstrated a positive culture and the sensitivity of dipstick testing was just 44%. All of these studies used CSU sampling.

The treatment of acute cystitis with antibacterial agents is firmly entrenched in clinical practice. Metanalysis of the available RCTs has demonstrated that compared to placebo, antibiotics provide superior symptom control and microbiological eradication, protect against recurrence and reduce the risk of upper tract infection (Falagas et al., 2009). Whilst a causal relationship has been forged between bacterial infection and the generation of acute symptoms commonly associated UTI the same cannot be said for chronic LUTS. The impact of antibiotic treatment in patients with chronic LUTS has never been explored, although the importance of excluding infection is cited universally in national and international guidance concerned with the management of LUTS (371-373). This includes the assessment of MS patients who present with urinary symptoms (104, 374).

The use of dipsticks in the diagnosis of UTI in MS patients with LUTS is grounded in data generated from patients with non-neurogenic urinary symptoms and expert opinion (104, 374). The UK consensus document on bladder management in MS makes its recommendations based on the results of one study in patients with non-neurogenic LUTS (375). The National Institute for Health and Care Excellence (NICE) advocates the use of dipstick testing in its guidance on urinary incontinence associated with neurological disease but this recommendation is based on opinion only. No research evidence is cited in its support (374).

The performance of microscopic pyuria and dipstick testing in patients with LUTS remains unclear. Existing data are few and employ catheter sampling of urine and bacterial culture thresholds rarely used in the UK (209-211). The results demonstrate that surrogate diagnostic measures perform poorly in patients with LUTS but the study methodologies used preclude the application of these data to routine practice in most clinical settings. There remains a need for the performance of these measures to be scrutinised when MSU sampling is employed and the microbiological criteria used to diagnose UTI reflect common clinical practice.

For patients with MS, a diagnostic accuracy study of surrogate markers of UTI would be best confined only to patients with MS and LUTS. Nonetheless, sample size calculations and the speed of patient enrolment encountered in other studies suggested that recruitment targets would be difficult to achieve. In this context, a controlled study of the diagnostic accuracy of microscopic pyuria and dipstick testing was undertaken in a large population of patients with non-neurogenic LUTS rather than MS patients.

4.2 Study overview

Adult patients presenting with one or more LUTS, and asymptomatic control subjects, provided clean-catch MSU samples for analysis. The urine was subject to microscopic pyuria quantification, dipstick urinalysis, and routine laboratory culture. The performance of microscopic pyuria and dipstick urinalysis as surrogate markers of UTI were reported using standard measures of diagnostic accuracy. Demographic data were stored in a secure clinical database. Urinary symptoms were recorded using validated questionnaires and a structured, electronic symptom matrix.

The study was conducted at the Department of Medicine, UCL Archway Campus. Ethical approval for this study was granted by the Whittington and Moorfields Research Ethics Committee (Ref: 07/H0704/74).

4.3 Study objectives

4.3.1 Primary objective

The primary objective of the study was to determine the following:

- The sensitivity/specificity and positive/negative predictive value of microscopic pyuria as surrogate marker of urinary infection defined by routine bacterial culture.

4.3.1.1 Secondary objectives

The secondary objectives of the study were to determine the following:

- The sensitivity/specificity and positive/negative predictive value of leucocyte esterase as surrogate marker of significant microscopic pyuria.

- The prevalence of positive, negative, and polymicrobial urine cultures amongst the study population.
- The magnitude of microscopic pyuria expression in samples with positive, negative, and polymicrobial urine cultures.

4.4 Study population

4.4.1 Recruitment of participants

Patients and control subjects were recruited as outlined previously (2.2.2).

4.4.2 Consent and eligibility

All participants provided written, informed consent prior to any study related procedures, and eligibility was checked before inclusion (2.2.3).

4.4.3 Inclusion and exclusion criteria

4.4.3.1 Patients with LUTS:

Inclusion and exclusion criteria are detailed in **Table 6**.

Table 6 *Inclusion and exclusion criteria for patients with LUTS.*

Inclusion criteria	
1	Adult patients presenting with one or more LUTS

Exclusion criteria	
1	Acute symptoms of upper or lower UTI, or new-onset LUTS (**Table 7**)
2	Patients who were pregnant or planning pregnancy
3	Patients using antibiotic therapy for any indication

Table 7 Symptomatic definitions of acute infective syndromes.

Exclusion criteria	Description
Symptoms of acute cystitis	Acute onset urinary frequency and/or dysuria
Symptoms of acute pyelonephritis	Acute onset loin or flank pain Fever or systemic upset
New-onset symptoms	LUTS of less than three months duration

4.4.3.2 Asymptomatic controls:

Inclusion and exclusion criteria are detailed in **Table 8**.

Table 8 *Inclusion and exclusion criteria for asymptomatic control subjects.*

Inclusion criteria	
1	Adults who did not describe urinary symptoms (***Table 9***)

Exclusion criteria	
1	Subjects who were pregnant or planning pregnancy
2	Subjects using antibiotic therapy for any indication

Table 9 *Symptom inclusion criteria for asymptomatic control subjects.*

Symptom complex	Description
Storage	No urinary urgency No perception of increased urinary frequency No urinary incontinence
Voiding	No voiding symptoms
Postmicturition	No post micturition symptoms
Pain	No pain attributed to the urinary tract

4.4.4 Study restrictions and concomitant medications

There were no additional restrictions for participants.

4.4.5 Discontinuation of subject participation

Patients could be withdrawn from the study at the discretion of the Chief Investigator. The criteria for subject withdrawal included: (1) non-compliance with the requirements of the protocol; (2) withdrawal on medical or administrative grounds. Patients could withdraw their consent to participate at any time without prejudice and it would not affect their medical care.

4.5 Study design

The study tested the diagnostic accuracy of surrogate markers of UTI against routine microbiological reference cultures. Compliance with the Standards for Reporting of Diagnostic Accuracy (STARD) checklist was ensured (376). Patients were recruited from a clinic specialising in the treatment of LUTS and asked to provide one or more MSU samples for analysis. Urine samples were collected when patients attended for clinic visits. Samples were assessed using microscopy and dipstick testing, and submitted for routine bacterial culture.

4.5.1 Duration of the study

The protocol allowed for the submission of multiple MSU samples from each enrolled patient. Patients who consented to study participation submitted samples at each clinic visit until they were discharged from the clinical service or the study closed. Patients were free to rescind their consent at any time and this would not affect their routine clinical care.

4.5.2 Blinding

All samples presented for analysis were identified only by a randomly generated, four-digit study number. Researchers responsible for urine microscopy were blinded to the results of the dipstick analyses.

4.6 Clinical and laboratory assessments

4.6.1 Clinical assessments

Patient identifiable data and demographics were recorded on an NHS database as previously described (2.3.1). Patient-reported urinary symptoms were recorded in a structured electronic matrix. The severity of urgency and pain symptoms was described using validated measures (2.14.2 and 2.14.3).

4.6.2 Laboratory assessments

Harry Horsley, Lisa Brackenridge and Sanchutha Sathiananthamoorthy provided assistance with urine microscopy and dipstick urinalysis.

4.6.2.1 Biological samples

All participants provided clean-catch MSU samples for analysis (2.6.1).

4.6.2.2 Laboratory analyses

The following analyses were conducted on all samples:

- *Microscopic pyuria count (2.9.1)*
- *Dipstick urinalysis (2.13)*

– *Routine laboratory culture (2.12.2)*

4.7 Data management

4.7.1 Data protection

The storage and protection of study-specific data was in accordance with GCP guidance for data management in clinical research (2.3.1). All study documents were kept in a locked cabinet in the Department of Medicine, UCL Archway Campus.

4.7.2 Data monitoring

The CRFs, source data records, and study documents, were monitored by regular internal audit operating in the Department of Medicine. This was in accordance with the Sponsor's standard operating procedure (SOP) for data monitoring in clinical studies. Data monitoring was conducted by designated research nurses, under the supervision of Mrs Elizabeth Denver, the Senior Clinical Research Nurse in the department.

4.8 Statistical methods and analysis

4.8.1 Sample size calculation

Data from existing studies has demonstrated that the standard deviation of the log leucocyte count is two (*sd*=2). A sample size of 400 in each group would yield 83% power to detect a clinically significant between-group difference of 0.5 at the 1% level (α=0.01), allowing for multiplicity.

4.8.2 Statistical methods

The performance of microscopic pyuria as a surrogate marker of UTI was determined by a comparison of microscopic leucocyte enumeration and routine laboratory urine culture. Positive microscopy was defined as the presence of ≥10 wbc μl^{-1} on microscopic assessment whilst the culture reference standard was the growth of $\geq 10^5$ cfu ml^{-1} of a single recognised uropathogen.

Samples that manifest low-level microscopic pyuria 1-9 wbc μl^{-1}, and cultures reported as 'mixed growth' were also reported. Pyuria 1-9 wbc μl^{-1} is currently dismissed as 'normal' and 'mixed growth' cultures are considered of 'doubtful clinically significance' (attributed to contamination, poor sampling, or failure to refrigerate the sample).

A separate analysis was conducted to determine the performance of dipstick urinalysis as a surrogate marker of microscopic pyuria. A positive dipstick was defined as ≥'trace' leucocyte esterase, which was compared against a microscopy reference standard of ≥10 wbc μl^{-1}.

The data were reported as counts and proportions where appropriate and summarised. Demographics and symptom scores were reported using standard descriptive statistics. Estimates of the sensitivity, specificity, and positive/negative predictive values of the tests were presented to illustrate diagnostic accuracy.

4.9 Results

Between October 2009 and November 2011, 1223 patients (F=1103; M=120; mean age=54; 95% CI=53-55) and 36 asymptomatic control subjects (F=28; M=8; mean age=41; 95% CI=36-46) provided 5081 MSU samples submitted for routine laboratory culture. After the exclusion of 706 samples, which were not subject to microscopic evaluation (*n*=257) or dipstick urinalysis (*n*=449), 4375 samples were included in the final analysis.

Patients demonstrated widespread urinary urgency (mean urgency score=3.38; *sd*=3.15) and incontinence symptoms (mean daily incontinence=0.93; *sd*=1.79) at presentation; mean 24hr urinary frequency was 9.35 (*sd*=5.00). Symptoms were longstanding (mean duration=4.6 years; *sd*=3.91). A full description of the symptoms reported by patients is reported in **Table 10**.

All MSU samples submitted by control subjects were negative for microscopic pyuria and routine laboratory culture (not tabulated). The diagnostic performance of microscopic pyuria in the detection of a positive routine laboratory culture of $\geq 10^5$ cfu ml^{-1} is presented in **Table 11**. Summary statistics are presented in **Table 12**. Microscopic pyuria did not perform well as a surrogate marker of UTI.

Almost identical proportions of positive, negative and mixed growth cultures demonstrated an inflammatory signal of ≥10 wbc μl^{-1}. This finding questions the assertion that polymicrobial cultures are the result of contamination and challenges the routine culture threshold of $\geq 10^5$ cfu ml^{-1}. Twenty six percent of symptomatic patients demonstrated low-level pyuria expression of 1-9 wbc μl^{-1}.

Table 10 *Lower urinary tract symptom prevalence amongst patients.*

Symptom complex	Description	Frequency (%)
Storage	Urinary urgency	64.8
	Urinary urgency incontinence	40.3
	Urinary frequency*	81.9
	Nocturia†	36.8
	Stress urinary incontinence	23.8
	Passive incontinence	6.1
Voiding	Hesitancy	15.2
	Reduced stream	16.1
	Intermittency	12.5
	Straining	4.1
	Terminal dribbling	11.7
Postmicturition	Incomplete emptying	13.7
	Post micturition dribbling	6.0
Pain	Pain or discomfort on bladder filling	9.9
	Pain or discomfort in the pubic area	2.9
	Burning or pain when passing urine	11.2
	Urethral pain	3.3
	Iliac fossa pain	2.1
	Loin pain	13.4
	Genital pain	2.6
	Pain radiating into the legs	12.2

**Urinary frequency defined as ≥8 episodes/24 hours; †Nocturia defined as ≥2 episodes.*

Almost two thirds of MSU samples that demonstrated microscopic pyuria ≥10 wbc μl^{-1} showed no leucocyte esterase on dipstick testing. These data and summary performance statistics are reported in **Table 13** and ***Table 14*****.** The use of leucocyte esterase dipstick testing failed to detect significant pyuria in the majority of samples included in this study.

A positive dipstick test, defined as ≥'trace' leucocyte esterase or positive nitrite failed to identify over half of urine cultures reported as positive. These data and summary performance are presented in **Table 15** and **Table 16**.

Table 11 *Diagnostic performance of microscopic pyuria ≥10 wbc μl^{-1} referenced against positive routine laboratory urine culture at ≥10^5 cfu ml^{-1}.*

	MSU result			**Totals**
	Negative*	Mixed growth†	Positive‡	
No pyuria (zero wbc)	1116 (42%)	141 (35%)	582 (45%)	1839
Pyuria 1-9 wbc μl^{-1}	875 (23%)	97 (24%)	173 (13%)	1145
Pyuria ≥10 wbc μl^{-1}	643 (35%)	168 (41%)	537 (42%)	1348
Totals	2364 (100%)	406 (100%)	1292 (100%)	4375

**Negative culture defined by bacterial growth of <10^5 cfu ml^{-1}; †Polymicrobial growth; ‡Positive culture defined by growth of a single recognised uropathogen of ≥10^5 cfu ml^{-1}.*

Table 12 *Summary statistics for microscopic pyuria ≥10 wbc μl^{-1} as a surrogate marker of urinary tract infection in patients with chronic LUTS.*

Index test	**Sensitivity (95% CI)**	**Specificity (95% CI)**	**PPV (95% CI)**	**NPV (95% CI)**
Microscopy*	0.42 (0.39-0.44)	0.73 (0.72-0.75)	0.40 (0.37-0.43)	0.75 (0.73-0.76)

**Positive diagnostic threshold* ≥10 wbc μl^{-1}; *PPV=positive predictive value; NPV=negative predictive value.*

Table 13 *Diagnostic performance of dipstick ≥'trace' leucocyte esterase in the detection of microscopic pyuria ≥10 wbc µl^{-1}.*

	Microscopic pyuria result			**Totals**
	No pyuria	Pyuria 1-9 wbc µl^{-1}	Pyuria ≥10 wbc µl^{-1}	
Dipstick negative	1566 (85%)	937 (82%)	840 (62%)	3343
Dipstick ≥ 'trace'	273 (15%)	208 (18%)	508 (38%)	989
Totals	1839 (100%)	1145 (100%)	1348 (100%)	4332

Table 14 *Summary statistics for dipstick ≥'trace' leucocyte esterase in the detection of microscopic pyuria ≥10 wbc µl^{-1}.*

Index test	**Sensitivity (95% CI)**	**Specificity (95% CI)**	**PPV (95% CI)**	**NPV (95% CI)**
Dipstick ≥ trace	0.38 (0.35-0.40)	0.84 (0.83-0.85)	0.51 (0.48-0.55)	0.75 (0.73-0.76)

PPV=positive predictive value; NPV=negative predictive value.

Table 15 *Diagnostic performance of a positive dipstick test in the detection of a positive routine laboratory urine culture at ≥10^5 cfu ml^{-1}.*

	MSU culture result		**Totals**
	Positive (%)	Negative (%)	
Dipstick negative	729 (55)	2235 (73)	2964
Dipstick positive	585 (45)	826 (27)	1411
Totals	1314 (100%)	3061 (27)	4375

Positive dipstick defined as ≥'trace' leucocyte esterase or positive nitrite.

Table 16 *Summary statistics for a positive dipstick test as a surrogate marker of urinary tract infection in patients with chronic LUTS.*

Index test	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)
Dipstick	0.45 (0.42-0.47)	0.73 (0.71-0.75)	0.41 (0.39-0.44)	0.75 (0.74-0.77)

PPV=positive predictive value; NPV=negative predictive value.

4.10 Discussion

Microscopic pyuria is our key diagnostic surrogate of UTI. Whilst microscopy remains an important component of urinalysis in most diagnostic laboratories, the dipstick has replaced the microscope in the clinic setting. Irrespective of which method is favoured, these data have important implications for those who employ these tests to guide their management. The results of this analysis cast considerable doubt on the veracity of both these methods as clinically useful indicators of infection in patients with LUTS who do not present with acute symptoms.

The UK consensus guidelines for MS bladder management promote the dipstick as an accurate screening tool for UTI (104). This recommendation is based on a single paper which cites the NPV of leucocyte esterase in the diagnosis of UTI as 99% which is far higher than any other published data (375). This performance statistic would provide the clinician with confidence that in the face of a negative dipstick, infection can be excluded.

Scrutiny of this evidence demonstrates that the reference standard for the diagnosis of infection required positive bacterial culture at $\geq 10^5$ cfu ml^{-1} and pyuria ≥5 leucocytes per high-power field (HPF), rather than a microbiological definition only. This is in contrast to almost every other published report addressing the performance of pyuria and other surrogate measures in the diagnosis of UTI.

Modern analysis no longer uses cell counts per HPF, which refers to the visual field at light microscopy when X400 magnification is employed. Nonetheless, in 1994 when the work referenced by the UK consensus guidelines was published, this measure was still used occasionally. It has now been superseded by cell counts expressed per microlitre. The conversion of leucocyte counts per HPF requires multiplication by a factor of approximately five to correct for the smaller volume of urine assessed (377). Thus, a pyuria threshold of ≥5 leucocytes HPF^{-1} described above would equate to around ≥25 wbc μl^{-1}.

The use of a reference standard for UTI that required a positive bacterial culture and associated pyuria ≥25 wbc μl^{-1} is of particular importance when considering the NPV of the dipstick in this study. Samples that tested negative for leucocyte esterase would almost certainly have tested negative for microscopic pyuria, and significant concordance between these two measures would be expected. Even if a sample then generated a positive bacterial culture, the absence of pyuria on microscopy would erroneously classify the urine as not infected. Thus, a negative dipstick test would be considered a true negative, despite a positive culture. This aspect of the study design would inflate the NPV of the dipstick artificially.

One of the strengths of the current study is sample selection: all enrolled patients were required to demonstrate chronic symptoms. Whilst the minimum permissible duration of symptoms was three months, the mean duration of LUTS amongst the sample was almost five years. The inclusion of only patients with chronic symptoms mitigates the effects of spectrum bias that commonly influence studies of diagnostic accuracy. The majority of published studies that assess the performance of surrogate markers of UTI fail to define their study population adequately or include patients with acute symptoms.

All subjects provided clean-catch MSU samples and microbiological reference cultures employed a diagnostic threshold of $\geq 10^5$ cfu ml^{-1}. The study design was pragmatic, using methods common to ordinary clinical practice. By contrast, existing studies of microscopic pyuria and dipstick testing in patients with LUTS utilise

different techniques (209-211). All employ catheter sampling, and lower microbiological thresholds to define urinary infection. Whilst rigorous, such methods are seldom used in routine clinical assessment in the UK.

This study has limitations. Different researchers conducted the urine microscopy to allow the analysis of a large number of samples and this may have influenced the findings. Whilst the microscopic evaluation of pyuria may have been subject to subtle inter-observer differences, the large sample size and narrow confidence intervals for the test performance statistics suggest that any variation was minimal. Patients were recruited from a single specialist centre, which could be a source of selection bias. Nonetheless, the distribution of LUTS in the study sample are comparable to those reported by large, population-based symptom prevalence studies (378). This ought to permit the application of these findings to the wider population of patients with LUTS.

The results of this study provide further evidence of the deficiencies of microscopic pyuria as a reliable surrogate of urinary infection in patients with chronic urinary symptoms. Microscopic pyuria, defined as ≥10 wbc μl^{-1}, failed to identify 58% of symptomatic patients with a positive culture of ≥10^5 cfu ml^{-1} (**Table 13**) and the dipstick erroneously classified a similar proportion of samples with a positive culture result as normal (**Table 15**). Other key performance data were similarly poor. These results suggest that the use of microscopic pyuria as a screening test for UTI in patients with LUTS needs to be reconsidered.

Dipstick leucocyte esterase ≥'trace' failed to identify microscopic pyuria ≥10 wbc μl^{-1} in 72% of the samples (**Table 14**). This was despite immediate microscopy and automated dipstick testing to avoid signal loss. The 'trace' leucocyte esterase reagent pad on the dipstick is calibrated to detect the enzyme in the presence of ≥15 wbc μl^{-1} which restricts the sensitivity of the dipstick to correctly identify all samples demonstrating ≥10 wbc μl^{-1}. Whilst some samples containing 10-15 wbc μl^{-1} may have been incorrectly reported as negative, it would seem extremely unlikely that

sufficient samples demonstrated leucocyte numbers in this narrow range to account for the insensitivity of the dipstick alone.

The detection of leucocyte esterase in urine is dependent on lytic and non-lytic release of the enzyme from neutrophils and other granulocytes. The existing literature describes the release of leucocyte esterase by both methods, but no experimental data explore the relative contribution of these two pathways to the final enzymic concentration in urine. Cell lysis is dependent on local conditions, particular osmotic, and the lytic release of leucocyte esterase could be retarded in more concentrated urine. Patients with chronic LUTS often engage in fluid restriction to attenuate symptoms whilst patients with symptoms of acute cystitis are commonly encouraged to increase their fluid intake, despite a lack of evidence to support this strategy (379). Whether osmolality might have a significant influence on the generation of leucocyte esterase in urine has not been explored.

The data comparing the prevalence of microscopic pyuria ≥10 wbc μl^{-1} according to culture status are worthy of discussion. Whether a positive or negative culture was reported, or the sample demonstrated polymicrobial growth, the proportion of samples with ≥10 wbc μl^{-1} was very similar. Comparable proportions of patients in each group also demonstrated no pyuria or low-level pyuria, defined as 1-9 wbc μl^{-1}. Whilst these results are difficult to explain using conventional dogma, data challenging the $\geq 10^5$ cfu ml^{-1} culture threshold (187-192), and the dismissal of polymicrobial cultures (190, 194-196), are relevant to these findings. These data suggest that bacterial growth below 10^5 cfu ml^{-1} and mixed cultures can be associated with an inflammatory signal in the urine of symptomatic patients.

Leucocyte contamination from the genital tract could be offered as an explanation for these findings. Whilst much of the published work on urine collection methods concerns paediatric patients and the elderly, only two studies have assessed the recovery of leucocytes in paired MSU and CSU samples in ambulatory women (380, 381). Both employed cleansing and urine collection methods almost identical to those used in this programme of study (2.6.1). One hundred and fifty-five paired

MSU and CSU samples demonstrated no statistically significant difference in leucocyte counts and both studies demonstrated 90% concordance between the two methods of urine collection. More data is required to ascertain the effects of different collection strategies on leucocyte salvage.

Over a quarter of symptomatic patients demonstrated low-level pyuria in the 1-9 wbc μl^{-1} range (**Table 11**), falling below the ≥10 wbc μl^{-1} threshold widely perceived to indicate infection (197). Whilst low-level pyuria is commonly dismissed as insignificant, cytokine data from two studies have demonstrated elevated levels of IL-6 in symptomatic patients with pyuria 1-9 wbc μl^{-1}, not evident in patients without pyuria or asymptomatic controls (259, 260). The controversial history of this diagnostic threshold, and finding of immune activation in patients with low-level pyuria, raise further questions about the validity of pyuria ≥10 wbc μl^{-1} as a surrogate of urinary infection. The implications of low-level pyuria expression in this population are unclear, although it could represent additional evidence of urothelial inflammation in patients with chronic LUTS.

Whilst the term 'infection' has long been synonymous with disease in clinical medicine, historical definitions of infection relate to the presence of an infecting organism in a host. They do not confer information about clinical sequelae. Infection is perhaps more usefully defined as 'the acquisition of a microorganism by a host' (382), and this event may result in colonisation by the infecting microbe or disease. Colonisation may drive subtle changes in the physiological processes of the host system, such as immune stimulation, but this would not be associated with damage of sufficient magnitude to cause disease. By contrast, disease is characterised by host-microbe interactions that initiate tissue damage and are often associated with the generation of symptoms.

Kass originally conceived his quantitative microbiological criteria to discriminate between true bacteriuria and contamination (186). From the outset, Kass acknowledged that significant bacteriuria was present in many asymptomatic individuals, indicating that bacteriuria was not always associated with disease.

Bacterial culture, irrespective of the diagnostic criteria used, identifies bacterial colonisation rather than a disease process. In common with other biological processes, the transition from colonisation to disease should be considered a continuum.

Whilst culture-based diagnosis provides information relating to colonisation, it does not inform us of the interaction between host and bacterium. Urinary nitrite is a surrogate of bacterial colonisation by some bacteria, but its presence does not indicate disease. By contrast, surrogate markers of infection such as microscopic pyuria and leucocyte esterase are measures of the inflammatory response rather than colonisation alone.

In clinical practice, the term UTI is used to describe symptomatic infection, rather than simply pathogen entry into the host. The relationship between bacterial infection, lower urinary tract inflammation and the acute onset of symptoms has been characterised in prospective clinical studies of women with recurrent UTI (258). The clinical presentation of acute UTI allows accurate symptom-based diagnosis, an approach that has been validated using sensitive microbiological methods (207).

The exclusion of UTI is universally advocated in patients who present with LUTS. This recommendation is not grounded in evidence, but borne of the perception that infection might be implicated in symptom generation. Whilst preliminary data has demonstrated evidence of bacterial colonisation and lower urinary tract inflammation in patients with OAB symptoms (211, 259, 260, 263, 265-267), the question of whether the symptoms are driven by infection is yet to be answered. The relationship between bacterial infection, inflammation and the generation of chronic LUTS should not be explored using diagnostic principles conceived in the study of acute infection. The spectrum of infecting uropathogens may be different, and the nature and magnitude of the inflammatory response distinct to that seen in acute infective syndromes.

Markers of urinary tract inflammation help to characterise the host-pathogen relationship when it has moved beyond simple colonisation. Preliminary evidence suggests that the use of microscopic pyuria as a continuous variable correlates with cytokine evidence of inflammation (259, 260), although pyuria may be absent even when inflammatory cytokine production is established. Whether dispatching the ≥10 wbc μl^{-1} threshold would enhance the utility of pyuria as a clinically useful measure of lower urinary tract inflammation is unclear.

This study provides data specific to the performance of pyuria and dipstick testing in patients with chronic LUTS. It employs methods common to ordinary clinical practice, and provides a practical assessment of surrogate measures that can be readily applied to similar patient populations. The study does not include patients with MS, although the distribution of LUTS described by the study population are very similar to those described by MS patients (55).

Whilst neuropathology is assumed to be the most common cause of LUTS in patients with MS, the role of urinary infection in the generation of LUTS remains unexplored. Epidemiological data is restricted to patient-reported, retrospective surveys. Whilst patients and clinicians cite UTI as a common cause of neurological deterioration, the impact of UTI on lower urinary tract function has been overlooked. The widespread use of catheters and immunomodulatory therapy by MS patients might be expected to increase levels of bacterial colonisation within the lower urinary tract, although data are lacking. How frequently colonisation evolves into infection is not known, although the incidence of patient-reported cystitis amongst MS patients with LUTS is threefold higher than that described in healthy female subjects (55, 163).

Current guidance on the management of LUTS in MS makes no recommendations relating to the utility of urine culture (104). Whilst advice on the assessment of bacteriuria in patients with MS is provided, how urine culture should be deployed in the assessment of patients with LUTS is not specifically described. National guidance on the management of urinary incontinence in neurological disease recommends submitting a urine sample for culture if dipstick analysis and symptoms suggest UTI

(374) although treatment should not be delayed. The culture results are therefore used to modify treatment if there is no clinical response to antibiotics, in the event an infecting organism demonstrates resistance. Dipstick testing is therefore promoted as the key diagnostic test for UTI in patients with MS and LUTS in the UK. The results of this analysis cast serous doubt on the reliability of microscopic pyuria and dipstick urinalysis as reliable indicators of urinary infection for these patients.

5 Bacterial infection, urothelial inflammation, and immune activation in patients with overactive bladder symptoms and multiple sclerosis

5.1 Background

The consequences of infection for patients affected by MS have been documented in the medical literature for three decades (152-154, 156, 171). Neurological deterioration in association with infection is widely recognised, although this reaction is not always synonymous with relapse. Urinary tract infection appears to be an important correlate of neurological deterioration in patients with MS (156, 171, 173) although the mechanisms by which infection mediates such effects remains poorly understood. Whilst the influence of UTI on neurological functioning is well documented, there are no data that explore the influence of infection on LUTS in patients with MS.

Recommendations made regarding the diagnosis and treatment of UTI in MS patients are contentious (104). Reliance on the dipstick leaves patients at risk of undiagnosed infection and its sequelae. These problems are confounded by the poor performance of standard culture-based diagnosis. Any deterioration in lower urinary tract function might be incorrectly attributed to neurological decline, corroborated by negative urine testing that may be inaccurate. Existing guidance describes the widespread occurrence of bacteriuria amongst those who use ISC and correctly asserts that it should not be treated in the absence of infective symptoms. Uncertainty relating to the nature of infective symptoms, especially in those patients with a numerous of pre-existing LUTS serves to make such 'clinical' diagnoses difficult. This could increase the risk of treatment being inappropriately withheld.

Whilst the role of bacterial infection in the generation of acute urinary symptoms has been clearly demonstrated, the same cannot be said for chronic LUTS. Nonetheless, preliminary data have provided evidence of bacterial colonisation, immune activation and urothelial inflammation amongst patients with OAB symptoms, not evident in asymptomatic controls (211, 259, 260, 263, 265-267). Whilst this work provides evidence of an infective, inflammatory aetiology in OAB, control subjects were much younger, and a greater proportion were male.

Bacteriuria and pyuria are known to be more prevalent in older subjects and females (179, 276-280), so carefully matched samples are required to confirm these findings.

In this study, culture of the spun urinary sediment was employed to characterise the microbiology of the lower urinary tract (266-270). The sediment culture uses centrifugation to concentrate the cellular content of the urine prior to quantitative culture (*RCF*=627 *g* for 5 minutes). This culture technique achieves greater bacterial growth and diversity than standard methods although the centrifugal forces used would not be expected to concentrate planktonic bacteria. Urothelial cells and inflammatory cells are readily concentrated by this method (383) but bacteria require much higher forces to pellet (384). Thus, planktonic bacteria remain in suspension. Host-tissue adhesion is the principal virulence factor demonstrated by uropathogens, allowing close contact with urothelial cells and perhaps entry to the intracellular space (214, 215, 225, 226). It may be that organisms associated with the urothelial cells are being preferentially salvaged by the technique, although this hypothesis has not yet been corroborated.

It is clear that no fixed bacteriological threshold is suitable for diagnosing urinary infection in all clinical circumstances. The thresholds adopted may have perverted our understanding of the ecology of urinary infection. Only by abandoning these diagnostic constructs can the microbiology of the lower urinary tract be characterised without existing tenets influencing our analysis. In this study, all bacteria isolated by culture of the spun urinary sediment were reported, irrespective of their magnitude of growth.

The expansion of a given population of bacteria would be expected to mediate increased host tissue damage if those bacteria are recognised pathogens. Pathogenic potential depends on organism, host and environmental factors. Pathogenicity stems from bacterial virulence such as toxins, exoenzymes, and adhesins (385), host immune defence (219), and the availability of nutrients, pH, temperature, and oxygen tension (386). Bacterial growth is a therefore a measure of reproductive success rather than disease activity. Whilst reproductive success might reflect an

organism's ability to exploit its environment, this might not be consistently associated with disease.

Classic symptoms of acute UTI such as urinary frequency and dysuria are accurate indicators of bacterial cystitis (207). Whilst acute symptoms must indicate interaction between bacteria and the host the significance of other LUTS is not known. To explore the relationship between bacterial infection and chronic LUTS, evidence of a microbe-host exchange should be sought. In this study, microscopic pyuria and urinary IL-6 were employed as measures of the inflammatory response in the lower urinary tract.

Immune activation results in urothelial cell exfoliation. Murine and human cell culture studies have implicated the UPEC Type 1 pilus adhesin FimH in this process. Uropathogenic E.coli bind to UP on urothelial cells, stimulating rapid apoptosis and exfoliation into the bladder lumen (387-389). Presumably, this mediates the clearance of urothelial cell-associated UPEC with bladder emptying. There are four principal UP subtypes and apoptosis in response to FimH appears dependent on the expression of UPIII. Uroplakin III is heavily expressed in well-differentiated urothelial cells at the luminal surface, known as umbrella cells. The binding of UPEC to these cells would be expected to trigger urothelial cell efflux into the urine which can be quantified. Increased urothelial cell exfoliation has been reported in patients with OAB compared to control subjects, although matching was poor (263). The expression of urothelial cells in human urine is greater in patients who demonstrate higher levels of microscopic pyuria (264). This suggests that urothelial cell enumeration could be a marker of immune activation in UTI.

The microscopic examination of centrifuged urinary sediments has demonstrated bacteria associated with exfoliated urothelial cells. These findings suggest that bacterial adherence to urothelial cells prevented the microbes from dispersion in the effluent during the Cytospin™ centrifugation process. Two studies have reported that patients with OAB demonstrate a greater proportion of shed urothelial cells with associated bacteria when compared to control subjects (271, 272). The study

groups demonstrated differences in demographic characteristics that could have been a source of bias.

Quantifying the prevalence of bacterial adhesion to exfoliated urothelial cells has potential advantages over standard bacterial culture in the assessment of UTI. Whilst both methods confirm the presence of bacteria, bacterial adherence to urothelial cells is a strong indicator of pathogenicity (390-392). The Type 1 pilus allows physical intimacy of the bacterium with the host. Whilst much attention has been focused on the entry of UPEC into the intracellular compartment, the close contact of bacteria with host cells is likely to potentiate the effects of other virulence factors including toxins and exoenzymes (385). Recent evidence from human urothelial cell culture studies has demonstrated that UPIII has a cytoplasmic signalling domain. The binding of FimH to UPIII mediates a significant increase in intracellular calcium (Ca^{2+}) from the sarcoplasmic reticulum and extracellular influx (223, 389). Elevated intracellular Ca^{2+} has been implicated in intracellular entry of UPEC via membrane trafficking, and urothelial cell apoptosis, processes that oppose one another. Thus, the success of UPEC in establishing an intracellular niche would seem to hinge on the dominance of internalisation rather than exfoliation. These processes may be moderated by other signals that impede apoptosis, or the variability of UPIII expression amongst urothelial cells at different stages of maturation. The interaction between UPIII activation and the innate immune system might influence this process but this has not been studied.

The release of ATP from host cells is believed to serve as a 'danger' signal. Lytic and non-lytic secretion have been observed in response to cell damage or stimulation (319-322). Bacteria are another potential source of ATP in the lower urinary tract and are able to generate ATP by similar mechanisms (323, 324). Although physiological data have been gleaned from experiments on tissue harvested from patients (67-69, 71, 74, 75, 262), the expression of ATP in urine has not been determined. Only one study has reported urinary ATP levels in vivo (334) but the analysis was not conceived to describe any differences in different patient groups.

Urinary ATP might seem an attractive surrogate marker of bacterial infection and urothelial distress, but at the time of writing, no data had explored such a function.

This primary aim of this analysis was to determine the prevalence of bacterial infection amongst MS patients with OAB symptoms and asymptomatic control subjects. Asymptomatic bacteriuria is perceived to be widespread amongst patients, so culture evidence alone would be insufficient to forge an association between UTI and OAB symptoms. Central to this work was measurement of the urothelial inflammatory response, characterised by microscopic pyuria and IL-6. Without the 'smoking gun' of urothelial inflammation, the role of infection in the generation of OAB symptoms in this group of patients would be in doubt.

Describing a microbe-host interaction is critical to this work. Whilst evidence of urothelial inflammation was sought using established methods, novel markers of immune activation, urothelial distress, and bacterial adhesion were also used to define the relationship between bacterium and host. Although the utility of these measures has not been confirmed, preliminary data suggest that they may prove to be useful markers of disease activity. Positive findings in this study would corroborate their use in the exploration of UTI and support the hypothesis at the centre of this work.

Control subjects were healthy adults without LUTS. The recruitment of MS patients without LUTS was considered, but the prevalence of urinary symptoms amongst patients would have made recruitment extremely difficult. In the absence of LUTS, there is no evidence to suggest that the disease process influences the ecology of the lower urinary tract. Catheter use has a significant association with UTI but would not be instituted in the absence of symptoms. The development of incomplete bladder emptying in patients without LUTS is possible but the relationship between elevated PVR volumes and UTI is unsubstantiated. Thus, the use of healthy asymptomatic control subjects was unlikely to have a significant effect on the comparative data.

Patients provided CSU samples for analysis whereas control subjects submitted a carefully collected MSU. The use of repeated CSU sampling would have been unacceptable for control subjects and the collection of a clean catch MSU difficult for patients, many of whom had significant disability. The use of different urine collection methods raises the possibility of genital contamination amongst control subjects influencing the prevalence of positive bacterial culture and microscopic pyuria.

Stamm (1982) used MSU, CSU, and suprapubic aspiration (SPA) in his seminal work on the diagnosis of acute UTI in ambulatory women (187). In his study, the bacterial colony counts achieved by clean-catch MSU sampling were compared to those yielded by CSU/SPA sampling. Almost 100 matched samples were analysed and the results demonstrated a strong correlation between the two methods (r=0.79). The influence of sampling methods on the detection of microscopic pyuria has been specifically studied in similar patient populations, and concordance between MSU and CSU samples was excellent (380, 381). These studies were undertaken in ambulatory females and bacterial and leucocyte contamination in samples submitted by males is likely to be lower if cleansing and careful technique are used. In the mixed gender study population employed here, the performance of MSU sampling ought to be better.

5.2 Study overview

These data represent a baseline analysis of a blinded, observational cohort study of MS patients with OAB and asymptomatic control subjects. Adult patients with MS and OAB symptoms provided a CSU, and asymptomatic control subjects submitted a clean-catch MSU. Patients and control subjects were matched for key demographic characteristics. Urine was subject to analyses scrutinising the microbiology of the lower urinary tract, and the inflammatory and immune response of the urothelium. Demographic data was stored in a secure clinical database. Symptoms were assessed using validated questionnaires, and lower urinary tract function was evaluated from bladder diary data.

The study was conducted at the Department of Medicine, UCL Archway Campus. Ethical approval for this study was granted by the National Research Ethics Service (NRES) Committee London, Queen's Square (Ref: 10/H0716/84).

5.3 Safety considerations

Patients provided CSU samples for analysis. Catheterisation is not painful generally but some patients describe minor discomfort associated with catheter insertion. The incidence of UTI associated with a single catheterisation has been estimated as less than 1% (393). The risks of catheterisation were documented in the patient information sheet for the study. The investigator also discussed the risks with patients at the time of consent.

5.4 Study objectives

5.4.1 Primary objective

– To evaluate the prevalence of urinary infection amongst patients and control subjects characterised by quantitative culture of the spun urinary sediment.

5.4.2 Secondary objectives

The secondary objectives of the study were to determine the following:

- To evaluate urothelial inflammation, characterised by microscopic pyuria and elevated pro-inflammatory cytokine secretion.
- To assess urothelial distress, characterised by heightened urinary purine expression.
- To evaluate immune activation, characterised by urothelial cell exfoliation.
- To detect bacterial colonisation of urothelial cells, evidenced by fluorescent microscopy.
- To assess the performance of routine bacterial culture in the hospital laboratory.
- To record the prevalence of LUTS using validated questionnaires.
- To evaluate lower urinary tract function from bladder diary data*.

**Data collected from patients only.*

5.5 Study population

5.5.1 Recruitment of participants

Patients and control subjects were recruited as outlined previously (2.2.2).

5.5.2 Consent and eligibility

All participants provided written, informed consent prior to any study related procedures, and eligibility was checked before inclusion (2.2.3).

5.5.3 Inclusion and exclusion criteria

The inclusion and exclusion criteria are described for patients in **Table 17** and asymptomatic controls in **Table 18**.

5.5.3.1 MS patients with OAB symptoms:

Table 17 *Inclusion/exclusion criteria for patients with MS and OAB.*

Inclusion criteria	
1	Adult patients with MS
2	Urinary urgency, with or without urgency incontinence
3	Ability to complete questionnaires and bladder diaries as required by protocol

Exclusion criteria	
1	Inability to provide informed consent
2	Patients who were pregnant or planning pregnancy
3	Patients with concurrent illnesses that in the opinion of the investigator were likely to compromise the validity of the data

5.5.3.2 Asymptomatic controls:

Table 18 *Inclusion/exclusion criteria for asymptomatic controls.*

Inclusion criteria	
1	Adults who did not describe urinary symptoms (***Table 19***).
2	Ability to complete questionnaires and bladder diaries as required by protocol

Exclusion criteria	
1	Inability to provide informed consent
2	Patients who were pregnant or planning pregnancy
3	Patients with concurrent illnesses that in the opinion of the investigator were likely to compromise the validity of the data

Table 19 *Symptom inclusion criteria for asymptomatic control subjects.*

Symptom complex	Description
Storage	No urinary urgency No perception of increased urinary frequency No urinary incontinence
Voiding	No voiding symptoms
Postmicturition	No post micturition symptoms
Pain	No pain attributed to the urinary tract

5.6 Study design

These cross-sectional data are derived from a baseline analysis of a blinded, prospective, observational cohort study. A full description of the observational cohort study design is reported elsewhere.

5.7 Study procedures

Eligibility was checked and consent confirmed. Bladder diary data was collected from patients. Any changes to concurrent medications and the occurrence of any adverse events were recorded. Patients completed questionnaires to assess the prevalence of urinary symptoms and their impact. Urine was submitted for analysis.

5.8 Clinical and laboratory assessments

5.8.1 Clinical assessments

Patient identifiable data and demographics (**Table 20**) were recorded in an electronic patient management system. This system was designed specifically for the assessment of patients with LUTS and protected by encryption (2.3.1).

Table 20 *Clinical and demographic data collected from patients and control subjects.*

Demographic data Age, gender, ethnicity, BMI, and menopausal status
History of lower urinary tract infection or instrumentation History of lower urinary tract instrumentation at any time, rUTI, or current catheter use
MS disease-specific data Disease subtype and time since diagnosis

**rUTI=Recurrent UTI.*

The following clinical assessments were also undertaken in accordance with protocol:

- Questionnaires, lower urinary tract:
  - *Whittington Urgency Score (2.14.2)*
  - *Whittington Pain Score (2.14.3)*
  - *ICIQ-LUTS (2.14.1)*
  - *ICIQ-LUTSqol (2.14.1)*

- Bladder diary data:
  - *Mean voided urinary volume (2.15)*
  - *Mean 24-hour urinary frequency (2.15)*
  - *Mean 24-hour urinary incontinence (2.15)*

5.8.2 Laboratory assessments

5.8.2.1 Biological samples

All patients provided CSU samples for analysis (2.6.2) and control subjects submitted a clean-catch MSU (2.6.1). Biological samples were stored securely on-site at the Department of Medicine, UCL Archway Campus (2.7).

5.8.2.2 Laboratory analyses

The following exploratory laboratory analyses were conducted on sampled urine:

Microscopic pyuria count (2.9.1)
Urothelial cell count (2.9.2)
Urothelial cells demonstrating associated bacteria (2.9.3)
Urinary IL-6 expression (2.10.1)
Urinary ATP expression (2.11.1)
Culture of the spun urinary sediment (2.12.1)
Routine urine culture in hospital laboratory (2.12.2)

5.9 Adverse events

Adverse event data were reported as outlined previously (2.5).

5.10 Data management

5.10.1 Data protection

The storage and protection of study-specific data was in accordance with GCP guidance for data management in clinical research (2.3.1). All study documents were kept in a locked cabinet in the Department of Medicine, UCL Archway Campus.

5.10.2 Data monitoring

The CRFs, source data records and study documents were monitored by regular internal audit (4.7.2). The findings, and any remedial action recommended as a result of the monitoring process, were submitted to the Sponsor, and the Director of Research and Development at the hosting organisation, Whittington Health NHS.

5.11 Statistical methods and analysis

5.11.1 Sample size calculation

The sample size calculation was undertaken using pilot data from a comparative observational study. Mean log bacterial growth, derived from culture of the spun urinary sediment, was reported amongst 26 asymptomatic control subjects, 41 patients with OAB symptoms, and 22 patients with OAB and microscopic pyuria ≥10 wbc μl^{-1}. Significant differences in log bacterial growth were detected between the groups (F=8.97; df=2; p<0.001).

The standard deviation of log bacterial counts across the dataset was 2 (sd=2.0). The standardised mean difference in log bacterial counts was 0.9 (f=0.9). A sample size of 20 subjects in each group would provide 80% power to detect a significant difference in log bacterial growth at the 5% level (α=0.05). The participants who provided the baseline data presented here also participated in a two-year prospective study with similar methods. Recruitment of ten additional patients and four control subjects was planned to accommodate attrition over the study term.

5.11.2 Statistical methods

Continuous data were assessed for normality using graphical methods, employing visual assessment of frequency distributions and Q-Q plots. Data that were not normally distributed were subject to transformation, allowing the use of parametric analysis methods if such transformation yielded a normal distribution. Non-parametric tests were employed in all other cases. Appropriate measures of central tendency and dispersion were calculated and the results presented in tabular and graphical form. The independent-samples t-test and Mann-Whitney test were used to assess between-group differences.

Categorical data were summarised in contingency tables and differences between the groups calculated using the chi-squared test or Fisher's exact test, dependent on sample characteristics. A Bonferroni-Holm correction was made to account for variable multiplicity in the secondary analyses (394). There were no missing data points.

Although non-parametric methods are used widely within the analysis, much of the data is displayed graphically after logarithmic transformation for ease of presentation.

The primary analysis determined the prevalence of urinary infection in the study groups. This was determined by the magnitude of bacterial growth, measured by quantitative culture of the spun urinary sediment, and an analysis of the dominant infecting bacterial isolates. Pilot data suggested that patients with OAB symptoms would yield bacterial colony counts in the region of 10^3 cfu ml^{-1} with recognised uropathogens emerging as the dominant infecting isolates (266). Control subjects were expected to demonstrate bacterial growth of 10^2 cfu ml^{-1} with Lactobacilli predominating.

The secondary analyses explored between-group differences in the prevalence of urothelial inflammation and distress, immune activation, and urothelial colonisation by infecting bacteria. The prevalence of infection, defined by a positive routine culture, was also assessed. The prevalence of symptoms, and symptom burden, were also evaluated.

5.11.3 Primary analysis

- Difference in bacterial growth defined by quantitative culture of the spun urinary sediment.

5.11.4 Secondary analyses

- Difference in microscopic pyuria counts.
- Difference in urothelial cell counts.
- Difference in proportion of urothelial cells demonstrating bacteria.
- Difference in urinary IL-6 expression.
- Difference in urinary ATP expression.
- Difference in proportion of positive routine cultures.

In the cohort study, questionnaire measures were used to demonstrate changes in symptom prevalence within patients over time. At baseline these measures were used only to describe the study population.

5.12 Results

Between February 2011 and June 2012, 62 patients and 22 asymptomatic control subjected were screened for study inclusion. Twenty-nine patients (F=26; M=3; mean age=54; *sd*=10.5) and 21 asymptomatic control subjects (F=15; M=6; mean age=50; *sd*=9.9) were recruited. Baseline demographic and clinical data are presented below. No significant differences in age and body mass index (BMI) (**Table 21**), gender (***Table 22***), menopausal status (**Table 23**) or previous lower urinary tract instrumentation (**Table 24**) were identified.

There were ethnic differences between the study groups and the patient group was comprised of caucasians only. Intermittent catheter use was confined to the MS group at the time of trial enrolment (*n*=9). Only MS patients described a history of recurrent UTI (rUTI) (*n*=8) defined as three or more symptomatic infections annually.

Table 21 *Comparison of age and BMI amongst patients and controls.*

Demographic data	**MS patients**	**Controls**	**Test statistic**	**Significance**
Age (years)	54.3 (*sd*=10.5)	50.0 (*sd*=9.9)	*t*=1.47 (*df*=48)	*p*=0.15
BMI (kg m^{-2})	24.3 (*sd*=3.5)	23.6 (*sd*=3.2)	*t*=0.80 (*df*=48)	*p*=0.43

sd=standard deviation; *df*=degrees of freedom.

Table 22 Comparison of gender amongst patients and controls.

Gender	**MS patients (%)**	**Controls (%)**	**Total**
Female	26 (90)	15 (72)	41
Male	3 (10)	6 (28)	9
Total	29 (100)	21 (100)	50

Fisher's exact test p=0.14

Table 23 *Comparison of menopausal status amongst patients and controls.*

Menopausal*	**MS patients (%)**	**Controls (%)**	**Total**
Yes	12 (46)	7 (47)	19
No	14 (54)	8 (53)	22
Total	26 (100)	15 (100)	41

Fisher's exact test p=0.61

*Includes physiological or surgical menopause.

Table 24 *History of previous lower urinary tract instrumentation at any time.*

Instrumented*	**MS patients (%)**	**Controls (%)**	**Total**
Yes	9 (31)	7 (33)	16
No	20 (69)	14 (67)	34
Total	29 (100)	21 (100)	50

Fisher's exact test p=0.55

*Includes lower urinary tract instrumentation of any type.

Table 25 *Comparison of ethnicity amongst patients and controls.*

Ethnicity	**MS patients (%)**	**Controls (%)**	**Total**
Caucasian	29 (100)	14 (67)	43
Asian	0 (0)	4 (19)	4
Afro-Caribbean	0 (0)	3 (14)	3
Total	29 (100)	21 (100)	50

Fisher's exact test p=0.001

Patients had a longstanding diagnosis MS (mean disease duration=13 years; *sd*=9.5; RRMS=48%; SPMS=38%; PPMS=14%) and demonstrated significant urinary urgency symptoms (mean urgency score=6.1; *sd*=2.3; range 1-10). Median voided volume was 168ml (interquartile range [*IQR*]=146-214), mean daily frequency 8.9 (*sd*=2.5), and median daily incontinence episodes zero (*IQR*=0.0-0.5). Lower urinary tract symptoms were widespread, and their impact on QoL significant (**Figure 12** and **Figure 13**).

Depression (*n*=7) and hypertension (*n*=6) were the two most common comorbidities amongst patients at study entry.

Figure 12 *ICIQ LUTS symptom scores amongst patients and controls.*

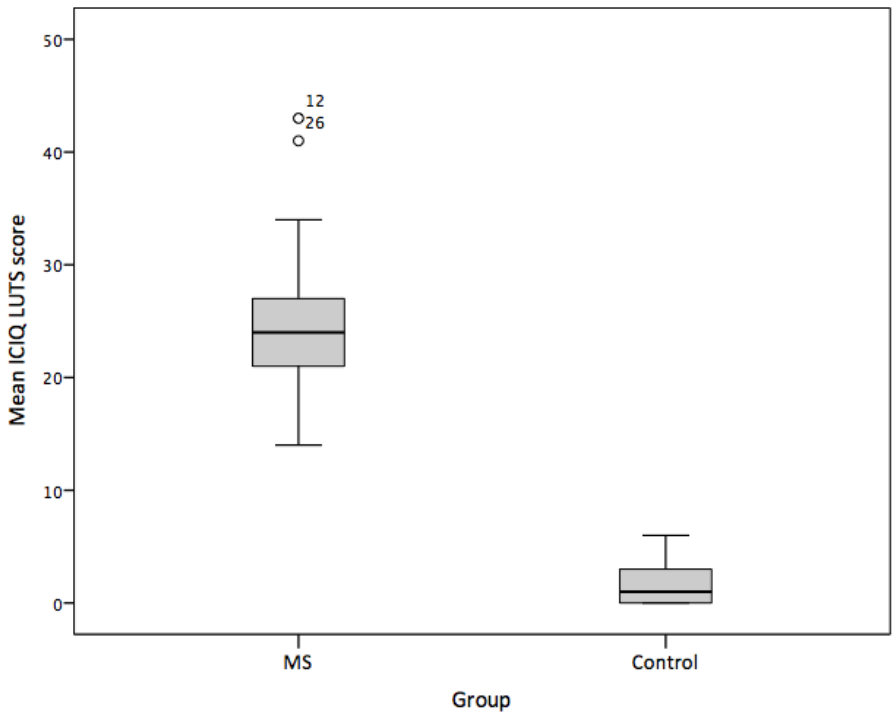


Figure 13 *ICIQ LUTSqol quality of life scores amongst patients and controls.*

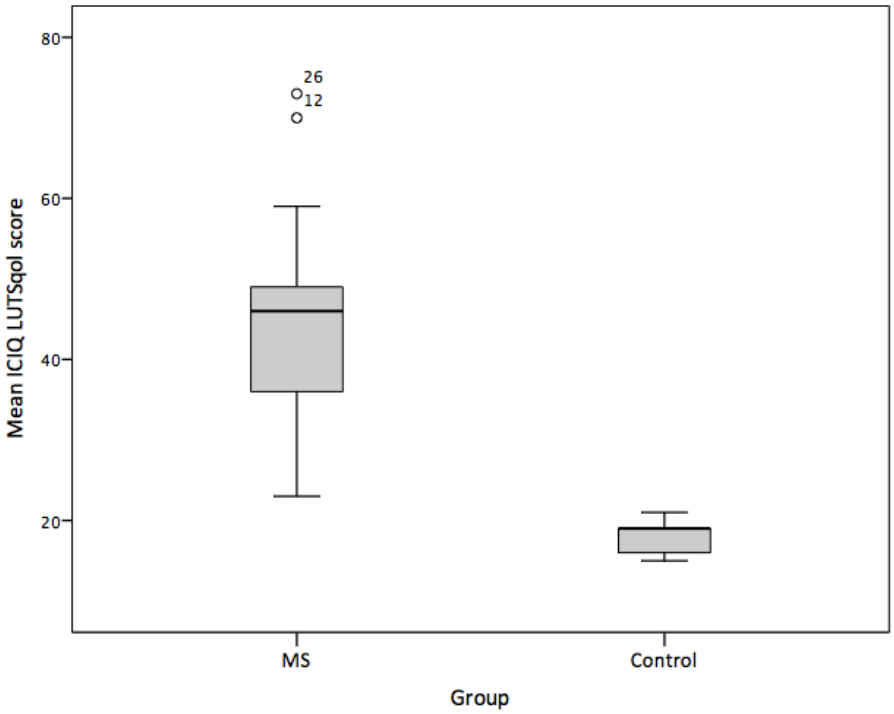
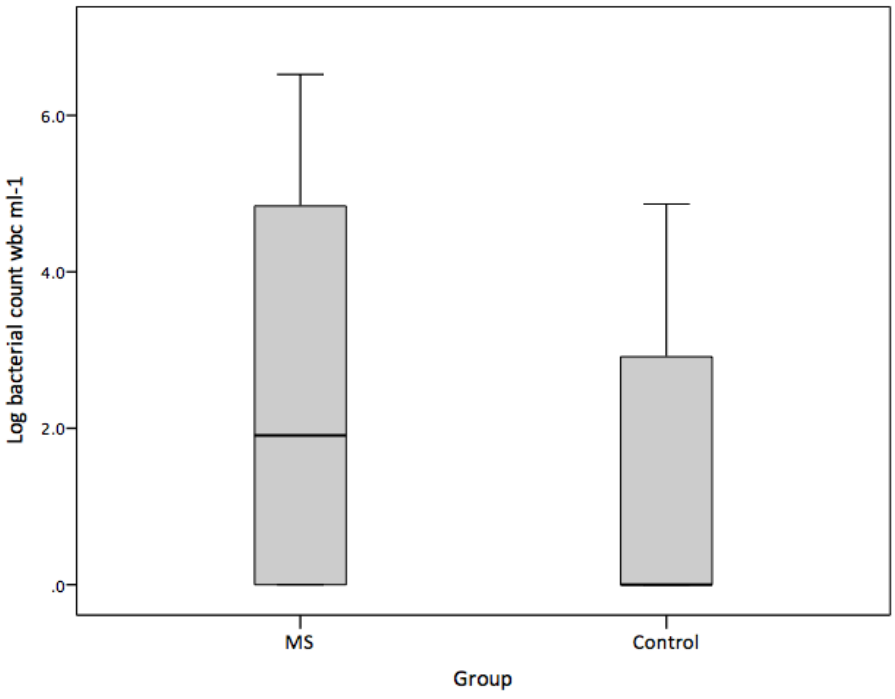


Figure 14 *Log bacterial growth amongst patients and controls.*



The primary outcome measure of log bacterial growth defined by culture of the spun urinary sediment demonstrated greater bacterial growth amongst patients than asymptomatic controls (U=209.00; p=0.047). Median growth amongst patient samples was 1.84 log cfu ml^{-1} but was zero amongst controls. The results are presented graphically in **Figure 14**. The spun sediment culture demonstrated a greater proportion of positive cultures, defined as any bacterial growth, amongst patients when compared to control subjects (patients=62%; controls=33%; p=0.042).

Culture of the spun urinary sediment demonstrated differences in the distribution of infecting bacteria amongst patients and controls. The dominant bacterial species isolated from these cultures, and the frequency with which they were isolated, is reported below (**Figure 15**). Similar results are presented for all bacterial species isolated in study samples, irrespective of their magnitude of growth (**Figure 16**).

In patient samples, *E.coli* was isolated as the dominant infecting bacterium more frequently than any other organism. Many other bacteria were isolated in patient cultures but at much lower frequencies. By contrast, *Streptococcus agalactaie* demonstrated dominance amongst control samples, with all but one of the positive cultures demonstrating this bacterium as its dominant isolate. Polymicrobial cultures were reported in 22% of positive patient cultures, and 14% of control samples.

In contrast to controls, patients demonstrated greater urinary leucocyte counts (U=105.00; p<0.001) and urinary IL-6 expression (U=143.00; p=0.003) indicative of urothelial inflammation (**Figure 17** and **Figure 18**). Urothelial cell exfoliation, suggestive of immune activation, was also more pronounced amongst patients (U=199.50; p=0.003) (**Figure 19**).

Figure 15 *The frequency and distribution of dominant bacterial isolates amongst patient and control samples, characterised by culture of the spun urinary sediment.*

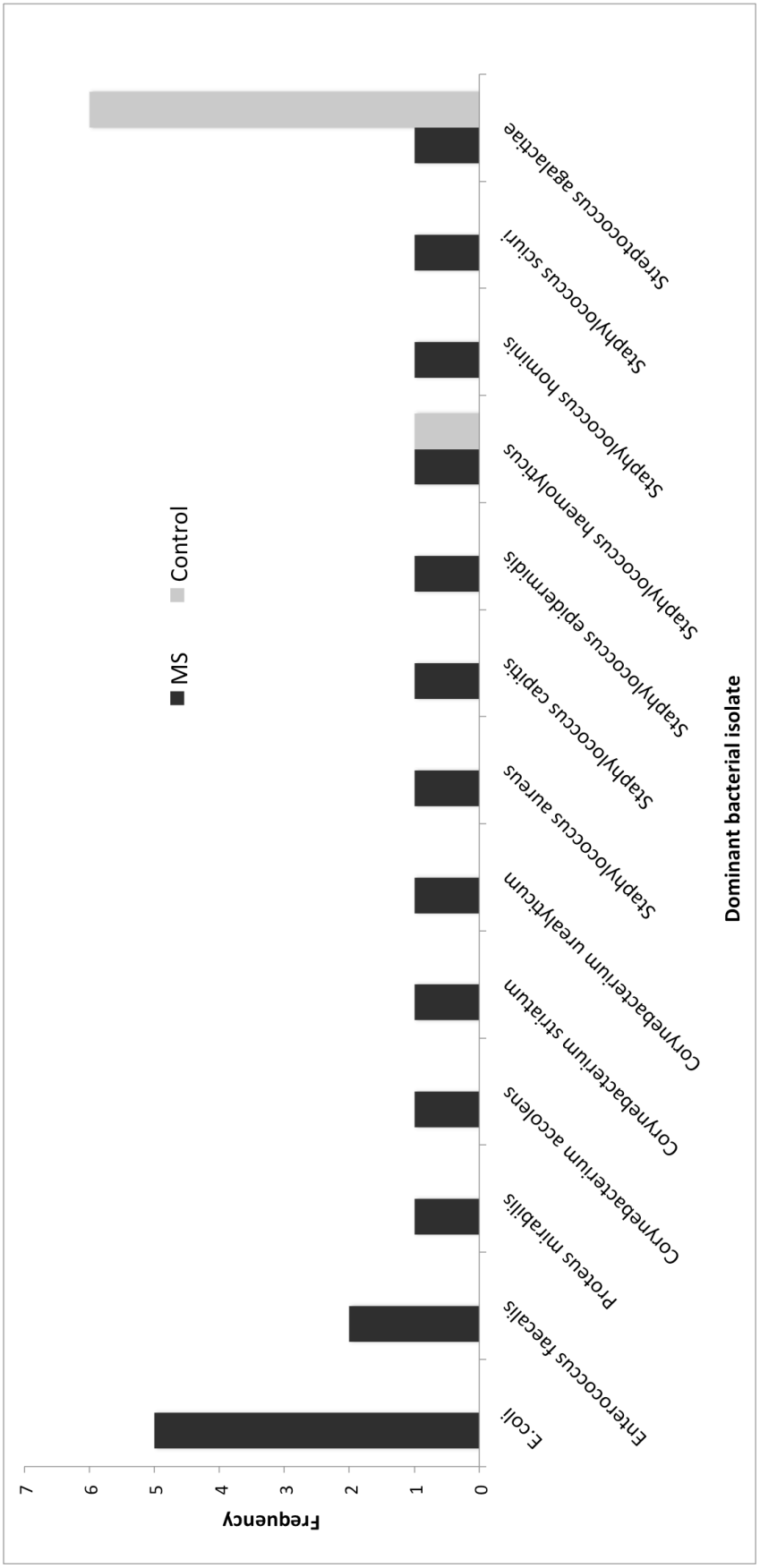


Figure 16 *The frequency and distribution of all bacterial isolates amongst patients and controls, characterised by culture of the spun urinary sediment.*

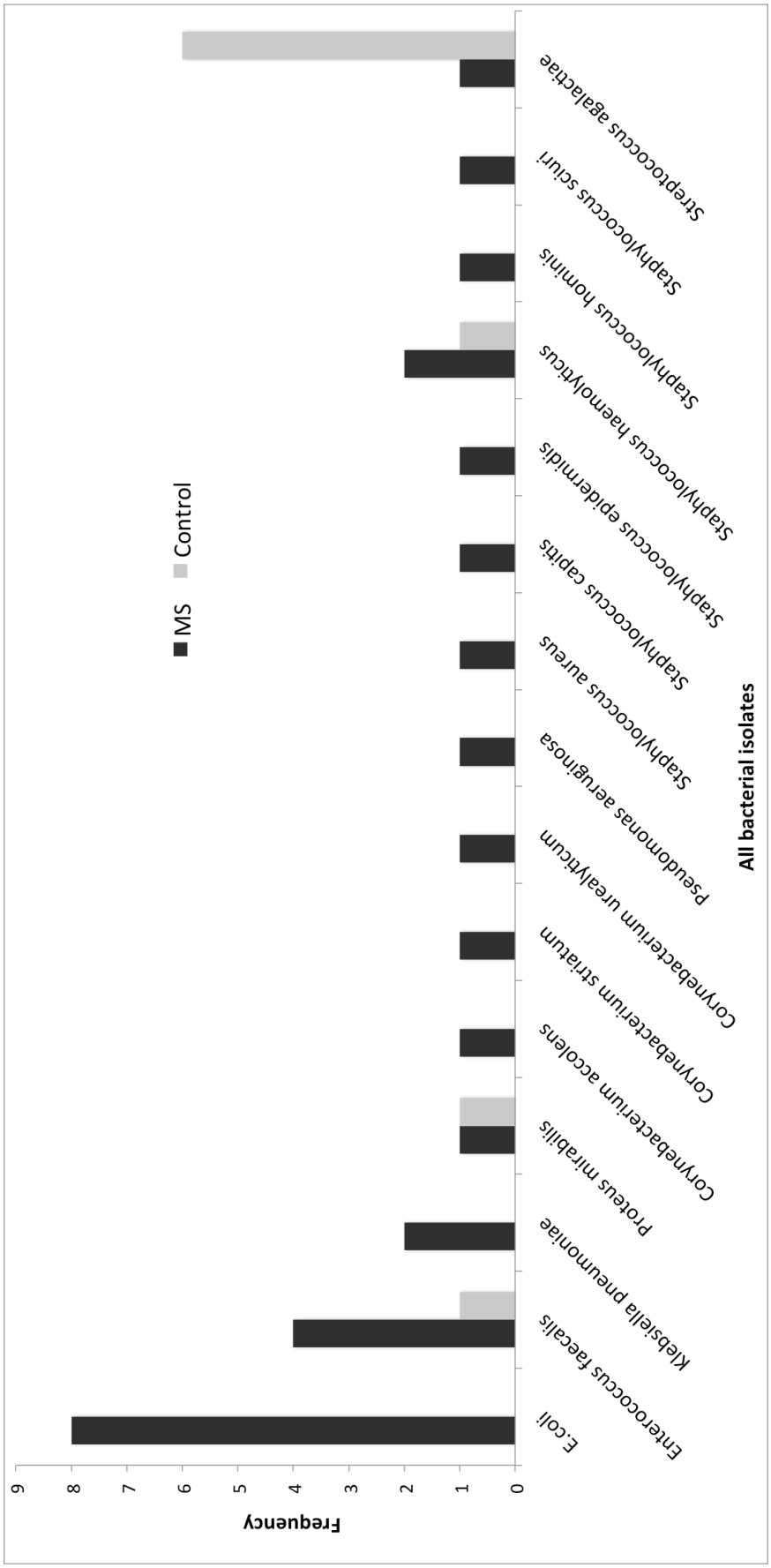


Table 26 Bacterial growth amongst samples that manifest a single bacterial isolate, stratified by study group.

Study group	Bacterial species	Bacterial growth cfu ml^{-1}
Patients	*C. striatum*	69.92
	C. urealyticum	24.8
	E.coli	2848000
	E.coli	2160000
	E.coli	1440000
	E.coli	9973.28
	E.coli	80.48
	E.coli	12
	E. faecalis	462400
	E. faecalis	105600
	S. capitis	45643.2
	S. epidermidis	100000
	S. haemolyticus	3344000
	S. sciuri	81.6
	S. agalactiae	29.6
Controls	*S. agalactiae*	73600
	S. agalactiae	6480
	S. agalactiae	1392
	S. agalactiae	1361.6
	S. agalactiae	790.4
	S. agalactiae	113.6

cfu=colony forming units.

Table 27 *Bacterial growth amongst polymicrobial cultures, stratified by study group.*

Sample origin	Bacterial species	Bacterial growth cfu ml^{-1}
Patient	*C. accolens*	*612.8*
	K. pneumoniae	*20.8*
	P. aeruginosa	*19.2*
	E. faecalis	*10.4*
Patient	*P. mirabilis*	*120*
	E.coli	*94.4*
	S. haemolyticus	*56.8*
	E. faecalis	*39.2*
Patient	*S. aureus*	*532.16*
	E.coli	*128.8*
Patient	*S. hominis*	*32*
	K. pneumoniae	*20.8*
Control	*S. haemolyticus*	*480*
	E. faecalis	*368*
	P. mirabilis	*3.2*

cfu=colony forming units.

Figure 17 *Log urinary leucocyte counts amongst patients and controls.*

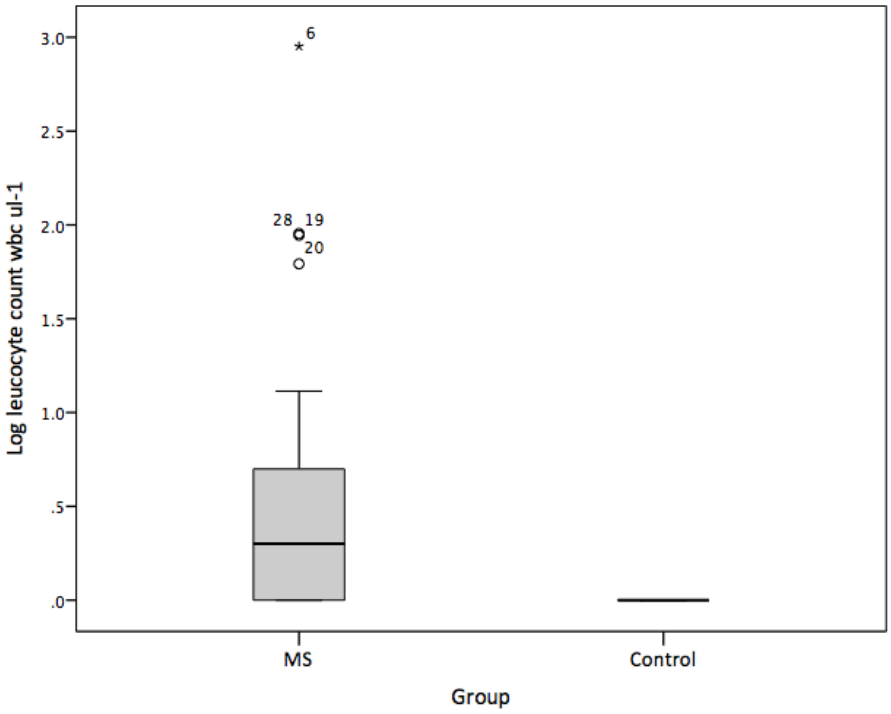


Figure 18 *Log urinary IL-6 levels amongst patients and controls.*

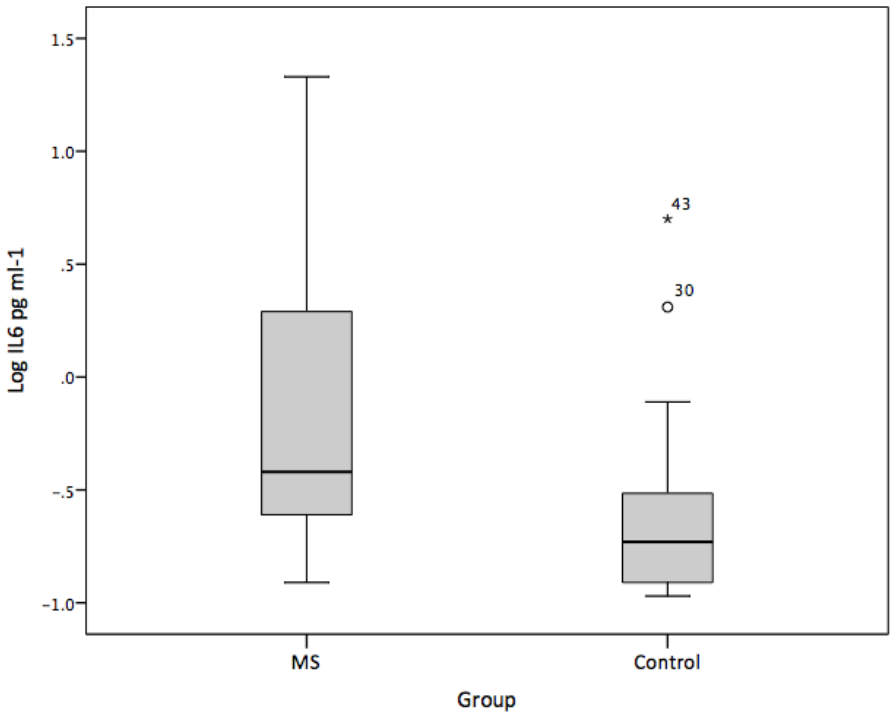
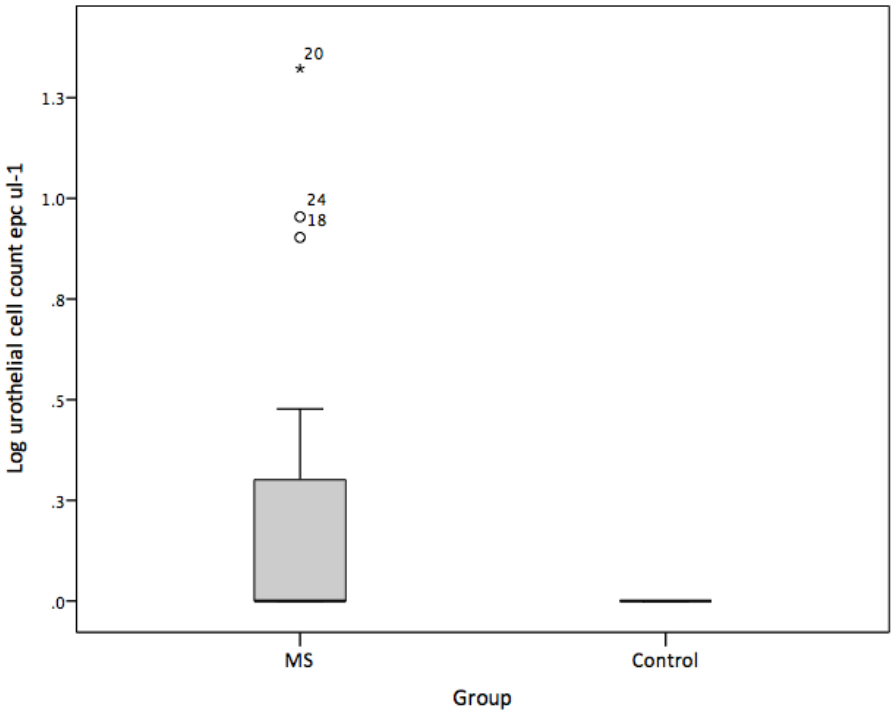


Figure 19 *Log urothelial cell counts amongst patients and controls.*



There was no between-group difference in the proportion of shed urothelial cells demonstrating bacterial colonisation (*U*=268.00; *p*=0.30) or mean urinary ATP levels amongst patients and controls (*U*=330.50; *p*=0.61).

Routine hospital laboratory culture with a diagnostic threshold of $\geq 10^5$ cfu ml^{-1} did not discriminate between the patient and control groups (**Table 28**). When the routine culture was reported as negative, the spun sediment culture yielded bacterial growth in 54% of samples (median growth=1.43 log cfu ml^{-1}). Of the samples that demonstrated a positive routine culture at $\geq 10^5$ cfu ml^{-1}, culture of the spun urinary sediment yielded median growth of 5.91 log cfu ml^{-1}.

All five of the positive routine cultures in the patient group yielded bacterial growth in excess of 10^5 cfu ml^{-1} when the spun sediment was cultured. The control samples that generated a positive routine culture in the hospital laboratory demonstrated growth of only 10^2 and 10^4 cfu ml^{-1} when the sediment was cultured. These data

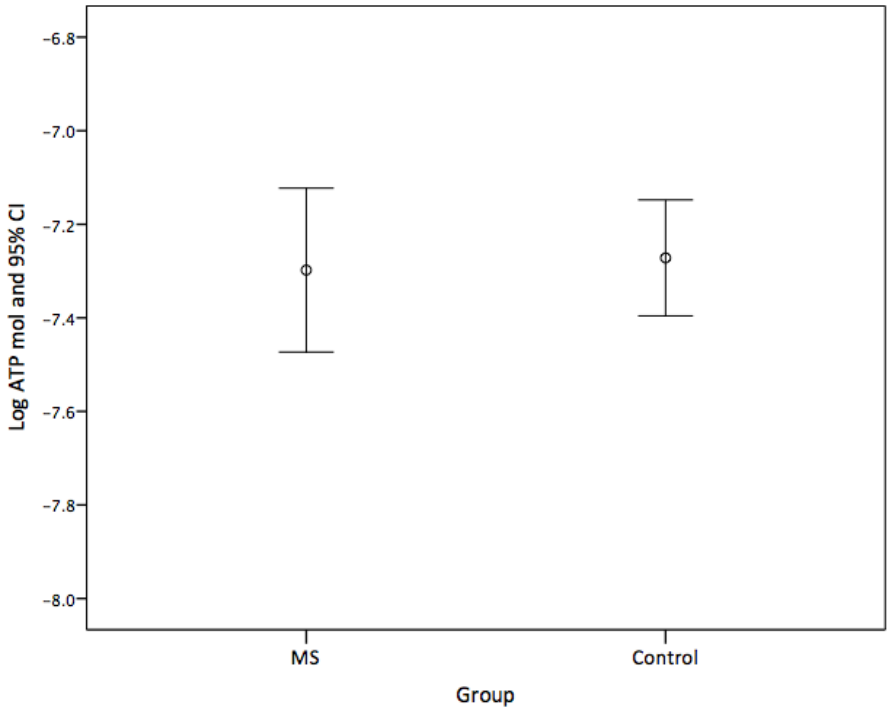
suggest that bacterial proliferation during storage and transit to central laboratories may contribute to false-positive culture results.

Table 28 *The proportion of positive routine bacterial cultures amongst patients and controls.*

Culture status	MS patients (%)	Controls (%)	Total (%)
Positive	5 (17)	2 (10)	7 (14)
Negative	24 (83)	19 (90)	43 (86)
Total	29 (100)	21 (100)	50 (100)

Fisher's exact test p=0.37

Figure 20 *Log urinary ATP levels amongst patients and controls*



5.13 Discussion

Recruitment allocation for dropout fell short for both groups but statistical power was maintained. Sixty percent of eligible patients that declined study entry cited problems with mobility and difficulties travelling. Nonetheless, over half of enrolled patients had progressive disease at the time of study entry. The recruitment of control subjects was retarded by the necessity for adequate matching.

The study groups demonstrated no differences in key demographic characteristics except ethnicity. The MS group comprised exclusively Caucasians, which reflects the epidemiological characteristics of MS. The control group demonstrated an ethnic mix that was very close to that described in the population of Greater London, from which the participants were sampled (395). Equal numbers of patients and control subjects described a history of lower urinary tract instrumentation. Only patients reported rUTI and current ISC use. Patients with MS and OAB demonstrated widespread urgency symptoms associated with multiple other LUTS and the impact of these symptoms on QoL was significant.

The primary outcome measure of bacterial growth derived from quantitative culture of the spun urinary sediment demonstrated greater bacterial growth amongst patients. Nonetheless, visual assessment of the distributions demonstrates that bacterial growth was common amongst patients and controls. By contrast, routine laboratory culture was unable to discriminate between patients and controls, with similar proportions of positive cultures in each group. These results illustrate the problems of standard culture techniques, constrained by diagnostic thresholds. These findings provide yet more evidence of the pervasive influence of fixed culture thresholds in the exploration of UTI.

In common with earlier work (266), the dominant infecting isolates differed by study group. *E. coli* was the most prevalent species amongst patients, and was the dominant infecting organism in five samples. *Enterococcus faecalis* was the second

most common species, recovered from two patient samples. Coagulase-negative staphylococci (CoNS) were isolated from five patients samples, although every sample yielded a distinct species. Other dominant bacteria included three species of corynebacteria, and one sample that demonstrated *P. mirabilis*. Amongst control subjects, *S. agalactiae* was the dominant bacterium in all but one of the positive cultures, with *Staphylococcus haemolyticus* isolated in the remaining sample. This is in contrast to a previous analysis that reported Lactobacilli as the most prevalent organism amongst asymptomatic controls (266).

The emergence of *E.coli* as the most prevalent infecting organism amongst patients mirrors epidemiologic data gleaned from the study of acute UTI in women (212). *Escherichia coli* are enteric organisms that colonise the perineum and vagina, initiating infection of the urinary tract by an ascending mechanism (396). Uropathogenic *E. coli* demonstrate a host of virulence factors, most notably adhesive structures including the Type 1 pilus, associated with urothelial cell attachment and internalisation (214, 215). Secreted toxins facilitate urothelial and immune cell damage (397). Intracellular entry and toxin-mediated tissue destruction liberates host nutrients, whilst siderophores scavenge iron, essential for bacterial growth (398).

Enterococcus faecalis, another gut commensal (399), was the second most prevalent bacterial species noted in patient cultures. In addition to the production of exotoxins and enzymes, enterococci are known for their ability to form biofilms that mitigate immune and pharmacological attack (400). Biofilm formation is dependent on pilus expression but other cell surface aggregation molecules contribute to this process (401, 402). These same molecules are thought to mediate intracellular entry into intestinal epithelial cells (403) but until recently, similar invasive capability had not been demonstrated in the human bladder.

Using a human urothelial cell culture system in this unit, Horsley (2013) demonstrated evidence of intracellular colonisation by *E. faecalis* (404). These findings are of particular interest, as the bacterial inoculum used in the experiments

was isolated from a patient who participated in this study. The patient demonstrated chronic OAB symptoms associated with a florid pyuria but no evidence of the organism on routine laboratory culture.

Staphylococci were a frequent isolate amongst patient samples. Positive cultures included a *staphylococcus aureus*, and five species of coagulase-negative staphylococci (CoNS). The primary habitat of *S. aureus* is the moist squamous epithelium of the nasal passages (405) although the organism also colonises the axillae in humans. Coagulase-negative staphylococci are widely distributed on skin, although some species exhibit tropism for specific sites (406). These organisms were long considered harmless commensals but are now recognised as important pathogens of the urinary tract and other systems (212, 406).

Staphylococcus saprophyticus is widely recognised as a leading cause of acute UTI in young women (164) although other CoNS are frequently isolated from infected urine samples (407, 408). In common with other uropathogens, virulence factors mediating cell adhesion and the formation of biofilms are highly prevalent amongst these bacteria (406). *Staphylococcus aureus* is perhaps the best known of the pathogenic staphylococci, a consequence of its vast array of virulence factors and the emergence of multi-drug resistant strains (409).

In humans, *Proteus spp.* are normally found in the gastrointestinal tract (410). *Proteus mirabilis* was named after Proteus, a sea-god and shapeshifter from Homer's 'Odyssey' (411). Proteus bacilli are able to differentiate into much larger multinucleate, flagellated organisms that demonstrate swarming motility. Whilst *P. mirabilis* demonstrates other virulence traits (412), the capability to swarm across biological and inert surfaces is a key virulence factor. The attenuation of *P. mirabilis* flagellar assembly is associated with a significant reduction in urinary tract colonisation and infection in a murine model (413).

Proteus mirabilis infection is associated with urinary crystal formation, a result of urease activity that elevates the urinary pH in the immediate vicinity of the

organism. This precipitates calcium phosphate and magnesium-ammonium phosphate into the urine (414). Bacterial capsular polysaccharides enhance crystal aggregation by binding metal cations, hastening the formation of urinary tract stones or mineral-rich biofilms (415). *Proteus mirabilis* has been specifically implicated in biofilm formation associated with urinary catheters systems and other indwelling devices in the urinary tract, causing chronic infection and outflow obstruction (416). Whilst *P. mirabilis* biofilm formation has been demonstrated in prostatic tissue (230-233) there are no reports of urothelium-associated biofilms in human subjects. Adherence of *P. mirabilis* organisms to urothelial cells in a murine model has been observed (417) but no data demonstrate the existence of mature *Proteus spp.* biofilms in vivo.

Corynebacteria are common commensals of the skin and mucus membranes in humans (396). *Corynebacterium urealyticum* is most commonly isolated from urine specimens (418, 419), although other species including *Corynebacterium striatum* may cause UTI (420). *Corynebacterium urealyticum* is a potent urea-splitting organism, and can be associated with urinary tract calculi and alkaline-encrusted cystitis, a chronic and often destructive infection of the lower urinary tract (421). Dense urothelial adherence of *C. urealyticum* has been observed in human cells harvested from urine (422) but the mechanism by which such adhesion occurs has not been fully described (423).

Streptococcus agalactiae (Group B streptococcus) was particularly prevalent amongst control subjects, accounting for all but one of the dominant isolates recovered from control urine samples. *Streptococcus agalactiae* is a common commensal of the urogenital and gastrointestinal tracts and has been isolated from 10-30% of healthy adults (424). Nonetheless, *S. agalactiae* has recognised uropathogenic potential and has been isolated from the urine of subjects with acute UTI (164).

The dominance of GBS as the primary isolate amongst controls contrasted with earlier data reporting Lactobacilli as the most prevalent isolate amongst the

asymptomatic (266). This finding might be explained by the considerable youth of the control group in that study (mean age=29; *sd*=12) when compared to the asymptomatic volunteers included in this work (mean age=50; *sd*=10). *Lactobacillus spp.* is a frequent coloniser of the urogenital tract amongst premenopausal women but its prevalence falls after the menopause (425).

Bacterial and host factors contribute to the virulence of uropathogens. Organism traits that confer virulence include the expression of adhesins, biofilm formation, the secretion of toxins and enzymes that cause tissue destruction, and systems that scavenge nutrients and iron. Uropathogens may also be capable of subverting the immune response and using morphological plasticity to avoid phagocytosis (214, 221). Toll-like receptor polymorphisms in the host may render some individuals more susceptible to bacterial infection than others (219, 220).

Whilst the presence or absence of specific virulence factors might explain many features of the host-microbe relationship, it seems likely that this exchange is much more complex. Purinergic signalling has been implicated in shaping the immune response but a complete understanding of this process remains elusive (326-329). The release of ATP as a danger signal could moderate the immune response to bacteria in the urinary tract. Other regulatory molecules have been proposed but the limited data are conflicting (218, 313, 426).

Specific bacterial serotypes have promoted as indicators of pathogenic potential. Some UPEC serovars express a wider array of virulence factors than intestinal colonisers (427) but the influence of individual serotypes on UPEC virulence requires further study. Data for Group B streptococci (GBS) illustrate that an extensive range of serovars can be associated with symptomatic UTI (428). At present, serotyping cannot reliably assess the pathogenic potential of urinary pathogens, so other indicators of the microbe-host interaction must be sought.

Fourteen percent of patient cultures and five percent of control cultures demonstrated polymicrobial growth. Whilst mixed growth cultures have long been

considered a result of contamination related to poor sampling or inadequate storage, this view has been convincingly challenged (194-196). Assumptions about the significance of polymicrobial growth are a vestige of Koch's postulates. These criteria are being revised in the face of new insights (429). The polymicrobial nature of biofilms, widely distributed in nature, serve as a reminder that communities of different bacterial species may cohabit to gain a survival advantage (430).

Measures of urothelial inflammation were significantly higher amongst patients than asymptomatic controls. None of the control subjects demonstrated pyuria at presentation. Whilst IL-6 levels were lower than those reported in a prospective study of acute cystitis in women (258), differences in experimental methods might account for this. A high sensitivity human IL-6 ELISA (R&D Systems, Abingdon, UK) with a detection range of 0.156-10 pg ml^{-1} was used for these experiments (although the minimum detectable concentration is cited as 0.04 pg ml^{-1}) (431). In the prospective study of acute cystitis (258) a Human IL-6 DuoSet™ (R&D Systems, Minneapolis, USA) ELISA was used, with a published detection range of 9.38-600 pg ml^{-1} (432). This is well outside the majority of reported IL-6 values in the paper (258). This could explain the absence of any detectable rise in cytokine levels in the days leading up to acute UTI, despite the establishment of pyuria and bacteriuria. Alternatively, escalating IL-6 secretion may be a late feature during the evolution of acute infection. Whilst there was no expectation that patients with MS and LUTS would demonstrate a cytokine response of similar magnitude to that seen in acute infection, differences in experimental conduct preclude any meaningful comparison.

The increased expression of urothelial cells in patient samples could represent additional evidence of immune activation related to bacterial infection. Accelerated exfoliation of infected urothelial cells has been demonstrated in the murine model of UTI and human bacterial cystitis (387-389). Catheter sampling in patients should have reduced contamination from the urethra and lower genital tract that could have influenced the results. Control samples did not demonstrate any epithelial cells, providing reassurance that clean-catch MSU sampling was associated with a very low

risk of contamination. These findings are strengthened by similar results for leucocyte expression amongst controls.

No differences in the proportion of urothelial cells colonised by bacteria were demonstrated between the groups. This is in contrast to two studies that demonstrated increased colonisation of shed urothelial cells in patients with LUTS compared with controls (271, 272). In both of these studies, control subjects were much younger, and the control groups included a greater proportion of male subjects. These differences could be a result of sample characteristics, insufficient statistical power related to the sample size in this study, or differences in the pathophysiology of LUTS between patients with MS and otherwise healthy adults.

There was no evidence of differential expression of urinary ATP amongst patients and controls. Scrutiny of the crude data suggested that urinary ATP levels only demonstrated marked increases in association with very significant urothelial inflammation. Nonetheless, in some patients who demonstrated other features suggesting marked lower urinary tract inflammation, ATP appeared to be paradoxically low.

Physiological data from human tissue biopsy studies have reported augmented purinergic neurotransmission in patients with lower urinary tract disorders including OAB (67-75, 92, 93). Data from human urothelial cell culture experiments have also demonstrated purine receptor upregulation in response to bacterial LPS and cytokine stimulation (262). Physiological data from human urothelium harvested from patients with OAB has demonstrated markedly increased basal ATP secretion (92, 93). Basal levels of ATP production in OAB biopsies were at least 20 times higher than control biopsies. Tissue stretch was associated with a two-fold rise in ATP production amongst patient and control samples.

The failure to identify elevated urinary ATP in patients with MS and OAB in this study may relate to numerous factors. The role of purinergic signalling in patients with MS and OAB may be different to that associated with OAB in otherwise healthy subjects.

The sample size may have constrained the power of the study to detect a difference in ATP between the groups. These data are cross-sectional and it is plausible that within patients, the expression of ATP may vary in response to host damage or distress. Only longitudinal sampling could identify such a relationship.

The strengths of this study include a carefully matched control group to attenuate the influence of confounding variables. Previous studies exploring the relationship between urinary infection and the generation of OAB symptoms were affected by inadequate matching of demographic characteristics amongst patients and controls. Whilst this work explores the association between infection and OAB symptoms in patients with MS, this work represent the first carefully controlled data scrutinising the relationship between these variables in any patient group.

Secondary outcome measures were carefully selected to explore immune activation and inflammation from different perspectives. Multiple variables examining similar outcomes were avoided. Existing studies, particularly those that examine cytokine responses in OAB, demonstrate significant variable multiplicity and inadequate matching of control subjects (315, 316). The use microarray of analysis to screen for huge panels of cytokines is common, often without any attempt to control the familywise error rate. In this study, a single measure was used to examine each different aspect of the host-organism relationship, and a Bonferroni-Holm correction was employed to reduce the risk of a type 1 error.

The use of different urine sampling strategies in the study groups could have affected the results. Whilst it is possible that the GBS positive cultures in the control group could be a result of contamination from the genital tract, existing data support the reliability of clean-catch MSU sampling in ambulatory adults (187). The higher prevalence of GBS positive cultures in this study compared to other work (276) might be accounted for by the decision to reject diagnostic thresholds entirely in this analysis. There is no evidence that the culture medium used in the study favoured preferential growth of any individual organism isolated in this work (286-289).

The assessment of bacterial adhesion to urothelial cells could have been affected by sampling strategy. Bacterial contamination from the lower genital tract could have liberated organisms into the urine that subsequently multiplied and colonised previously uninfected urothelial cells in the sample. The immediate refrigeration of sampled urine and the processing of these samples within one hour of collection reduce this risk.

The available evidence suggests that MSU sampling does not significantly influence leucocyte enumeration when compared to the use of a CSU (380, 381). Whilst genital contamination is possible, the absence of leucocytes and urothelial cells in control samples militates against this. There is no evidence that the use of catheterisation increases cell salvage from the bladder. The influence of catheter sampling on ATP or IL-6 levels in urine is unknown. Whilst ATP could be liberated as a result of direct trauma during catheterisation, IL-6 is a transcription product. Thus, the production of IL-6 would be extremely unlikely to reach significant concentrations in the urine during catheterisation and sample collection.

In this study, the only demographic differences amongst the study groups were ethnicity, the current use of ISC, and a history of rUTI. The ethnic profile of the patient group, which was exclusively white British, reflected the epidemiological characteristics of the disease (4). Multiple sclerosis is uncommon in Afro-Caribbean and Asian populations. Thirty-one percent of patients were using ISC at enrolment, comparable to a rate of 26% reported by a large population-based study from North America (433). Twenty-eight percent of patients described a history of rUTI, which is similar to the rate of infection reported in the literature (55).

It could be argued that the use of ISC is responsible for the increased rates of bacterial infection amongst the patients in this study. Proponents of this argument would uphold that a sample of patients who did not use ISC or describe rUTI would provide more reliable data. This argument could paradoxically introduce bias, as the use of ISC and the prevalence of UTI are themselves a consequence of MS. Any

attempt to modify the sample to correct for these variables could increase the probability of a type 2 error and attenuate the external validity of the data (434). Whilst the study was not powered for the purposes of a subgroup analysis, no differences in bacterial colonisation (U=47.00; p=0.32), routine culture results (p=0.53), pyuria expression (U=53.00; p=0.53), ATP (U=68.00; p=0.83) or IL-6 (U=71.00; p=0.70) were demonstrated when patients who used ISC were compared to those that did not.

This study demonstrated that there was a statistically significant difference in the distribution of log bacterial growth amongst patients and controls. Despite this observation, bacterial growth was relatively common in both groups. By contrast, the distribution of infecting taxa amongst the study groups was distinct. The increased expression of inflammatory markers in patient samples points to greater microbe-host interaction. The relationships between these measures and symptoms will be further explored in the next chapter.

6 The relationship between urothelial inflammation, bacterial infection, and symptom generation in patients with overactive bladder symptoms and multiple sclerosis

6.1 Background

Analysis of cross-sectional data has demonstrated that the distribution of bacterial growth amongst MS patients with OAB symptoms and asymptomatic controls is distinct. Nonetheless, these data indicate that the presence of bacteria in sampled urine amongst patients and controls is commonplace. Moving away from quantitative assessment, the quality of infecting organisms in the two groups was distinct. These ecological differences were associated with evidence of increased urothelial inflammation amongst patients.

Historically, it has been assumed that the bladder is a sterile environment. A urine culture reported as 'negative' is not synonymous with sterile urine. The use of dichotomous culture thresholds that classify UTI in binary terms have likely propagated this misconception. Our understanding of the bacterial ecology of the bladder has been heavily influenced by these quantitative diagnostic criteria. The majority of laboratories in the UK dismiss any organism that has not grown a recognised urinary pathogen in pure culture at $\geq 10^5$ cfu ml^{-1}. Polymicrobial growth is considered clinically insignificant. Bacteria that fail to satisfy these requirements are overlooked.

The term microbiota is defined as all of the microorganisms that exist in a specified habitat or environment. The microbiome has no agreed definition, but relates to the specified environment as a whole, including host and microbial genetic material, their products and local conditions (435). The influence of the microbiota of human systems on health is best described in the gut, where changes in the composition of polymicrobial communities have been linked to significant disease (436). The microbiota of the bladder remained unexplored until recently, although several groups have now published data on the ecology of the lower urinary tract (437-444). Notably, the bladder was not included in the Human Microbiome Project which was initiated in 2008 (445).

Whilst data are limited, two published reports have explored the microbiology of women with OAB symptoms compared with control subjects (442, 443). One hundred and eighty women were recruited into these studies, with approximately equal groups of patients and controls. There was no matching of study groups. Significant differences in the bacterial ecology of the lower urinary tract were reported amongst patients and control subjects, although the bacterial profiles varied between studies.

One other report has examined the microbiology of patients with neurogenic LUTD and healthy controls (438). The age and gender of the study groups were comparable. One-third of patients in the study used ISC or an indwelling catheter. *Escherichia spp., Klebsiella spp.* and Enterococci were reported as the three most common isolates amongst patients with neurogenic bladder dysfunction. These bacteria were significantly more prevalent amongst patients than controls. Whilst direct comparisons are difficult, these data are similar to the findings reported in this work.

Enhanced culture techniques have been deployed to characterise the bacterial ecology of the lower urinary tract. These methods use multiple culture media and extended incubation under a variety of environmental conditions (442, 443). Compared to standard methods, a significant improvement in performance has been reported. Whilst the adoption of more complex culture systems would be expected to increase bacterial yield, some viable bacteria in the lower urinary tract cannot be cultured, even in optimal conditions (446). This 'viable but not culturable' (VBNC) state is well recognised and many bacteria may enter the VBNC state in response to environmental stressors (447, 448). Cell damage, nutrient deficiency, excursions of temperature, and changes in oxygen tension or osmotic gradient may promote the adoption of a VBNC state.

In the context of these data and the limitations of standard culture techniques, non-culture methods have been promoted as the natural successor to urine culture. Whilst fluorescence in-situ hybridisation (FISH) has been successfully employed in

the detection of urinary tract bacteria (449, 450) nucleic acid-based techniques (NAT) have been used in the majority of published studies which examine the microbiota of the urinary tract (437-441, 444). These techniques target the bacterial 16S ribosomal ribonucleic acid (rRNA) gene that may permit species-specific bacterial identification. The RNA is then amplified using PCR and the base sequence analysed to identify the bacterium. High-throughput sequence analysis can read thousands of sequences in parallel, reducing processing time and ultimately cost. These advances, coupled with improved bioinformatic software support, have made these techniques more accessible for researchers. Quantitative methods of analysis can also supplement the taxonomic data generated by culture-independent techniques.

Whilst molecular methods of analysis undoubtedly represent a technological advance in microbiological exploration of the lower urinary tract, their application in this field has been limited. Non-culture techniques have been deployed (437-441, 444) but direct comparisons with culture-based methods are scarce. In one study, high-throughput sequencing was compared with routine and enhanced culture methods in the analysis of urine (443). Enhanced culture methods, using multiple media and incubation conditions, demonstrated bacteria in almost 80% of samples. Sequencing isolated bacterial genes in only 65% of samples. Over a quarter of samples that did not yield bacteria using culture-independent analysis demonstrated a positive cultures. Six percent of samples that were culture-negative demonstrated evidence of bacteria when sequenced.

Another study used similar culture methods to those employed in this comparative analysis and recovered bacteria from a similar proportion of samples (442). A study of urine culture and genetic sequencing in patients with rUTI demonstrated similar dominant taxa (444). Variable rates of bacterial recovery using non-culture techniques are reported in the literature, likely a result of method selection and sample characteristics (437-444). It seems improbable that the failure to recover bacterial genetic material from the lower urinary tract represents a true ecological void.

The sensitivity of enhanced culture techniques used in these analyses is consistently reported as superior to standard culture methods. This is not unexpected, as the use of multiple growth media and diverse culture conditions ought to improve performance. However, the enhanced and standard culture protocols used in these studies deserve closer scrutiny. The standard culture methods employed a detection threshold of 1000 cfu ml^{-1} whilst the enhanced culture reported any growth of an organism (442, 443). This difference in the reporting threshold precludes meaningful conclusions being formulated relating to the utility of more complex culture media and conditions.

One of these studies compared culture without a detection threshold and sequencing analysis (443). Both methods failed to identify any bacteria in a significant proportion of samples. The bacterial profiles generated by both techniques were similar, although sequencing identified a greater number of taxa overall. This is consistent with other studies that have examined urinary microbiota using culture-independent techniques (437-441, 444). Whilst the detection of greater bacterial diversity afforded by molecular techniques has been welcomed, the implications for researchers need to be considered.

Although non-culture methods are capable of generating large amounts of bacteriological information, the clinical application of these data poses new challenges. Examining causation will become more complicated as the number of previously uncultivable organisms rapidly expands. More importantly, existing culture-independent data demonstrate significant variation amongst samples and between studies. Until more data are generated on a large scale, the existence of comparable bacteriological profiles amongst different groups cannot be assumed. Non-culture methods promise a more comprehensive description of the microbiology of the lower urinary tract but whether these data will prove to be of benefit to patients remains to be determined.

These data are relevant to this work, as proponents of molecular diagnosis would argue that culture-based methods should be confined to the history books. At the

current time, there is no direct evidence that non-culture methods should be ubiquitously deployed in studies that explore the ecology of the lower urinary tract and urological disease. Indeed, the baseline microbiological analysis reported in this work supports the utility of culture-based methods. The between-group differences in bacterial ecology identified in this study were achieved using simple culture techniques and overnight incubation.

The differences in bacterial ecology and urothelial inflammation amongst patients and controls in this work were derived from cross-sectional data. These findings do not provide evidence of causation. Whilst OAB symptoms have been the target of antibiotic therapy, there remains no conclusive evidence that treatment directed at aerobic bacterial uropathogens (273) or atypical organisms (243-246) palliate symptoms. Although there are biologically plausible mechanisms by which bacterial infection and urothelial inflammation might be implicated in the overactive bladder, the interaction between these variables remains undetermined. Specifically, no controlled, longitudinal data have been reported.

In order to determine the relationship between bacterial infection and symptom generation, prospective data were collected from patients with MS and OAB symptoms and asymptomatic controls. The study groups were carefully matched for key demographic characteristics. Statistical analysis was conducted within a mixed models framework to allow analysis of fixed and random effects. This technique permits the influence of variation amongst individual subjects and visits to be modeled in addition to fixed effects or predictor variables.

6.2 Study overview

A blinded, prospective cohort study was undertaken to explore the relationship between bacterial infection, markers of inflammatory and immune activity in the lower urinary tract, and urinary symptoms. Adult patients with MS and OAB symptoms, and asymptomatic control subjects, were invited to participate. These data represent an extension of the analysis reported previously (5).

Patients and controls attended a screening visit to complete the consent process and check eligibility. Subject to successful screening, participants were required to attend eight study visits, conducted every 12 weeks. Symptoms were assessed using validated questionnaires, and measures of lower urinary tract function derived from bladder diary data. At each visit, patients provided a CSU and control subjects submitted a clean-catch MSU. Urine was subject to analyses that scrutinised the microbiology of the lower urinary tract, and the inflammatory and immune response of the urothelium.

The study was conducted at the Department of Medicine, UCL Archway Campus. This study was approved by the National Research Ethics Service (NRES) Committee London, Queen's Square (Ref: 10/H0716/84).

6.3 Safety considerations

Patients provided CSU samples for analysis. Catheterisation can sometimes be associated with minor discomfort, and rarely UTI (5.3).

6.4 Study objectives

6.4.1 Primary objective

- To evaluate the relationship between microscopic pyuria and bacterial infection characterised by quantitative culture of the spun urinary sediment.

6.4.2 Secondary objectives

The secondary objectives of the study were to determine the following:

- To explore the relationship between markers of urothelial inflammation, immune activation, and bacterial infection.
- To compare the results of routine laboratory culture with culture of the spun urinary sediment.
- To explore the relationship between urothelial inflammation, symptom generation, and lower urinary tract function.

6.5 Study population

6.5.1 Recruitment of participants

Patients and control subjects were recruited as outlined previously (2.2.2).

6.5.2 Consent and eligibility

All participants provided written, informed consent prior to any study related procedures, and eligibility was checked before inclusion (2.2.3).

6.5.3 Inclusion and exclusion criteria

Inclusion and exclusion criteria have been previously described (5.5.3).

6.5.4 Study restrictions and concomitant medications

There were no restrictions on the use of anticholinergic drugs, DDAVP and tricyclic agents commonly employed in the treatment of LUTS. The use of all concomitant medication was recorded in the study documentation. Any change to regular medication was documented at each visit. This included all prescription drugs, over-the-counter medications, herbal remedies, vitamins, minerals and supplements.

6.5.5 Discontinuation of subject participation

Patients could be withdrawn from the study at the discretion of the Chief Investigator. The criteria for subject withdrawal included: (1) non-compliance with the requirements of the protocol; (2) an adverse event after which continued participation would present an unacceptable risk; (3) withdrawal on medical or administrative grounds; (4) if continued participation would not be in the patient's best interests; (5) pregnancy. Patients could withdraw their consent to participate at any time and this would not affect their clinical care.

6.5.6 Treatment of urinary tract infection

Patients participating in the study were closely monitored for evidence of UTI. Urinary tract infection was diagnosed if a patient reported symptoms or signs indicative of UTI, microscopic pyuria or a positive routine urine culture. Evidence of urinary infection was reported to the Principal Investigator, Professor Malone-Lee, who advised on treatment. All episodes of UTI were recorded in the study documentation and the patient's general practitioner was informed.

6.6 Study design

6.6.1 Summary of study design

This was a blinded, prospective, observational cohort study of adult patients with MS and OAB symptoms, and asymptomatic control subjects. The study design is summarised in **Figure 21**.

6.6.2 Duration of the study

The expected duration of the study from screening to the end of blinded treatment was approximately 84 weeks.

6.7 Blinding

All study samples were identified only by a three-digit study number.

6.8 Study procedures

6.8.1 Time and events schedule

A time and events schedule is presented in **Figure 22**.

Figure 21 *Schematic of study design.*

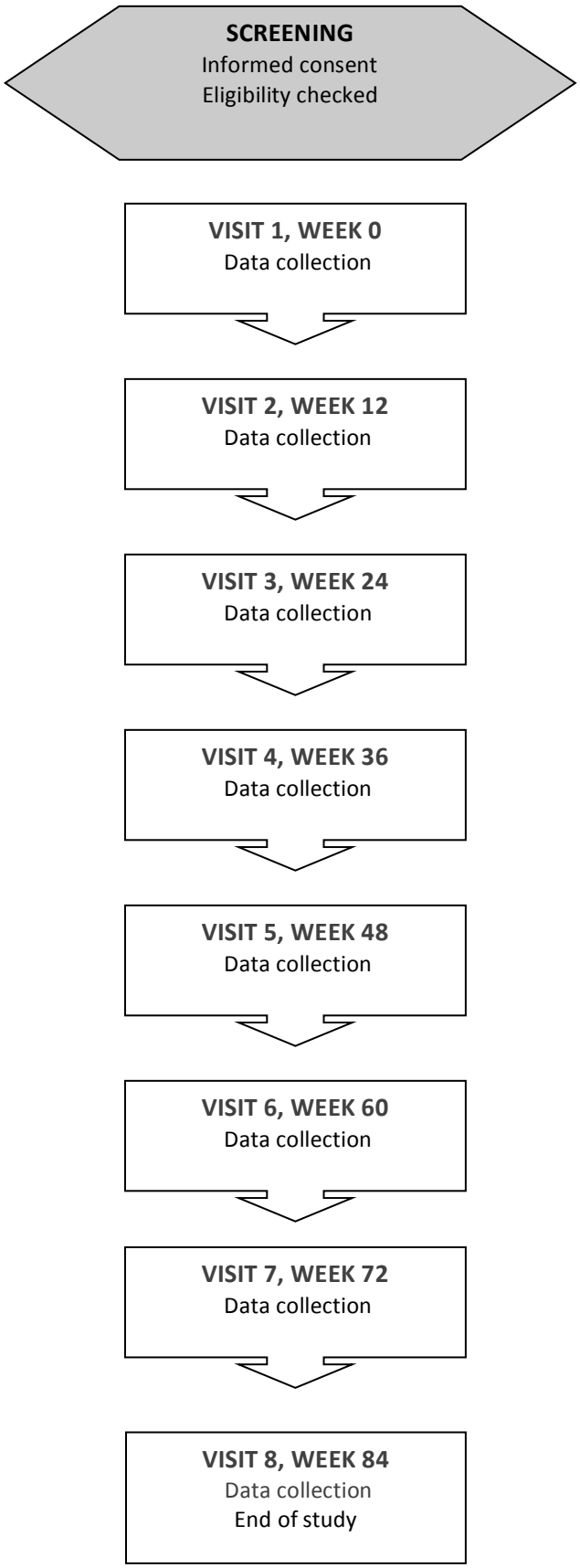


Figure 22 *Time and events schedule.*

	Screening	Study visits								
		Visit 1, Week 0	Visit 2, Week 12	Visit 3, Week 24	Visit 4, Week 36	Visit 5,Week 48	Visit 6, Week 60	Visit 7, Week 72	Visit 8, Week 84
Informed consent	X	X	X	X	X	X	X	X	X
Eligibility criteria	X	X	X	X	X	X	X	X	X
Medical history	X	X	X	X	X	X	X	X	X
Urine sample, exploratory		X	X	X	X	X	X	X	X
Bladder diary collection*		X	X	X	X	X	X	X	X
Urgency score		X	X	X	X	X	X	X	X
Pain score		X	X	X	X	X	X	X	X
ICIQ-LUTS		X	X	X	X	X	X	X	X
ICIQ-LUTSqol		X	X	X	X	X	X	X	X
Concurrent medications	X	X	X	X	X	X	X	X	X
Adverse events	X	X	X	X	X	X	X	X	X

**Bladder diaries were collected from patients only.*

6.8.2 Screening

Patients and control subjects who wished to participate were sent a patient information sheet and then contacted by phone. Patients who attended were required to provide informed consent prior to any study related procedures. A full medical history was taken and concurrent medications were recorded. Eligibility was checked. A bladder diary was provided for completion and the first study visit scheduled.

6.8.3 Study visits

All study visits were identical in the patient-related activities undertaken and data collected. Eligibility and consent were checked. Changes to medication and any adverse events were recorded. The following patient-reported measures were used to assess bladder symptoms:

- Whittington Urgency score.
- Whittington Pain score.
- ICIQ-LUTS.
- ICIQ-LUTSqol.

A urine sample was collected and subject to analysis in accordance with the protocol. A bladder diary was provided for completion and the next study visit was scheduled. On the final visit, the patient left the study.

6.9 Clinical and laboratory assessments

Clinical and laboratory assessments have been previously described (5.8).

6.10 Adverse events

Adverse event data were reported as outlined previously (2.5).

6.11 Data management

The data management strategy for the study has been previously described (5.10).

6.12 Statistical methods and analysis

6.12.1 Study population

The study population included all patients and control subjects. Only outcome data for participants who attended a minimum of four study visits was included in the statistical analysis.

6.12.2 Sample size calculation

The primary analysis evaluated the association between microscopic pyuria and bacterial infection in the study population. Whilst the occurrence of microscopic pyuria was expected to fluctuate, data from 4641 patient attendances in this centre suggested that pyuria was evident at approximately 50% of visits. A previous comparative study reported the standard deviation of the log bacterial count amongst patients with OAB and asymptomatic controls as 2.0 (sd=2.0). The standardised mean difference in log bacterial counts was 0.9 (f=0.9). An intraclass correlation of 0.7 was imputed for the sample size calculation.

Power was fixed at 80% to detect a significant difference in log bacterial growth at the 5% level (α=0.05). A sample size of 10 subjects in each group would permit the detection of a significant within-group difference within a repeated measures

design. The recruitment of 20 subjects in each arm was required to power the baseline calculations, allowing the analyses described here to be achievable within the confines of the study population.

6.12.3 Statistical methods

The repeated measures design generated hierarchical data. Visits were nested within individuals, and individuals were assigned to one of two study groups. All analyses were conducted using linear mixed modelling. Normality was evaluated by graphical assessment of Pearson residuals. Standardised residuals were plotted against predicted values to assess homogeneity of variance. Collinearity amongst independent variables was checked by assessment of the variance inflation factor and collinearity diagnostics. A Bonferroni-Holm correction was made to account for variable multiplicity in the secondary analyses (394). No corrections were made for missing data.

The primary analysis included two comparisons of interest. In both cases the dependent variable was the log bacterial count characterised by quantitative culture of the spun urinary sediment. The first of these comparisons used only the data generated from the MS group. The magnitude of bacterial growth was calculated when the patients demonstrated pyuria and when the urine was clear. A second comparison included all of the data and reported bacterial growth amongst controls and cases when pyuria was present.

The secondary analyses were conducted to evaluate the relationship between markers of urothelial inflammation, immune activation, bacterial growth, urinary symptoms, and lower urinary tract function. In addition, culture of the spun urinary sediment was used to determine the magnitude of bacterial growth in routine laboratory cultures reported as positive and negative.

Continuous data that were analysed outside the mixed models framework were assessed for normality using graphical methods. Data that were not normally distributed were subject to transformation to permit the use of parametric analysis methods if a normal distribution was produced. Non-parametric tests were employed in all other cases. Data were summarised using standard descriptive statistics.

6.12.4 Primary analysis

- Difference in bacterial growth amongst patients when microscopic pyuria was present and when the urine was free of pyuria.
- Difference in bacterial growth amongst patients and controls when microscopic pyuria was present.

6.12.5 Secondary analyses

- Relationship between bacterial growth and markers of urothelial inflammation, immune activation, urothelial distress, and bacterial colonisation of urothelial cells amongst patients and controls.
- Bacterial growth, derived by culture of the spun urinary sediment, in positive and negative routine cultures, amongst patients and controls.
- Relationship between bacterial growth, urinary symptoms, and lower urinary tract function amongst patients.

6.13 Results

Between February 2011 and June 2012, 62 patients and 22 asymptomatic control subjects were screened for study inclusion. Twenty-nine patients (F=26; M=3; mean age=54; *sd*=10.5) and 21 asymptomatic control subjects (F=15; M=6; mean age=50; *sd*=9.9) were recruited. The study groups were matched for key demographic

characteristics. Baseline attributes and disease-specific data have been presented previously (5.12).

There were 226 protocol deviations. Mistimed or missed study visits were responsible for the majority of protocol deviations (*n*=148). The non-completion of bladder diaries by patients largely accounted for the remainder (*n*=67). Patients with MS were often unable to attend scheduled visits due to intercurrent illness, although fatigue, problems with mobility and transport issues were also cited.

Twenty-four patients and twenty control subjects attended the specified minimum of four study visits for data inclusion. Eight of the patients who completed the study were using ISC. Two-thirds of patients who completed the study attended at least seven visits (*n*=16). Two patients left the study as they no longer wished to participate and two were withdrawn, unable to comply with the demands of the protocol. One patient was lost to follow-up. There were no differences in any of the demographic or disease-specific variables amongst the patient population included for analysis and those who were withdrawn. The flow of participants through the study is summarised in **Figure 23**. There were no recorded adverse events during the study. Data for bacterial adhesion studies were not included in any of the analyses as the majority of the samples were damaged in transit when the laboratory moved to new premises.

None of the control subjects who participated described any urinary urgency or pain symptoms during the course of the study. Minor fluctuations in the ICIQ-LUTS and ICIQ-LUTSqol measures were noted amongst asymptomatic control subjects, mostly a consequence of variable urinary frequency that is often sensitive to fluid intake. The distribution of responses amongst patients and control subjects for the ICIQ measures are summarised in **Figure 24** and **Figure 25**.

Figure 23 *Flow of participants through the study.*

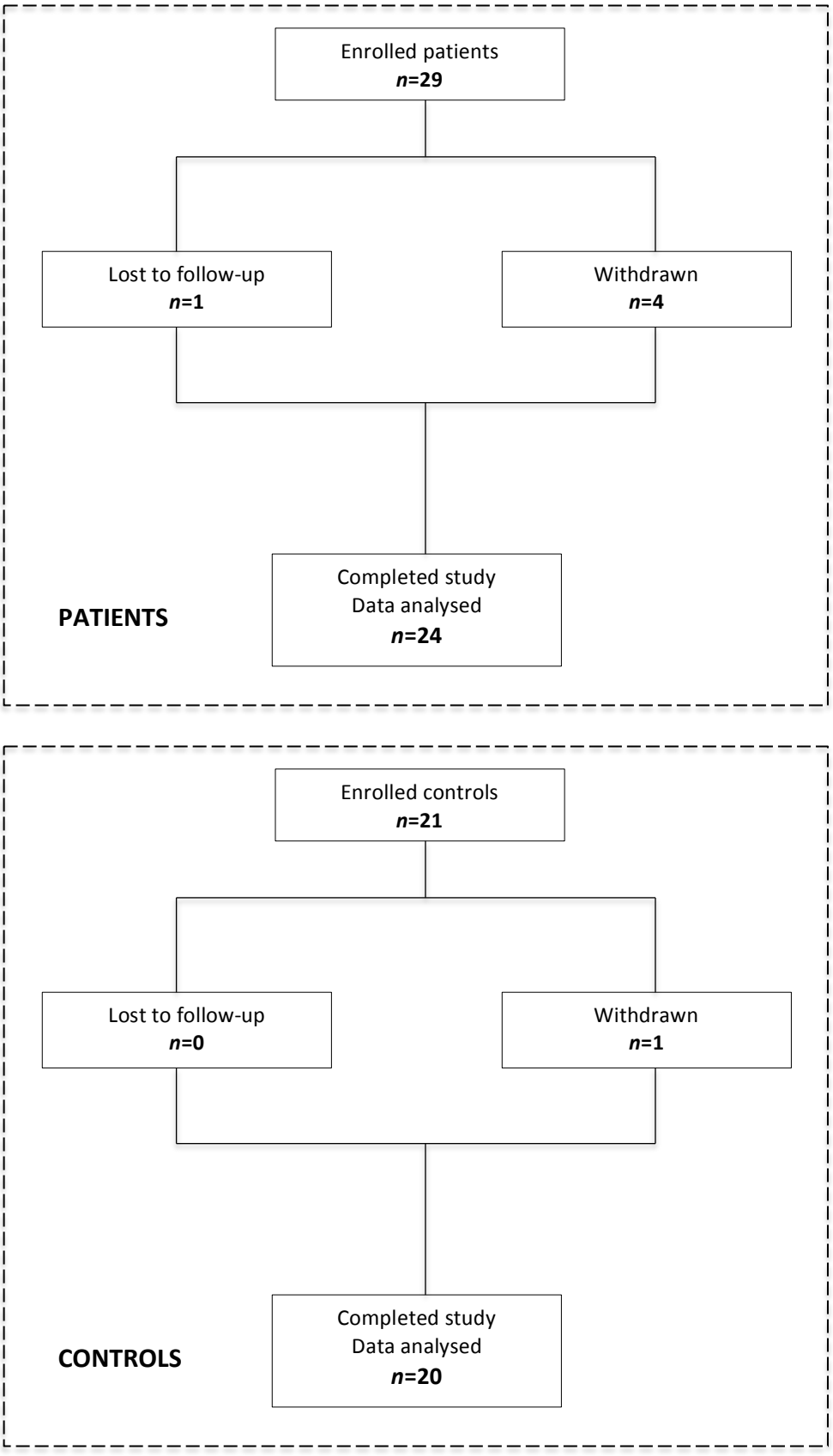


Figure 24 *ICIQ-LUTS scores reported by patients and controls.*

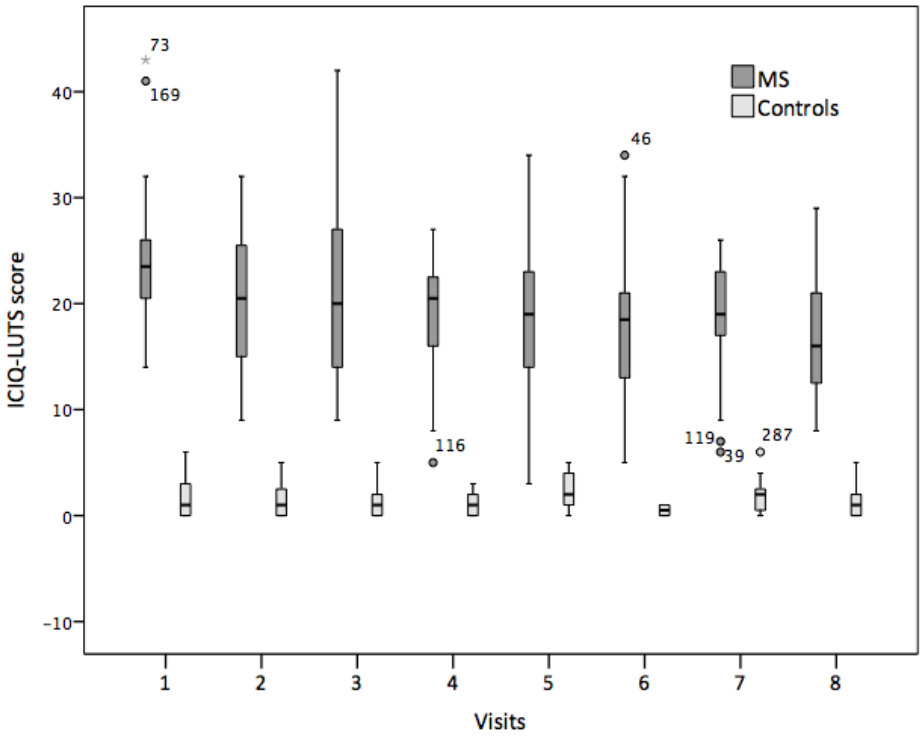
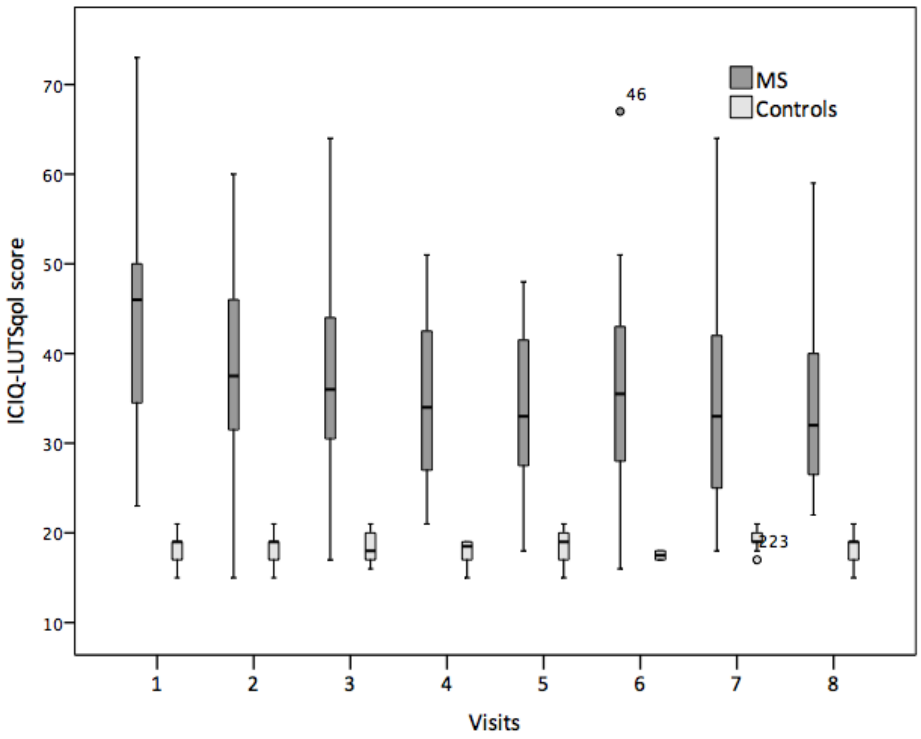


Figure 25 *ICIQ-LUTSqol scores reported by patients and controls.*



Patients who completed the study demonstrated microscopic pyuria at 40% of study visits. Only one patient had clear urine throughout the study. These findings corroborate existing data and support the assumptions of the sample size calculation. By contrast, asymptomatic controls demonstrated pyuria at only 7% of visits. Eighty-five percent of controls had clear urine at every visit.

Across the study as a whole, patients demonstrated greater bacterial growth, and higher levels of pyuria, urinary IL-6 and ATP when compared to controls. These differences remained significant despite patients receiving antibiotic treatment for urinary infection (**Table 29**).

Table 29 *Increased bacterial load and urinary inflammation amongst patients when compared to asymptomatic controls across the study.*

Dependent variable	Parameter estimate*	Significance
Bacterial growth§	0.75 (95% CI=0.19−1.30; *t*=2.66; *df*=231.98)	*p*=0.008
Pyuria count†	0.29 (95% CI=0.15−0.42; t=4.39; df=178.14)	*p*<0.0005
Urinary IL-6‡	0.29 (95% CI=0.14−0.43; t=3.90; df=203.89)	*p*<0.0005
Urinary ATP**	0.13 (95% CI=0.14−0.43; t=3.90; df=203.89)	*p*<0.0005

***Parameter estimate:** Increase in magnitude of dependent variable demonstrated by patients compared with control subjects during the study; **§Bacterial growth:** log cfu ml^{-1}; **†Pyuria count:** wbc ul^{-1}; **‡ Urinary IL-6:** log pg ml^{-1}; ****Urinary ATP:** log nmol l^{-1}.*

The first model generated for the primary analysis used only data from MS patients with OAB symptoms. Log bacterial growth characterised by culture of the spun urinary sediment was selected as the dependent variable and the presence of pyuria was selected as a fixed effect. Individuals and visits were selected as random effects. Model residuals were graphically assessed and normally distributed. There was no evidence of heteroscedasticity amongst the data. Model assumptions were satisfied.

Amongst patients with OAB symptoms, the presence of pyuria was associated with higher levels of bacterial growth (β=2.20; 95% CI=1.56–2.85; *t*=1.95; *df*=146.01; *p*<0.0005). The parameter estimate indicated that bacterial growth was 2.20 log cfu ml^{-1} greater when pyuria was present compared to when it was absent. This indicates that the emergence of pyuria is associated with a greater magnitude of bacterial growth in symptomatic patients.

A second comparison was planned to determine any differences in bacterial growth amongst patients and controls when pyuria was present. Log bacterial growth characterised by culture of the spun urinary sediment was selected as the dependent variable. The interaction between the pyuria and group membership was selected as a fixed effect. Individuals and visits were selected as random effects. Model assumptions were checked. Normality of the model residuals and homogeneity of variance were confirmed. Group membership did not have a significant effect on log bacterial growth when pyuria was present (β=-1.33; 95% CI=-3.15–0.50; *t*=-1.44; *df*=101.03; *p*=0.153). The very small number of control subjects who exhibited pyuria during the study limits the power of these findings.

The secondary objectives of the study were to examine the relationship between microscopic pyuria, bacterial growth, inflammation, immune activation, urothelial distress, urinary symptoms and bladder function.

In the first of these planned analyses, all of the data was used in the calculations. Log bacterial growth was selected as the dependent variable in the model. Log pyuria, log urinary IL-6, log urinary ATP and the log urothelial cell count were selected as fixed effects. Individuals and visits were selected as random effects. Model assumptions were satisfied. Increases in IL-6, ATP, and urothelial cell expression were not predictive of the bacterial colony count. Only pyuria was significantly associated with bacterial growth (β=1.98; 95% CI=1.55–2.41; *t*=9.32; *df*=65.51; *p*<0.0005).

A second planned analysis was undertaken to examine the influence of study group membership and routine culture status on the relationship between pyuria and bacterial growth. When these interaction terms were introduced into the analysis, the resultant model residuals were not normally distributed precluding further interpretation. The analysis was conducted again with the dependent variable and fixed effects unchanged but with the calculations confined to only patients with a negative routine culture. The results were comparable to the previous analysis and demonstrated that amongst patients with a negative routine culture, increasing pyuria is associated with increased bacterial growth (β=1.78; 95% CI=1.09–2.47; t=5.16; df=52.55; p<0.0005).

Exploratory models were constructed to explore the relationships between these variables further. Only the patient data was included. In the first of these models, log pyuria was selected as the dependent variable. Log bacterial growth, log urinary IL-6, log urinary ATP and log urothelial cell expression were selected as fixed effects. Individuals and visits were selected as random effects. The model residuals were normally distributed and there was no evidence of heteroscedasticity. Collinearity was not significant. Increasing bacterial growth (β=0.15; 95% CI=0.11–0.19; t=8.00; df=115.14; p<0.0005), IL-6 (β=0.18; 95% CI=0.02–0.33; t=2.27; df=92.75; p=0.020) and ATP (β=0.68; 95% CI=0.46–0.90; t=6.02; df=115.18; p<0.0005) were predictors of pyuria. Urothelial cell exfoliation did not correlate with pyuria.

In a second model, log bacterial growth was selected as the dependent variable. Log pyuria, log urinary IL-6, log urinary ATP and log urothelial cell expression were selected as fixed effects. The model residuals were normally distributed and homoscedasticity was confirmed. Collinearity was not significant. Only increasing pyuria (β=1.98; 95% CI=1.56–2.40; t=9.32; df=76.14; p<0.0005) was a predictor of bacterial growth. None of the other measure predicted bacterial expansion.

When these models were run using only control data, pyuria predicted bacteriuria (β=1.40; 95% CI=0.62–2.20; t=3.51; df=62.14; p=0.001) although the parameter estimate was of a slightly smaller magnitude that that calculated for patients. Model

assumptions were violated when pyuria was selected as the dependent variable, as the model residuals were not normally distributed. Numbers were small as very few control subjects demonstrated pyuria during the study.

A further analysis detailed in the statistical plan for the study was conducted to determine log bacterial growth, characterised by culture of the spun urinary sediment, amongst positive and negative routine cultures. Three hundred and fifty-two samples from patients and controls were included in the analysis. The results are reported in **Table 30**.

Table 30 *Bacterial colony counts amongst positive and negative routine cultures.*

MSU result	**Bacterial growth* log cfu ml^{-1}**		
	Median growth	IQR[§]	Range
MSU positive†	6.33	1.81	0.00−7.35
MSU negative‡	0.00	2.58	0.00−7.04

**Characterised by culture of the spun urinary sediment; †Defined as the pure growth of a recognised uropathogen at ≥10^5 cfu ml^{-1}; ‡ Polymicrobial culture or growth below 10^5 cfu ml^{-1}; IQR= interquartile range.*

Table 31 *Proportion of positive and negative cultures demonstrating any bacterial growth on spun sediment culture.*

MSU result	**Proportion of cultures (%)**	
	No growth*	Any growth*
MSU positive†	7.4	92.6
MSU negative‡	53.1	46.9

**Characterised by culture of the spun urinary sediment; †Defined as the pure growth of a recognised uropathogen at ≥10^5 cfu ml^{-1}; ‡ Polymicrobial culture or growth below 10^5 cfu ml^{-1}.*

Almost half of the negative routine cultures demonstrated bacteria when subject to culture of the spun urinary sediment (**Table 31**). These cultures demonstrated mean

growth of 2.75 log cfu ml^{-1} (95% CI=2.47–3.02). Significant bacterial infection is being missed by routine culture methods with fixed diagnostic thresholds.

An analysis to explore the relationships between bacterial infection, lower urinary tract function and urinary symptoms was specified in the study protocol. Only patient data was included. The log bacterial count was defined as the dependent variable with urgency and pain scores, ICIQ-LUTS and ICIQ-LUTSqol questionnaire responses, and bladder diary data entered as fixed effects. Model assumptions were checked but the residuals deviated significantly from a normal distribution. There was also evidence of multicollinearity amongst some of the symptom measures. Attempts to transform the data did not yield normally distributed model residuals. These violations precluded meaningful interpretation of the results using the statistical model specified in the study protocol.

An alternative statistical approach was employed in which symptom measures and bladder diary data were entered as dependent variables in individual models. Measures of urinary infection, inflammation and urothelial distress were selected as fixed effects. The analysis was limited to the urgency score and bladder diary data in order to constrain model multiplicity. Urgency is the hallmark of OAB, and although it is commonly associated with urinary frequency and incontinence, urgency remains the principal treatment target in symptomatic patients.

Urgency scores, 24-hour urinary frequency and incontinence episodes, and mean voided volume were selected as dependent variables in individual models. Log bacterial growth, log pyuria, log urinary IL-6 and log urinary ATP were selected as fixed effects. The influence of anticholinergic drugs, DDAVP and tricyclic agents (TCA) were accounted for in the models. Individuals and visits were selected as random effects. The model residuals were normally distributed and significant heteroscedasticity was excluded. There was no evidence of collinearity amongst the fixed effect variables.

Only pyuria demonstrated an association with urinary urgency and lower urinary tract function. Bacterial growth, urinary IL-6 and urinary ATP did not influence these

measures in any of the models. The findings were independent of routine culture status. Increasing pyuria was associated with worsening urinary urgency and frequency, and a reduction in voided volume, but it did not appear to influence incontinence episodes (**Table 32**). Similarly, the use of anticholinergics, DDAVP or TCA agents did not mediate any significant effects in this group of patients.

Table 32 *Influence of log pyuria count on urinary urgency, urinary frequency and incontinence episodes.*

Dependent variable	Parameter estimate*	Significance
Urgency score§	0.70 (95% CI=0.23−1.16; *t*=3.01; *df*=52.16)	*p*=0.004
Voided volume†	-38.99 (95% CI=-59.57—18.42; *t*=-3.78; *df*=73.87)	*p*<0.0005
24-hr frequency‡	2.07 (95% CI=1.17−2.98; *t*=4.60; *df*=54.44)	*p*<0.0005
24-hr incontinence‡	0.08 (95% CI=-0.02−0.17; *t*=1.60; *df*=106.65)	*p*=0.112

****Parameter estimate:*** *Change in magnitude of dependent variable for each unit increase in log pyuria;* ***§Urgency score:*** *Whittington urgency score (range 1-10);* ***†Voided volume:*** *Expressed in millilitres;* ***‡ Frequency and incontinence:*** *Episodes/24 hours.*

6.14 Discussion

Protocol deviations were frequent but minor. Mistimed or missed visits were common, but there were no concerns that such deviations affected the integrity of the data. Patients often failed to submit complete bladder diary data, although all patients completed a minimum of four diaries, as demanded by the protocol. The study groups were matched at baseline and the demographic and disease-specific variables amongst patients who left the study were similar to those who were included in the analysis. Statistical power was maintained well within the constraints of the sample size calculation. At baseline and over the course of the study, there were significant differences in terms of bacterial load and urothelial inflammation amongst patients and controls. No adverse events were reported.

The primary analysis demonstrated that amongst patients, the emergence of pyuria was associated with a significant increase in bacterial growth. This relationship was strengthened by the results of the secondary analyses. Rising pyuria was associated with increasing bacterial numbers. When the analysis was confined to patients with negative routine culture the results were very similar. These findings demonstrate that the relationship between urothelial inflammation and bacterial infection is not contingent on a positive routine bacterial culture.

These data provide evidence that the emergence of pyuria is associated with higher bacterial colony counts. As the magnitude of urothelial inflammation increases, bacterial growth increases. However, the data that describe the interaction between pyuria and bacteriuria do not appear to demonstrate a simple proportional relationship. When exploratory models were generated to scrutinise the effect of rising bacterial counts on pyuria amongst patients, the results were quite different. Using the model parameter estimates to illustrate these findings, an increase in the leucocyte count of 1 log wbc μl^{-1} was associated with a mean rise in bacterial count of approximately 2 log cfu ml^{-1}. By contrast, an increase in the bacterial colony count of 1 log cfu ml^{-1} was associated a mean increase in pyuria of 0.2 log wbc μl^{-1}.

Pyuria is a measure of urothelial inflammation. When pyuria increased it was accompanied by an increase in colony count. These data demonstrate that in the presence of lower urinary tract inflammation, increasing inflammation appears to be associated with increased bacterial numbers. When pyuria was selected as the dependent variable in the model, significant increases in bacterial growth were associated with only very small increases in pyuria expression.

These findings hint at the complex relationship between bacterial proliferation and inflammation in the lower urinary tract. In the first model, increasing urothelial inflammation is associated with increasing bacterial load. Pyuria reflects stimulation of the innate immune system and implies a pathogen-host interaction. In the second model, rising bacterial colony counts have a comparatively marginal effect on leucocyte expression. In contrast to pyuria, bacterial load is not synonymous with a

proportionate microbe-host exchange. Thus, bacteria appear to be capable of considerable expansion without eliciting a significant inflammatory response.

When the analysis was confined to control subjects only, increasing pyuria was associated with proportionately greater increases in bacterial growth. This association was comparable to the parameter estimate generated for patients. Amongst controls, the effect of rising bacteriuria on pyuria was not calculated as model assumptions were violated. The number of controls demonstrating pyuria was very small, hampering statistical efforts to evaluate this relationship.

These data seem to illustrate that although bacteria may proliferate without targeting their host, once an incursion on the host is established, further population expansion leads to increased tissue damage and consequent inflammation. Whilst the relationship between increased bacterial load and greater host damage seems intuitive, an apparent paradox exists in that marked bacterial expansion may occur without the host being attacked. If the relationship between host tissue damage and bacterial proliferation is not directly proportional, how is pathogenicity controlled? Mounting evidence suggests that quorum sensing may mediate this process.

Quorum sensing is a term that describes intercellular communication between bacteria. This process allows microbes to share information relating population numbers and preferentially modify the expression of individual genes in response to changes in cell density and community composition (451). Bacteria are microscopic organisms and individually they are unable to manipulate host systems through virulence factors, irrespective of the potency of such agents. In much greater numbers, the concerted efforts of large bacterial populations may be able to gain advantage over their host. Advances against a host, for example to secure nutrients, are associated with significant risk as bacteria face immune attack and energy expenditure. If an attack on the host is unsuccessful it may threaten the survival of the community. Quorum sensing appears to restrict such operations by constraining the expression of genes that code for virulence factors until a critical biomass is

reached (452). Such a strategy might maximise the likelihood of a successful interaction with the host.

Bacteria communicate by releasing small molecules termed autoinducers (AIs). In conditions of low cell density, these AI molecules diffuse without detection by other microbes as they have no 'near neighbours'. In conditions of high cell density, the local concentration of AIs may become extremely high, binding to receptors on adjacent cells and mediating changes in gene expression (453). In addition, the binding of AI molecules to receptor sites further stimulates AI production by bacteria, forming a positive feedback loop (454). These insights may explain the complex relationship between bacterial expansion and pyuria noted previously. Some organisms that possess potent virulence factors might successfully initiate an attack on their host at lower cell densities than microbes with lesser pathogenic potential. Such traits would be propagated within a population by natural selection.

Quorum sensing systems are not only for intra-species communication. Bacteria from different species and genera produce universal AI signalling molecules that impart information about the population density of other organisms (455, 456). Within biofilms, communication between different cohabiting bacteria might facilitate cooperation within these communities to gain a survival advantage (457). Biofilm formation in some species appears to be contingent on an intact quorum sensing system (458, 459) and identical bacterial species can express quite different patterns of gene expression within the biofilm itself (460, 461). In contrast to a symbiotic function, some microbes utilise information from quorum sensing systems to neutralise competing populations. The production of antibiotics to kill other bacteria, or manipulation of quorum sensing molecules to constrain virulence amongst competitors has been described (462-465).

No association between ATP and bacterial load was demonstrated but increases in urinary ATP were associated with greater pyuria. Adenosine triphosphate belongs to a group of endogenous molecules that are released in response to tissue damage or physiological stress. These molecules are known as danger-associated molecular

patterns (DAMPs) and are able to trigger or augment the inflammatory cascade by activating pattern recognition receptors (PRRs) on innate immune target cells. These data suggest that the primary source of purine release associated with lower urinary tract inflammation might be host cells rather than bacteria.

Urinary IL-6 had no detectable association with bacterial growth. Increased urinary IL-6 secretion has been demonstrated in prospective studies of acute UTI (258) but these increases were not evident until late in the disease course. A detectable rise in IL-6 was observed only when patients manifest dysuria associated with frequency or urgency, pyuria ≥15 wbc μl^{-1} and bacteriuria ≥10^3 cfu ml^{-1}. Whilst there are some doubts relating to the sensitivity of the IL-6 assay used in that study, the data generated suggest that significant urinary IL-6 secretion may be a late feature in acute cystitis, perhaps contingent on significant immunostimulation of the host.

Rising levels of IL-6 were associated with increasing pyuria, although the magnitude of this effect was small. Although IL-6 and CXCL-8 may be induced through TLR activation via the NF-κB signalling pathway, these molecules have distinct roles. Whilst pyuria and urinary IL-6 are indicative of urothelial inflammation, the control of leucocyte chemotaxis is not the main function of IL-6 which is a key mediator of the acute phase response (249, 466). Leucocyte chemotaxis is primarily mediated through chemokines such as the prototypical CXCL-8, although the process of leucocyte migration is complex. Vascular transmigration and movement to sites of inflammation involves numerous other chemoattractants, lipid mediators, and DAMPs (249). Interleukin-6 augments the production of chemokines such as CXCL-8 and other mediators of vascular adhesion and migration, but does not play a central role in neutrophil trafficking (466).

There is evidence that neutrophil entry into the lower urinary tract may occur independent of any detectable IL-6 response. In a prospective study, 23 patients with a history of recurrent UTI submitted serial CSU samples over periods extending to 18 months (220). In an analysis confined to periods when the patients did not report acute symptoms, bacteriuria was associated with pyuria and increased urinary

CXCL-8 production without any elevation in urinary IL-6. In another small inoculation study, eight women with a history of recurrent UTI were inoculated with UPEC (467). No association between the entry of leucocytes into the lower urinary tract and IL-6 secretion was identified. Whether these patients developed symptoms after inoculation is unclear.

Data from a prospective study of recurrent UTI in women suggests that the initial IL-6 response in patients who develop acute symptomatic UTI is pronounced (258). How this IL-6 response changes as symptomatic infection evolves or resolves remains unclear. The IL-6 response after the onset of symptoms was not reported in this work. The nature of the inflammatory response associated with bacterial infection could differ amongst patient groups with different symptomatic presentations.

Hedges (1991) did report the mucosal secretion of IL-6 following inoculation, although it is unclear whether these patients were symptomatic (467). Interleukin-6 was secreted in an intermittent fashion over the first 48 hours after inoculation, despite sustained colonisation. Further data from prospective studies of UTI are needed to corroborate these findings. Interleukin-6 production is subject to a complex regulatory system governed by membrane-bound and soluble receptors, and modulated by a host of other influences (468). The intermittent secretion of IL-6 following inoculation may reflect these regulatory systems moderating IL-6 production. It is known that some strains of UPEC have developed strategies to circumvent the immune response by suppressing cytokine responses to infection (469-471). Whether bacterial factors could influence the nature of IL-6 secretion described above is not known. Whilst the onset of acute bacterial cystitis appears to be associated with an increase in urinary IL-6 secretion (258), the nature of any continuing cytokine response requires further study. Whether IL-6 will prove to be a useful marker of disease activity in UTI is unclear.

The statistical plan for this study specified a multilevel analysis to explore the relationship between bacterial growth, urinary symptoms and lower urinary tract

function. When the analysis was conducted, model assumptions were violated precluding meaningful interpretation of the results. Whilst the magnitude of parameter estimates in mixed model analysis are unaffected by deviations from normality, the variance of these estimates are often rendered inaccurate (472). This makes interpretation of statistical significance unreliable.

The relationship between these variables was subsequently approached from another perspective. Symptoms and lower urinary tract function data were entered as dependent variables in individual models. Markers of infection and inflammation were selected as fixed effects. This approach allowed the analyses to control for the effects of pharmacological agents used to attenuate LUTS. From a design perspective, this was a deviation from the planned analysis, but the alternative would have been to abandon any assessment of the relationship between infection and the generation of symptoms. In order to minimise the number of models used to scrutinise this relationship, the dependent variables were confined to urinary urgency, which defines the OAB syndrome, and bladder diary data.

Accounting for variable multiplicity in each of the models, only pyuria demonstrated a relationship with symptoms and function. Pyuria predicted urgency scores, 24-hour urinary frequency and voided volume, although there was no association with incontinence episodes. These findings were independent of routine culture status. Whilst pyuria repeatedly demonstrated a relationship to urgency and bladder function, none of the other measures of infection, inflammation, or urothelial distress were associated with these outcomes. The consensus amongst the results provides reassurance that the use of more than one model to describe these relationships did not yield a type 1 error.

The use of anticholinergic drugs, DDAVP, and TCA agents was not associated with significant changes in urgency or bladder function data. Whilst anticholinergic medications are often advocated to treat OAB symptoms in MS, such recommendations are extrapolated from the study of patients with OAB symptoms

without neurological disease (157). Whilst widely used, there remains insufficient data to support the efficacy of these drugs in patients with MS (158).

The minimal clinically important difference (MCID) is a term used to define the minimum change in a symptom measure that a patient would identify as clinically important. In validation studies conducted in patients with idiopathic OAB, a reduction in urgency score of two points was associated with the patient reporting that they were 'better' rather than 'no better' or 'worse' (473). The MCID is subject to spectrum bias and whether a comparable a value is applicable to MS patients with OAB symptoms is unclear. There are no intervention studies that have employed the urgency score solely in MS patient samples. Nonetheless, a reduction in pyuria of a magnitude that would mediate a clinically meaningful change in urgency ought to be achievable based on clinical experience treating urinary infection associated with pyuria.

Log pyuria predicted 24-hour urinary frequency, although in common with urinary urgency, any clinical effect would be expected to correlate with the fall in pyuria afforded by treatment. Nonetheless, even a small decline in pyuria would be expected to mediate a reduction in urinary frequency greater than that associated with anticholinergic medication. When used to treat idiopathic OAB symptoms, anticholinergic drugs confer a mean reduction in urinary frequency of around 0.5 voids/24 hours when compared to placebo (157).

An increase in voided volume associated with falling pyuria was also demonstrated. A reduction in pyuria of 1 log wbc μl^{-1} would be expected to be associated with an increase in voided volume of 39ml (95% CI=18-60ml). This is comparable to the effect of treatment with the class-leading anticholinergic drug in patients with non-neurogenic OAB symptoms (474). An effect size of this magnitude could conceivably be achieved employing antibiotic therapy in patients with bacterial infection and associated urothelial inflammation.

Pyuria did not demonstrate any significant association with incontinence episodes. Existing data have demonstrated that samples with average daily incontinence episodes of less than one are likely to be insensitive to treatment effects or the influence of other variables (475). At baseline, median daily incontinence amongst patients in this study was zero (*IQR*=0.0-0.5). Based on these data, the analysis presented here was underpowered to demonstrate an association between incontinence and pyuria, if one exists.

Atypical and anaerobic organisms were not targeted by the culture methods used in this study and the impact of this strategy on the study findings is unclear. From an analytical perspective, the cultivation of additional bacteria afforded by more complex culture media would be expected to strengthen the relationship between infection and inflammation, rather than weaken the associations reported here. Some patient samples in this work demonstrated pyuria but no bacterial growth. Whether the use of media and culture conditions to capture fastidious and anaerobic microbes would have implicated bacterial infection in the generation of urothelial inflammation in these samples is not known.

Enrolled patients were invited to receive their clinical care at the study centre, subject to the agreement of their general practitioner. The outpatient service has particular expertise in the management of the MS bladder. Where evidence of urinary infection was identified in association with LUTS, treatment with an extended course of urinary antibiotic was initiated at full dose for up to six months. This approach contrasts with the provision of short courses of antibiotic offered in most clinical services, or low-dose prophylaxis recommended for recurrent infections. The nature and duration of antibiotic treatment was moderated by clinical assessment that included a systematic symptom review and urinalysis for pyuria. This approach evolved through clinical observation and data from one prospective study (273).

It is unlikely that the method of antibiotic treatment administered to patients influenced the study findings. Protracted courses of antibiotic might attenuate

bacterial growth for long periods, mediating a sustained reduction in urothelial inflammation, but there would be no expectation that the nature of treatment could manipulate the interaction between these variables. It is the antimicrobial property of antibiotics that is assumed to reduce the bacterial load in the lower urinary tract thereby restraining tissue damage and inflammation.

Some antibiotics do exhibit anti-inflammatory effects. Macrolides, quinolones and tertacycline antibiotics have been shown to suppress some immune responses in animal studies and human cell line experiments, although in vivo data are limited. The available data have been extensively reviewed by Labro (2000) and Tauber (2008) (476, 477). Only macrolide antibiotics have been shown to mediate beneficial immunomodulatory effects in clinical studies. Seventy-one percent of the antibiotics used by patients during this study were nitrofurantoin or beta-lactam antibiotics such as cefalexin. There is no evidence that these agents are able to mediate immunosuppression (476, 477). Some patients used ciprofloxacin, which is a quinolone, and doxycycline, a tetracycline antibiotic, but clinical studies have not yet demonstrated that these agents have a detectable immunosuppressive effect. Based on these data, the bactericidal activity of antibiotics is likely to eclipse any theoretical immunomodulatory effects on urothelial inflammation.

Although variations in the provision of antibiotic therapy treatment would not be expected to influence the relationships described in this work, the nature of treatment might affect symptom palliation. If the link between bacterial infection and the generation of symptoms is causal, clinical studies are needed to determine how this problem is best approached. This was not an intervention study and whilst the data generated provide evidence of a link between infection, urothelial inflammation and symptom generation, they do not inform on how these patients are best managed.

Whilst the use of an extended antibiotic treatment period appears capable of attenuating infection and symptom generation in this group of patients, the superiority of this approach to standard treatment remains unproven. Whilst single-

dose and three-day courses of antibiotic are often recommended for acute cystitis, longer courses of treatment are associated with lower bacteriological recurrence rates. Two meta-analyses, including 48 RCTs, have demonstrated that bacteriological recurrence within eight weeks of treatment for bacterial cystitis is significantly lower when antibiotics are administered for two weeks (478, 479). Future studies that explore the antibiotic treatment of patients with chronic urinary symptoms should specifically examine the efficacy of standard and extended treatment protocols. Recent data have demonstrated the capability of uropathogenic bacteria to establish an intracellular niche in the lower urinary tract that may confer protection from immune and antibiotic attack (224-226). It is tempting to implicate such intracellular infection in the generation of chronic LUTS but this is no more than speculation at present. The inherent resistance of these organisms to antimicrobials would support clinical observations that only prolonged courses of treatment supported persistent symptom palliation but this requires further study. Intracellular bacterial colonisation has been demonstrated in patients with chronic symptoms (270, 404) but the prevalence of intracellular infection amongst patients with LUTS and their role in symptom generation is not known.

Whilst these data demonstrate an association between bacterial infection, urothelial inflammation and the overactive bladder, a precise description of how these symptoms might be generated remains unclear. In the neurogenic bladder, the loss of higher neural control mechanisms and the emergence of automatic voiding reflexes were traditionally thought to mediate the symptoms of OAB. This view has been challenged by data demonstrating functional changes to afferent as well as efferent signalling, and even urothelial barrier dysfunction associated with CNS damage (89, 105, 121, 122).

In patients without neurological disease, uncontrolled detrusor contractions are widely perceived to generate the symptoms of OAB. The cause of this unregulated activity has not been elucidated but hypotheses including spontaneous ‘myogenic’ activity arising within the detrusor itself (480) and reduced CNS inhibition of the sacral parasympathetic nuclei (481) have been offered. Urodynamic studies are

widely employed in patients with OAB symptoms to detect these unsolicited detrusor contractions and corroborate the history with an objective 'urodynamic' diagnosis. However, in a study of 843 women with OAB symptoms, only 46% of patients demonstrated detrusor contractions during testing (482). In another urodynamic study, only half of patients who demonstrated detrusor contractions experienced a sensation of associated urinary urgency when these contractions ccurred (483). These data challenge the notion that detrusor overactivity generates the symptoms of OAB in patients without neurological damage.

Anticholinergic drugs have been the mainstay of treatment for OAB symptoms (157) although the recent development of an adrenoceptor agonist has offered an alternative (484). These drugs are thought to mediate their therapeutic effects by suppressing detrusor contractions. However, in the doses used in human studies there is no evidence that they block the neuromuscular junction or have a direct effect on muscle relaxation (485, 486). The available urodynamic data from clinical studies of anticholinergic agents suggest that their therapeutic effects are mediated by a reduction in afferent outflow from the lower urinary tract rather than direct inhibition of the detrusor (485). Moreover, the presence or absence of detrusor overactivity on urodynamic testing has not been shown to determine treatment success in patients with OAB (487). Whilst these data relate to patients with non-neurogenic OAB symptoms, they are of relevance to the findings of this study. They convincingly challenge the belief that the syndrome is a disease of the motor system, driven by damage to neural circuits in the case of neurological disease, or the emergence of autonomous detrusor activity in patients without a neurological disorder. Afferent signalling in the lower urinary tract appears to play a significant role in the generation of OAB symptoms.

The activation of TLR4 by bacterial LPS is known to initiate the production of cytokines via the NF-κB signalling pathway (216-218). Toll-like receptor activation has recently been shown to stimulate the transcription of inflammatory cytokines independent of NF-κB by mediating rapid increases in intracellular $Ca^{2+}$and cyclic adenosine monophosphate (cAMP) (488). The binding of the bacterial pilus

component FimH to UPIII on urothelial cells is also known to induce elevations in cytosolic Ca^{2+} (223, 389). Increases in intracellular calcium are a common consequence of receptor activation in mammalian cells (489) and the release of urothelial ATP is associated with elevated cytoplasmic Ca^{2+} (490, 491). Whether Ca^{2+} signalling is implicated in the generation of OAB symptoms is unknown.

Adenosine triphosphate is released in response to stretch, cell damage and bacterial LPS (262, 319-322). Augmented purinergic neurotransmission has also been demonstrated in bladder tissue isolated from patients with lower urinary tract disorders including OAB (67-75, 92, 93). Whilst ATP has been shown to activate bladder afferents and influence sensory outflow from the lower urinary tract (95), evidence for a paracrine effect in the human bladder was unproven until recently. It has now been demonstrated that urothelial-derived ATP is able to influence the detrusor smooth muscle behaviour through a paracrine mechanism, and influence the function of other mucosal components (492). Thus, purinergic signalling in the lower urinary tract might moderate sensory outflow and detrusor function. The urothelium, lamina propria and detrusor are in close proximity, and there is evidence of cell-to-cell coupling mediated by gap junctions (493). These structural features might facilitate direct communication between the mucosa and the detrusor. Whether the paracrine action of ATP influences the response of detrusor smooth muscle to neurological inputs or is capable of generating spontaneous contractile activity that might manifest as OAB symptoms is not known.

The available evidence illustrates that lower urinary tract function is governed by a complex interplay of local and central influences. Whilst the neuropathology of MS disrupts the coordinated control of lower urinary tract function, a growing body of evidence suggests that other influences may shape the evolution of symptoms. This study suggests that urothelial inflammation might contribute to the generation of OAB symptoms, although the mechanism by which this might occur remains to be elucidated.

The interaction between the neurogenic component of lower urinary tract dysfunction and any effects mediated by infection and inflammation may vary between patients. It is conceivable that some patients have a pronounced infective, inflammatory component to their symptomatology that is potentially treatable. In others, the symptoms may be generated primarily as a result of neurological damage. In these patients, infection and urothelial inflammation might be palliated with antibiotic treatment without symptomatic benefit due to fixed neurological deficits affecting lower urinary tract function. Nonetheless, the data presented in this study suggest that those patients who demonstrate pyuria might benefit from treatment.

7 A randomised, double-blind, placebo controlled, crossover trial of AIMSPRO for the treatment of overactive bladder symptoms in patients with secondary progressive multiple sclerosis

7.1 Background

Whilst a minority of patients with MS will demonstrate progressive disease at diagnosis, most will present with the relapsing-remitting form of the condition. Unfortunately, at least half of patients with relapsing-remitting MS (RR-MS) will have entered a secondary progressive phase at 10-15 years (51-53). The onset of secondary progressive MS (SP-MS) is associated with a continuous and irreversible decline in neurological function. Progressive disease, primary or secondary, is associated with the vast majority of disability for patients.

Since the first clinical trial of interferon (IFN) β−1b in RRMS was published over 20 years ago, there has been continued progress in the treatment of this disease variant (494). Disease modifying agents that reduce the relapse rate have been shown to attenuate the accumulation of disability (495). There is also recognition that early introduction of these drugs in patients with a single demyelinating episode may delay the onset of clinically definite MS (496).

In contrast to relapsing-remitting disease, there is no conclusive evidence that any treatment halts neurological progression associated with SP-MS. The European Trial in SP-MS (EUSPMS) tested IFNβ−1b against placebo but despite an apparent reduction in disease progression in the IFN arm these results were not corroborated by another large North American study (497). Further analysis has suggested that the initial positive findings were likely a result of differences in the study populations. The European study enrolled younger patients with higher rates of relapse and a greater prevalence of enhancing lesions on MRI (498). These data suggest that the impact of treatment on disability was mediated through relapse suppression rather than a direct influence on progression. Based on these data and others (499), the use of IFN in SP-MS is usually confined to patients with persistent relapses.

Mitoxantrone is an antineoplastic agent that mediates a reduction in B- and T-lymphocyte numbers through interference with DNA replication and repair (500).

The efficacy of mitoxantrone has been examined in studies with mixed populations of RR-MS and SP-MS (501, 502). Whilst progression of existing disability was retarded in the active treatment groups, these effects were likely mediated through relapse suppression rather than a direct effect on the underlying disease course. No firm conclusions can be made relating to the efficacy of this agent in patients with SP-MS who do not experience ongoing relapses. Long-term use of the drug can be associated with cardiac failure in around 10% of users and therapy-related acute leukaemia in around 0.5% (503).

Relapsing-remitting MS appears to demonstrate differences in its pathophysiology when compared to progressive disease variants. Relapsing-remitting MS is characterised acute episodes of demyelination, mediated by T lymphocytes, and as a result of macrophage activity. By contrast, patients with progressive MS demonstrate diffuse neuroaxonal loss (30) and new focal demyelination is much less common (34, 35). Axonal loss may occur as a consequence of continuous, grumbling inflammation within the CNS, with nitric oxide and glutamate implicated in this process (37-39). The eventual failure of damaged but fragile axons may also contribute to permanent loss of function (40). The lack of efficacy demonstrated by immunomodulatory agents in progressive disease may be explained by these insights.

Major histocompatibility complex Class II proteins are key antigen presenting molecules in humans. Interferon β, which has a complex effect on immunological function, is thought to exert some of its therapeutic effect in MS through suppression of MHC Class II activity (504, 505). Other drugs used in relapsing remitting disease also disrupt T-lymphocyte recognition of self-peptide. Glatiramer acetate achieves this by binding directly to MHC Class II proteins (506) whilst mitoxantrone suppresses lymphocyte numbers thus reducing the magnitude of the adaptive immune response (500).

In 1999, Capralogics Incorporated, a research facility in Massachusetts, USA, was contracted to assist in the development of a polyclonal neutralising antibody against

HIV. Goats were initially inoculated with HIV antigens and the sera analysed to determine whether HIV antibodies could be generated at high levels. The product was subsequently found to contain extremely low antibody titres to HIV but high titres to MHC Class II proteins. The resulting medication was less effective than conventional therapy for HIV but its potential anti-inflammatory effects made it a candidate drug for chronic autoimmune and inflammatory conditions. Daval International, a UK-based biotechnology company, purchased the technology and continued to develop the product as AIMSPRO® (an acronym for Anti-inflammatory IMmunoSuppressive PROduct).

Subsequent analysis by Daval demonstrated that AIMSPRO® contains a complex of molecules that may have immunomodulatory effects (507). The product contains hypothalamo-pituitary-adrenal (HPA) regulatory molecules including corticotrophin-releasing hormone (CRH), arginine vasopressin and β-endorphin. AIMSPRO® also contains Pro-opiomelanocortin (POMC), the peptide precursor of a number of pituitary hormones, and the cytokines IL-1β and IL-10. Daval claim that AIMSPRO® can modify HPA axis function, resulting in the endogenous production and regulation of melanocortins including ACTH, CRH, β-endorphin and vasopressin.

Daval have reported that patients administered AIMSPRO® demonstrate a shift in their cytokine profile favouring anti-inflammatory effects (508). They propose that these changes are mediated by modulation of the HPA axis leading to enhanced glucocorticoid secretion and the suppression of innate and adaptive immune responses. The influence of the HPA axis on inflammation is well recognised (509). The action of anti-inflammatory cytokines, elevated production of melanocyte stimulating hormone (MSH), and direct effects on immune cell function have been proposed as mechanisms by which AIMSPRO® might mediate any anti-inflammatory effects (507).

Neurophysiological data from threshold tracking experiments have suggested that AIMSPRO® might be able to improve conduction in damaged nerves. In one patient with Chronic Demyelinating Inflammatory Polyradiculoneuropathy, enhanced nerve

excitability was reported after treatment. This was implied by a reduction in the triggering voltages of sodium channels in peripheral nerves and a prolongation of channel opening after AIMSPRO® administration (510). This observation has been corroborated in one other report (511).

Over the last decade, Daval has promoted AIMSPRO® as a putative treatment for a variety of human diseases without direct evidence of efficacy. Although unlicenced, it is registered as a 'special' with the MHRA, allowing its provision on a named patient basis when clinical need cannot be met by a licenced product. Responsibility for the use of these drugs lies solely with the prescribing physician and any adverse drug reactions must be reported (512). Outside the UK, similar schemes that permit the use of unlicenced medications in this manner are known as 'compassionate use' or 'expanded access' programmes (513).

Data relating to the therapeutic use of AIMSPRO® are limited to clinical observations and case reports (275, 514-520). Whilst only two published reports describe the use of AIMSPRO® in MS (517, 519), over 400 patients with the condition have now taken AIMSPRO® on compassionate grounds for periods of up to five years. Objective improvement has been claimed in some cases, but these observations are uncontrolled, and documented in personal communications between prescribing clinicians and Daval (514). Daval also claim improvements in fatigue and bladder control, colour vision, balance and mobility that they suggest may manifest rapidly after administration (514). Daval has proposed that these perceived improvements are a consequence of: (1) the general anti-inflammatory effects of the agent (507, 508); and (2) the enhancement of sodium channel activation in damaged nerves (510, 511).

Since AIMSPRO® was first granted authorisation for compassionate use, Daval has never been far from controversy. The first randomised study of AIMSPRO® in patients with SP-MS was halted after a dispute between Daval and researchers at St George's Hospital, London, over standards of preservation of the study drug. Disability data were collected from all 47 enrolled patients but the results were not

made public as the dispute was never resolved. A second much smaller trial in patients with MS and a previous episode of optic neuritis was conducted at the John Radcliffe Hospital, Oxford. No evidence of benefit was demonstrated in the primary or secondary outcome measures (521). Allegations of improper conduct relating to the marketing and provision of AIMSPRO® were reported in the UK press, and Daval was accused of misrepresenting study data for commercial gain (522, 523). These claims were investigated by the MHRA, but after a lengthy consultation, no charges were brought against Daval.

A recent review of compassionate use drug programmes in ten EU countries has highlighted the potential problems with such schemes (513). Whilst these programmes were conceived to try and help patients for whom licenced medications offer no hope of palliation, those seeking such treatments represent a vulnerable group. Such individuals may be more likely to pursue expensive drug therapies despite a lack of data relating to efficacy and safety. In contrast to clinical trials, these programmes do not offer safeguards to patients including ethical review, informed consent and insurance for adverse events.

Compassionate use programmes must not offer an easy method of collecting efficacy and safety data on a new product. They are not a substitute for clinical trials, necessary to satisfy regulatory authorities and bring effective therapies to a wider population of patients. Randomised studies also offer guaranteed access to a study drug for patients who benefitted once a trial has ended. Furthermore, if the drug is found to be safe and effective, positive results catalyse expanded access to the treatment whilst licencing is being sought. Whilst compassionate use programmes were conceived to help those whose suffering cannot be palliated by conventional means, patients must be protected.

During open-label observations in patients with MS, one of the most consistent benefits of AIMSPRO® administration was a reported improvement in lower urinary tract function that appeared rapid in onset. In view of these reports, Daval approached Professor James Malone-Lee to evaluate the efficacy and safety of

AIMSPRO® in the treatment of OAB symptoms in patients with MS. After some hesitation, Professor Malone-Lee met with Daval regarding the proposal. He agreed to take on the trial because he believed that he could offer a rapid and effective means of resolving the controversy surrounding AIMSPRO®. His agreement was subject to the study meeting all MHRA regulatory requirements and the production of AIMSPRO® to Good Manufacturing Practice (GMP) standards. Whilst efficacy was unproven, AIMSPRO® appeared to be safe. Records from Daval documented the provision of more than 19,000 individual vials of product to patients, apparently administered without any notable adverse effects (524). No significant adverse reactions were reported in either of the randomised studies that were undertaken in Oxford or London (525).

Large, parallel-group randomised studies are often deployed to test new treatments for LUTS. A recent phase IIa randomised study that evaluated a new treatment for OAB symptoms enrolled hundreds of patients in several countries (526). The costs of large RCTs can be prohibitive for smaller biotechnology companies looking to develop new treatments (527, 528). In 2003, Professor James Malone-Lee and Alex Yaroshinsky, a Roche Bioscience statistician, developed a randomised, crossover trial design that could be used for proof of concept studies using a small sample size of 20 patients (475). The sample size calculation is powered to detect a mean increase in voided volume of 60ml, which has been associated with clinically significant improvements in lower urinary tract function reported by patients (529). This design contrasts with large parallel-group studies that are often powered to detect smaller effect sizes that might be statistically significant but of questionable clinical value.

The design permits the rapid assessment of new treatments that is cost-effective. Failure to detect an effect using this approach implies that the drug would be unlikely to demonstrate a clinically useful response. This would allow its development to be halted at an early stage, minimising investment in a treatment unlikely to make it to market. This method has been used successfully in the evaluation of new treatments for OAB, mitigating the need for larger studies in early drug development programmes (530-532).

The crossover study has two short treatment phases, separated by a 'washout period' during which the participant remains free of investigational drug. The use of a crossover design is contingent on the absence of any measurable 'crossover effect'. Thus, the investigational drug must have the potential to moderate clinical manifestations of the disease rapidly, but these effects must be reversible on its withdrawal. The agent must ameliorate disease-related symptoms only, not effect a permanent cure, and must be rapidly eliminated after therapy is discontinued. This is dependent on pharmacokinetic characteristics and the proposed duration of the washout period. Based on the presumed pharmacology of AIMSPRO® and clinical observations, a randomised crossover design was deemed a suitable means of evaluating the effects of the drug on lower urinary tract function.

Not unexpectedly, there was significant resistance to the study. Concerns about the alleged conduct of Daval and a litigious approach to critics stalled the development of the trial for almost five years. Despite ethical approval being granted by the NRES, the research ethics committee at the NHS Trust that was approached to host the trial rejected the study. This prompted the Chairman of the NRES committee that provided ethical approval to seek legal advice and appeal to the Health Research Authority. Although local ethical objections to the study were overturned, the Chief Executive of the NHS Trust then vetoed the study. The trial was finally accepted by another Trust in London.

Whilst attempts to prevent this study being undertaken were perhaps inevitable, they were not ultimately in the patient interest. Irrespective of concerns about the alleged conduct of Daval, clinical trial data were required to address their assertions and provide answers for patients. These aims cannot be achieved by an embargo. Previous clinical trials of AIMSPRO® had unfortunately failed to resolve the claims and counter-claims relating to the efficacy of AIMSPRO®. Proof of concept studies conducted in this centre have repeatedly demonstrated that such disputes can be resolved quickly whilst maintaining scientific standards and limiting financial burden. Professor Malone-Lee believed that the controversies relating to AIMSPRO® could be dealt with by these means. This was a view shared by The MS Society in the UK (533)

although the problems encountered during previous controlled trials of AIMSPRO® remained a concern. Whilst Daval sponsored the study, Professor Malone-Lee worked with complete autonomy for the duration of the trial. Liaison with Daval's Medical Director was necessary to satisfy regulatory requirements but no other contact between investigators and Daval was sanctioned.

In addition to bladder-related treatment effects, Daval wished to collect data on disease status, neurological function and measures of the inflammatory response. They argued that if the study demonstrated evidence of an effect, these analyses would help to characterise the physiological mechanisms mediating such an effect if it existed. An open-label extension of the RCT was also agreed to evaluate the safety of AIMSPRO® during extended administration. Measures of bladder and neurological function were also collected during the open-label phase, in order to identify any effects associated with the prolonged administration of the drug. If evident, such data might justify longer controlled trials of AIMSPRO® in the future.

It was made clear from the outset that secondary outcome measures relating to neurological function, exploratory analyses and data from open-label observations would be used to guide future research only. Any positive findings amongst these measures were not to be reported by Daval as evidence of AIMSPRO® modifying the neurological course of SP-MS without further study. The trial was powered only to detect a clinically significant effect on the primary outcome that assessed lower urinary tract function. All other findings were to be treated with caution. This was agreed by all parties and recorded explicitly in the study protocol. Nonetheless, the collection of exploratory data was in the wider interest.

In view of proposed physiological effects of AIMSPRO®, any improvements in lower urinary tract function in MS might be mediated by two complementary pathways: (1) drug effects on CNS inflammation and nerve conduction mediating an improvement in neurological function; and (2) local immunomodulatory and anti-inflammatory effects in the bladder. The second of these hypotheses was apposite to this programme of study. If the administration of AIMSPRO® was associated with an

improvement in bladder function, cytokine analyses and neurological testing might help to elucidate the mechanisms by which such effects were mediated.

7.2 Study overview

Adult patients with SP-MS participated in a phase IIa randomised, double-blind, placebo controlled trial to determine the efficacy and safety of AIMSPRO® in the treatment of OAB symptoms. Participants had to comply with the inclusion and exclusion criteria set out in the protocol and demonstrate no evidence of UTI using methods common to ordinary clinical practice. A crossover design was employed, with two treatment phases lasting 28 days, separated by a washout period of 42 days. During each of the treatment phases, the investigational drug or placebo was administered twice weekly as a sub-cutaneous injection, under double-blind conditions.

All patients attended a screening visit to assess eligibility, including an assessment by a consultant neurologist to verify the diagnosis of SP-MS prior to inclusion. Eligible patients were assessed at three visits during each treatment phase. Lower urinary tract function was evaluated using bladder diary data and urinary symptoms were assessed by validated questionnaires. Disability and neurological status were evaluated using a combination of functional testing and questionnaire data. Threshold tracking experiments were conducted to assess peripheral nerve excitability after administration of AIMSPRO®. An exploratory analysis of serum and urine cytokine expression was conducted to evaluate the effects of the drug on the inflammatory response. Safety was evaluated by serial laboratory analysis of haematological, biochemical and immunological indices, clinical examination and adverse event analysis.

An open-label extension of the study was approved to assess the safety of AIMSPRO® during prolonged administration. Observational data evaluating lower urinary tract function, urinary symptoms, general disability and neurological function were also collected during this period.

The study was conducted at the Department of Neurophysiology, Royal Free London NHS Foundation Trust. Ethical approval was granted by the NRES Committee London, Queen's Square (Ref: 10/H0716/84).

7.3 Safety considerations

The only adverse reactions reported in association with AIMSPRO® administration are cutaneous reactions at the injection site. These reactions are typically erythematous, pruritic and are usually two to three centimetres in maximum diameter. Around 10% of users report injection site reactions that are usually alleviated with oral antihistamines (525). Clinical experience would suggest that these reactions are rare once the duration of therapy has exceeded two months.

No significant adverse reactions were identified in either of the previous randomised trials of AIMSPRO®. At the time of writing, no adverse effects had been logged through the MHRA "Yellow Card" system in the UK, or reported to Daval by prescribing physicians in Canada, New Zealand and Australia (525). Prior to the submission of the study protocol, 14 patients who had been receiving AIMSPRO® for between two and five years were examined by an independent physician. No significant clinical or laboratory abnormalities were identified in any of the patients (534).

7.4 Study objectives

7.4.1 Primary objective

- To evaluate whether the regular administration of AIMSPRO® improves OAB symptoms, demonstrated by a significant increase in average voided volume derived from bladder diary data.

7.4.2 Secondary objectives

The secondary objectives of the study were to determine the following:

- The effects of AIMSPRO® administration on urinary frequency and incontinence episodes derived from bladder diary data.
- The effects of AIMSPRO® administration on symptoms of urinary urgency and bladder-related QoL using validated questionnaires.
- The effects of AIMSPRO® administration on the physical and psychological health of patients using validated questionnaires.
- The effects of AIMSPRO® administration on ambulatory and cognitive impairment using functional composite testing.
- The effects of AIMSPRO® administration on colour vision and visual acuity using the Fansworth-Munsell and LogMAR tests.

7.4.3 Tertiary objectives

The tertiary/exploratory objectives of the study were to determine the following:

- The effects of AIMSPRO® administration on peripheral nerve conduction determined by threshold tracking studies.
- The effects of AIMSPRO® administration on serum and urine biomarkers.

7.4.4 Safety objectives

The safety of AIMSPRO® was assessed by means of adverse event reporting throughout the blinded and open-label phases of the study. The frequency and nature of all adverse events were summarised and classified using standard methods.

7.5 Study population

7.5.1 Recruitment of participants

Patients and control subjects were recruited as outlined previously (2.2.2).

7.5.2 Consent and eligibility

All participants provided written, informed consent prior to any study related procedures, and eligibility was checked before inclusion (2.2.3).

7.5.3 Inclusion and exclusion criteria

Ambulant adult patients with SP-MS, urinary urgency, and 24-hour urinary frequency ≥8 were eligible for inclusion. The diagnosis of SP-MS was made using the modified McDonald diagnostic criteria, published in 2005 (535). The exclusion of UTI using standard urinalysis methods was essential prior to study inclusion. No more than one relapse in the last 12 months was permitted. Any relapse in the six months prior to screening precluded participation. A full list of inclusion and exclusion criteria are described in **Table 33** and **Table 34**.

7.5.4 Study restrictions and concomitant medications

The use of any immunosuppressive therapy during the study was prohibited, although a short course of corticosteroid therapy, oral or intravenous, was permitted for a disabling relapse of MS when recommended by a neurologist.

The use of all concomitant medication was recorded in the study documentation. This included all prescription drugs, over-the-counter medications, herbal remedies, vitamins, minerals and supplements.

Table 33 *Inclusion criteria.*

Inclusion criteria	
1	Adult patients with MS
2	Agreement to use adequate birth control measures for the duration of the study and for six months after receiving the last dose of the study drug
3	A diagnosis of clinically definite SP-MS
4	The ability to walk, with or without walking aids
5	No more than one relapse within the last 12 months and no relapse within the last 6 months
6	Urinary urgency with or without urgency incontinence
7	24-hour urinary frequency ≥8
8	A documented MRI of the brain or spinal cord demonstrating features consistent with MS
9	Screening laboratory tests demonstrating the following parameters: haemoglobin ≥ 9.5 g dl^{-1}; leucocytes ≥ 3.5 x 10^9 l^{-1}; neutrophils ≥ 1.5 x 10^9 l^{-1}; and platelets ≥ 100 x 10^9 l^{-1}
10	Baseline liver function, thyroid function, and serum electrophoresis levels must be within the normal range for the laboratory conducting the test
11	Ability to adhere to the study visit schedule and other protocol requirements
12	Ability to provide written informed consent prior to study inclusion

7.5.5 Discontinuation of subject participation

Patients could be withdrawn from the study at the discretion of the Chief Investigator. The criteria for subject withdrawal included: (1) non-compliance with the requirements of the protocol; (2) an adverse event after which continued participation would present an unacceptable risk; (3) withdrawal on medical or administrative grounds; (4) if continued participation would not be in the patient's best interests; (5) pregnancy. Patients could withdraw their consent to participate at any time without prejudice but would be invited for follow-up visits to monitor safety data.

The protocol demanded that patients who withdrew in first four weeks of the study should be replaced. If a patient withdrew as a result of an adverse event, they would remain under review until the adverse event resolved or stabilised.

Table 34 *Exclusion criteria.*

Exclusion criteria	
1	Pregnant or lactating women and women
2	Women who are planning pregnancy within 12 months of screening
3	The use of desmopressin for nocturia
4	The use of urinary catheters
5	Acute symptomatic urinary infection
6	Positive urine culture
7	No clear progression of disability in the last 12 months
8	Previous administration of AIMSPRO®
9	The receipt of any investigational drug within 30 days of screening or within five half-lives of the drug, whichever is longer
10	The receipt of any immunosuppressive therapy
11	The use of the anticonvulsant lamotrigine or the anti-arrhythmic drug flecainide, both of which are potent sodium channel blocking agents
12	A history of severe allergy or any history of allergy to animal proteins
13	Any other neurological condition in addition to SP-MS
14	Established renal, hepatic, haematologic, gastrointestinal, endocrine, pulmonary, cardiac disease or malignant disease that would affect the interpretation of study data
15	Any history of serious infections (such as pneumonia or pyelonephritis) within three months of screening (less serious infections such as acute upper respiratory tract infection or simple urinary tract infection, should be followed to their conclusion or treated, as appropriate, prior to inclusion)
16	Patients with opportunistic infections, including but not limited to evidence of active cytomegalovirus, active Pneumocystis carinii, Aspergillosis, histoplasmosis or atypical mycobacterium infection within six months of screening
17	Any history of tuberculosis
18	Any history of lymphoproliferative disease including lymphoma, or signs and symptoms suggestive of lymphoproliferative disease, such as lymphadenopathy or splenomegaly
19	Any organ transplant, with the exception of a corneal transplant undertaken at least three months prior to screening
20	Clinically significant substance abuse
21	Poor tolerability of venepuncture or lack of adequate venous access for study requirements
22	Inability to complete bladder diaries, questionnaires or other study documents

If a patient withdrew as a result of pregnancy, they would continue to be reviewed for the duration of the pregnancy. Daval who sponsored the study, NRES and the medical monitor would be notified. The patient's General Practitioner would also be informed.

7.5.6 Discontinuation of the Study

The Chief Investigator, Daval's medical expert, and the independent medical monitor reviewed safety data throughout the study. Criteria that would have triggered a review of continued study activity included: (1) the occurrence of one or more SUSARs or SAEs; (2) a high frequency of AEs that individually may not have been significant but collectively raised concerns about safety.

7.6 Study design

7.6.1 Summary of study design

The study was a phase IIa randomised, double-blind, placebo controlled crossover study to determine the efficacy and safety of AIMSPRO® in the treatment of OAB symptoms in patients with SP-MS. This was a proof of concept study. A schematic of the study design is presented in **Figure 26**.

7.6.2 Screening

Patients who wished to enrol and were provisionally identified as suitable participants were invited for screening. Screening processes were conducted up to 42 days prior to randomisation and initiation of the study.

7.6.3 Study visits

If screening was successful, eligible patients were required to participate in two blinded treatment phases, separated by a washout period. During each of the treatment phases, the patient would administer AIMSPRO® or placebo by subcutaneous injection in a twice-weekly dosing schedule. Nine doses of would be given in each phase.

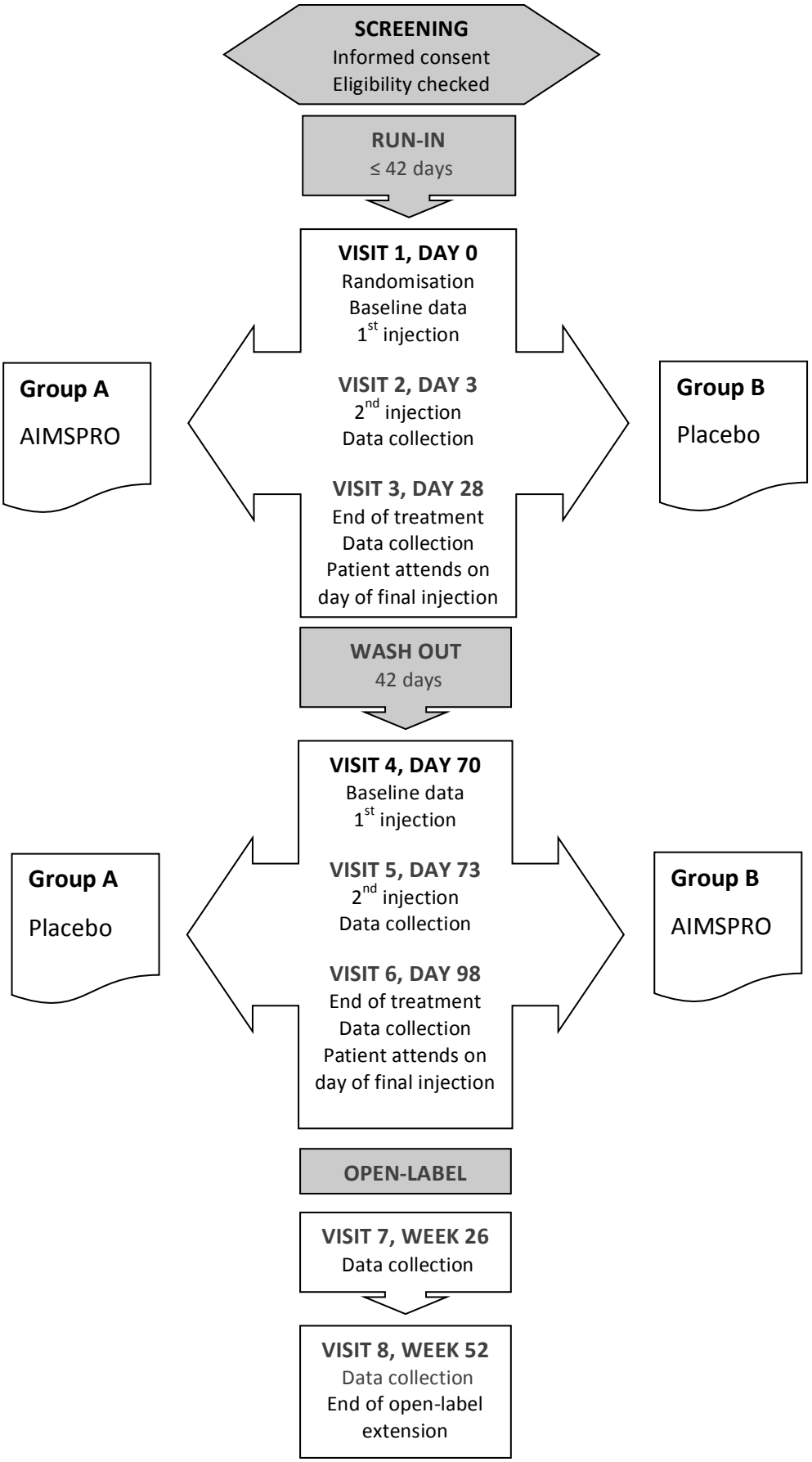
The first two injections of each treatment phase were administered under supervision at the study centre. This was to allow the patient or their carers to be trained in injection technique and monitor for acute allergic reactions. All subsequent treatments were administered at home in the morning on rising. The final injection in each phase was given on the morning of the last study visit prior to attending the study centre. Patients and their carers were asked to keep a drug administration log that was collected at the end of each treatment phase and filed in the CRF.

At the end of the blinded phase of the study, patients were invited to continue administering AIMSPRO® on an open-label basis. Two further study visits were scheduled during this phase of the study. During the open-label phase, study visits were scheduled to allow AIMSPRO® to be injected on the morning of the visit.

7.6.4 Duration of the study

The expected maximum duration of the study from screening to the end of blinded treatment was 20 weeks. The open-label phase of the study had a maximum duration of 38 weeks.

Figure 26 *Schematic of study design.*



7.7 Investigational medicinal product

7.7.1 Production

AIMSPRO® is a polyclonal hyperimmune serum product supplied as a frozen liquid for injection (AIMSPRO® 4.5mg/ml). The drug is produced from pooled serum obtained from goats raised and housed at facility in Tasmania (Selborne Biological Services Pty. Ltd.). The facility is registered with the Therapeutic Goods Administration, a division of the Department of Health, Australia. The animals are vaccinated using a heat and detergent inactivated HIV viral lysate. Serum is shipped frozen to Biotec Services International Ltd. (Bridgend, UK) who holds the MHRA 'specials' manufacturing licence. The product is purified, which includes viral filtration at 35 nanometres and released after sterility and endotoxin testing. All manufacturing processes are conducted to GMP standards.

The placebo for this study was provided by Biotec Ltd as 4.5% human albumin (Baxter Healthcare, Newbury, UK) vialed in identical glass containers to those in which AIMSPRO® was provided.

7.7.2 Storage and accountability

AIMSPRO® must be stored frozen at -18°C or below. Daval transported the drug from the manufacturer to study site frozen. Electronic temperature monitors travelled with the product at all times. On arrival at the study site the IMP was transferred to a dedicated freezer in the hospital pharmacy provided by Daval. The unit was subject to 24-hour temperature monitoring and fitted with an alarm.

The IMP (AIMSPRO® 1ml/placebo) was provided in packs of nine vials for each treatment phase. The first two doses of the study drug were administered at the study site and were dispensed by the clinical trials pharmacist immediately prior to injection. They remained frozen on dry ice until administration. The remaining seven

vials of frozen product were transported to the patient's home with a validated electronic temperature log. Marken Logistics (London, UK) was responsible for the distribution of IMP to patients. Strict frozen conditions were maintained at all times with continuous temperature monitoring. On arrival, a box containing the remaining seven vials of frozen product was deposited into a desktop freezer unit provided by Daval. At the end of each treatment phase, the box containing the electronic temperature log and the empty vials was collected and deposited at the study site before being returned to Daval.

7.7.3 Administration of the study drug

Patients were instructed on the handling and administration of the IMP prior to administration at home. The IMP was removed from the freezer unit immediately prior to each injection. It was thawed by rolling the vial between the hands. The contents were usually ready for administration after one to two minutes. The liquid was then drawn up and injected sub-cutaneously. The process had to be completed within three minutes of removal of the vial from the freezer. Any recognised injection sites could be used but rotation was recommended. The patient was supplied with alcohol skin swabs, syringes, and a sharps box.

Anaphylaxis has not been reported in association with AIMSPRO® administration. Nonetheless, the first two injections were administered under medical supervision at the study site. The clinical area was fitted with couches and an emergency call button. Resuscitation equipment, including emergency drugs for the treatment of anaphylaxis were immediately accessible within the department. All research staff had resuscitation training annually.

7.7.4 Overdose

The effects of AIMSPRO® overdose are unknown. The protocol made no specific contingencies in this regard. Any such event would have been assessed immediately and reported to the Chief Investigator, medical expert at Daval, and the independent medical monitor.

7.8 Randomisation and blinding

7.8.1 Randomisation

Enrolled patients were randomised at their first study visit to one of two treatment groups (AIMSPRO® or placebo). The randomisation sequence was generated from random number tables by Datapharm Australia (Drummoyne, Australia) who provided data management support for the study. The allocation sequence was sent to Biotec Services International Ltd. who manufactured the study medication. The study packs were prepared in chronological order using the study numbers 001-020 and the random allocation table employed to allocate the medication sequence. Patients were randomised on a 1:1 ratio so half of the group started with either the active drug or placebo.

7.8.2 Blinding

This was a double-blind study. Neither the investigators nor the patients were aware of treatment allocation. The randomisation code was held in a sealed envelope at the offices of Verius Ltd. (Cambridge, UK) who specialise in pharmacovigilance services. The active drug and placebo were identical in appearance and volume, and were manufactured in identical vials.

7.8.3 Unblinding

An independent medical monitor at Verius was contactable 24 hours a day by telephone. In the event of an emergency requiring unblinding, the Chief Investigator would be contacted to manage the process.

7.9 Study procedures

7.9.1 Time and events schedule

A time and events schedule summarising study activity is presented in **Table 35**.

7.9.2 Screening

Patients who wished to participate were sent a patient information sheet and then contacted by phone. Screening was undertaken from Day -42 to Day -1. Written, informed consent was taken and patients were then assessed by Dr Richard Orrell, a Consultant Neurologist at the Royal Free Hospital, to verify the diagnosis of SP-MS. This assessment was a protocol requirement. A full medical history and examination were undertaken and serum samples sent for laboratory analysis. Urinalysis was conducted. Concurrent medications and any adverse events were recorded. Inclusion and exclusion criteria were checked and the results of laboratory analyses were reviewed prior to confirming eligibility. A bladder diary was provided for completion before the next visit. The first study visit was scheduled pending satisfactory laboratory screening results.

Table 35 *Time and events schedule.*

	Screening	Blinded phase								Open-label phase	
	Day -42 to -1	Visit 1, Day 0		Visit 2, Day 3	Visit 3, day 28	Visit 4, Day 70		Visit 5, Day 73	Visit 6, day 98	Visit 7, Week 26	Visit 8, Week 52
		Pre-dose	Post-dose			Pre-dose	Post-dose				
Informed consent	X	X		X	X	X		X	X	X	X
Neurological history and examination	X										
Eligibility criteria	X	X		X	X	X		X	X	X	X
Medical history	X	X		X	X	X		X	X	X	X
Physical examination	X	X	X	X	X	X	X	X	X	X	X
Serum sample, screening and safety	X	X			X	X			X	X	X
Serum sample, exploratory		X	X		X	X	X		X	X	X
Urine sample, screening and safety	X	X			X	X			X	X	X
Urine sample, exploratory		X	X		X	X	X		X	X	X
Bladder diary collection		X		X	X	X		X	X	X	X
Urgency score		X			X	X			X	X	X
I-QOL		X			X	X			X	X	X
SF-36		X			X	X			X	X	X
EDSS		X			X	X			X	X	X
MSIS-29		X			X	X			X	X	X
MSWS-12		X			X	X			X	X	X
MSFC		X	X		X	X	X		X	X	X
Colour vision and acuity		X	X		X	X	X		X	X	X
Threshold tracking		X	X		X	X	X		X	X	X
Concurrent medications	X	X	X	X	X	X	X	X	X	X	X
Adverse events	X	X	X	X	X	X	X	X	X	X	X

7.9.3 Study visits

7.9.3.1 Visit 1

Visit 1 was conducted on Day 0. Bladder diary data was collected from the patient and baseline bladder function was reviewed. Eligibility was rechecked and consent confirmed. Any changes to concurrent medications and the occurrence of any adverse events were recorded. A physical examination was conducted.
The following patient-reported measures were used to assess bladder symptoms, general health status, the impact of MS on physical and psychological wellbeing, and mobility:

- Whittington Urgency score.
- I-QOL.
- SF-36.
- MSIS-29.
- MSWS-12.

The neurologists Dr John McHugh and Dr Kishore Kumar completed the following assessments of general disability, ambulation, cognitive function, visual function and peripheral nerve excitability:

- EDSS.
- MSFC.
- Farnsworth-Munsell 100 Hue Colour Vision Test.
- LogMAR visual acuity testing.
- Threshold tracking.

Blood and urine were sampled for safety evaluations and exploratory analyses. The patient was then administered the study drug and injection technique was taught. The patient was observed for one hour to monitor for any adverse reaction.

After this period of observation, further blood and urine samples were taken for exploratory analyses only. Neurological and neurophysiological testing undertaken prior to dosing was repeated:

- EDSS.
- MSFC.
- Farnsworth-Munsell 100 Hue Colour Vision Test.
- LogMAR visual acuity testing.
- Threshold tracking.

These repeat assessments were conducted before and after administration of IMP to identify any immediate effects of administration on the cytokine profile and neurological function. A bladder diary was provided for completion before the next study visit and the patient was asked to return on Day 3.

7.9.3.2 Visit 2

Visit 2 was conducted on Day 3. The completed bladder diary was collected from the patient. Eligibility was rechecked and consent confirmed. Any changes to concurrent medications and the occurrence of any adverse events were recorded. A physical examination was conducted. The second dose of IMP was administered by the patient under the supervision of the investigator. Injection technique was checked. The patient was observed for one hour to monitor for any adverse reaction. Arrangements were made for the delivery of the remaining seven vials of IMP to the patient's residence. A bladder diary and a drug administration log were provided for completion. The patient was then allowed home and asked to return on Day 28.

7.9.3.3 Visit 3

Visit 3 was conducted on Day 28. The completed bladder diary and drug administration log were collected. Eligibility and consent were reviewed and any changes to concurrent medications or adverse events were recorded. A physical examination was undertaken. Blood and urine were sampled for safety evaluations and exploratory analyses.

The following patient-reported measures were used to assess bladder symptoms, general health status, the impact of MS on physical and psychological wellbeing, and mobility:

- Whittington Urgency score.
- I-QOL.
- SF-36.
- MSIS-29.
- MSWS-12.

The neurologists Dr John McHugh and Dr Kishore Kumar completed the following neurological and neurophysiological assessments:

- EDSS.
- MSFC.
- Farnsworth-Munsell 100 Hue Colour Vision Test.
- LogMAR visual acuity testing.
- Threshold tracking.

A bladder diary was provided for completion before the next study visit. Arrangements were made for the next study visit on Day 70 following a six-week washout period.

7.9.3.4 Visits 4-6

Visits 4-6 in the second blinded treatment phase were conducted as described for visits 1-3 in the first blinded phase. Upon completion of the blinded phase at Visit 6, patients were invited to continue administering AIMSPRO® in an open-label extension. For those patients who agreed, arrangements were made for the delivery of the product to the patient's residence. A bladder diary and a drug administration log were provided for completion.

7.9.3.5 Visits 7-8 (open-label phase)

Visits 7 and 8 of the open-label phase were scheduled at 26 and 52 weeks respectively. These visits were conducted employing the same processes described in Visit 3 of the blinded phase of the study. Patients were provided with supplies of AIMSPRO® throughout this period by Daval. At 52 weeks no further supplies of the drug were made available and participating patients left the study.

7.10 Clinical and laboratory assessments

7.10.1 Clinical assessments

The following clinical assessments were undertaken in accordance with protocol:

- Bladder diary data:
  Mean voided urinary volume (2.15)
  Mean 24-hour urinary frequency (2.15)
  Mean 24-hour urinary incontinence (2.15)

- Questionnaires, lower urinary tract:
  Whittington Urgency Score (2.14.2)
  I-QOL (2.14.4)

- Questionnaires, MS health status and disability:

  SF-36 (2.16.1)

  MSIS-29 (2.16.2)

  MSWS-12 (2.16.3)

  EDSS (2.16.4)

- Neurological assessments:

  MSFC (2.16.5)

  Farnsworth-Munsell 100 Hue Colour Vision Test (2.16.6)

  LogMAR visual acuity testing (2.16.7)

  Threshold tracking (2.16.8)

7.10.2 Laboratory assessments

7.10.2.1 Biological samples

All patients provided a clean-catch MSU for analysis (2.6.1). Biological samples were stored securely on-site in the Department of Rheumatology, Royal Free Hospital, London (2.8).

7.10.2.2 Laboratory analyses

The following exploratory laboratory analyses were conducted as per study schedule:

- Urine sample, screening and safety:

  Urinary dipstick testing (2.13)

  Routine urine culture in hospital laboratory (2.12.2)

- Urine sample, exploratory:
  Urinary IL-6 expression (2.10.1)
  Urinary ATP expression (2.11.1)

- Serum sample, screening and safety:
  Full blood count (FBC)
  Urea and electrolytes (U&E)
  Liver function tests (LFT)
  Calcium profile
  Thyroid function tests (TFT)
  Erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP)
  T-cell subsets
  Serum protein electrophoresis
  Hepatitis B/C and Human Immunodeficiency Virus (HIV)

- Serum sample, exploratory:
  Cytokine analysis

7.11 Adverse events

Adverse event data were reported as outlined previously (2.5). Dr Brian Youl was the authorised medical expert and Medical Director at Daval. Verius Ltd. provided independent pharmacovigilance services and medical monitoring for the study.

7.12 Data management

7.12.1 Data protection

The storage and protection of study-specific data was in accordance with GCP guidance for data management in clinical research (2.3.1). All study documents were kept in a locked, fireproof cabinet in the Department of Neurophysiology, Royal Free

Hospital, London. Dr Anthony Kupelian, study investigator, and Mrs Elizabeth Denver, Senior Clinical Research Nurse at the Department of Medicine, held the only keys. Access was granted to study documentation for monitoring and audit purposes by authorised bodies only.

7.12.2 Data monitoring and audit

Study conduct and all associated documents were subject to regular external monitoring and audit by PSR Group (Hoofdorp, Netherlands). The study complied with International Conference on Harmonisation (ICH) GCP standards (European Union Clinical Trials Directive 2001/20/EC).

7.13 Statistical methods and analysis

7.13.1 Study population

Twenty adult patients with SP-MS and OAB symptoms who met the eligibility criteria for inclusion were invited to participate. The efficacy analyses were conducted on an intention to treat (ITT) basis. The ITT population included all patients who received any study drug and generated outcome data at one or more subsequent study visits. Safety data were analysed from all patients who received study drug.

7.13.2 Sample size calculation

Previous data from the study of MS patients with OAB has demonstrated that a clinically significant change in average voided volume is 60 ml (*sd*=90) (529). The primary objective of the study was to test the null hypothesis that the mean difference in average voided volume between active treatment and placebo was zero. Given these data, a sample size of 20 subjects would provide 80.7% power to detect statistically significant result at the 5% level (α=0.05).

7.13.3 Statistical methods

Demographic and disease-specific data were summarised using standard descriptive statistics. Continuous data were assessed for normality using graphical methods, employing visual assessment of frequency distributions and Q-Q plots. Appropriate measures of central tendency and dispersion were calculated.

The efficacy analysis was conducted to compare the difference in outcome measures during the two treatment phases. For the first treatment phase, data from Visit 1 and Visit 3 were compared. For the second treatment phase, data from Visit 4 and Visit 6 were compared. All efficacy measures were analysed within a mixed models framework. The study drug and treatment group were entered into the model as fixed effects whilst the voided volume at baseline in each phase was entered as a covariate. Patients were entered as random effects. Any interactions between the study drug and treatment phase were reported. A Bonferroni-Holm correction was made to account for variable multiplicity in the secondary and tertiary analyses (394). No corrections were made for missing data.

7.13.4 Primary efficacy analysis

- Change in average voided volume.

7.13.5 Secondary efficacy analyses

- Change in 24-hour urinary frequency.
- Change in 24-hour urinary incontinence.
- Change in Whittington Urgency Score.
- Change in I-QOL.
- Change in SF-36.
- Change in MSIS-29.
- Change in MSWS-12.

- Change in EDSS.
- Change in MSFC.
- Change in Farnsworth-Munsell 100 Hue Colour Vision Test.
- Change in LogMAR visual acuity testing.

7.13.6 Tertiary efficacy analyses

- Change in peripheral nerve excitability.
- Change in serum and urine biomarkers.

7.13.7 Safety Analysis

Safety data were continually reviewed throughout the study. All adverse events were reviewed by the Chief Investigator. A safety monitoring meeting was called every three months when the trial was in progress. The Chief Investigator, Daval's medical expert and the independent medical monitor reviewed the data. Meeting outcomes were communicated to the study investigators.

7.13.8 Interim and subgroup efficacy analyses

No interim or subgroup efficacy analyses were conducted.

7.13.9 Protocol deviations

All protocol deviations were recorded and graded in the study documentation.

7.14 Results

Between April 2010 and December 2010, 81 patients underwent screening. Twenty patients were eligible (F=16; M=4; mean age=50; *sd*=9.7) and participated in the study. The blinded phase of the study was completed in May 2011. The majority of patients were Caucasian (*n*=19) and the mean BMI of participants was 28 kg m^{-2}.

Patients demonstrated a longstanding history of MS symptoms (mean symptom duration=18.7 years; *sd*=7.8) and had established secondary progressive disease (SP-MS duration=5.7 years; *sd*=5.9). At baseline, only three patients had experienced a relapse in the preceding 12 months. There were no relapses in any patient within six months of study participation. Almost all patients used walking aids (*n*=18).

Urinary urgency symptoms were significant (mean urgency score=6.5; *sd*=2.6; range 3-10). Patients demonstrated reduced functional bladder capacity (mean voided volume=151 ml; *sd*=7.8), frequent voiding (mean 24-hour frequency=9.4; *sd*=7.8) and incontinence (mean daily incontinence episodes= 1.7; *sd*=2.5).

Gastrointestinal and psychiatric disorders were the two most common comorbidities amongst patients. Constipation and depression were reported most frequently (*n*=10). Prior to screening, the use of treatments to palliate OAB symptoms, including anticholinergic agents and DDAVP, was widespread (*n*=10). The protocol required that these medications were withdrawn before enrolment.

All randomised patients received at least one dose of study drug and submitted at least one bladder diary after treatment with IMP was initiated. Thus, 20 patients were included in the safety and efficacy analyses. All but one of the randomised patients completed both blinded phases of the study. The withdrawal was due to an AE deemed unrelated to IMP administration part way through the first treatment phase.

There were 82 protocol deviations during the blinded phase of the study, the vast majority of which were minor. In the main, these deviations were the result of mistimed or omitted assessments and laboratory safety samples not being processed. None of these deviations had any effect on the integrity of the study data or patient safety. Study drug compliance was 100% during the blinded phase of the trial.

A summary of the primary and secondary efficacy analyses is reported below (**Table 36**). Treatment with AIMSPRO® was not associated with a significant change in the primary outcome measure of mean voided volume. None of the secondary outcome measures indicated any effect on lower urinary tract function, bladder symptoms, or any non-bladder measures including disease status or neurological functioning.

Twenty-three serum biomarkers were assayed in the tertiary analysis, selected on the basis of laboratory and pre-clinical investigations of AIMSPRO®. These included hormones, growth factors, cytokines and other regulatory molecules. Urinary ATP and IL-6 expression were reported. These results are tabulated below (**Table 37**).

After correction for variable multiplicity, only corticotrophin releasing hormone (CRH) demonstrated a statistically significant increase associated with AIMSPRO® administration. Several other molecules also demonstrated increased expression including adrenocorticotrophic hormone (ACTH), β-Endorphins, met-enkephalin and interleukin-23 (IL-23) but none of these met the criteria for statistical significance after correction for the familywise error rate.

AIMSPRO® did not have any detectable effects on peripheral nerve excitability and the data are not presented.

Table 36 *Summary of primary and secondary efficacy analyses.*

Primary outcome measure	**Mean difference (95% CI)**	**Significance**
Mean voided volume (ml)	0.1 (-33.2−33.3)	*p*=0.99

Secondary outcome measures	**Mean difference (95% CI)**	**Significance**
Urinary frequency (episodes/24 hrs)	-0.3 (-1.0−0.5)	*p*=0.51
Urinary incontinence (episodes/24 hrs)	0.1 (-0.7−0.9)	*p*=0.73
Whittington Urgency Score	0.0 (-1.2−1.2)	*p*=0.99
I-QOL*	1.8 (-4.5−8.1)	*p*=0.57
MSWS-12	4.0 (-6.7−14.8)	*p*=0.47
MSFC§	0.3 (-0.1−0.6)	*p*=0.14
MSIS-29 (physical)†	0.2 (-6.2−6.6)	*p*=0.94
MSIS-29 (psychological)†	1.3 (-6.3−8.8)	*p*=0.74
SF-36 (BP)‡	-8.4 (-22.5−5.7)	*p*=0.24
SF-36 (GH)‡	-1.7 (-7.7−4.2)	*p*=0.58
SF-36 (MH)‡	-1.7 (-11.9−8.5)	*p*=0.74
SF-36 (PF)‡	-0.6 (-6.5−5.3)	*p*=0.84
SF-36 (RE)‡	8.9 (-5.2, 23.0)	*p*=0.25
SF-36 (RP)‡	6.1 (-8.8−21.0)	*p*=0.43
SF-36 (SF)‡	-4.8 (-21.1−11.5)	*p*=0.56
SF-36 (VT)‡	-2.4 (-8.6, 3.88)	*p*=0.49
Farnsworth-Munsell 100 Hue Test	-11.2 (-33.8−11.4)	*p*=0.35
LogMAR visual acuity	-0.1 (-0.2−0.1)	*p*=0.25

****I-QOL** total score presented – separate analysis of subscales (1) avoidance & limiting behaviour; (2) psychosocial impact; and (3) social embarrassment did not alter the strength of the findings.*

***§MSFC** total score presented – separate analysis of components (1) 9HPT; (2) TWT; and (3) PASAT3 did not alter the strength of the findings.*

***†MSIS-29** subscales (physical and psychological impact) are presented separately as using a combined outcome score is not recommended.*

***‡SF-36** domains presented individually as the results cannot be presented with a single summed score: BP=bodily pain; GH=general health; MH=mental health; PF=physical functioning; RE=role emotional; RP=role physical; SF=social functioning; VT=vitality (higher scores indicate better function in each scale).*

Table 37 *Summary of tertiary biomarker analyses.*

Tertiary biomarker measures	Mean difference (95% CI)	Significance
POMC (ng ml^{-1})	-0.44 (-0.93−0.05)	*p*=0.103
OPN (ng ml^{-1})	3.31 (-2.16−8.78)	*p*=0.263
CRH (ng ml^{-1})	1.87 (0.87−2.87)	*p*=0.002
ACTH (ng ml^{-1})	0.29 (0.03−0.55)	*p*=0.047
β-Endorphins (ng ml^{-1})	0.19 (0.07−0.31)	*p*=0.006
Met-enkephalin (ng ml^{-1})	10.36 (4.12−16.60)	*p*=0.005
MSH (ng ml^{-1})	0.09 (-0.07−0.25)	*p*=0.277
BAFF (ng ml^{-1})	-59.01 (-120.4−2.38)	*p*=0.082
FGFβ (pg ml^{-1})	5.16 (-5.31−15.63)	*p*=0.359
TGFβ (pg ml^{-1})	0.014 (-3.93−3.96)	*p*=0.995
EBNA-1 (U ml^{-1})	25.68 (-48.09−99.45)	*p*=0.514
KLK1 (pg ml^{-1})	-7.46 (-36.9−21.98)	*p*=0.634
KLK6 (ng ml^{-1})	-4.57 (-8.40−0.74)	*p*=0.035
IFNα (pg ml^{-1})	2.10 (-0.05−4.25)	*p*=0.062
IL-1β (pg ml^{-1})	-0.03 (-2.08−2.02)	*p*=0.977
IL-4 (pg ml^{-1})	1.22 (-1.03−3.47)	*p*=0.312
IL-5 (pg ml^{-1})	-3.57 (-11.96−4.82)	*p*=0.427
IL-6 (pg ml^{-1})	9.73 (-9.41−28.87)	*p*=0.344
IL-10 (pg ml^{-1})	3.13 (-2.62−8.88)	*p*=0.313
IL-17 (pg ml^{-1})	0.38 (-0.35−1.11)	*p*=0.331
IL-17F (ng ml^{-1})	144.51 (-27.1−316.08)	*p*=0.125
IL-23 (ng ml^{-1})	1.52 (0.60−2.44)	*p*=0.006
IL-33 (ng ml^{-1})	3.95 (-0.86−8.75)	*p*=0.134
Urinary IL-6 (pg ml^{-1})	-5.27 (-15.85−5.31)	*p*=0.368
Urinary ATP (nmol l^{-1})	-142.89 (-906.64−618.85)	*p*=0.706

POMC *Pro-opiomelanocortin;* ***OPN*** *Opiomelanocortin;* ***CRH*** *Corticotrophic releasing hormone;* ***ACTH*** *Adrenocorticotrophic hormone;* ***MSH*** *Melanocyte stimulating hormone;* ***BAFF*** *B-cell activating factor;* ***FGFβ*** *Basic fibroblast growth factor;* ***TGFβ*** *Transforming growth factor β;* ***EBNA-1*** *Epstein-Barr virus nuclear antigen 1;* ***KLK1*** *Kallikrein 1;* ***KLK6*** *Kallikrein 6;* ***IFNα*** *Interferon α;* ***IL-1β*** *Interleukin 1β;* ***IL-4*** *Interleukin 4;* ***IL-5*** *Interleukin 5;* ***IL-6*** *Interleukin 6;* ***IL-10*** *Interleukin 10;* ***IL-17*** *Interleukin 17;* ***IL-17F*** *Interleukin 17F;* ***IL-23*** *Interleukin 23;* ***IL-33*** *Interleukin 33;* ***ATP*** *Adenosine triphosphate.*

Four patients reported an AE prior to the administration of any drug. Mild hypertension was noted in one patient and another three required treatment for UTI before dosing. Follow-up cultures were performed in all cases. One patient reported pseudomembranous colitis after screening which required the run-in to be extended. Screening processes were repeated prior to study entry.

Treatment-emergent AEs affected three-quarters of the patients. Twelve patients reported at least one AE during treatment with AIMSPRO® and eight patients reported at least one AE whilst administering placebo. Injection site reactions were the most common AE associated with AIMSPRO® treatment (n=9). Transient elevations in transaminases (n=2), pain (n=1) and MS relapse (n=1) were also reported. During placebo administration, UTI (n=3) and rhinitis (n=2) were the most commonly reported AEs. Adverse events that were considered by the investigator to be related to IMP use are tabulated below (**Table 38**). One patient withdrew from the trial due to general bodily pain whilst administering AIMSPRO®. There were no SAEs.

Nineteen patients who completed the blinded phase of the study were invited to continue using AIMSPRO® during an open label extension of the study. Eighteen patients accepted this invitation and one patient declined citing a lack of perceived efficacy in the blinded phase. Three of these patients withdrew prior to Visit 7 and declined any further assessments. Of the 15 patients who attended Visit 7, seven did not attend for Visit 8. Six of these patients withdrew citing lack of efficacy and did not wish to attend for any further assessments. One patient was lost to follow-up. The flow of participants through the study is summarised below (**Figure 27**).

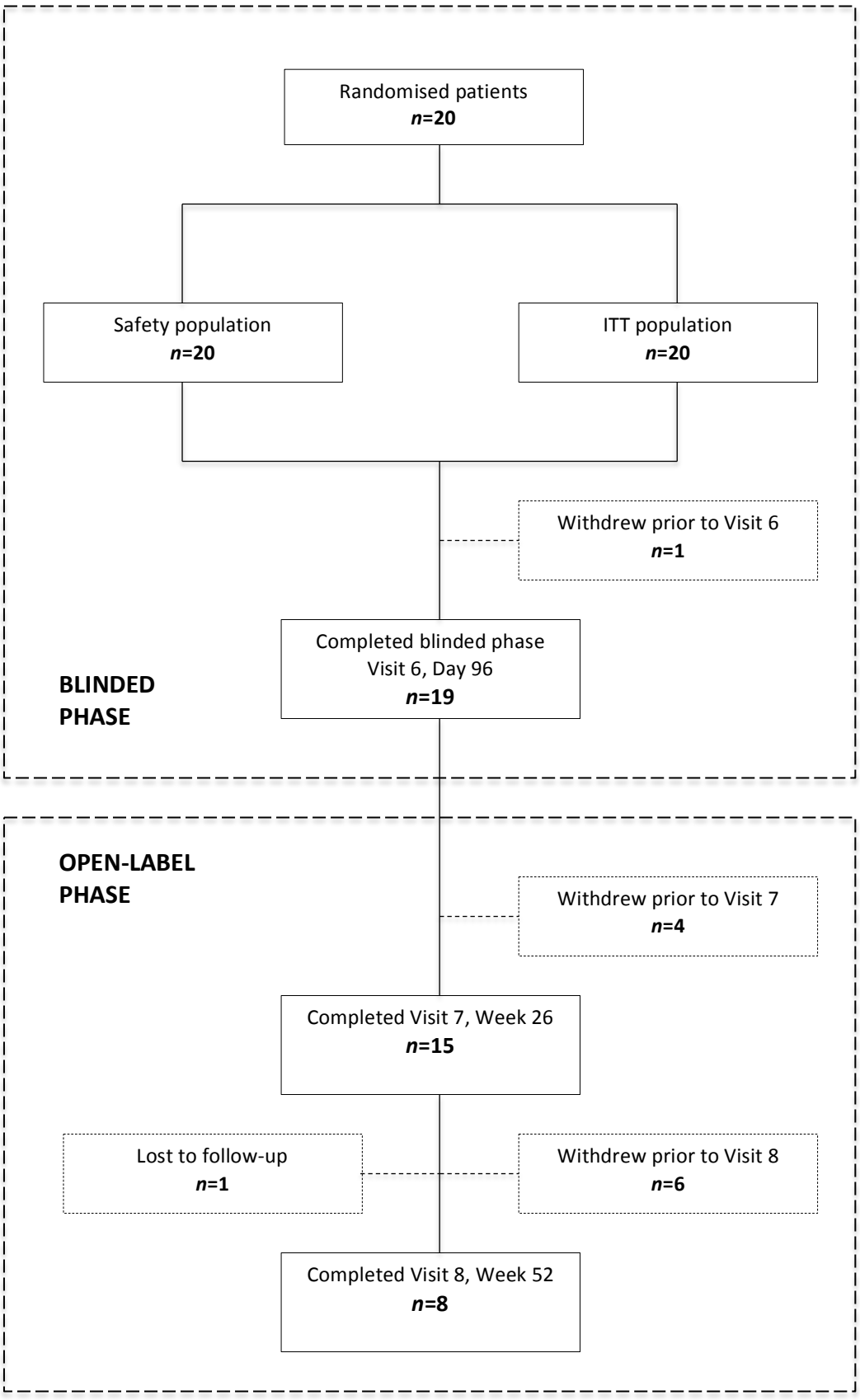
Table 38 *Treatment-emergent adverse events considered related to study drug administration.*

Treatment-emergent AEs*	**AIMSPRO® (%)**	**Placebo (%)**
All AEs	**12 (60)**	**3 (15)**
Injection site reactions/general disorders	**10 (50)**	**0 (0)**
Injection site erythema	6 (30)	0 (0)
Injection site inflammation	1 (5)	0 (0)
Injection site urticaria	1 (5)	0 (0)
Injection site eczema	1 (5)	0 (0)
Pain	1 (5)	0 (0)
Central nervous system disorders	**1 (5)**	**0 (0)**
MS relapse	1 (5)	0 (0)
Cardiovascular disorders	**1 (5)**	**0 (0)**
Hypertension	1 (5)	0 (0)
Laboratory and clinical assessment	**2 (10)**	**2 (10)**
Elevated serum creatinine	0 (0)	1 (5)
Elevated serum transaminases	2 (10)	0 (0)
Elevated body mass	0 (0)	1 (5)

****AE**=adverse event; Causation defined as 'possibly', probably' or 'definitely' related to IMP use.*

In the open-label phase, median exposure to AIMSPRO® was 105 days (range 17-343 days). Treatment-emergent AEs affected half of participating patients. Most were recognised symptoms of MS and very minor abnormalities identified on laboratory testing. Two patients reported upper respiratory infections and two culture-positive UTIs were treated. Adverse events that were classified as possibly related to AIMSPRO® use included one report of optic neuritis and one episode of unscheduled vaginal bleeding.

Figure 27 *Flow of participants through the study.*



One SAE occurred in the open-label extension. Six weeks into open-label administration of AIMSPRO®, a 36 year old female patient was admitted to hospital with worsening mobility. This worsening in neurological function had been slow and in keeping with underlying disease progression. The patient was treated with physiotherapy and home modifications to assist with mobility and safety were put in place. The patient was able to return home after a prolonged period of rehabilitation and formally withdrew from the study shortly after discharge. She cited lack of efficacy as her reason for leaving the study. This patient had experienced progressive problems with mobility that predated her participation in the study. This SAE was not classified as related to the study drug.

High rates of attrition were observed during the open label phase of treatment and almost two-thirds of patients did not complete the study. All patients who contacted the investigators prior to leaving the study cited a lack of efficacy as their reason for withdrawal. Nonetheless, AIMSPRO® appeared to be safe during extended administration amongst this cohort of patients.

7.15 Discussion

Patients with SP-MS often accumulate significant disability that might limit their capacity to engage in clinical trials with more demanding protocols (536). Patient interest in this study came from across the UK, but the visit schedule was deemed too challenging for many potential participants. Four subjects were screened for every patient successfully recruited to the study. The recruitment target of 20 patients was achieved although one patient did not complete the blinded phase of the trial. Despite this loss, the efficacy analyses were conducted on an ITT population of 20 subjects, as specified in the study protocol. The safety population included all 20 patients.

Protocol deviations were frequent but minor and there was no expectation that they would influence the results of the trial. Most deviations related to insignificant delays associated with the collection of biological samples, study drug administration and visit timing. Study drug compliance was 100% in the blinded phase of the trial and all efficacy outcome measures were met. Occasionally, serum samples for safety monitoring were misplaced after being deposited in the NHS laboratory at the study site but such events were infrequent.

In the blinded phase of the study and during extended open-label administration, AIMSPRO® appeared to be safe. Cutaneous injection site reactions were commonplace but almost always self-limiting. Minor fluctuations in laboratory markers were observed in a minority of patients. No specific intervention was required and there was no need to interrupt administration of the study drug. One patient relapsed in the open-label phase having developed optic neuritis. One SAE was recorded in the open-label extension, the result of ongoing neurological decline noted prior to study entry. This was not classified as related to AIMSPRO® use.

During the blinded phase of the study, the administration of AIMSPRO® was not associated with any effects on bladder function, lower urinary tract symptoms or

bladder-related QoL. None of the secondary outcome measures detected any disease-specific effects or changes in general health status. The tertiary biomarker data did identify an increase in CRH associated with AIMSPRO® use, which is a principal component of the drug based on laboratory analysis (507). Increases in the pituitary hormone ACTH, the endogenous opioid peptides met-enkephalin and β-Endorphin, and the pro-inflammatory cytokine IL-23 were noted but these changes were not found to be statistically significant after correction for variable multiplicity. No changes in the expression of urinary cytokines were noted in association with AIMSPRO® administration.

The endogenous production of ACTH and β-Endorphin from the pituitary might be expected in response to increased CRH levels. Both molecules are cleavage products of POMC (537), secreted in response to rising levels of CRH. Thus, AIMSPRO® could plausibly enhance the pituitary production of these peptides. Adrenocorticotrophic hormone drives the production of glucocorticoids that are known to suppress the immune response (509) and it is a universal agonist of the melanocortin receptor, expressed on numerous immune cells (538). The use of ACTH as an immune modulator has been specifically studied in relapsing MS with encouraging results (539).

β-Endorphin is an endogenous opioid that is capable modulating the immune response through inhibition of T-cell activation (540). Whilst synthesised through a distinct pathway, Met-enkephalin is another opioid peptide that may have immunomodulatory effects. In animal studies, met-enkephalin demonstrates phagocyte and T-cell suppression (541, 542) and may be able to influence the HPA axis directly (543).

In contrast to HPA peptides and met-enkephalin that demonstrate immunosuppressive effects, IL-23 is recognised pro-inflammatory cytokine (544). Interleukin-23 has been convincingly implicated in the pathogenesis of autoimmunity and linked to numerous human diseases including MS (545-550). If increased expression of IL-23 associated with AIMSPRO® administration was

detected in future studies, it would difficult to reconcile in view of the claimed anti-inflammatory effects of the drug (508).

Whilst the open-label phase of the study generated additional safety data during extended drug administration, no definitive conclusions relating to efficacy can be drawn from such an analysis. Whilst measures of bladder and neurological function were collected during the extension phase, it was made clear in the study protocol these data were not to be used as proof of a treatment effect. If significant changes in any of these data were detected during open-label administration, the findings were only to be used to guide further research.

Amongst those patients who completed the open-label phase, and accounting for multiple testing, sustained and statistically significant improvements were noted in scores for the psychological subscale of the MSIS-29, and the PASAT-3 and 9-HPT components of the MSFC. No consistent bladder effects were noted over the course of the open-label phase. Nearly two-thirds of the patients who completed the blinded phase of the study withdrew from the open-label phase of the study prior to the last scheduled visit at 12 months. Almost all cited a lack of efficacy as their reason for withdrawal. The loss of more than half of the subjects in this phase of the study limit the utility of these data for shaping future research.

The PASAT-3 and 9-HPT components of the MSFC are sensitive to 'practice effects' which describe increased performance in the completion of a task related to habituation rather the influence of an intervention that is being tested (such as a study drug). Practice effects associated with the PASAT-3 and 9-HPT have been reported to persist at testing intervals of up to three months (551, 552). Whilst the first open-label visit was only three months after the last visit of the blinded phase, the final visit in the open-label phase was conducted six months after the previous visit. Whether a practice effect for these measures could be sustained over this period is not known.

Whilst no changes in physical functioning were detected in the physical domain of the MSIS-29 questionnaire, sustained and significant improvements in the psychological subscale of this measure were noted amongst patients who completed the open-label phase. The psychological subscale of the MSIS-29 includes nine items that include an assessment of anxiety, worry, confidence and depression (302). It is plausible that the psychological impact of medicating with AIMSPRO® in the open-label phase could have conferred improvements in MSIS-29 psychological responses through mechanisms independent of a direct effect on the underlying disease process.

Much has been written in the press and social media relating to the perceived benefits of AIMSPRO®. When interest in AIMSPRO® was perhaps at its greatest, patients delivered a petition to Downing Street containing tens of thousands of signatures calling for NHS backing of the drug (553). The impact of this media coverage and the perceived benefits of the drug cannot be dismissed. It has been demonstrated that the magnitude of a placebo effect can be increased or decreased by modifying patient expectations (554).

The selection of threshold tracking as an exploratory measure of nerve function in this study could be questioned. Threshold tracking is conducted on peripheral nerves (312) whilst MS is a disorder of the central nervous system. Visual evoked potentials (VEP) are an established method to assess the functional integrity of visual pathways in the brain and they are widely employed in the diagnosis of MS (535). The use of VEPs might seem a more appropriate method of studying nerve function in patients with MS, as MS does not affect peripheral nerves.

The selection of peripheral nerve threshold tracking as a means of investigating axonal function after AIMSPRO® administration was made on the basis of the proposed neurophysiological effects of the drug. Initial observations suggested that the administration of AIMSPRO® was associated with a reduction in the triggering voltages of sodium channels in nerves and a prolongation of channel opening (510, 511). Whilst threshold tracking does not test central nervous system function

directly, any effects mediated by the drug on peripheral nerve sodium channel function would be expected to be manifest by nerves in the CNS. No such effects were detected after AIMSPRO® administration under double-blind conditions.

Prior to the inception of this study, there was great censure of Daval in the MS community. This related not only to their alleged conduct and perceived exploitation of patients, but their handling of previous studies of AIMSPRO®. The prospect of another clinical trial of AIMSPRO® was eschewed by many in the MS community, although efficacy data were required to guide clinicians and patients. During this period, AIMSPRO® continued to be prescribed to patients and Daval attributed the collapse of the London study to failures on the part of the investigators.

The randomised phase of this trial took 12 months to complete. The study demonstrated that a crossover design, powered on a clinically relevant effect size, was able to provide a conclusive result whilst inflicting much lower costs than a traditional parallel group RCT. By contrast, the administrative and bureaucratic conflict encountered during the development phase of this project took years to overcome. The Research Governance Framework in the UK provides clear guidance relating to the legal duties of sponsors and investigators in research studies. The successful completion of the study provides reassurance that these mechanisms should be trusted to protect the interests of all parties in clinical research.

Access to the internet can be extremely helpful for those affected by disability (555). It allows patients to connect to provide support and information to one another, and internet forums provide opportunities for group discussion and debate. This can be a vital resource for many patients, especially those who may be isolated as a result of physical and geographical constraints. Whilst patients may benefit from enhanced access to information afforded by the internet, its impact is not universally positive. A recent paper has highlighted the dangers of research findings being propagated through a media disconnected from the scientific community (556). The authors discuss the emergence of chronic cerebrospinal venous insufficiency (CCSVI) as a putative cause of MS, although the analysis is relevant to any treatment that has

been promoted through the media, particularly social, without good evidence of safety and efficacy. There has never been a greater need for clinicians and scientists to engage with the public on these issues.

AIMSPRO® continues to be provided to patients on a named basis in the UK through its registration as a 'special' with the MHRA. In other countries, 'expanded access' programmes permit provision to patients where clinical need cannot be met by a licenced product. Whilst responsibility for the provision of unlicenced medications in these programmes is taken by the prescribing clinician, regulatory requirements vary considerably. In the UK, clinicians must maintain prescribing records and report any adverse events to the MHRA, although in some member states, no legislation is in place (512, 513). The MHRA requires these medicines to be manufactured in accordance with GMP standards but there is no requirement for safety or efficacy data, or ethical review (557). This is the case in most European states (513).

The administration of AIMSPRO® twice weekly for four weeks was not associated with any detectable effect on lower urinary tract function amongst patients with SP-MS and OAB symptoms. No disease-specific effects were identified amongst the secondary outcome measures but only an extended RCT of AIMSPRO® would reliably determine whether long-term use might confer benefit. A recent RCT of AIMSPRO® in the treatment of systemic sclerosis, a multisystem autoimmune disorder, was recently completed in the UK. The trial demonstrated that whilst AIMSPRO® appears to be safe, there was no concrete evidence of therapeutic effect, although a non-significant trend towards improvement was noted (558).

Whilst this study was opposed in some quarters, the MS Society supported the efforts of Professor Malone-Lee to complete the study. They recognised the importance of clinical trial data in dealing with the ongoing controversy surrounding AIMSPRO®. The data generated from this study provide no evidence that short-term use of the drug has any beneficial effects.

8 General discussion and conclusion

8.1 Background

Lower urinary tract symptoms are almost ubiquitous in patients with a longstanding diagnosis of MS. Their impact is comparable to loss of mobility (54) and for some patients urinary symptoms are the most oppressive element of their disease. Chronic LUTS are compounded by urinary infections that are frequently recurrent (55) and aggravated by high rates of catheter use (433). The results of this interaction are not self-limiting. Urinary tract infection exacerbates existing LUTS and has been implicated in functional deterioration and relapse (171, 173, 174). Overactive bladder symptoms are the most prevalent and disruptive LUTS amongst patients (55).

Serious concerns have been raised over the performance of diagnostic tests commonly used to exclude UTI (187-192, 205-208). This is particularly relevant to patients with MS. Emerging data also question whether undisclosed infection may generate OAB symptoms (250, 259-261, 263, 264, 271, 273).

This study evaluated the performance of current tests used to diagnose UTI in patients with chronic urinary symptoms. Controlled data were gathered to determine whether patients with MS and OAB symptoms demonstrate evidence of urinary infection and inflammation dismissed by routine testing. The relationship between bacterial infection, urinary inflammation and OAB symptoms was also explored. The opportunity to test a novel treatment for OAB symptoms associated with MS arose and this prospect was grasped.

8.2 Limitations and weaknesses

Patients with non-neurogenic LUTS, rather than patients with MS, were recruited to evaluate the diagnostic accuracy of surrogate markers of UTI. The volume of participants required to satisfy the sample size calculation precluded the exclusive recruitment of MS patients. The study population demonstrated LUTS of a similar

distribution and chronicity to those reported by MS patients. Nonetheless, the sampling strategy could limit the external validity of the data.

The use of different urine sampling methods amongst patients and controls could have influenced the data. The use of catheter sampling in control subjects would have been unacceptable, particularly as the data were collected prospectively over multiple visits. Despite the lengths taken to minimise contamination amongst control samples, the introduction of bias as a result of sampling strategy cannot be excluded. The culture methods used in this work were directed at the cultivation of aerobic uropathogens. Media and culture conditions to detect fastidious and anaerobic organisms were not used and this may have influenced the findings.

An unplanned analysis was executed to determine the relationship between urinary infection and symptom generation from the longitudinal data. The data model specified in the study protocol was abandoned after statistical assumptions were violated precluding further interpretation. The use of alternative modelling strategies was limited to constrain the impact of multiplicity. Nonetheless, it is possible that this deviation could have influenced the results.

8.3 Diagnostic testing for UTI

Existing evidence suggests that current diagnostic tests for UTI are inadequate. The problems affect the culture methods used to define UTI and their surrogate measures. The culture criteria defined by Kass (1957) fail to account for spectrum bias and were devised to detect infection in patients with pyelonephritis (186). The importance of polymicrobial infection has also been overlooked (194, 559). It is now recognised that much lower culture thresholds should be employed to detect acute infection confined to the lower urinary tract (187-192). These diagnostic constructs may not apply to chronic symptoms (183) and data relating to the performance of dipstick testing and culture methods in patients with chronic LUTS are few (209-211).

National guidance for the screening of UTI in MS recommends the dipstick as the primary tool to exclude infection (104, 374). This work provides evidence that the performance of the dipstick and other surrogates of urinary infection in patients with chronic LUTS are extremely poor, even before the controversies relating to bacterial culture are considered. The failure of these tests could leave UTI untreated, mediating significant negative effects on lower urinary tract function.

Whilst not the focus of this work, infection has been associated with neurological deterioration and relapse in MS (152-154, 156, 171). Urinary infection has been implicated in this interaction and UTI is frequently associated with hospital admission (156, 171, 173, 174). The failure to diagnose and treat UTI in patients with MS might increase the risk of upper tract involvement and systemic infection. This could have a considerable impact on neurological function and even trigger relapse.

8.4 Urinary infection and inflammation

Infection is being disregarded by standard culture methods and the ecology of urinary infection is being concealed through the use of fixed diagnostic thresholds. Using a new culture technique devised in this centre, urine samples were subject to much closer microbiological scrutiny than current tests allow (266-269). Evidence of urothelial inflammation was sought by quantitative assessment of pyuria and urinary IL-6. Experimental measures of urothelial cell colonisation and distress were explored. These data were carefully controlled and subjects were matched on key demographic characteristics.

When diagnostic thresholds were abandoned and more sensitive culture methods employed, cultures from patients and controls demonstrated distinct ecology. Whilst quantitative differences in bacterial growth were observed, differences in the infecting taxa amongst the groups were striking. In the patient group, recognised uropathogens including *E. coli and Enterococcus spp.* were isolated. Amongst controls, *S. agalactaie* was the dominant isolate in all but one of the positive

cultures. Routine urine culture did not detect these differences. These ecological findings were associated with evidence of increased urothelial inflammation amongst patients, implying that these microbes were more than harmless contaminants.

8.5 Infection, inflammation and symptoms

The proposed relationship between urinary infection and the generation of OAB symptoms has been forged from cross-sectional studies. These data were often subject to inadequate matching of patients and asymptomatic controls. Prospective data were needed to test this hypothesis and the judicious selection of controls was a necessity. An observational cohort study was undertaken to achieve these aims. Patients and controls were matched for key demographic characteristics and monitored at eight study visits over a two-year period.

The emergence of pyuria was associated with increased bacterial colony counts and increasing pyuria expression was associated with a commensurate rise in bacterial load. These findings were independent of routine culture status. Nonetheless, the interaction between pyuria and bacteriuria did not appear to be a simple reciprocal relationship. These data imply that once the inflammatory response is established, escalating inflammation is mediated by expanding bacterial numbers. By contrast, significant bacterial expansion may occur without inciting an inflammatory response.

Quorum sensing mechanisms might restrain the expression of virulence factors until a critical biomass is reached. Thus, bacterial numbers may increase significantly without an attack on the host. The cell density at which this occurs is likely to vary between genera and species. Asymptomatic bacteriuria exemplifies this phenomenon. Increasing pyuria was associated elevated levels of urinary ATP and IL-6, indicative of urothelial distress and inflammation, although the strength of these relationships varied.

Increasing pyuria was associated with deterioration in urinary urgency, frequency and voided volume. These relationships were independent of routine culture status. The estimated effect size for each of these variables is likely to be of clinical relevance to patients. These controlled, prospective data are the first to demonstrate a significant association between bacterial infection, urothelial inflammation and the generation of OAB symptoms.

Whilst the influence of neuropathology on lower urinary tract function is undisputed, it does not appear to be the sole determinant of lower urinary tract function in MS. The interaction between infection, inflammation and neurological damage on bladder function is likely to be variable amongst patients. Nonetheless, these data suggests that the treatment of presumed bacterial infection in the presence of pyuria might confer significant symptomatic benefit.

8.6 Testing a novel treatment for the overactive bladder

In the development phase of this proposal, the opportunity to test a novel therapy for OAB symptoms in MS patients presented itself. Daval International, the manufacturer of AIMSPRO®, has promoted the drug as a putative treatment for a number of autoimmune and inflammatory conditions. Nonetheless, the agent is unlicenced and evidence of efficacy is limited to clinical observations and reports (275, 514-520).

At the time of writing, hundreds of patients with MS had used the drug for periods of up to five years (524). Improvements in lower urinary tract function had been reported during AIMSPRO® administration and these benefits could be seen very soon after initiating treatment (514). AIMSPRO® is claimed to have immunomodulatory effects and a drug that could palliate LUTS by such a mechanism would be apposite to the central hypothesis of this work.

The proposal was controversial. Daval International, the manufacturers of AIMSPRO®, had been accused of improper conduct relating to the marketing and provision of the drug, although these claims were investigated and no charges were made (522, 523). Despite these concerns, and attempts to stop the study being undertaken, it was completed successfully. The crossover design used in the trial generated an unequivocal result without the need for a larger parallel-group trial. Such studies are financially prohibitive for smaller biotechnology companies and may restrain the development of new candidate drug therapies.

The use of AIMSPRO® for four weeks was not associated with any improvements in lower urinary tract function. There were no detectable changes in neurological status or neurophysiological parameters in the blinded phase of the trial. Only an extended study of AIMSPRO® could reliably determine whether long-term use of the agent might confer benefit for patients with SP-MS. A recently reported RCT of AIMSPRO® in the treatment of systemic sclerosis failed to demonstrate efficacy after six months of treatment, although there was a trend towards improvement in the primary outcome measure and drug appeared to be safe (558).

8.7 Future work

Our approach to the diagnosis of UTI needs to be revised and the disease should no longer be viewed in binary terms. Rather than pursuing new diagnostics to define the presence or absence of the condition, we should seek to develop models that identify those patients most likely to benefit from treatment. This will rely on a fuller description of the interaction between bacteria and host. Studies to explore the prevalence of atypical bacterial infection in these patients would complement this work. The longitudinal data derived from this study indicate that patients with MS and OAB symptoms who demonstrate pyuria might benefit from antibiotic treatment. Intervention studies are now required to demonstrate that antibiotic therapy is effective in palliating symptoms and determine how such treatment is best delivered.

Existing data suggest that UTI associated with fever may be associated with relapse. Whilst UTI without features of systemic involvement may mediate deterioration in neurological function, such infections have not been convincingly implicated in relapse. More work is needed to establish the influence of urinary infection on the disease in general. Whether apparently localised infection can influence the course of MS is not known and studies that examine the immune response to such infections are needed. This is particularly important in view of the prevalence of undiagnosed UTI amongst patients. Whether the peripheral activation of T-cells or cytokine production in the absence of systemic infection might drive neurological progression is unclear.

This study provides the first prospective data that support a link between bacterial infection, urothelial inflammation and the generation of OAB symptoms in patients with MS. These findings may also be of relevance to patients with non-neurogenic lower urinary tract dysfunction. Future studies should include this group.

8.8 Conclusion

This programme of study was conceived to test the hypothesis that bacterial infection of the lower urinary tract goes undetected by routine diagnostic testing and contributes to the generation of LUTS in patients with MS.

The aims of the study were as follows:

(1) To determine the diagnostic performance of routine urinalysis methods used to detect urinary tract infection in patients with chronic lower urinary tract symptoms.

(2) To determine the prevalence of urinary tract infection and inflammation in patients with multiple sclerosis and overactive bladder symptoms, employing sensitive bacteriological methods and measures of the urothelial inflammatory response.

(3) To determine the relationship between bacterial infection, urothelial inflammation, manifest by pyuria and elevated local pro-inflammatory cytokines, and lower urinary tract symptoms in patients with multiple sclerosis and overactive bladder symptoms.

(4) To test the effects an immunomodulatory agent on lower urinary tract functioning in patients with multiple sclerosis and overactive bladder symptoms, without evidence of urinary tract infection on routine testing.

The study aims were explored successfully and the results support the central hypothesis of this work. Urinary tract infection is dismissed by current diagnostic tests and infection, associated with urothelial inflammation, is commonplace amongst patients with MS. Inflammation of the lower urinary tract, in addition to neurological damage, appears to be associated with the generation of overactive bladder symptoms. Whilst antimicrobial therapy might be a candidate treatment for this condition, further study is required. The immunomodulatory agent tested in this work did not have any effect on lower urinary tract symptoms or any disease-specific outcomes in MS.

The risks of UTI for patients with MS are considerable and the initiation of ISC is associated with a significant risk of chronic and recurrent infection (165, 167, 168). Current guidance recommends the initiation of ISC at low residual volumes in asymptomatic patients without any supportive evidence (104). This recommendation needs to be reconsidered, as it may be associated with harm.

9 References

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