# UCL

# MD Thesis

# An Experimental study to find out the Significance of sterile Pyuria and evidence of Inflammation in Overactive Bladder

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## Declaration

I, RAHUL LUNAWAT 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

**Purpose:** It has been reported that over 33 % of patients with OAB present with pyuria (≥10 wbc µl-1) on urine microscopy, but under a third of these have bacteriuria. To clarify this situation, an accomplished comparative scrutiny of the inflammatory state of the urothelium in OAB, was carried out.

**Materials and Methods:** This was a prospective, blinded, observational study of idiopathic OAB patients compared with controls. CSU samples were obtained and submitted to fresh urine microscopy, routine culture and microscopic examination of spun sediment. Another sample of OAB patients and asymptomatic controls provided cystoscopic bladder biopsies for histopathology and electron microscopy.

**Results:** 178 OAB patients and 21 controls provided spun sediment samples. 75 (42 %) of these OAB patients had microscopic pyuria (≥1 wbc µl-1) with 25 of which (33 %) were culture-positive. None of the controls had pyuria, nor bacteriuria. In a 20 $mm^2$ spun deposit there was an average of 48 wbc (95 % CI 26 to 589) in OAB with pyuria; 12 wbc (95 % CI 10 to 15) in OAB without pyuria; and 4 wbc (95 % CI 3 to 7) in controls. Biopsies from 79 OAB patients, showed chronic inflammation and hyperplasia in 69 (87 %; 95 % CI=78 to 92: only 20 % had pyuria) and none in 5 controls. EM of 22 OAB patients showed increased basement membrane thickness compared to 2 controls ($H=48$, $df=2$, $p<0.001$)

**Conclusions:** The study shows evidence of chronic cystitis and urothelial hyperplasia associated with OAB irrespective of pyuria or bacteriuria. The phenomenon has been confirmed by 3 different methods.

## List of Publications and Presentations

1) Khasriya R, Khan S, Lunawat R, Bishara S, Bignall J, Malone-Lee M, Ishii H, O'connor D, Kelsey M, Malone-Lee J. The Inadequacy of Urinary Dipstick and Microscopy as Surrogate Markers of Urinary Tract Infection in Urological Outpatients with Lower Urinary Tract Symptoms without Acute Frequency and Dysuria, J Urol. 2012 May; 187(5):1938

2) Lunawat R, Khasriya R, Maraj B, Falzone M, Malone-Lee J. Mixed inflammatory cell infiltration in urinary spun sediments in OAB patients with or without pyuria compared to normal controls, UKCS, Swansea, 15-17 April 2009

3) Lunawat R, Khasriya R, Maraj B, Malone-Lee J. The evidence of cystitis as the cause of Overactive bladder, RSM 2009, London, Nov 2009

4) Lunawat R, Khasriya R, Maraj B, Falzone M, Malone-Lee J. Histological evidence for urothelial inflammation at the heart of the OAB, UKCS, Swansea, 15-17 April 2009

5) Lunawat R, Khasriya R, Maraj B, Falzone M, Malone-Lee J. Urothelial Metaplasia and OAB symptoms – data supporting chronic urothelial stress, ICS society, Cairo, 20-24 April 2008

6) Lunawat R, Khasriya R, Khan S, Bishara S, Maraj B, Malone-Lee J. Routine MSU culture in patients with symptoms of OAB may be missing many genuine infections, ICS society, Cairo, 20-24 April 2008

7） Malone-Lee J, Ghei M, Lunawat R, Bisahara S, Kelsey M. Urinary white cells and the symptoms of the overactive bladder. Neurourol Urodyn 2007;26(5):656-7

8） Lunawat R, Khasriya R, Khan S, Maraj B, Malone-Lee J. Urinary Nitrite Test is Useless, UKCS, Birmingham, April 2007

## Table of Abbreviations

- OAB Overactive Bladder
- MSU Mid Stream Urine
- CSU Catheter Specimen of Urine
- UTI Urinary Tract infection
- LUTS Lower Urinary Tract Symptoms
- IC Interstitial Cystitis
- DO Detrusor Overactivity
- PAP Papanicolou Stain
- MGG May Grunwald Giemsa Stain
- EM Electron Microscopy
- ICS International Continence Society
- EAU European Association of Urology
- AUA American Urological Association
- ICS International Continence Society
- NICE National Institute of Clinical Excellence
- CRP C reactive protein
- NGF Nerve growth factor
- PgE Prostaglandins E
- NANC Non-adrenergic Non-cholinergic
- NO Nitric oxide
- CP Chronic Prostatitis
- CPPS Chronic Pelvic Pain Syndrome
- PBS Painful Bladder Syndrome

- ATP Adenosine Tri Phosphate
- ADP Adenosine Di Phosphate
- AMP Adenosine Mono Phosphate
- IJP Inhibitory Junctional Potential
- EJP Excitatory Junctional Potential
- CNS Central Nervous System
- PNS Peripheral Nervous System
- ACh Acetyl Choline
- IPSS International Prostate Symptom Score
- ICI International consultation on Incontinence
- ICP Integrated Care Pathway
- PFMT Pelvic Floor Muscle Training
- E. Coli Escherichia Coli
- NIDDK National Institute of Diabetes and Digestive and Kidney Diseases
- CRF Case Record form
- BM Basement Membrane
- H & E Haematoxylin & Eosin

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# Lower Urinary Tract Symptoms

# CHAPTER 1

# LOWER URINARY TRACT SYMPTOMS

The term lower urinary tract symptoms (LUTS) describe storage, voiding, and post micturition symptoms. According to two recent multinational population-based studies, the prevalence of LUTS is 3–10 % in men aged 40–49 years, rising to 24–29 % in those aged 70–80 years (1),(2). Problems with storage are commoner (men, 51 %; women, 59 %) than voiding difficulties (men, 25 %; women, 19 %). Both sexes are similarly affected by post micturition symptoms (men, 17 %; women, 14 %). The prevalence of overactive bladder (OAB) causing frequency, urgency and/or incontinence is 11 %. All these symptoms are more common in late life (2). Chronic prostatitis (CP), also called "Chronic pelvic pain syndrome" (CPPS) has a 9 % prevalence in men (3). Interstitial Cystitis (IC) also called "Painful bladder syndrome (PBS) occurs in between 5 and 16 per 100,000 (4). Symptomatically, all these conditions overlap substantially and clear diagnostic compartmentalization is very difficult (5).

## 1.1 Overactive bladder

### 1.1.1 What is it?

The International Continence Society (ICS) defines Overactive Bladder (OAB) as "Urgency, with or without urge incontinence, usually with frequency and nocturia" with the synonyms the overactive bladder syndrome, urge syndrome or urgency-frequency syndrome. These terms may be used provided that there is "no proven infection or obvious pathology" (6). "OAB-dry" is a term that describes individuals with symptoms of OAB but without urge incontinence. Similarly "OAB-wet" is a term that describes individuals with symptoms of OAB with urge incontinence. The key symptom of OAB is urgency - the sudden, compelling desire to void that is difficult to defer. Frequency is defined as the patient complaining of voiding 8 times or more in a day, and nocturia is the complaint that the individual wakes up more than once at night to void. Frequency and nocturia can occur with or without urge incontinence (7;8). Urinary incontinence is the complaint of any involuntary leakage of urine. Urge incontinence is the involuntary loss of urine accompanied by or immediately preceded by urgency (6). A debate persists about the appropriateness of the term OAB as some doubt whether it is really a syndrome, particularly because the symptoms frequency, urgency and urge incontinence do not imply a single disease. In point of fact they are symptoms of several different pathological states which includes; detrusor overactivity, detrusor hypersensitivity, infravesical obstruction, bladder neck insufficiency and polyuria (7).

Cystitis is left off this list because it is always accepted that diagnosis has been excluded, assuming the accuracy of the tests used to achieve this.

The symptoms of OAB are usually attributed to involuntary contractions of the detrusor muscle (9). Detrusor overactivity; whether neurogenic (arising from neuropathology);

myogenic (arising from detrusor muscle disease); or idiopathic (no known cause), is characterized by involuntary contractions of the detrusor during the filling phase that may be spontaneous or provoked. Involuntary detrusor contractions may occur at any bladder volume. The ICS describes 2 types of detrusor overactivity: phasic and terminal. Phasic detrusor overactivity is defined by a characteristic waveform and may or may not lead to urinary incontinence; it is the pattern typically seen in most idiopathic detrusor overactivity. Terminal detrusor overactivity is characterized by a single involuntary detrusor contraction that occurs at cystometric capacity (when the normal, controllable desire to void is experienced), which cannot be suppressed and results in incontinence, often leading to complete bladder emptying (6).

### 1.1.2 Anatomy of Lower urinary Tract

The lower urinary tract consists of the urinary bladder, the urethra, the internal urinary sphincter, the bladder outlet, the external urethral sphincter, and the striated muscles of the pelvic floor. The structures of the pelvic floor are similar in males and females; however, in males, the bladder neck is stronger and the prostate and rhabdosphincter offer support. In females, the levator ani muscles and the external sphincter are the primary bladder support structures (10). The bladder wall is lined with bundles of smooth muscle fibers that make up the detrusor bulk. The fibers are interspersed with connective tissue, giving the bladder its compliance – which describes the relationship between change in bladder volume and change in detrusor pressure. The internal urinary sphincter consists of the bladder neck and the proximal urethra itself. The urethra and the entire internal urinary sphincter are surrounded by striated muscle fibers. The bladder outlet consists of the internal sphincter and its surrounding striated musculature and is supported by the pelvic floor muscles (10;11). The periurethral

striated muscle fibers and those surrounding the entire internal sphincter constitute the external urethral sphincter.

### 1.1.3 Innervation

The lower urinary tract is innervated by an integrated afferent and efferent neuronal complex of peripheral neural circuits involving sympathetic, parasympathetic, and somatic neurons (12). The efferent pathways are heavily myelinated, whereas the bladder afferent pathways consist of myelinated A-δ fibers and unmyelinated C-fibers. The normal micturition reflex is initiated by signals from the A-δ fibers; the C-fibre afferent neuron signals are not essential for normal voluntary voiding (13).

The sympathetic nerves arise in the lateral horns of spinal segments T1–L2. These preganglionic fibers branch off and enter the sympathetic ganglia. The sympathetic nervous system stimulates sphincter closure in the urethra as well as relaxation of the detrusor muscle during filling (10). Conversely, the parasympathetic nervous system is responsible for the contraction of the detrusor muscle during micturition while simultaneously relaxing the urethral sphincter. Parasympathetic innervation arises from the $2^{nd}$, $3^{rd}$, $4^{th}$ sacral segments of the spinal cord.

The somatic efferent motor neurons arise from the $2^{nd}$, $3^{rd}$, $4^{th}$ segments of the sacral spinal cord. Somatic innervation maintains tone in the pelvic floor musculature and provides excitatory innervation to the striated muscles of the external urethral sphincter. Bladder control also comes from the higher centres, including the brain stem and cerebral cortex. The cortex exerts a predominantly inhibitory influence, while the influence of the brain stem is facilitatory.

### 1.1.4 Neurotransmission

Acetylcholine activation of muscarinic receptors to stimulate contraction of the detrusor appears to be the most important physiologic control system for urinary bladder contraction (9;14;15). Muscarinic receptors, found on presynaptic nerve terminals, may be excitatory or inhibitory. There are atleast 5 types of muscarinic receptors but to date, ≥3 muscarinic receptor subtypes ($M_1$ to $M_3$) have been identified in the human bladder by receptor-binding assays (15). Very small numbers of $M_1$ receptors appear to facilitate the release of acetylcholine in the bladder (16). $M_2$ receptors predominate numerically in the human bladder, but $M_3$ receptors mediate the cholinergic-induced contractions of the detrusor (15). It seems that the $M_2$ and $M_3$ receptors are functionally coupled and act synergistically (17;18). Activation of $M_2$ receptors inhibits sympathetically mediated detrusor relaxation; therefore, coactivation of $M_2$ receptors may enhance the detrusor contraction in response to $M_3$ receptor activation (12). There is some evidence that $M_2$ receptors may also partially stimulate bladder contraction directly (18). The relative contribution of the different muscarinic receptors may be altered during aging, disease, or neurogenic lesions; muscarinic receptor functions may upregulate mechanisms that normally have little clinical importance, and contribute to the pathophysiology of OAB (16).

The sympathetic nervous system also contributes through the influence of a noradrenergic innervation (19).

For many years it has been recognised that there is also some non-adrenergic, non-cholinergic (NANC) innervation of the bladder (20;21).This has been identified as mediated via the influence of ATP acting on purinergic receptors (22). NANC transmission has been

particularly described in animal models and it was thought to contribute very marginally to human bladder function, which may not be the case in pathological states.

### 1.1.5 The History of NANC Transmission

Burnstock et al. in 1962 recorded inhibitory junctional transmission between enteric neurones and smooth muscle cells of the gut. These inhibitory junction potentials (IJPs) were first attributed to stimulation of sympathetic nerve terminals releasing noradrenaline. The discovery that the IJP could not be blocked with the adrenergic blocking drugs, that this transmission was identified as nonadrenergic and noncholinergic, hence introduction of the acronym NANC. At the same time that NANC inhibitory junctional transmission was described, an atropine-resistant excitatory junctional potential (EJP) to the same smooth muscle of the gut was discovered and so that an excitatory NANC transmission was identified. Much work has gone into to identify the NANC transmitters responsible for these properties. In the gut the IJP has been found to arise from nitric oxide signaling and the EJP result from tachykinins. Burnstock et al. has always promoted the view that purines are important transmitters for the EJP in many of the pelvic viscera and blood vessels. The nerves that use ATP as the principal transmitter were named 'purinergic'. It was Burnstock who proposed a model of storage, release, and inactivation of ATP (22) and this has indeed been supported by subsequent experimental data (23;24) despite initial opposition to the idea. Understandably biochemists saw ATP as an intracellular energy source and balked at the idea that this could be used for inter-cellular signaling as well. This stands true to the Darwin theory of evolution about grabbing a remarkable opportunity. ATP was one of the 1$^{st}$ biological molecules to appear and, so it is very reasonable that it has been co-opted for extracellular and intracellular purposes by evolution (25).

### 1.1.5.1 NANC Transmitters

A large number of NANC transmitters are recognized and the 3 important classes are - Neuropeptides (e.g. Angiotensin, Opioids), Purines (e.g. ATP, Adenosine, ADP, and AMP) and NO (Nitric oxide). Nitric oxide is unique in being produced on demand. It is not pre-stored in vesicles.

### 1.1.5.2 Purinergic Receptors

2 main types of purinoceptor have been identified as P1 for adenosine and P2 for ATP/ADP (26) (27). In both cases, sub-groups of these classes have been identified (28). In 1985 a pharmacological properties were used to distinguish 2, P2 receptors P2X and P2Y (26). In 1994, Abbracchio and Burnstock, following signal transduction experiments and molecular biological sequencing proposed that purinoceptors should be divided into 2 families: a P2X family of ligand-gated ion channel receptors and a P2Y family of G-protein-coupled receptors. This has been widely adopted and nowadays 7, P2X subtypes and 8, P2Y receptor subtypes are recognized. These now include some receptors responsive to pyrimidines as well as purines (28) (29). 4 subtypes of P1 G-protein-coupled receptors have been cloned, namely, $A_1$, $A_{2A}$, $A_{2B}$, and $A_3$. 7 subtypes of P2X receptors have been identified. The P2X subunit class occurs in 2 different size clusters, one about 1 μm diameter and the other about 0.4 μm diameter all smooth muscle studied to date, including the urinary bladder (30). The subunits $P2X_2$, $P2X_3$, and $P2X_5$ are commonly found in clusters in the detrusor, but fewer $P2X_4$ and $P2X_6$ (31).

### 1.1.6 Co-Transmission

Burnstock et al (32) proposed the cotransmitter hypothesis. By this he meant that nerve terminals could produce more than one neurotransmitter. Nowadays it is accepted that many nerves signal via more than one molecule. ATP is a cotransmitter with classical transmitters (ACh and NA) in most nerves of the peripheral (PNS) and central nervous systems (CNS) (33). Parasympathetic nerves supplying the urinary bladder use ACh and ATP as cotransmitters, in variable proportions in different species (27;34).

## 1.2 Pathophysiology of OAB

An understanding of the pathophysiology of OAB should assist effective treatment. Normal urinary storage and voiding function depends on interactions between the cerebral cortex, pons, spinal cord and autonomic nervous system. There is scope for many different pathological processes to affect the nervous control and tissue function of the system at numerous locations (9;10;35).

OAB may be classified according to the presumed aetiology of the lesion: neurogenic (e.g., spinal cord injury), myogenic (e.g., obstruction secondary to benign prostatic hyperplasia), inflammatory (e.g., interstitial cystitis), or idiopathic. (36).

Regardless of the underlying disease, OAB manifests some typical behaviour. These include spontaneous intravesical pressure rises during bladder filling, spontaneous contractions of organ-bath mounted detrusor, a tendency to fused tetanic contractions, and alterations in sensitivity to neurotransmitters.

### 1.2.1 Electrical properties of the detrusor

The spontaneous contractions occurring in isolated detrusor strips been documented in tissue from obstructed unstable bladders (37) and neuropathic bladder (38). It has been observed that in response to obstruction there is increased sensitivity to muscarinic stimulation and to potassium chloride, but have reduced contraction to tetrototoxin (TTX) sensitive electrical stimulation (39)(40). In idiopathic overactivity, isolated strips show increased responsiveness to potassium chloride but not cholinergic stimulation. However, reduced contraction to tetrototoxin (TTX) sensitive electrical stimulation is also shown (41). If denervated, detrusor

will express increased quantities of M3 receptors (42).The smooth muscle bundles of the detrusor rely on dense innervations in order to achieve synchronous contractions through simultaneous stimulation. Whereas, overactive bladder may exhibit increased cell-to-cell coupling i.e better coupled electrically. This generates uninhibited contractions. Given the dense neuronal innervation of the cells it is difficult to be confident that it will contribute significantly to the disease process (43).

### 1.2.2 Morphologic changes in the detrusor

There have been a limited number of histological studies of bladder obtained from patients with OAB. The morphological changes described include patchy denervation, hypertrophy and fibrosis (44). 1 study has reported at an ultrastructural changes but further experiments failed to reproduce these findings (45).

### 1.2.3 Neuroplasticity

It has been found that the nervous system is capable of changing neurotransmitters, reflexes, and synapse function in response to disease. This is now referred to as neuroplasticity. This has been invoked to explain some of the pathological responses that are exhibited by the bladder (46). This particularly seems to involve the enhancement of ATP activity. The significance of these changes mean that in diseased states a different set of neuroactive drugs would be required to modify function than those implied through studies of the health state.

### 1.2.4 Ischaemic changes

The symptoms of OAB amongst the elderly are associated with reduced bladder capacity, slower voiding and incomplete bladder emptying. Recently these manifestations have been induced in animal models by the induction of bladder ischaemia (47).

It has been found the nerves are more susceptible to ischaemic damage than is the detrusor. It is notable that the patterns of bladder overactivity measured in the elderly have been found to parallel those identified in younger patients with neurological disease (48).

### 1.2.5 Central nervous system

Spinal cord transection results in detrusor hyperreflexia. This features highly reproducible reflex bladder contractions that can be induced reliably by the infusion of the same volume of saline into a bladder. Cord transection is also associated with urinary retention arising from detrusor sphincter dyssynergia and a reduced ability to sustain a detrusor contraction (49-51). After spinal injury, there is some delay before the micturition reflex re-establishes and during this period there is complete urinary retention (52;53)(46). With time the micturition pathway is reorganized forming a spino-bulbo-spinal or spinal loop, which dominates the life of the bladder through primitive reflex voiding. It is notable that C-fiber (unmyelinated) afferents in particular can trigger micturition contractions in the spinal bladder, whereas this is not a feature of normal bladders (54;55).

### 1.2.6 The urothelium

Nowadays it is becoming evident that the urothelium plays a more active role in bladder physiology than a simple barrier. The urothelium is able to communicate with afferents on the basal surface that would seem to convey important signals related to bladder filling. Urothelium releases Ach, NO, but a development of considerable significance is the recognition that the human urothelium can generate ATP, which acts on suburothelial purinergic afferents. For a long time it was assumed that the human bladder produced very little ATP and this is probably the case in normal circumstances, However in the presence of inflammation there is a considerable potential for a great enhancement of ATP production in

response to urothelial stretch and if this were to be shown to be the case it would greatly alter the current appreciation of the bladder pathophysiology (56).

### 1.2.7 Other factors

There have been a plethora of hypotheses about the pathophysiology of OAB that have implicated in various ways i.e. nerve growth factor, local tissue acidosis, hyperkalaemia, hyperosmolality, neurotrophins and cytokines. Much of this is drawn from animal models and there is a regrettable lack of human data. Perhaps this problem could be alleviated by greater use of the human tissue samples that are available from different sources, including the urine and flexible cystoscopic biopsies. Given the advances in scientific methods it may be that these previously limited options may prove to be capable of narrating a more sophisticated tale.

## 1.3 Epidemiology

LUTS describes storage, voiding, postmicturition and pain symptoms. According to 2 recent multinational population-based studies, the prevalence of LUTS is 3–10 % in men aged 40–49 years, rising to 24–29 % in those aged 70–80 years (1;57;58). Problems with storage are commoner (men, 51 %; women, 59 %) than voiding difficulties (men, 25 %; women, 19 %). Both sexes are similarly affected by postmicturition symptoms (men, 17 %; women, 14 %). The prevalence of overactive bladder (OAB) causing frequency, urgency and/or incontinence is 11 %. All these symptoms are more common in later life (2). Chronic prostatitis (CP), also called "Chronic pelvic pain syndrome" (CPPS) has a 9 % prevalence in men (3). Interstitial Cystitis (IC) also called "Painful bladder syndrome (PBS) occurs in between 5 and 16 per 100,000 adults (4). Symptomatically, all these conditions overlap considerably and their pathophysiology is not clear (5). The burden is illustrated by OAB, the commonest, which is independently associated with falls and fractures; urinary tract and skin infections; sleep disturbances and depression. A survey of 16,776 interviews, conducted in 6 European countries, found a prevalence of overactive bladder symptoms of 16.6 % in individuals aged ≥ 40 years. Frequency (85 %) was the most commonly reported symptom, followed by urgency (54 %) and urge incontinence (36 %). The prevalence of OAB increased with advancing age (59). These symptoms overlap with those experienced in urinary infection (UTI), "frequency-urgency-dysuria", "Painful bladder syndrome" and "Interstitial cystitis". The latter has an estimated prevalence of 0.31 %; 94 % female (Kidney and Urologic Diseases Statistics USA, 1988-1994). The lifetime risk of UTI for women is between 40 % and 60 %, and recurrent UTI occurs in 16 to 40 % of women who have had 1 UTI. In some, recurrent UTI is a serious problem (60).

One study has estimated that OAB cost 5 EEC countries in excess of €4.2 billion in the year 2000 (61). Urinary tract infection is one of the commonest infectious diseases worldwide (62). Below is a table reproduced with permission from a New England Journal of Medicine review written by J.Ouslander (2004) (9).

## 1.4 Conditions that may cause or contribute to symptoms of OAB?

Displayed with permission - from JG Ouslander (9)

| Conditions | Mechanism or Effect | Implications for management |
|---|---|---|
| Lower urinary tract conditions – Both sexes | | |
| Urinary Tract Infection | Inflammation/activation of sensory afferent | Treat infection before other interventions are considered |
| Obstruction | Can contribute to detrusor overactivity or urinary retention | Consider surgical intervention |
| Impaired bladder contractility | Reduced functional bladder capacity | Avoid drugs contributing, teach to enhance voiding, clean Intermittent catheterization may be helpful |
| Bladder abnormalities (tumor/stone/ interstitial cystitis) | Can precipitate Detrusor overactivity | Sterile haematuria/risk factors shall be evaluated |
| Women | | |
| Estrogen deficiency | Atrophic vaginitis / Urethritis can contribute to symptoms | Topical estrogen may help |
| Sphincter weakness | Leakage of urine into proximal urethra may precipitate urgency | Pelvic musc, periurethral injections or surgery needed |
| Men | | |
| Prostate enlargement | Benign or malignant disease can contribute to detrusor overactivity | Screen for prostate cancer, consider Alpha blocker, 5-alpha reductase inhibitor or surgery |

| | | |
|---|---|---|
| Neurologic conditions | | |
| **Brain** – stroke, dementia, Alzheimer's, Parkinson's, multiple sclerosis | neurogenic detrusor overactivity | Management include means of compensation for impaired cognition or mobility, or both |
| **Spinal cord** – lumbar stenosis, disc herniation, spinal cord injury, multiple sclerosis | neurogenic detrusor overactivity / urinary retention | Necessary evaluation +/- Urodynamic testing deemed necessary |
| **Peripheral innervation** – diabetic neuropathy, nerve injury | Low functional bladder capacity / urinary retention | Further evaluation for nerve injury. |
| Systemic conditions | | |
| Heart failure, venous insufficiency | Volume overload can cause increase frequency and nocturia | Timing of diuretics, salt restriction |
| Diabetes mellitus | Osmotic diuresis and polyuria | Good blood glucose control |
| Sleep disorders | Can cause nocturia | Further evaluation |
| Abnormalities of arginine vasopressin | Can cause Polyuria and nocturia | May benefit from desmopressin therapy |
| Functional and behavioral conditions | | |
| Excess intake of caffeine / alcohol | Polyuria and urinary frequency can result | Modification of fluid intake is critical |
| Poor bowel habits / constipation | Fecal impaction may contribute to symptoms | Appropriate bowel regime |
| Impaired mobility | Interfere with toileting ability and precipitate urge incontinence | Treating underlying disorder including physical therapy |
| Psychological conditions | Anxiety / voiding dysfunction can cause | Further evaluation |

| | | |
|---|---|---|
| | symptoms | |
| Side effects of medication | | |
| Diuretics | Causes rapid increase in bladder volume | Altering the timing of dose or changing the diuretics |
| Narcotics, calcium channel blockers | Affects bladder contractility and capacity | Consider discontinuation if feasible |
| Cholinesterase inhibitors | Can contribute to detrusor overactivity | No clinical studies as yet |

## 1.5 Diagnostic Evaluation

Effective treatment of patients depends on an accurate evaluation. Nowadays there are guidelines for the management of urinary incontinence (63) and benign prostatic hyperplasia (64). Clearly, the clinical history is all-important and it is common place for disease-specific symptom questionnaires to be deployed. The International Prostate Specific Symptom Score (IPSS) is recommended by the American Urological Association (AUA) and the European Association of Urologists (EAU) (65;66). There are a variety of questionnaires addressing the broader lower urinary tract symptoms and these are now being consolidated by the International Consultation on Incontinence (ICI), this work being supported by a bespoke website (http://www.iciq.net/). A desire to measure urgency so as to detect between treatment groups differences in change prompted the development of a particularly sensitive measurement scale for urgency by Al-Buheissi et al. (67;68). Bladder diaries are also recommended to describe the frequency, volume, and daily rhythm of voiding (69;70). The texts recommend a detailed physical examination that includes genitourinary, pelvic, and rectal examinations although the evidence that, in the absence of leading symptoms, such invasive assessment provide important data is lacking. For the sake of the patients, the underlying assumptions merit critical testing.

Tests to exclude infection form an important part of the assessment. The science related to these methods is covered in the next section.

Chronic urinary retention should be checked for in patients at specific risk of this; such advice would apply to diabetics, spinal injury sufferers, persons with MS, benign prostatic enlargement and some elderly. This can be accomplished by sterile residual catheterization with a Jacques catheter. There are some portable ultrasonographic devices for non-invasive

identification of clinically significant residual urine (>100 ml). The latter term “clinically significant residual urine” brings into focus another conundrum. The relief of incomplete bladder emptying, by intermittent catheterisation or surgery, evinces good evidence of benefit for patients with neurological disease, men with prostatic obstruction and following Botox injections. In other circumstances there are no data to justify a threshold of a significant residual of ≥100 ml nor is the evidence that treating such residuals convincing. In some cases we do not know which is causative, the infection or the retention. The EAU guidelines accept an untreated residual of up to 350 ml (71). Despite this conundrum it has been accepted that any residual urine volume is only significant if it is symptomatic.

Patients with haematuria on dipstick merit this being checked by microscopy since the dipsticks are very sensitive. Patients with haematuria associated with urinary infection should be followed up after resolution of the infection. Persistent haematuria, identified by microscopy and without proteinuria merits screening for bladder cancer. Based on the risk factors identified, urine cytology, imaging and cystoscopy may be indicated.

The EAU Guidelines recommend Cystoscopy for patients with a history of recurrent urinary tract infection. It has been claimed that cystoscopy should be offered to all patients with overactive bladder symptoms. The literature lacks a clear explanation of what the cystoscopist should be looking for. Additionally there is a lack of data on the sensitivity and specificity of cystoscopy in these circumstances. If the majority of examinations are negative, the positive predictive value will be extremely low and weak specificity will drive up management costs and add to patient distress.

It is possible that a young man could present with LUTS, or even OAB symptoms. Possibility should not be confused with probability. In the absence of evidence, it should be remembered that rarity will push down the positive predictive value and specificity of a PSA will generate adverse repercussions.

The role of urodynamic testing in the evaluation of patients with OAB symptoms has been questioned (72). The evidence implies that symptoms are the key to diagnosis. It has to be said the investigation of other LUTS by urodynamics has a very meager fund of evidence (73). As far as OAB symptoms are concerned, none of modern guidelines recommend urodynamics as part of the primary assessment. But, Urodynamics does have a role in clinical practice after primary assessment and treatment failure.

It is germane that the evidence in support of the tests to rule out infection in this situation is no less threadbare (74-77).

## 1.6 Care Pathways for Management of Incontinence

The cost of treating urinary incontinence in UK, community-dwelling adults, aged over 40 was estimated as £536 million in 1999/2000 prices with cost to the individuals of £207 million (78). There is considerable interest in reducing these sums. One option, that has been vociferously promoted, has been the use of structured pathways of care.

"Integrated Care Pathways" (ICP), synonymous with "Clinical Pathways" (CP), were introduced in the UK and the USA during the early 1990's. They are being increasingly adopted throughout the developed world. ICP's are structured, multi-disciplinary plans of care designed to support the implementation of clinical guidelines and protocols. They are supposed to improve clinical, resource and risk management whilst enhancing clinical effectiveness, audit and financial control. The pathways provide detailed guidance for each stage in the management of a patient with a specific condition, and include measures of progress and outcome.

Pathways have been designed for over 45 conditions and one of those is urinary incontinence. There are several proposals in the literature and some are also designed under the sponsorship of the NHS Research and Development of the Health Technology Assessment Programme. The National Institute of Clinical Excellence (NICE) is also in the process of working out an ICP for urinary incontinence and another has been proposed by the International Continence Society (ICS) in the 3rd Consultation on Incontinence.

The various pathways exhibit a common thread with the patients broadly undergoing five types of assessment; 1) clinical history 2) validated scales 3) physical examination 4) simple investigations 5) advanced investigations.

At least 5 publications have reported the benefit of the nurse led services (79-83). Most do not describe a comparator. Where controls were reported, they were non-specialist clinicians in primary or secondary care. The efficacy of specialist medical incontinence expertise in early management has not been disproved.

A meta-analysis comparing nurse practitioner with doctors in providing care at first contact for minor illnesses reported more patient satisfaction with a nurse practitioner and no differences in outcome (84). “Patient satisfaction” is not necessarily a reliable assessment of the quality of care provided. Horrocks et al. also reported that nurses had longer consultations and arranged more investigations than doctors. No differences were found in prescriptions, return consultations, or referrals. The longer consultation time by nurse practitioners has also been shown in other studies (85-87). The length of consultation (88;89) has been shown to be a good predictor of patient satisfaction. Increased consultation times are not assuredly beneficial; they increase costs and waiting times

Staffing accounts for 70 % of NHS spending, so altering skill and grade mix is attractive. Some fear the erosion of standards (90;91) and ask whether we really understand the properties of knowledge, skill and experience. (92).

### 1.6.1 Are structure pathways suitable for diagnosis?

There are very few trials on the diagnostic accuracy of assessment pathways. They rely on Boolean logic, which lies at the heart of the digital revolution and much database searching. By contrast, we teach diagnosis as an interchange between deduction, induction, abduction and Bayesian reasoning. The tools are knowledge, practise, experience and continuing

education. There is a contradiction at the heart of the ICP and problems with the veracity of the assumptions that underlie the pathways: For example, one of the earliest tests any ICP is some forms of urinalysis to exclude urinary tract infection. As will be discussed later, there are legitimate serious doubts about the validity of the tests being used to achieve this. If the tests are as insensitive as reported, the ICP falls at an early hurdle. Boolean decision nodes introduce additional serious errors: They stipulate a "yes" or "no" response without consideration of positive and negative predictive values, or the germane probabilities. There is no opportunity to consider the decision in the light of prior data or to revisit with posterior data. They therefore obviate skilled, professional diagnostic reasoning.

A skilled, experienced clinician is capable of making accurate diagnostic and therapeutic decisions very rapidly, using a minimum dataset, collected through selective assessment. This does not employ the regimented steps in the standard ICP, actions being selective according to the symptom complex. This is less time consuming and less expensive. There are good data supporting the cost-effectiveness of this approach (93). One of the key factors in the economic success of Kaiser Permanente is the deployment of their skilled workforce at the front (94).

## 1.7 Conservative treatments first?

The pathways appear to rest on assumptions that have not been proven. Most propose a pyramidal structure that is contingent on the majority of persons with urinary incontinence being remedied by simple not medical-interventions. There are no data that validate this belief one-way or the other. All ICP's recommend the use of "conservative" measures management, prior to investigative assessment. The evidence of the efficacy of conservative measures is sparse and contradictory.

Below are reproduced the conclusions of the Cochrane reviews on conservative treatments for urinary incontinence in adults. The best claims are "weak evidence" or "suggestive evidence". This means that there are no RCT data on the efficacy of these methods. Despite this, there remains a widespread belief that patients are better off if managed with conservative methods first. It is reasonable to ask "Where is the evidence for this dogma?"

**Bladder training for urinary incontinence in adults (Cochrane Review) (95)**

Definitive research has yet to be conducted: more research is required.

**Complementary and miscellaneous interventions for nocturnal enuresis in children (Cochrane Review) (96)**

There was weak evidence to support the use of hypnosis, psychotherapy, acupuncture and chiropractic but it was provided in each case by single small trials, some of dubious methodological rigour. Robust randomised trials are required with efficacy, cost-effectiveness and adverse effects carefully monitored.

**Habit retraining for the management of urinary incontinence in adults (Cochrane Review) (97)**

Data on habit retraining are few and of insufficient quality to provide a firm basis for practice.

**Mechanical devices for urinary incontinence in women (Cochrane Review) (98)**

Currently there is little evidence from controlled trials on which to judge whether their use is better than no treatment and a large well-conducted trial is required for clarification

**Pelvic floor muscle training versus no treatment, or inactive control treatments, for urinary incontinence in women (Cochrane Review) (99)**

Overall, the review provides some support for the widespread recommendation t PFMT be included in first-line conservative management programmes for women with stress, urge, or mixed, urinary incontinence.

**Physical therapies for prevention of urinary and faecal incontinence in adults (Cochrane Review) (100)**

There is insufficient evidence to determine whether physical therapies can prevent incontinence in childbearing women, or men following prostate surgery. Further, better quality research is needed.

**Prompted voiding for the management of urinary incontinence in adults (Cochrane Review) (101)**

There was insufficient evidence to reach firm conclusions for practice. There was suggestive, although inconclusive, evidence of short-term benefit from prompted voiding and from adding the muscle relaxant, Oxybutynin to prompted voiding.

**Prevention and treatment of urinary incontinence after stroke in adults (Cochrane Review) (102)**

There was suggestive evidence that specialist professional input through structured assessment and management of care and specialist continence nursing may reduce urinary incontinence after stroke. Data from trials of other physical, behavioral, complementary and anticholinergic drug interventions are insufficient to guide continence care of adults after stroke.

**Timed voiding for the management of urinary incontinence in adults (Cochrane Review) (103)**

The data were too few and of insufficient quality to provide empirical support for or against the intervention of timed voiding.

**Weighted vaginal cones for female stress urinary incontinence (Cochrane Review) (104)**

This review provides some evidence that weighted vaginal cones are better than no active treatment in women with stress urinary incontinence and may be of similar effectiveness to PFMT and electrostimulation. This conclusion must remain tentative until further larger high quality studies are carried out using comparable and relevant outcome measures.

## 1.8 Drug therapy

Medications used to treat urge incontinence and OAB symptoms are aimed at inhibiting the involuntary contraction of the bladder and improving bladder function. There are several types of medications that may be used alone or in combination:

- ✓ Anticholinergic agents (oxybutynin, tolterodine, solifenacin, trospium, darifenacin, propiverine, fesoterodine)
- ✓ Tricyclic antidepressants (imipramine, amitriptyline)

The anticholinergic medications work by blocking the M3 and M2 receptors on the detrusor neuromuscular junction. All the members of these groups have additional properties that include calcium channel blocking activity and local anesthetic properties. Whether these extra effects bear any relationship to the efficacy of the drugs in clinical practice is not known. The anticholinergic groups have been a fertile source of "me-too" manufacture and there is a plethora of publications seeking to identify significant differences in efficacy or side effect profile. There is no convincing evidence of striking differences and efficacy does seem to be very similar in comparative clinical trials that have taken place.

Atropine-resistant, nerve induced detrusor contractions have long been recognised and efforts are being made to find alternatives to the antimuscarinics. Attention focused on β3-sympathomimetic agents and P2X purinergic receptor antagonists. Botulinum toxin, superior to antimuscarinic agents, inhibits co-transmission of acetylcholine and ATP. Noradrenaline acting at β3 sites inhibits detrusor contractions. Serotonin is also a known inhibitor of bladder contractions (189). It should be possible to augment the effect of an antimuscarinic by using another drug that works synergistically at these alterantive targets, but will be some years before these molecules come out of development.

Imipramine hydrochloride has been used in the in the treatment of childhood enuresis for a long time. Over 20 years ago it was recommended as a treatment for the overactive bladder in adults, but efficacy was limited and it was superseded by the advent of oxybutynin and others of this class (105).

Imipramine inhibits reuptake of noradenaline and serotonin equally. Changes in postsynaptic beta-adrenergic receptor sensitivity and increased responsiveness of the adrenergic and serotonergic systems contribute to the mechanism of action. The drug partially blocks ionic currents mediated through P2X receptor channels (105).

56 randomised trials of tricyclic drugs for nocturnal enuresis, involving 3624 children have been published. Cochrane report the quality of many of these trials as poor. Treatment with most tricyclic drugs (such as imipramine, amitriptyline, viloxazine, nortriptyline, clomipramine and desipramine) was associated with a reduction of about one wet night per week. Only 1 study of Imipramine in adults has been published, it is was conducted in the elderly but was underpowered (106).

## 1.9 Surgery

The goal of any surgery to treat bladder overactivity is aimed at increasing the storage ability of the bladder while decreasing the pressure within the bladder. Surgery is reserved for patients who are severely debilitated by their incontinenc and who have an overactive bladder symptoms and poor ability to store urine.

For many years the augmentation cystoplasty was the most frequently performed surgical procedure for severe urge incontinence. In this reconstructive surgery a segment of the bowel is, de-tubed and then sewn into the bladder which has been opened like a clam. The bowel patch so placed separates the two halves of the bladder.

Possible complications include those of any major abdominal surgery, including bowel obstruction, blood clots, infection, and pneumonia. There is a risk of developing urinary fistulae, urinary tract infection, and urinary retention. Augmentation cystoplasty is also increased risk of developing carcinoma in the patch. It can also lead to metabolic disturbances and stone formation (190).

Nowadays it is more usual for the intervention to be a botulinum toxin injection. There are 7 immunologically distinct antigenic subtypes of botulinum toxin, of which type A is the most widely used. Botulinum toxin A (Botox) is a purified neurotoxin complex, which blocks the release of acetylcholine and other transmitters from presynaptic nerve endings. Thus nerve-mediated contractions of the detrusor become inhibited. The effect is not permanent and tends to dissipate over the ensuing 6 months leading to a requirement for repeat injections. Botox cannot cross the blood–brain barrier and hence has no CNS side effects. A Cochrane review of Botulinum toxin injections for adults with overactive bladder syndrome (107) published in

2007 concluded “Intravesical botulinum toxin shows promise as a therapy for overactive bladder symptoms, but as yet too little controlled trial data exist on benefits and safety compared with other interventions, or with placebo.” Since then several RCT’s have been published with good results (108-112).

The side effects of this treatment include dry mouth, dry eyes, gastro-oesophageal reflux, constipation, and not often urinary retention. Botox can lead to transient hypoasthenia that resolves by itself.

The role of has been included under the Surgery section as it is classified as a surgical procedure though mostly it can be undertaken as an outpatient procedure under local anaesthetic.

# Chapter 2

# Urine Infection

# CHAPTER 2

# URINE INFECTION

## 2.1 Acute urine Infection

The American Foundation for Urological Diseases reports that acute bacterial cystitis affects 8 – 10 million Americans a year and most of these patients are women. The National Institute of Diabetes and Digestive and Kidney Diseases notes that acute cystitis prompts about 9.6 million doctor visits annually; $4.5 billion is spent on 11 million antibiotic prescriptions. Occurring in otherwise healthy young women with no congenital, neurological or structural abnormalities, acute, symptomatic, uncomplicated urinary tract infection (UTI) or acute female cystitis (AFC) is the most common infection (113;114) next to the common cold.

25 to 40 % of females will experience an acute UTI in their lives, and up to 6 % of women will have one or more UTI in a given year (115;116). After an initial infection, most women have sporadic recurrences, and a quarter to half have another infection within one year. 3 to 5 % have recurrent urinary tract infections - that is, symptomatic infections that follow the clinical resolution of a previous episode, generally (but not necessarily) after treatment (117).

The probability of cystitis in a woman with dysuria, urinary frequency, or gross haematuria is about 50 % in primary care settings (118). Symptoms suggesting vaginitis or cervicitis, such as vaginal irritation or discharge, reduce the likelihood of a diagnosis of cystitis by about 20 %. Specific combinations of symptoms (e.g., dysuria and frequency without vaginal discharge or irritation) raise the probability of cystitis to more than 90 %. When a woman who has previously had cystitis has symptoms suggesting a recurrence, there is an 84 to 92 % chance that an infection is present (119;120).

## 2.2 Chronic / Recurrent Urine Infection

Chronic urinary tract infection is a disorder involving repeated or prolonged bacterial infection of the bladder or lower urinary tract (urethra). The term “Cystitis” refers to the bladder which is most commonly affected. This occurs when the normally sterile lower urinary tract is infected by bacteria and an inflammatory response is induced. The symptoms of acute cystitis usually disappear within 24 - 48 hours after treatment begins. Recurrent cystitis may be either a re-infection (after successful eradication of infection) or a relapse after inadequate treatment. Chronic or recurrent urinary tract infection includes repeated episodes of cystitis, there being more than 2 in 6 months, or urinary tract infection that does not respond to antibiotic treatment or lasts longer than 14 days (121). In young girls, recurrent urinary tract infections may be an indication of a urinary tract abnormality, such as vesico-ureteral reflux, and hence imaging assessments are recommended. Urinary tract infections in boys are extremely uncommon in the absence of urinary tract abnormalities so a single episode of cystitis should result in imaging studies.

Amongst the elderly, cystitis acute and chronic is much more common due to incomplete emptying of the bladder, prostatism, immunoparesis, diabetes, vascular disease, low fluid intake, faecal incontinence, decreased mobility, and the use of indwelling catheters are the most commonly proposed aetiological factors although the truth is yet to be verified with rigor.

In truth, this entity is no more than a clinical definition based on arbitrary time frames. The literature is signally sparse in explaining the pathophysiology. Hultgren et al. have discovered that in their murine model, populations of E.Coli can persist in the bladder for months on end, during which time they exist in a quiescent reservoir (122).

## 2.3 Aetiology of UTI

The microbial aetiology of urinary infections has been regarded as well established and reasonably consistent. Escherichia coli remains the predominant uropathogen (80 %) isolated in acute community-acquired uncomplicated infections, followed by Staphylococcus saprophyticus (10 to 15 %). Klebsiella, Enterobacter, Proteus species, and Enterococci infrequently cause uncomplicated cystitis and pyelonephritis. Fungal pathogens like Candida albicans or other Candida species, account for up to 10 % of positive urine cultures in tertiary care (123), but most of these are in complicated UTI's. The aetiology of UTI is also affected by underlying host factors that complicate UTI, such as age, diabetes, spinal cord injury, and catheterization. It has been found that the most common organism grown deliberately in the laboratory is the E. coli (124;125) (126). The most common organisms isolated in children with uncomplicated UTI is E.coli but also Enterobacteriaceae group (127).

## 2.4 Pathogenesis of E. Coli UTI

A critical initial step in the pathogenesis of both acute and recurrent UTI involves colonization of the urinary tract or vaginal introitus with E. coli. Uropathogenic E. coli strains generally possess filamentous surface adhesive organelles called fimbriae or pili. In case of type 1 fimbriae, the adhesin molecule Fim H, which is located at the tip of the type 1 pilus, directly interacts with host receptors and facilitates bacterial attachment on the luminal surface of the bladder epithelium. This may also mediate the internalization of bacteria into epithelial cells, where E. coli can replicate and escape host defense mechanisms. Research has shown that women with Recurrent UTI have 3-fold more E. coli adhering to vaginal, buccal, and voided urothelial cells than women without recurrent infection. Further, women with Recurrent UTI's also have longer durations of vaginal colonization with uropathogenic E. coli (128), even during asymptomatic periods (129). Each E. coli clone has distinct characteristics, including O and K serotype, similar outer membrane protein patterns, and hemolysins. Some O and K serotypes of E. coli are more common uropathogens than others. Nevertheless, P fimbriation appears to be a virulence factor. Further, there appears to be a correlation between colonization of the gastrointestinal tract with P-fimbriated E. coli and acute pyelonephritis (130). In some patients with diabetes, Tamm-Horsfall protein is markedly reduced, allowing adherence and cell entry of type 1 fimbriated E. coli (131). Whereas P fimbriation and hemolysin production have been identified as important virulence factors among patients with diabetes (132), there is as yet, no evidence identifying differences in bacterial virulence factors for E. coli among patients with or without diabetes.

## 2.5 Pathogenesis of Non – E. Coli UTI

In contrast to the pathogenesis of E. coli UTI, S saprophyticus and enterobacterial species adhere to urothelial cells through different adhesive mechanisms. After attachment, Proteus spp., K pneumoniae, and S saprophyticus each produce urease, which catalyzes the hydrolysis of urea in urine and causes the release of ammonia and $CO_2$. As a result, the urinary pH is elevated causing the alkalinisation of urine that leads to the formation of bladder and kidney stones.

## 2.6 Interstitial Cystitis/ Painful Bladder syndrome

Skene et al. first muted the term interstitial cystitis in 1887 (133), but widely acceptable diagnostic criteria for interstitial cystitis was developed in 1987, at a meeting convened by the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) in the USA (134). The mucosal ulcers were a preliminary criterion, which was first identified by Hunner et al. in 1914 (135). Pain on bladder filling that was relieved by emptying as well as suprapubic, pelvic, urethral, vaginal or perineal pain and/or glomerulations (urothelial haemorrhages elicited by bladder hydrodistention) were deemed positive factors for Interstitial Cystitis, of which 2 were required for diagnosis.

Pain was a prominent part of the case definition discussion at the 1987 NIDDK meeting. Gillenwater et al. introduced Interstitial Cystitis by referring to "painful bladder disease." Holm-Bentzen et al. presented extensive data on "the painful bladder syndrome."(134). Held et al. surveyed 127 board certified urologists about their experiences with patients with interstitial cystitis and pain was ranked as the single most common criterion for diagnosing, while "pain on filling and relieved by emptying" was ranked $4^{th}$ and non-pain symptoms were ranked lower, including urgency as $7^{th}$, nocturia as $17^{th}$ and waking frequency as $19^{th}$ (136). Pain exacerbation can be due to stress, sexual intercourse, tight clothing, as well as due to consumption of acidic beverages, coffee and spicy foods.

Use of the phrase painful bladder syndrome (PBS) goes back at least to 1951 (137). In 1987 in a clinical trial of "interstitial cystitis and painful bladder disease" Holm-Bentzen et al. described PBS in terms similar to those for IC (138). Since 1976, the ICS has standardized the terminology of lower urinary tract diseases and in 2002 it reported PBS for the first time. "Painful bladder syndrome is the complaint of suprapubic pain related to bladder filling,

accompanied by other symptoms such as daytime and night time frequency, in the absence of proven urinary infections or other obvious pathology." (138;139).

The footnote on a report also recommended that interstitial should be defined as a condition that is symptomatically the same as PBS in terms of pain and other symptoms, such as frequency, but must be confirmed clinically. "The ICS believes this to be a preferable term to 'interstitial cystitis.' Interstitial cystitis is a specific diagnosis and requires confirmation by typical cystoscopic and histological features." (138;139)

These features typically include ulcers or glomerulations observed on the bladder wall. Other committees recently came to similar conclusions about IC and PBS, and several suggested that the names should be combined into one term (4;140) (141)

## 2.7 Excluding urinary infection

Unfortunately, in diagnosing OAB the exclusion of UTI poses a problem of great significance. Standard laboratory methods operate a diagnostic threshold of $\geq 10^5$ colony forming units (cfu) $ml^{-1}$. But Stamm et al. (142;143) found $\geq 10^2$ cfu $ml^{-1}$ was appropriate for persons with acute frequency/dysuria and that $\geq 10^5$ cfu $ml^{-1}$ missed 50 % of infections. The dipstick leukocyte esterase and nitrite tests have been calibrated to $\geq 10^5$ cfu $ml^{-1}$. The OAB literature, reassured by these tests, may not have addressed infection adequately.

At the moment the best surrogate marker of urinary infection, superior to routine culture and dipstick analysis, is the detection of ≥10 white blood cells (wbc) per $\mu l^{-1}$ of fresh, unspun urine, examined in a haemocytometer (144). This has been well validated.

### 2.7.1 The history of pyuria as a surrogate marker of infection

Since 1957 the diagnosis of urinary tract infection (UTI) from culture of a mid-stream urinary specimen (MSU) has rested on criteria described by Kass et al. (1957) in his classic paper (125). He reported that the MSU's of 25 patients with chills fever, flank pain and dysuria had grown more than $10^6$ bacteria colony forming units per ml (cfu $ml^{-1}$). After studying the MSU in asymptomatic people, he concluded that "For survey purposes a count of $10^5$ cfu $ml^{-1}$ of a known urinary pathogen should be designated, arbitrarily as the dividing line between true bacilluria and contamination". Kass never claimed to define a threshold for use with cystitis symptoms. However $10^5$ cfu $ml^{-1}$ has been widely adopted in clinical practise.

In 1982 Stamm et al. (143) published work conducted on acutely dysuric women. He discovered that in the presence of the classical symptoms of acute cystitis, a culture result of

$10^2$ cfu $ml^{-1}$ was a more appropriate threshold for diagnosis. There are no data on a threshold applicable to patients with other lower urinary tract symptoms.

The MSU nowadays is usually, processed using semi-automated methods. These are generally set to a threshold of $10^4$ cfu $ml^{-1}$ which is higher than Stamm's threshold of $10^2$ cfu $ml^{-1}$ for cystitis (113). Hooton & Stamm et al. have reported that laboratory MSU cultures miss over 50 % of genuine infections (145).

An alternative method of diagnosing urine infection is the identification of significant pyuria. The determination of the excretion rate of white blood cells in urine was first described by Hottinger et al. in 1893. Nevertheless, the technique used is usually attributed to Addis et al., who published his method in 1925, referred to as the "Addis count" (146). The method is laborious and impractical.

Hamburger et al. in 1950 (147), and Houghton and Pears et al. in 1957 (148) described modifications of the Addis count which made it simpler but reliable, although a catheter was used to collect the urine. The leucocytes were enumerated by examining the urinary sediment in a haemocytometer.

Hamburger, Houghton and Pears et al. obtained the sediment by centrifugation of 10 ml of urine at 1500 rmp (radius 12 cm) for three minutes. 9 ml of supernatant was removed and the 1 ml of sediment remixed with a Pasteur pipette before spreading on the haemocytometer chamber to count the leucocytes. Having used this technique in volunteers, they concluded that the normal rate of leucocyte excretion varied between 18,000 and 196,000 per hour.

The upper limit of the normal leucocyte excretion rate per hour was established as 200,00 wbc $h^{-1}$ to 400,000 wbc $h^{-1}$ by Houghton and Pears at al. in 1957 (148-151) all of whom demonstrated excretion rates of >400,000 wbc $h^{-1}$ in patients with proven, symptomatic urinary infection. Little et al. (1962) successfully evaluated the mid-stream urine (MSU) as an alternative to catheter collection. In his method, there was no interruption of the urinary stream during collection (151).

In 1968, Mabeck et al. attempted to simplify evaluation by counting the white cells per high powered field (x40 objective x10 optical = x400pf) using the unresuspended, stained, sediment. All patients showing 3 or more wbc per high powered field (x, hpf) excreted more than 400,000 wbc $h^{-1}$, but fewer than 3 wbc phpf did not exclude excretion rates of ≥400,000 wbc $h^{-1}$. The spun sediment underestimated the leucocyte excretion rate, presumably because of cell destruction during centrifuging (152).

Mond et al. (1965) examined unspun fresh urine and counted the white cells using a haemocytometer, finding that ≥10 wbc $mm^{-3}$ were noted in all patients with symptomatic acute cystitis and bacteriuria ($10^5$ cfu $ml^{-1}$) (153). In 1968 Gadeholt et al. compared the examination of spun and unspun specimens and showed that the calculated excretion rates on uncentrifuged specimens showed higher values (154;155). This confirmed earlier suspicions that centrifugation caused loss of cells. Gadeholt et al. (1968) showed that all patients with ≥10 wbc $mm^{-3}$, of unspun urine, excreted >400,000 wbc $h^{-1}$; This was confirmed by Mabeck in 1969 (156) and by Baerheim et al. (1989), all of whom identified the problem with centrifugation. Thus the finding of ≥10 wbc $mm^{-3}$ (µl) , of unstained, unspun urine examined on a haemocytometer, became established as the most effective method for diagnosing urinary infection (157).

Latham et al. (1984) publishing with Stamm reported that the detection of pyuria ≥10 wbc $mm^{-3}$ was the most accurate and efficient method of identifying urinary tract infections in ambulatory women with symptoms of acute cystitis (158).

Whilst Stamm et al. has achieved much in clarifying the diagnostic criteria useful with classical symptoms, there remains a problem with non-dysuric lower urinary tract symptoms. It is no longer reasonable to rely on the reassurances of bacteriuria of $<10^5$ cfu $ml^{-1}$.

A convenient alternative to MSU analysis is the dipstick test for leucocyte esterase test. Using a diagnostic threshold of $10^5$ cfu $ml^{-1}$, this has a reported sensitivity of 75 to 90 % in detecting significant pyuria. There are no data related to a culture threshold of $10^2$ cfu $ml^{-1}$ in the presence of cystitis symptoms (159-161).

There have been 2 meta-analyses of urinary dipsticks, used to assess acute frequency/dysuria, in adults (162;163) and 1 in children (164). Hurlburt and Littenberg et al. concluded that dipsticks cannot exclude infection reliably in most clinical settings. Deville et al. (162) reported leucocyte esterase sensitivity of 0.76 (95 % CI 0.6 to 0.98) and specificity 0.46 (95 % CI 0.32 to 0.68) for the diagnosis of urinary infection and a nitrite sensitivity of 0.49 (95 % CI 0.38. to 0.62) and specificity of 0.85 (95 % CI 0.73 to 1.0) in primary care.

In relation to OAB in contrast to acute cystitis or acute frequency/dysuria there are no published data on an appropriate threshold for the diagnosis of pyuria. Therefore, for the purposes of this thesis, it was thought wise to err on the conservative side and describe pyuria as ≥1 wbc $\mu l^{-1}$.

It will be prudent to discuss the role of other inflammatory markers in the diagnosis of urine infection, although it is not a part of this thesis. The role of Urothelium and the inflammatory markers have been discussed briefly in the chapter of pathophysiology, earlier. It has been described about elevated Interleukins in upper urinary tract infections in children (191). Similarly elevation of serum C-reactive protein and urinary Nerve Growth factor has been shown to be elevated in states of chronic inflammation of the urinary bladder (192).

# Chapter 3

# Squamous Metaplasia

# CHAPTER 3

# SQUAMOUS METAPLASIA

It would be inappropriate to discuss any chronic inflammatory condition affecting the bladder without introducing the matter of metaplasia.

Metaplasia is the reversible replacement of one type of adult tissue by another type.

Squamous metaplasia refers to benign changes in the epithelial lining of certain organs within the body. These cells assume a more squamous morphology. Common sites for squamous metaplasia include the bladder and cervix. Smokers often exhibit squamous metaplasia in the linings of their airways. These changes don't signify a specific disease, but represent the body's response to stress or irritation.

Replacement of urothelium by stratified squamous epithelium could entail non-keratinised (v) and keratinized subtypes of squamous cells.

The non-keratinized subtype is found commonly in the trigone area and the keratinized subtype in the rest of the bladder.

The presence of non-keratinised squamous metaplasia in the bladder is also called pseudomembranous trigonitis, trigonal cystitis, urethra-trigonitis, and vaginal metaplasia of trigone. It should be no surprise that this plethora results in much confusion.

The keratinized squamous metaplasia in the bladder is called as leukoplakia and is closely associated with Vit. A deficiency (165).

Pseudomembranous trigonitis commonly affects the female trigone and is defined as partial or complete replacement of urothelium by mature squamous epithelium and it is diagnosed macroscopically by the presence of white patches or plaques visible on the trigone endoscopically (166).

The condition was first described as “Cystitis Trigoni” by Heymann et al. in 1905.

We do not know whether squamous metaplasia should be considered a normal variant of trigonal urothelium in women. Wiener et al. (167) detected non keratinised squamous metaplasia in 40 % and 5 % of female and male trigone’s, respectively in “normal bladders” at autopsy. They are far from explicit in how they defined a normal bladder for their purposes.

The ratio of mitotic cells to resting cells, the mitotic index, of pseudomembranous trigonitis is 0.17 %, which is significantly higher than normal (≈ 0%) (168).

Leukoplakia undergoes three successive stages – squamous cell modulation, squamous cell metaplasia and squamous cell metaplasia with keratinisation. It has been shown to have precancerous potential (168).

## 3.1 Histopathology

Normal trigonal urothelium consists of three cell layers (basal, intermediate and superficial) whereas pseudomembranous trigonitis constitutes many layers of stratified squamous epithelium.

The urothelium forms effective permeability barrier and can withstand repeated stretch and contractions (169). To perform these functions the apical surface of urothelium becomes highly specialised as it is covered almost completely for 16 nm depth by uroplakin particles that are packed hexagonally (170).

In squamous metaplasia, the basal cells contain prominent nuclei with condensed chromatin, nucleoli and nuclear bodies. The basal region is two to three cells in thickness and consists of the smallest cells. The shape of cells in this basal region is cuboidal or columnar. The cytoplasm of these cells is rich in mitochondria. The profiles of the urothelial cells become progressively elongated, nuclei increasingly smaller and the content of cell organelles gradually reduced as the luminal surface is approached.

The squamous surface cells, linked by desmosomes, retain many longitudinally arranged fine filaments, together with an occasional degenerate nucleus and a pale staining cytoplasm.

When the glycogen is abundant and there is vacuolisation, the metaplastic urothelium may resemble the vagina.

Whilst this non-keratinized squamous metaplasia is considered to be benign, there is a report of atypical hyperplasia found in two cases of non-keratinized metaplasia (171).

## 3.2 Inflammatory Features

The incidence of inflammatory features has been assessed as about 30 % (171). These manifest lymphoid follicles and/or plasma cells within the tunica propria and prominent venous channels forming a plexus about the urethral orifice. There was also oedema and vascular dilatation underlying areas of non-keratinized metaplasia (166).

## 3. Clinical correlations

Pseudomembranous trigonitis may be related to frequency and urgency symptoms (172). Brawn et al. (1984) proposed that squamous metaplasia may be less efficient in maintaining the barrier between blood and the urine. In normally differentiated three-layered trigonal urothelium, the retention in the upper strata of some internal cytoskeleton, external membranous and desmosomal connections might be functionally important as blood urine barriers (168).

## 3.4 Concomitant pathology

Non-keratinised squamous metaplasia of the trigone was considered an adaptive response to inflammation of the bladder in many earlier reports (173). Other reports suggest that Non-keratinised squamous metaplasia may predispose to the development of chronic cystitis.

Davies and Hunt et al. demonstrated bacilli adherent to the surface epithelium of the bladder trigone in a woman with recurrent urinary tract infections. Others have concluded that there is a variation in the histological appearance of vaginal metaplasia with phases of menstrual cycle under the hormonal influence of oestrogen (174-176).

# Chapter 4
# Experiment Plan and Hypotheses

# CHAPTER 4

## 4.1 Experimental plan which led to this study

Malone-Lee et al .(177) studied 785 patients ( mean age ± SD = 54 ± 19 years: 717 females and 68 males) with overactive bladder symptoms over a four year period (April 2003 to March 2007; (1). Using the more stringent criteria to examine fresh, unspun urine samples by light microscopy with a haemocytometer it was found that 58% (n=452) of the specimens demonstrated significant pyuria ≥10 wbc $\mu l^{-1}$. Of these, only 12 % (n=53) showed a significant pure growth on culture and 9 % (n=40) showed a mixed growth. Thus the MSU culture was sterile in 79 % (n= 359) of urine samples showing pyuria ≥10 wbc $\mu l^{-1}$. 68 % of the specimens (n=535) tested "Trace positive" or greater to dipstick leucocyte esterase and of these 83 % (n=446) were sterile and 69 % (n=371) showed pyuria ≥10 wbc $\mu l^{-1}$ on microscopy.

Of the 452 specimens, which showed pyuria ≥10 wbc $\mu l^{-1}$, only 8 % (n=36) were dipstick nitrite positive. Of the 55 specimens, which showed a pure bacteriuria (≥ $10^4$ cfu $ml^{-1}$), only 16 % (n=9) were dipstick nitrite positive.

Between those with pyuria ≥10 wbc $\mu l^{-1}$ and those without there was a significant difference in average 24-hour urinary frequency and incontinence episodes (Pyuria ≥10 wbc $\mu l^{-1}$ versus no pyuria (zero wbc $\mu l^{-1}$): Urinary frequency 95 % CI -0.96, -0.15, F=6.5 p=0.011; Incontinence episodes 95 % CI -0.28, -0.07, F=25, p<0.001).

During the observation period 74 % (n=579) of the study population demonstrated significant pyuria ≥10 wbc $\mu l^{-1}$ at some time, with this being slightly more common in older persons;

70 % of patients aged ≥50 years showed pyuria ≥10 wbc $\mu l^{-1}$ compared to 63 % under 50 years.

Despite sterile MSU cultures, the pyuric patients were treated with antibiotics for varying lengths of time until the pyuria ≥10 wbc $\mu l^{-1}$ cleared (Median days of antibiotic treatment: 154 Interquartile range = 164 days). Patients being treated with antimuscarinics had worse symptoms if they had pyuria ≥10 wbc $\mu l^{-1}$ than if they did not (95% CI Diff frequency -1.0, -0.05, F= 6.6 p=0.01 and 95 % CI Diff incontinence -0.3, -0.03, F=17, p<0.001).

When pyuric only 8 % (n=46) of patients described dysuria. More common dysaesthesic symptoms were; loin (30 %) and suprapubic discomfort (29 %) and vague abdominal discomfort (23 %). The symptom complex had much in common with those described in Interstitial Cystitis.

Thus is preliminary work demonstrated a urinary inflammatory reaction in more than 50 % of patients with OAB symptoms, associated with worse symptoms, particularly incontinence, despite antimuscarinics treatment.

The slow response to antibiotics compliments an important experiment by Anderson et al. (2004) (122;179). Using a mouse model of urinary tract infection, they demonstrated the ability of E. coli to colonise the superficial umbrella cells of the bladder with intracellular biofilms, exhibiting striking antibiotic and immune resistance. Electron microscopic studies showed these biofilms forming pod-like protrusions on the cell wall with the bacterial encased polysaccharide rich matrix surrounded by a protective shell of uroplakin. This could

explain the Malone-Lee et al. (177) , experience that sustained antibiotic treatment was necessary to clear infection effectively.

The sensory symptoms elicited from the patients fit well with current discoveries about ATP. Activation of P2X 2/3 and P2X7 receptors by ATP, released in response to inflammation, has been implicated in atypical dysaesthesia. The symptoms experienced by the patients in the Malone-Lee et al.(177) series compliment these discoveries.

Sterile pyuria accords with Stamm's discoveries. An apparent slow response to antibiotics chimes with contemporary mouse experiments (122;179). The phenomenon might explain antimuscarinics resistance, fluctuant symptoms and recovery on placebo. Although Malone-Lee et al. (177) obtained a large sample, with blinding between microbiology and clinician, these were only observational data requiring validation by rigorous methods.

## 4. The Objectives

It has been shown by these means that pyuria would seem to be under-diagnosed in patients with OAB symptoms because of the methods that have been used in its assessment. The data indicate that it is an extremely common complication. Whilst the earlier experiments identified pyuria as ≥10 wbc $\mu l^{-1}$, this threshold was worked out in the context of acute urinary tract infection symptoms and may not be appropriate to OAB group so from this point in time, pyuria is going to be taken as ≥1 wbc $\mu l^{-1}$.

There are a number of questions that need to be resolved through a sequence of clinical experiments to find out the significance of pyuria –

1) What is the morphological profile of the inflammatory reaction?

2) Could the detection of a urinary inflammatory response be enhanced by analysis of urinary spun sediments prepared using methods developed for cytology?

3) Is there confirmatory histological evidence of urothelial inflammation associated with culture-negative pyuria or OAB symptoms without pyuria?

4) Is there evidence of undue urothelial stress; manifest by increased urothelial cell denudation, hyperplasia and metaplasia, in patients experiencing persistent pyuria ≥1 wbc $\mu l^{-1}$ and overactive bladder symptoms?

5) What additional information does electron microscopy throw onto the pathophysiology of the urothelium in OAB symptoms associated with pyuria?

## 4.3 Hypotheses tested

Five hypotheses were formulated for testing. OAB symptoms with pyuria ≥10 wbc $\mu l^{-1}$ are associated with the following pathophysiological phenomenon –

(1) Urothelial inflammation results in a coherent chronic/acute inflammatory cell response.

(2) A Shandon Cytospin™ preparation suitably stained will prove a more sensitive detector of urinary inflammatory activity in patients with OAB symptoms, than current routine clinical methods.

(3) Urothelial inflammation, oedema and cell denudation should be detected by histology of H&E stained biopsies obtained from affected patients.

(4) The patients should demonstrate increased urothelial cell denudation; hyperplasia and metaplasia detected by histology of H&E stained biopsies and urinary spun sediments.

(5) The patients should demonstrate e of morphological alterations in the urothelium detected by transmission electron microscopy

# Chapter 5
# Methods

# CHAPTER 5

# METHODS

## 5.1 Sampling

The study was submitted for approval to The East London Research Ethics Committee which reviewed proposal study and approved its enactment.

The study was initiated to test the hypotheses related to urothelial inflammation associated with OAB symptoms that were articulated at the end of the last section. The experiments required recruitment and screening of the patients referred to the incontinence clinic at the Department of Medicine, Whittington Campus, UCL Medical School. Control patients were recruited from hospital staff and medical students for the provision of urine samples whereas control biopsy material was obtained from wholly asymptomatic patients attending a one-stop haematuria clinic at the same site.

## 5.2 Study Population

### 5.2.1 Subject Inclusion Criteria

1. Adults aged ≥18 years
2. Males
3. Females
4. Symptoms of frequency ≥ 8 per day; urgency with or without urge incontinence i.e. OAB symptoms
5. Pyuria of ≥1 wbc $\mu l^{-1}$ (OAB Pyuria patients)
6. No Pyuria of zero wbc $\mu l^{-1}$ (OAB Non-Pyuria patients)
7. A group of asymptomatic normal volunteers matched for age and sex termed "Normal controls"
8. A group of asymptomatic patients undergoing routine flexible cystoscopy because of an episode of microscopic haematuria, termed "Asymptomatic control patients"
9. Able to complete a validated symptom questionnaire

### 5.2.2 Subject Exclusion Criteria

1. Adults aged <18 years
2. Inability to consent
3. Suffering from a significant concomitant urinary tract pathology likely to cause urinary infection of the bladder in its own right; such conditions would include chronic obstruction, catheterisation, neurological disease, prior radiotherapy, anatomical defects or the presence of implanted devices
4. Pregnancy

5. Bleeding disorders / on Anticoagulants

6. Diabetes

7. Urinary Tract Infection

## 5.3 Enrolment Procedures

### 5.3.1 Screening

Eligible patients were identified from Professor Malone-Lee's incontinence clinic and the routine one stop haematuria clinic at the Whittington Hospital NHS Trust. The "Asymptomatic Normal controls" were identified via the recruitment of normal healthy volunteers from the staff at the same institution.

### 5.3.2 First Contact

Suitable patients were written to with a brief lay description of the study asking whether they would be willing to consider helping.

### 5.3.3 Visit One - Initiation visit

The patients were seen in the Incontinence clinic. Urine samples were collected on verbal consent as this formed a part of normal routine clinical practice. However, all patients had a written explanation of urine analysis that was planned. The patient's symptoms were collected by use of a standardised validated questionnaire (67). The data were stored onto a bespoke clinical database and in a case record form (CRF). A unique ID number was then assigned from a chronological sequence. A catheter specimen of urine (CSU) and/or meticulous midstream urine specimen (MSU) only from male were collected. A CSU is the preferred sampling method in the normal clinical practice because of the superior quality of the specimen. The specimen was then processed as follows –

1. An urine aliquot was tested with dipsticks and the results recorded onto Artemis and the CRF.
2. An urine aliquot was examined using a haemocytometer, the constituent cells counted, and the data recorded onto Artemis and the CRF.
3. An urine aliquot was sent for routine microscopy, culture and sensitivity to the microbiology laboratory (threshold of $10^5$ cfu $ml^{-1}$).
4. An urine aliquot sample was centrifuged in a Shandon Cytospin™ to prepare a single cell layer preparation of the spun sediment. These were stained by Giemsa and Pap stain.

All patients, not "Normal controls", were then asked about undergoing a cystoscopy and bladder biopsy using local anaesthetic and a flexible cystoscope, as a day case procedure. Those patients agreed to undergo this procedure were issued with an additional information sheet and another attendance was booked. The majority who agree to this were to undergo Botox injections and a biopsy at the time of this event was a much less inconvenient imposition.

### 5.3.4 Visit Two – Pre-biopsy

An informed consent form was signed. The usual urological preoperative assessment was accomplished and the attendance date for cystoscopy and biopsy was booked.

### 5.3.5 Visit Three – Day care unit

At the time of admission in the day-care unit the surgical staffs were warned of the need for a research-related tissue biopsy and the appropriate preparation was effected. The patient's

willingness to provide a gift of a tissue biopsy was re-confirmed at the time of admission by the undertaking clinician (myself).

The biopsy sample was collected according to the instructions, which were placed in the medical notes. The collection of the specimen and the reception by the researcher and the team was recorded in the hospital notes. The study patient completion page was finished and signed by the researcher/investigator and the fact recorded in the hospital notes.

### 5.3.6 Visit Four – Follow up visit

The patients were seen in the Incontinence clinic so as to follow their progress and to ensure that they had not encountered any adverse effects from the research biopsy. The patient's symptom data was collected and recorded onto, a bespoke clinical research database called "Artemis" that is used for all of the clinical studies conducted in this centre.

Their future care was arranged by supervision in the incontinence clinic.

## 5.4 Clinical Evaluations

### 5.4.1 Concomitant Medications

These were recorded in the Artemis database at the first visit.

### 5.4.2 Concomitant Treatment

This was also recorded in the Artemis database at the first visit.

## 5.5 Diagnostic Category

Categorisation of bladder function according to one of the following diagnostic categories utilising the patient's clinical history and medical notes:

1) OAB with zero pyuria
2) OAB & pyuria $\geq$1 wbc $\mu l^{-1}$
3) Asymptomatic Normal controls

## 5.6 Laboratory Evaluations

### 5.6.1 Clinical Laboratory Evaluations

1. Fresh urine light microscopy using a Haemocytometer
2. Chemical urinalysis: dipstick urinalysis
3. CSU / MSU to the microbiology laboratory for routine culture
4. Shandon Cytospin™ sediment preparations
5. Giemsa and Pap staining of the prepared Cytospin™ slides
6. Bladder biopsy for histology after H&E staining
7. Bladder biopsy for transmission electron microscopy

### 5.6.2 Urological Intervention

1. Flexible cystoscopy and bladder biopsy

## 5.7 Procedures

### 5.7.1 Collection of a Catheter specimen of Urine (CSU)

The CSU was collected from the patients attending the incontinence clinic.

**Equipment**

Medicated swab (saturated with isopropyl alcohol 70 %)

Sterile saline

Universal specimen container

Disposable gloves

Single use catheters esp. Jacques

**Procedure**

After explaining the procedure to the patient, appropriate consent was obtained. The hands were washed using soap and water, dried thoroughly and the operator wore disposable gloves to minimise the risk of cross-infection. The perineum was cleaned with sterile normal saline solution and Jacques ("in and out") size 12 Fr catheter (Mediquip, Devon, UK) was inserted in the urinary bladder under aseptic conditions using "No Touch technique". The initial 5 ml was discarded and the rest of the urine was collected into a universal container. The urine specimen was divided into 3 different aliquots. (A) 1st aliquot was labelled and dispatched to the laboratory as soon as possible after sample was taken for routine culture. (B) The 2nd aliquot was used for dipstick testing and fresh urine microscopy, (C) The 3rd aliquot of urine was subjected to cytocentrifuge in a Shandon Cytospin™ to prepare two slides of single cell layer preparation for subsequent staining with Giemsa and Pap stain.

The type of specimen taken was documented in the patient's notes.

## 5.7. Collection of a meticulous mid-stream specimen of Urine (MSU)

The MSU was collected from male patients attending the incontinence clinic or from the normal controls.

**Equipment**

Universal specimen container

**Procedure**

Verbal consent was obtained. The patients were told to provide meticulous clean catch mid stream uninterrupted urine. They were asked to initiate voiding into the toilet and after a pause pass the universal container into the stream to collect a specimen and then out of the stream so that voiding could be completed into the toilet. The urine specimen was divided as described above (CSU section).

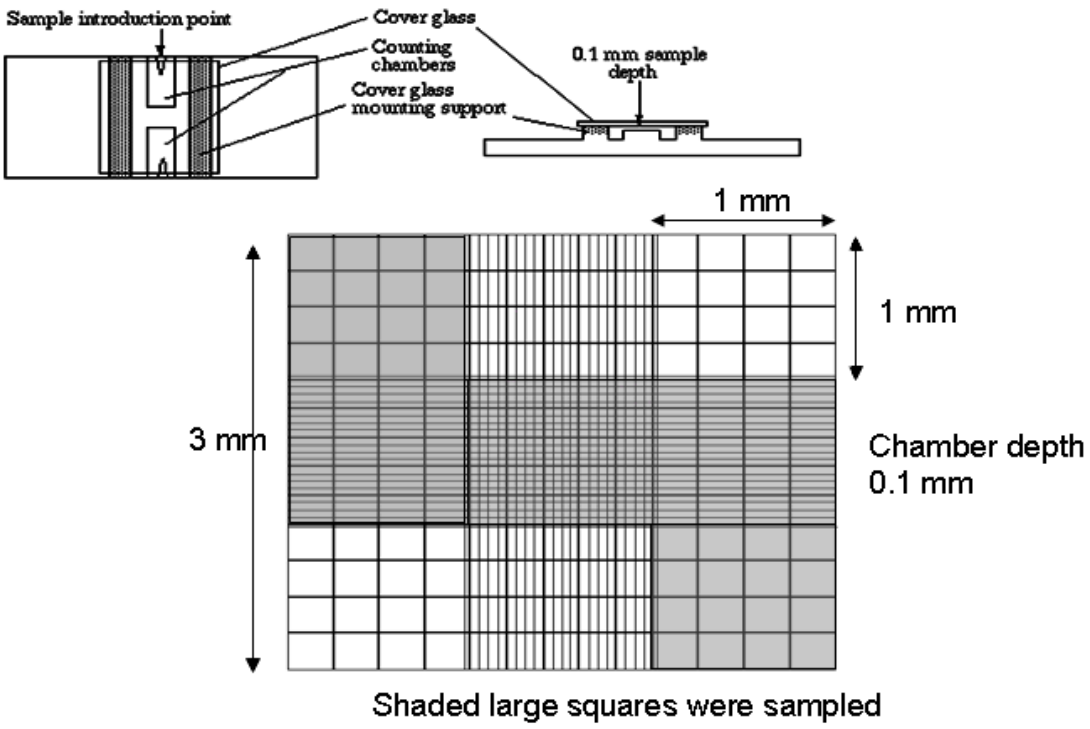
The type of specimen taken was documented in the patient's notes.

## 5.7.3 Fresh urine Microscopy using Haemocytometer

The fresh unspun specimen of urine collected was subjected to microscopy using haemocytometer to evaluate for pyuria $\geq$1 wbc $\mu l^{-1}$. The Haemocytometer is a special microscope slide. It is thicker than normal and has a "chamber", which is 0.1 mm deep, cut into it. The chamber has a grid etched onto it, which can be seen under the microscope. The grid is made of squares, rather like graph paper. Each tiny square is 0.05mm x 0.05mm. The "medium" squares (4 x 4 tiny squares) are 0.2 mm x 0.2 mm and the "large" squares are 1 mm x 1mm (The volume of 1 large square = 0.1 x 1 x 1 = 0.1 $mm^3$ or 0.1 µl). 5 large squares

have a volume of 0.5 $mm^3$ or 0.5 µl. The pus cells/ white blood cells (wbc) were counted in the 5 large squares and then doubled up to give the count of pus cells per µl. If a cell overlapped a ruled line engraving, it was counted "in" if it overlapped the top or right ruling; ignored as "out", if it overlapped the bottom or left ruling. This was standard counting to avoid any discrepancy. The image of Haemocytometer grid is obtained from online resource www.ruf.rice.edu/.../cellcounting.html and www.jove.com/index/details.stp?ID=262

**Figure 1 - A Haemocytometer grid** (HPA Culture collections, Salisbury, UK)




## 5.7.4 Dipstick urine analysis

The fresh urine collected was subjected to dipstick analysis. The urine was dipped by a Multistix® 8 SG and read by a Clinitek Status colorimeter (Bayer Healthcare, Newbury, UK). The nitrite test pad sensitivity of the dipstick was stated to be 13-22 µmol/L (0.06-0.1 mg/dl) nitrite ions. Therefore if the urine sample contained nitrite ion in the range specified, the pad

would turn pink indicating a positive result. The leukocyte test pad sensitivity of the dipstick ranges between trace to > 2000 mg/dl.

### 5.7.5 Routine culture method

A urine aliquot collected either by CSU or MSU was labeled appropriately and conveyed to the hospital laboratory. The specimen was treated fresh, or after overnight storage at $4^0$ C. 1 µl of unspun urine was transferred by loop to a chromogenic media, CPS ID2 ( bioMerieux, Hampshire, UK ). The plate was incubated for 24 hrs at $37^0$ C. Bacterial colonies were identified by colour change and size. The result was taken as positive if greater than $10^5$ colonies generated per ml after 24 hrs culture.

## 5.8 Cytocentrifuge

### 5.8.1 Shandon Cytospin™ 2

**Principle**

This centrifuge is used to concentrate the cells from either small volumes of urine or from centrifuged samples with negligible cell content. The cells are concentrated into a small area on a glass slide with the excess fluid absorbed onto a cardboard filter.

A small amount of urine, approximately 5 drops, is placed in either disposable or reusable cuvettes, which fit into a sealed, removable rotor head. The cuvettes must always be loaded inside the safety cabinet and the rotor head must always remain sealed outside the safety cabinet.

**Equipment**

**Shandon Cytospin™ 2**

(Woodley Equipment Company LTD, Bolton, UK)


**Procedure**

1. The slides were labelled with the patient's study number.
2. After assembling, the slide, white cardboard filter and cuvette were clipped together using the metal holder ensuring that the hole in the cuvette lined up with the hole in the filter.
3. The cuvette assemblies were placed in the rotor head in the slots provided, ensuring that they balanced, using a dummy assembly as required.
4. A disposable plastic pipette was used to place five drops of urine into the base of each cuvette assembly. This procedure was performed in the safety cabinet.
5. The rotor head was closed and transferred the sealed assembly to the Cytospin.

**Operation**

The sealed rotor head was placed in the Cytospin and the lid was closed to run at 850 rpm for 5 minutes using HI acceleration.

**Health and safety**

- Equipment should always be used according to the manufacturer' instructions.
- Safety devices must always be used and never overridden.
- In the event of failure, the fault must be reported immediately and the equipment taken out of use.
- Any electrical work on equipment should be performed by a competent electrician i.e. someone from the works department or a service engineer.

**Disinfection**

The interior working parts and operating panel of each centrifuge was wiped with hypochlorite once a week. Any reusable equipment e.g. cuvettes was immersed in hypochlorite overnight.

Spillage was cleaned and the area wiped with hypochlorite according to the disinfection policy.

## 5.9 Stains

The following 2 stains were used which is described in detail below –

### 5.9.1 May Grunwald Giemsa Stain (MGG) (180) –

**Principle –** It gives a good nuclear and cytoplasmic differentiation. Nuclei stain purple and cytoplasm stain gray to blue.

**Methods –** The cytospun slide was stained with 50 % May Grunwald (Sigma-Aldrich, St. Louis, USA) and then rinsed in distilled water, counterstained with 10 % Giemsa solution for 20 minutes and then finally rinsed in buffer (phosphate buffer tablet - pH 6.8, mix in 1 litre of distilled water - Sigma-Aldrich, St. Louis, USA) for 30 seconds. It was then dried on a hot plate ready to be mounted with a cover slip.

**Health and safety –**

Methanol in MGG stock solution is highly flammable and toxic

### 5.9.2 Pap stain – Papanicolou stain (181)

**Principle –** It is a polychrome staining method, which depends on degree of cell maturity and cellular metabolic activity. The three main advantages of this staining procedure are:

(1) Good definition of nuclear detail.

(2) Cytoplasmic transparency.

(3) Indication of cellular differentiation of squamous epithelium.

**Methods –** The various agents were placed in the different chambers in the Shandon Varistain-24-3 staining machine. This was then run though a prefixed programme to produce a correctly Pap stained slide. The programme of staining is described below –

| Station | Reagents | Memory A |
|---|---|---|
| 1 | 80% Alcohol | Pass |
| 2 | 70 % Alcohol | Pass |
| 3 | 50 % Alcohol | 5 sec |
| 4 | Tap water | 5 sec |
| 5 | Harris Haematoxylin | 2 min 30 sec |
| 6 | Tap water | 45 sec |
| 7 | 0.25% Acid water | 10 sec |
| 8 | Tap water | 3 min |
| 9 | 95% Alcohol | 20 sec |
| 10 | OG 6 | 2 min |
| 11 | 95% Alcohol | 20 sec |
| 12 | EA 50 | 3 min |
| 13 | 95% Alcohol | 20 sec |
| 14 | Isopropanolol | 60 sec |
| 15 | Xylene | 30 sec |

**Items required** – Prepared cytospun slide, cover slip, sterile pipettes, various % of Alcohol, 0.25% Acid alcohol, Haematoxylin, EA50, OG 6, Xylene, Canada balsam and slide warmer (Sigma-Aldrich, St. Louis, USA).

**Health and safety**

- OG 6 is flammable, toxic and irritant
- EA 50 is flammable and oxidising agent
- Alcohol and Isopropanolol is also flammable and oxidising agent
- Acid water is irritant and corrosive
- Xylene is flammable, toxic and is narcotics if used in high dosage. It is also harmful by ingestion and inhalation.

### 5.9.3 Reading Stained Slides

Once the cytospun urine sediment was obtained, it underwent Giemsa and Pap staining. For quantification purposes, the inflammatory cells i.e. neutrophils and lymphocytes in the Giemsa stained slides were counted over the whole field. Similarly the Pap stain slides were counted for the whole of the stained area to quantify the different urothelial cells including metaplastic squamous cells. The slides were examined under light microscope using a x20 objective with x10 eyepiece.

## 5.10 Flexible Cystoscopy

The procedure was explained to the patient and an informed consent was obtained. All the patients were willing to undergo a bladder biopsy so as to provide a gift of bladder tissue for research purposes. The procedure was performed by the researcher and by a colleague engaged in parallel research.

**Procedure**

A chlorhexidine cleaning solution and local anaesthetic – 2 % lignocaine gel (Care Fusion, Basingstoke, UK) were used to disinfect, numb and lubricate the urethra to make the passage into the bladder as comfortable as possible. A cystoscope (Dantec Medical, UK) was inserted into the bladder via the urethra. Attached to the instrument was a telescopic lens, a light source and a bag of sterile water to fill the bladder so that the bladder wall could be inspected. Once the instrument was in place, the examination took only few minutes to complete. The majority of the patients were undergoing intravesical injections of Botox, which was administered after the biopsy. The injections took about 20 minutes.

.

Most patients experienced slight discomfort during the procedure a minority found it painful. Practice and allowing more time for local anesthetic activity reduced the experience of pain. Once inspection, biopsy and the Botox injection were complete, the instrument was removed, and the patients were informed of the findings and the need for any further management.

A nurse remained with the patient throughout the procedure.

**Side effects and risks**

There are potential complications with any procedure. Although rare, it is important to be aware of them.

**Common**

- Mild burning or bleeding on micturition, which settles down itself.

**Occasional**

- Infection of the bladder requiring antibiotics.

**Rare**

- Delayed bleeding secondary to infection requiring antibiotics or catheter insertion with irrigation to remove clots.
- Injury to the urethra.
- Urinary retention post procedure requiring temporary insertion of a catheter.

## 5.11 Bladder biopsy specimens

The bladder sample was removed during local anaesthetic flexible cystoscopy. 2 sets of bladder biopsy sample were taken from the dome of the bladder, after the informed consent of the patient. There was no need to apply diathermy to stop bleeding, as the samples were small and shallow having been taken with a flexible cystoscope. Both samples were collected, as the bladder mucosa was utilised. One of the biopsy samples was stored in formalin solution and sent for routine histology after H&E staining and the other biopsy sample was stored in 1.5 % glutaraldehyde solution and sent for Transmission Electron Microscopy.

## 5.12 Preparation of tissue for H & E stained Histology slide

**Procedure**

**Reception of specimen**

The sample was transferred from the theatre to pathology laboratory where various checks were undertaken to make sure that the details from the specimen matched with the request form. The specimen was given an electronic number so that the details could be retraced from the computer database. Because the processing of these specimens formed a part of patient care it was required by the pathology department that the specimens be processed for histology by trained pathology technicians. However, the researcher did witness the process, was informed of the methods used and could accomplish them.

**Cutup stage**

The macroscopic description of the bladder biopsy specimen was recorded at this stage including dimensions, colour pigmentation and any artefact.

**Processing of the specimen**

The tissue was processed in a Vacuum Infiltration processing machine (VIP), which takes 12 hours. Here the principle is dehydration of tissue with alcohol and xylene. The tissue specimen was the subject to various agitation mechanisms whereby the tissue was impregnated with paraffin wax a biocompatible material, which gave a firm structural support matrix to the tissue.

**Embedding**

The tissue was orientated in a particular manner to achieve maximum visibility upon slicing the tissue. It was then embedded in $60^0$ C molton paraffin wax and a cassette was placed on top to create a block

**The blocking cut stage**

The surgical block was matched to the appropriate form and the surgical number. Standard laboratory quality control was conducted. Because these specimens were part of clinical care they had to be prepared by certified NHS laboratory technicians

**Microtomy**

The tissue was expertly cut at 4 µm (thickness of one cell) and creases eliminated by floating the slice in a $35^0$ C water bath and then trapping the slice on a glass slide which had previously been numbered correctly. The slide was then dried on the hot plate at $60^0$ C for 10 minutes.

**Staining of slide - H & E**

The slides were processed by a staining machine for the Haematoxylin and Eosin stain (H & E) (Sigma-Aldrich, St. Louis, USA), which took 15 minutes. The wax in the slides was melted in a $50^0$ C oven, with further dewaxing by dipping in a series of solutions of xylene and alcohol followed by a water wash. The slide was then stained by Haematoxylin to define cellular constituents of tissue (e.g. Chromatin). This was then differentiated in acid/alcohol to give sharp nuclear stain. Then the slide was plunged into water and counterstained with eosin (for cytoplasm). The tissue was again dehydrated via various strengths of alcohol and finally washed with xylene, which helped the coverslip to adhere.

**Coverslip**

The adhesive Dpx was used which has the same refractive index as the microscope (1.5).

The slides were reviewed by the researcher, supervisor and the histopathologist.

**Health and Safety**

- Alcohol is flammable
- Xylene is flammable, toxic and irritant

## 5.13 Preparation of tissue for Transmission Electron Microscopy

**Procedure**

The electron microscopy services were insistent that the tissue be prepared by trained technicians because of the need to ensure quality of the product.

The tissue was fixed in 1.5 % paraformaldehyde/1.5 % glutaraldehyde in phosphate buffer, washed with phosphate buffered saline [Oxoid] and postfixed using 1 % osmium tetroxide / 1.5 % potassium ferricyanide, washed with distilled water and dehydrated using graded alcohols 30 %, 50 %, 2 x 70 % and 3 x 100 %, followed by infiltration with 50 % alcohol / 50 % Lemix (TAAB) epoxy resin mixture overnight and the next day infiltrated with 100 % Lemix resin for a minimum of 6 hours, followed by polymerisation at $70^0$ C overnight.

Sections were cut on a Reichert-Jung ultracut E microtome and collected on 300HS, 3.05mm copper grids (Gilder). The sections were stained with saturated uranyl acetate in 50 % ethanol (TAAB) and Reynolds lead citrate.

Sections were viewed and photographed using a Philips CM120 transmission electron microscope (John Hopkins, Baltimore, USA). This was reviewed by the electron microscopist, researcher and the supervisor.

**Reagents**

**Glutaraldehyde**

(20mls 20 % paraformaldehyde [Analar BDH] + 16mls 25 % glutaraldehyde [TAAB] + 59 mls phosphate buffered saline [Oxoid])

**Osmium tetroxide**

1 % osmium tetroxide [Analar BDH] + 1.5 % potassium ferricyanide [BDH] in PBS [Oxoid]

**Toluidine blue stain**

1 % toluidine blue [Raymond Lamb] with 0.2 % pyronine [Raymond Lamb] in 1 % sodium tetraborate [Analar BDH].

**Reynolds lead citrate**

Dissolve 1.33g lead nitrate [BDH] in 15mls distilled water and 1.76g sodium citrate [BDH] in 15mls distilled water, mix solutions together and dissolve the resulting precipitate with 8 mls of 1M sodium hydroxide [BDH], make up to final volume of 50mls

**Lemix epoxy resin**

Lemix A (25mls) + Lemix B (55mls) + Lemix D (20mls). Pour into plastic resin bottle and add 2 mls of BDMA, mix well.

**Health and Safety**

- Glutaraldehyde - Harmful by inhalation. Harmful if in contact with skin. Prolonged skin contact may cause dermatitis.
- Osmium tetroxide - Harmful by inhalation and if swallowed. Corrosive to eyes and skin causing burns. Stains skin black. Low concentrations of vapour affect the eyes. Use only in a fume cupboard
- Potassium ferricyanide - Low toxicity. May be harmful if ingested in large quantities. Irritating to eyes, respiratory system and skin

- Alcohol 74OP - Flammable. Toxic by inhalation, in contact with skin and if swallowed.
- LEMIX A - Mild skin and eye irritant.
- LEMIX B - May cause slight irritation to eyes and skin. Low toxicity.
- LEMIX D - Harmful by ingestion, inhalation and skin contact. May be irritating to skin and eyes.
- BDMA - Irritating to eyes, skin and respiratory system. Harmful by ingestion.

## 5.14 Light Microscopy

A light microscope is a sophisticated optical instrument that provides high-resolution images. The four basic components of a microscope addresses resolution, illumination, magnification and imaging. An Olympus CX41 Light Microscope was used to count the cells using x20 objective with x10 eyepiece (GX Optical, Suffolk, UK).

## 5.15 Statistical Analysis

The data were checked for normality using Q-Q plots and by histograms. Normally distributed data were analysed using the parametric method for comparing means - ANOVA.

Where the distributions were found to be non-normal no attempt was made to transform by logarithmic conversion because the data consisted of discontinuous counts of cells. Therefore non-parametric tests of significance were achieved. Where comparison between two groups was being achieved the Mann-Whitney U test was used. In circumstances where more than two groups were compared the Kruskal-Wallis H test was used. In all cases the 95 % level of confidence was used (alpha=0.05). The data distributions were shown on graphs as the medians with 95 % confidence intervals.

Ordinal data were analysed in contingency tables using the Chi-square ($X^2$) test at the 95 % level of confidence ($\alpha$=.05)

It was not possible to calculate a suitable sample size at the inception of this study because the subject matter was so inchoate. It was therefore thought best to sample within the limits of patient availability, consent and time.

Giemsa Stain – In the event with a mean total inflammatory cell count of 29 (sd=33) in 178 patients and the same in 21 controls with 6 (sd=5.6), this study had 100% power at alpha of 5%, so that the probability of a type II error was vanishingly small when the probability of a type I error was 0.05.

Pap Stain – Similarly with a mean deep transitional cell count of 67.27 (sd=162) in 157 patients and the same in 29 controls with a mean deep transitional cell count of 18.86 (sd=18.36), this study had a 97.6% power at alpha of 5%.

Histopathology and Electron Microscopy – It was not possible to calculate the sample size for this study as the subjects were few in numbers, especially the controls.

## 5.16 Experience of Methods

This section provides an operational account of the application of the methods and the experiences that resulted.

### 5.16.1 Sampling and Recruitment of patients

As the patients were recruited from the Incontinence clinic at the Department of Medicine, Whittington Campus, UCL Medical School there is some overlap of patients providing the urine sample for different analysis. There was no random selection of patients but the samples and data were collected consecutively from those attending the Incontinence clinic. The stringent recruitment criteria which were applied during the screening made it essential that all comers were considered for inclusion in the study if the study was to achieve the necessary sample sizes. The normal controls were obtained from the hospital staff and the medical students. It was essential to have control data from persons without any symptoms at all, including no nocturia and it was found more pragmatic to obtain such from groups who could clearly understand the strictures rather than general population. We had already discovered the cost and time demands of finding normal controls randomly in the general population.

### 5.16.2 Urine Collection

The CSU collection was a part of the routine patient care but nevertheless there were a few occasions when patients refused to provide with a sample at all or were insistent on providing only MSU. These persons were not included in our study. Because of ethical problems a MSU from the normal controls had to be obtained, although a small number of CSU's could be obtained from patients undergoing gynaecological procedures. The controls providing the MSU's were carefully instructed in the production of a meticulous MSU and many already

clearly understood the principles. The moment urine was collected; 5 ml of the sample was mixed with 5 ml of 70 % alcohol immediately to help preserve the cells and to achieve better Cytospin™ preparation. At times there was very little urine collected in the sample, this was especially the case when collecting CSU's. If the urine volume was less than 5 ml, it was not included in the study because of the very high risk of contamination. The CSU/MSU was sent for routine culture to the laboratory as soon as possible, within 3 hours. On 2 occasions the laboratory was unable to process the culture request because of urine leakage from the specimen pot. Because the samples were anonymised for processing through research methods, utmost care was needed to avoid any mismatch of labels on the urine sample pots.

### 5.16.3 Cytospin™ Preparation

The researcher took some time and considerable practice before achieving entirely satisfactory urinary sediment preparations, and ultimately that depended on the Cytospin™ equipment. Initially researcher took 10 ml of urine which was centrifuged at 2000 rpm for 6 minutes. The supernatant fluid was discarded and the sediment was spread manually over a glass slide with another glass slide. Despite many hours the researcher was unable to achieve reliably, a single cell layer preparation. The stacking of cells on the slide prevented from obtaining an even stain of the preparation. This was presented to the cytologists at this centre who suggested the use of the Shandon Cytospin™ 2. A second-hand device was purchased and after some practice the researcher began to achieve beautiful single-layer cell preparations very consistently. These proved very well to be able to take up the various stains evenly. Initially single frosted slides were used but then chose to use super frosted versions since these are known to be made of a higher quality glass and thereby result in a much clearer image. It was extremely important to store the slides in closed, clean containers before use because they had a tendency to attract contaminants onto both surfaces.

### 5.16.4 Staining

The staining was an essential part of this experiment and it was vital to get it correct. The Giemsa stain was used to describe the differential morphology of white cells discriminating lymphocytes and polymorphonuclear leucocytes. The Pap stain was used for a different purpose in that it permits proper discrimination between epithelial cells. Whilst the Pap stain is taken up by inflammatory cells so that they are indentified as contrasting from epithelial cells they look very similar and it is not possible to discriminate lymphocytes from polymorphs. Initially, the Pap stain was used manually but found to be extremely difficult to achieve uniform results and felt that the interpretation could not be accepted as reliable. Therefore; the manually stained slides were not included in the analysis. In order to overcome the problem of consistency a Shandon Varistain machine was obtained. This has the capacity to achieve a number of automated staining protocols. Once a defined programme had been entered into the machine it proved well able to achieve very consistently stained Pap slides with sets of slides being processed in bulk. There were instances when machine got stuck and sometimes faulty transitions caused slides to break, in which case replacement preparations were made up and processed. The Giemsa staining was done manually since it involved fewer steps and provided very consistent results without any need for automation.

### 5.16.5 Reading slides

Once the slide was stained, they were fixed with a cover slip mounted using Canada balsam. The slides were then examined under a light microscope in order to evaluate the nature of the cells and to count them. This proved to be the most difficult task and proved to be the most time-consuming. The cells over the whole circle of sediment were counted. This required several traverses of the slide surface and particular concentration to ensure that the traverse

was not duplicated by accident. On an average, it took an hour to read a slide for each of the stains. It took a considerable amount of practice to become proficient at focusing on the field of interest as paucicellular preparations could demand a high level of persistence before the counting zone was assimilated. The area of interest was a circular area in the centre of a slide which was 5 mm in diameter. It was found that if the counting zone area was encircled with a red marker pen it became much easier to get the bearings. In order to be consistent the slide was moved so that the objective sampled in traverses of the vertical plain, going from top to bottom then bottom to top, thus sampling columns as it moved across the slide from left to right.

It soon became evident that the consistency of the Cytospin™ machine meant that the spun circles of sediment offered the opportunity of semi-quantitative analysis of the cells. Initially morphological distribution counts of samples of 100 cells, was tried. However, it was found out that some slides had fewer than 100 cells in the sediment area whereas others had well in excess. This meant that a limit of 100 cells counting imposed an arbitrary maximum achievable total. It was therefore decided to count all of the cells in the circle of sediment thereby producing a meaningful total cell count along with a differential analysis. At the time, it was not expected that the total cell count to exhibit between disease state differences. It was assumed, falsely as it turned out, the differential ratios would be the salient elements.

### 5.16.6 Flexible cystoscopy and Bladder biopsy

As most of the patients were undergoing Botox injection at the time of providing bladder biopsy, the procedure lasted for approximately 20-25 minutes. At first it was found that a few patients reported pain when the biopsy was obtained. Thereafter the method was modified so as to wait for an extra five minute after instilling 2% lignocaine gel into the urethra. From

that time, the procedure was better tolerated although complete avoidance of some discomfort could not be achieved. The probability of this was made clear in the patient's information sheet, and very grateful to the patients who agreed to this biopsy for research purposes. The biopsy destined for routine histology after H&E staining was collected in formalin solution. The biopsy for electron microscopy was initially collected in the normal saline for immediate transfer to the EM laboratory. This process was then modified so that the specimen was collected into glutaraldehyde solution which preserved the specimen thereby obviating the need for immediate transportation.

An interesting experience arising out of this work was that initially the histopathologist challenged the utility of a superficial bladder biopsy. It transpired that their appreciation of this process was that it was deployed to seek out evidence of malignancy. Surprising though this may be, it turned out that the notion of taking an urothelial sample in order to look for evidence of inflammation and reactive urothelial changes was a novel notion. In fact up to that point the reports had been classifying biopsies as "normal" if no malignancy or dysplasia was seen. When archived slides classed as "normal" were reviewed, in many cases inflammatory infiltrates were identified. An engaging consequence of this work is that routine histological reporting of urothelial specimens has changed at this centre.

### 5.16.7 Interpretation of Histology slides

Once the procedure with the histopathologist was agreed, the researcher examined the slide preparations with his supervisor. The slides were examined and reported independently of each other. Had it been different outcomes a consensus view was negotiated. In the event a divergent view never occurred. Having recorded the findings these were cross-checked with those provided by a histopathologist. The fact that there was an agreement, it demonstrates

the very straightforward nature of the inflammatory changes observed. In particular normal preparations looked very different from those exhibiting inflammation in this series.

When these analyses had been completed, the slides were crosschecked with another consultant histopathologist at a different hospital.

### 5.16.8 Interpretation of Electron Microscopy slides

The methods section described the preparation of the epoxy resin fixed slides used for EM. An experienced electron microscopist prepared sets of photomicrographs obtained from the sections relevant to each biopsy. The EM pictures confirmed the appearances that had already been described by light microscope. The only novel observation, additional to that, which could not be seen under light microscopy, was an apparent thickening of the basement membranes of the urothelium obtained from patients with OAB. Because of the difficulties in obtaining bladder biopsy material from normal controls, the biopsies were taken from 3 persons with no symptoms and normal controls but one of the samples was too small for processing so EM photomicrographs were obtained from two controls.

There were 100 different measurements taken to assess for basement membrane thickening and the average was recorded. To maintain the standard measurement all the photomicrograph with a magnification of x8800 were included.

### 5.16.9 Quality Assurance

This was particularly important for Giemsa and Pap stain slides. There were approximately 400 slides, which were subjected to total cell count of different morphological origin depending on the type of stain used. This was extremely laborious and time consuming as

each slide took approximately an hour to read and considerable practice to be able to recognise the various cells. It would be very difficult to find someone to review all the 400 slides.

Therefore random samples of slides were reviewed to ensure quality control.

# Chapter 6

# Results

# CHAPTER 6

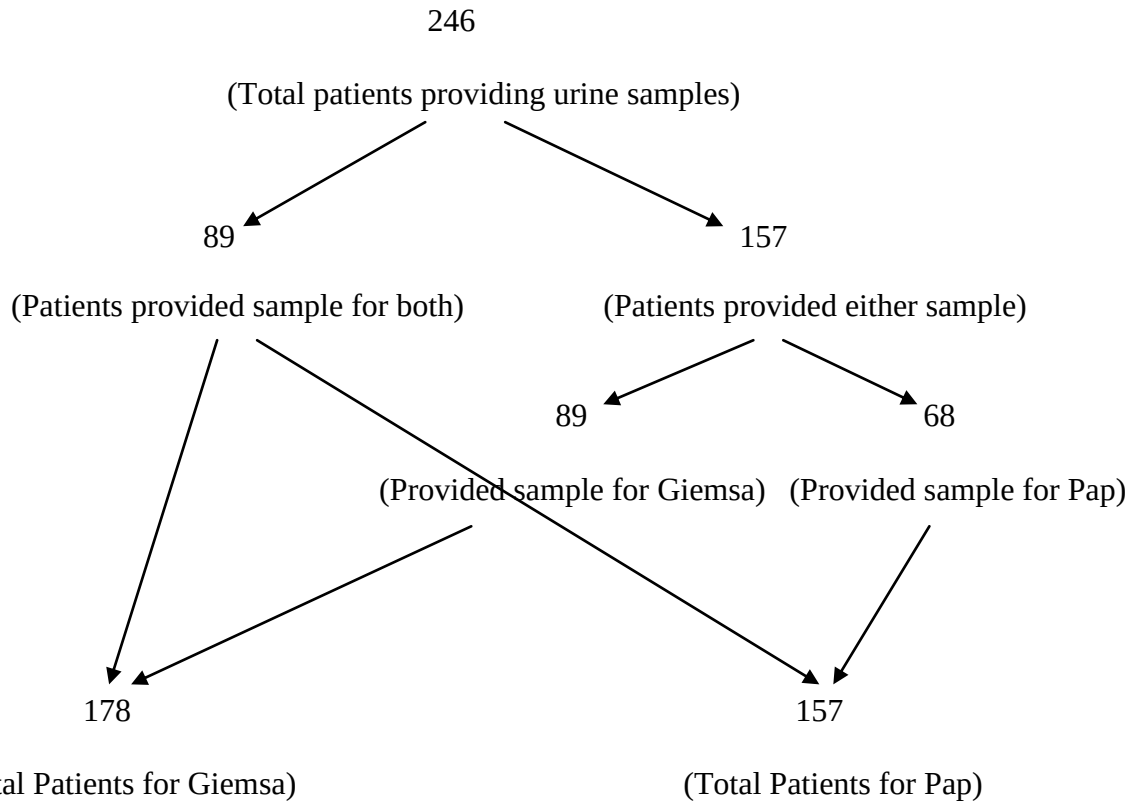
# RESULTS

The patients attending the Incontinence clinic at the Department of Medicine, Whittington Campus, UCL Medical School were recruited for the study. This study involved the deployment of 4 different methods for evaluating the inflammatory reactions associated with symptoms of OAB. The methods were applied to samples of different numbers of patients but there was some overlap so that patients could contribute data into more than one method.

(1) The first data set was obtained from 246 patients, 234 females (95 %) and 12 males (5 %), mean age of 57 (range 22-91), sd =19, with symptoms of OAB. These patients provided a catheter specimen of urine, which was processed so as to provide Cytospin preparation. Initially the slides were stained by the Pap method and subsequently, when differential white cell analysis was required, the Giemsa stain was adopted. 89 Patients provided Pap and Giemsa stained samples; the other 157 patients provided either a Pap preparation or a Giemsa version. Thus 178 patients provided Giemsa preparations and 157 Pap stained slides.

(See flow chart below)

## Flow Chart




(2) The second data set was obtained from 79 patients, 66 female (84 %) and 13 male (16 %), mean age 56 (range 25-81), sd=15, with OAB symptoms. They provided bladder biopsies for paraffin block section, H&E stained, histology. The majority of these patients donated a biopsy at the time of intravesical Botox injection for OAB.

(3) The third data set was obtained from 22 patients all of whom were suffering from OAB. 18 of these also contributed bladder biopsies for the H&E histology data set. This third group provided biopsies for electron microscopic analysis (EM) analysis. This sample consisted of 16 females (73 %), 6 males (27 %) and their mean age was 50 (range 27-71), sd=13.

(4) 43 normal controls provided urine samples; 36 MSU and 7 CSU. 21 samples provided Giemsa preparations and 29 provided Pap stained preparations. The 7 CSU samples were

used to achieve Pap and Giemsa slides. This sample consisted of 24 females (56 %), 19 males (44 %) and their mean age was 33 (range 20-64), sd=10.

5 normal controls (all males) provided bladder biopsies for histology and 2 normal controls (all males) provided biopsies for EM comparisons. It was extremely difficult to recruit normal controls for the experiments that required biopsy. Whilst many patients undergoing routine cystoscopy were very happy to offer biopsies for research purposes, finding those without urinary symptoms was the key impediment. Though the demographics were different between the control and the patient group, as the controls were all males and younger than the patient group but utmost care was taken to make sure they were devoid of lower urinary tract symptoms and their urine was free of pyuria and urine culture clear.

These patients and controls were characterised according to their symptoms and whether or not they exhibited pyuria $\geq$1 wbc $\mu l^{-1}$. Since all of the controls showed zero pyuria they provided a single clinical description. In the patient groups all had symptoms of OAB some with pyuria $\geq$1 wbc $\mu l^{-1}$ and some without.

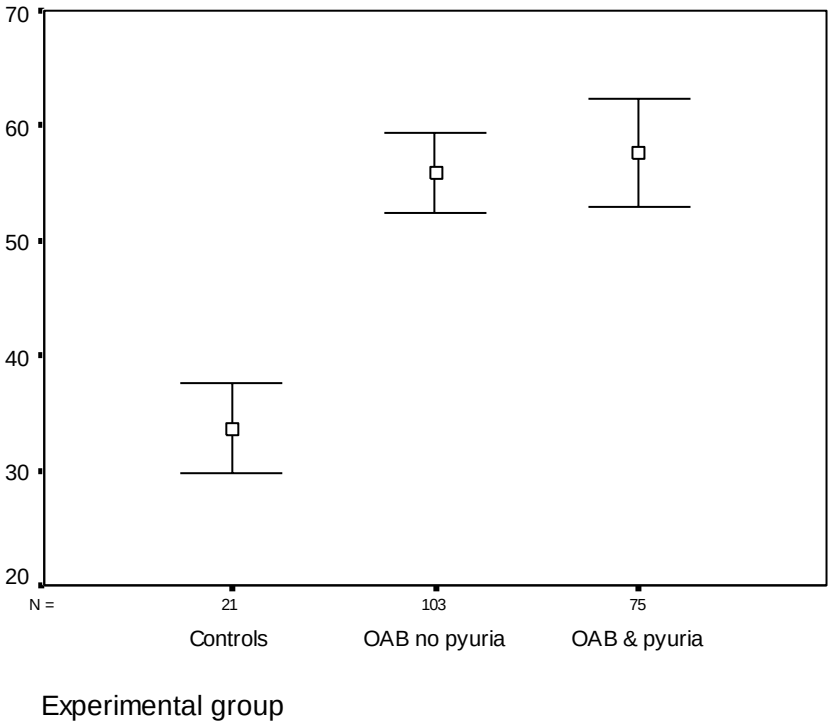
Whilst all the controls were significantly younger than the patients, the 2 patient groups, OAB symptoms zero pyuria and OAB symptoms & pyuria $\geq$1 wbc $\mu l^{-1}$, did not differ in respect to age, sex and symptoms; frequency, incontinence and urge score. This is further described in details in individual section.

## 6.1 Giemsa staining Results

The aim of this study was to examine the differential inflammatory cell count in the cytospun preparation from patients with OAB symptoms compared with normal controls. The patients with OAB symptoms were further sub-classified on the presence or absence of pyuria ≥1 wbc $\mu l^{-1}$ as OAB symptoms & pyuria or OAB symptoms zero pyuria.

In total, 178 patients with OAB symptoms (94 % female) and 21 controls (76 % female) were studied. The controls were younger (mean age 34, 95 % CI 30 to 38, sd=9) as compared to patients with OAB symptoms (mean age 57, 95 % CI 54 to 60, sd=19). The age distribution is shown in figure 6.1.

**Figure 6.1 – The mean age with 95 % CI for the experimental groups**



The distributions of the symptoms of frequency, urgency and incontinence were checked for normal distributions using Quantile-Quantile (QQ) plot available in SPSS and found to be appropriately normal and ANOVA was used to analyse the between group differences.

The patients with OAB symptoms & pyuria ≥1 wbc $\mu l^{-1}$ reported an average 24-hour frequency of 10 (sd=6, 95 % CI 8 to 12) and average 24-hour incontinence of 1 (sd=2, 95 % CI 1 to 2). Their mean urgency score was 5 (sd=5, 95 % CI 3 to 6), whereas patients with OAB symptoms zero pyuria reported an average 24-hour frequency of 8 (sd=4, 95 % CI 7 to 10) and average 24-hour incontinence of 1 (sd=2, 95 % CI 1 to 2). Their mean urgency score was 4 (sd=3, 95 % CI 3 to 5).

Formal comparisons of the two groups OAB symptoms & pyuria ≥1 wbc $\mu l^{-1}$ and OAB symptoms zero pyuria, showed no significant differences in symptoms: 24-hour frequency ( p=0.056, 95 % CI of difference -0.1 to 5), 24-hour incontinence (p=0.406, 95 % CI of difference -1 to 0.5) and urgency score (p=0.532, 95 % CI of difference -2 to 1).

Despite the differences in age and gender the symptoms of the MSU group did not differ from those of the CSU group.

CSU group: Average 24-hour frequency = 9 episodes (sd=5); average 24-hour incontinence = 1episodes (sd=2); mean urgency score = 4 (sd=4)

MSU Group: Average 24-hour frequency = 6 episodes (sd=1); average 24-hour incontinence = 0 episodes, sd=0; mean urgency score = 1 (sd=0)

**Comparisons**

24-hour frequency: p=0.257, 95 % CI of difference -9 to 2

24-hour incontinence p=0.286, 95 % CI of difference -3 to 1

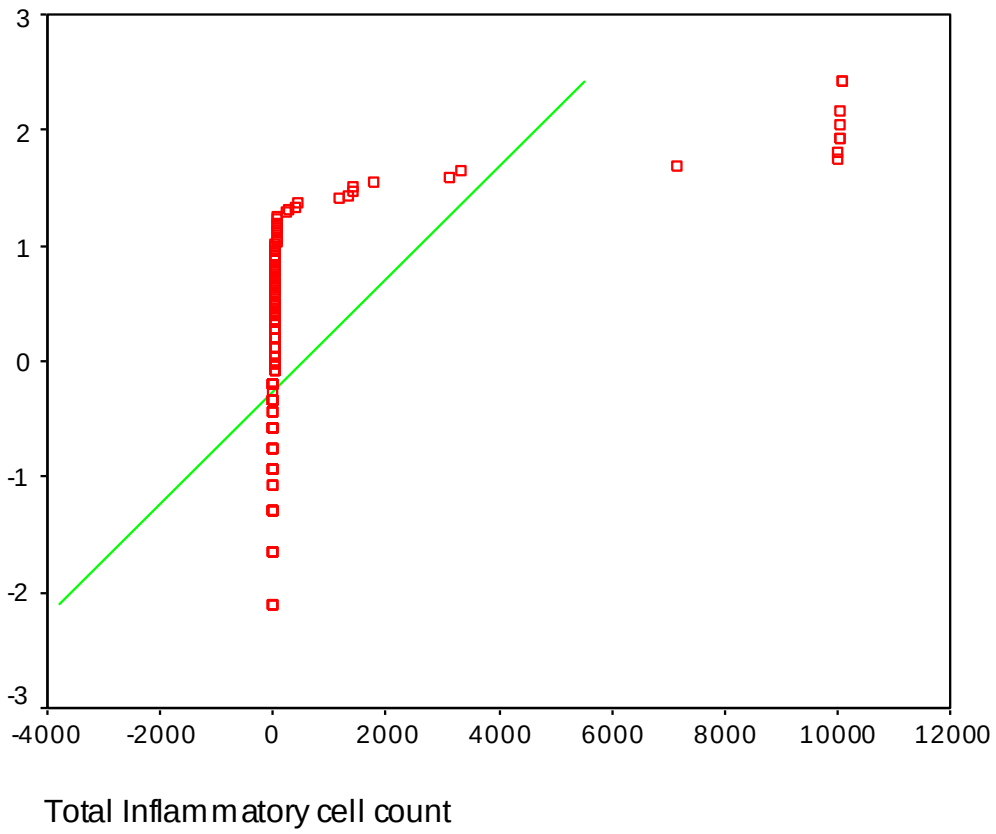
Urgency score p=0.152, 95 % CI of difference -7 to 1

**Analysis of cell counts**

The data describing the cell counts were first checked for normality using the Quantile-Quantile (QQ plot available in SPSS. It was shown that these data were far from normally distributed. Figure 6.2 illustrates the QQ plot and if the data were normally distributed the points would fall juxtaposed to the linear function plotted for comparison. The lie of the data points show that the experimental data deviate substantially from the ideal.

Because of this, non-parametric tests were used for the analysis. There were 3 groups; (1) controls, (2) OAB symptom zero pyuria and (3) OAB symptom & pyuria $\geq$1 wbc $\mu l^{-1}$ so the Kruskal-Wallis test was used and the test statistic H was recorded along with the degrees of freedom (=2) and p value. The data distributions were illustrated by plotting the median count and 95 % CI of the median which were calculated by using the statistical package Minitab.

**Figure 6.2 – Normal QQ plot of the Total White cell count**




In the above graph, the X axis shows total inflammatory cell count and the green perpendicular line suggest the expected normal lie of the data, and the linearity of points (small red boxes) suggests that the data are not normally distributed.

75 of 178 (42 %; 95 % CI = 36 % to 48 %) patients with OAB symptoms showed pyuria ≥1 wbc $\mu l^{-1}$. 103 of 178 (58 %; 95 % CI = 50 % to 65 %) patients did not show pyuria zero wbc $\mu l^{-1}$. None of the 21 controls had pyuria ≥1 wbc $\mu l^{-1}$. As has already been described, the 2 OAB symptom groups were similar in age & sex.

Because of the decision to identify pyuria as ≥1 wbc $\mu l^{-1}$ for the purpose of this thesis, a sub-analysis was conducted on these data to check for differences between Pyuria = 0 wbc $\mu l^{-1}$,

pyuria = 1 to 9 wbc $\mu l^{-1}$ and pyuria $\geq$10 wbc $\mu l^{-1}$.There was a difference in urine culture results ($X^2$ =34, df=2, p<0.001).

7 of 103 (7 %) of OAB zero pyuria were positive.

4 of 30 (13 %) of OAB & pyuria < 10 (1 to 9 wbc $\mu l^{-1}$) were positive.

21 of 45 (47 %) of OAB & pyuria $\geq$10 $\mu l^{-1}$ were positive.

1 of 21 (5 %) of controls was culture positive.

There were significant differences between all the groups in the number of neutrophils, lymphocytes and therefore the total white cell counts. These are shown in figures 6.3 to 6.5. It can be seen that as might be expected, the patients with OAB and pyuria $\geq$1 wbc $\mu l^{-1}$ stand out. However it should also be noted that OAB patients without pyuria had significantly higher inflammatory cell counts in their spun sediments than did normal controls. This implies that the Cytopsin method is unmasking an inflammatory reaction in the urine of OAB patients that would not be detected by ordinary microscopy of the urine.

**Figure 6.3 – The median Neutrophil count and 95 % CI between groups**

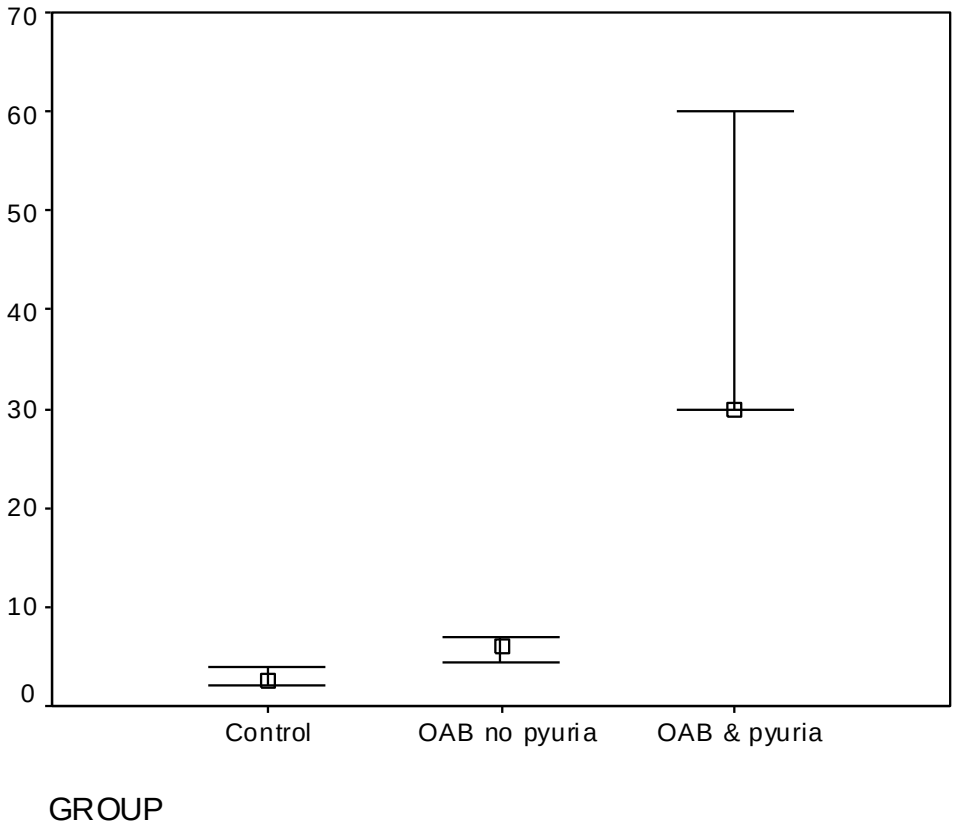



**Figure 6.3a – Picture of Neutrophil**

**(Olympus light microscope, X20 Magnification, Giemsa stained slide)**

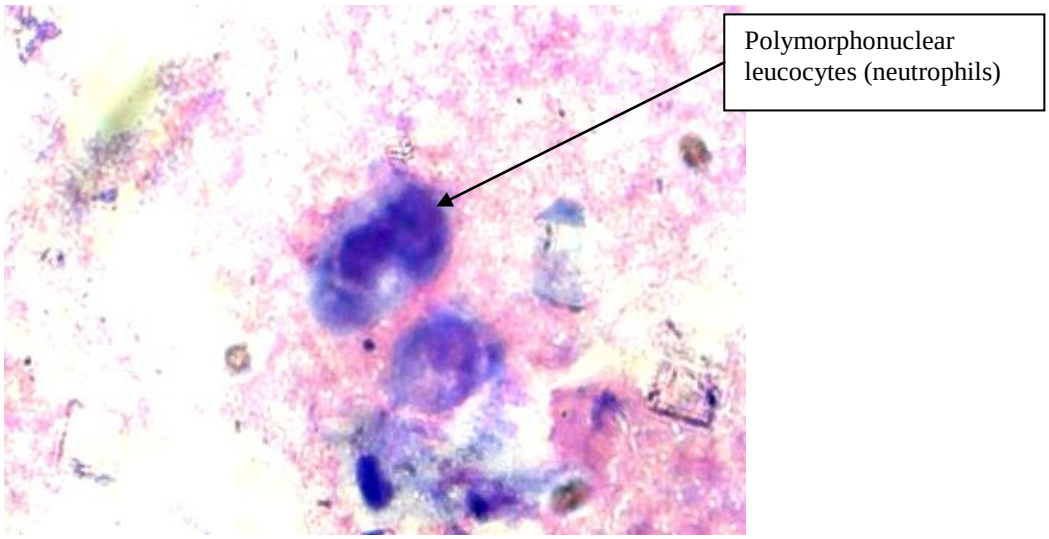


**Figure 6.4 – The median Lymphocyte count and 95 % CI between groups**

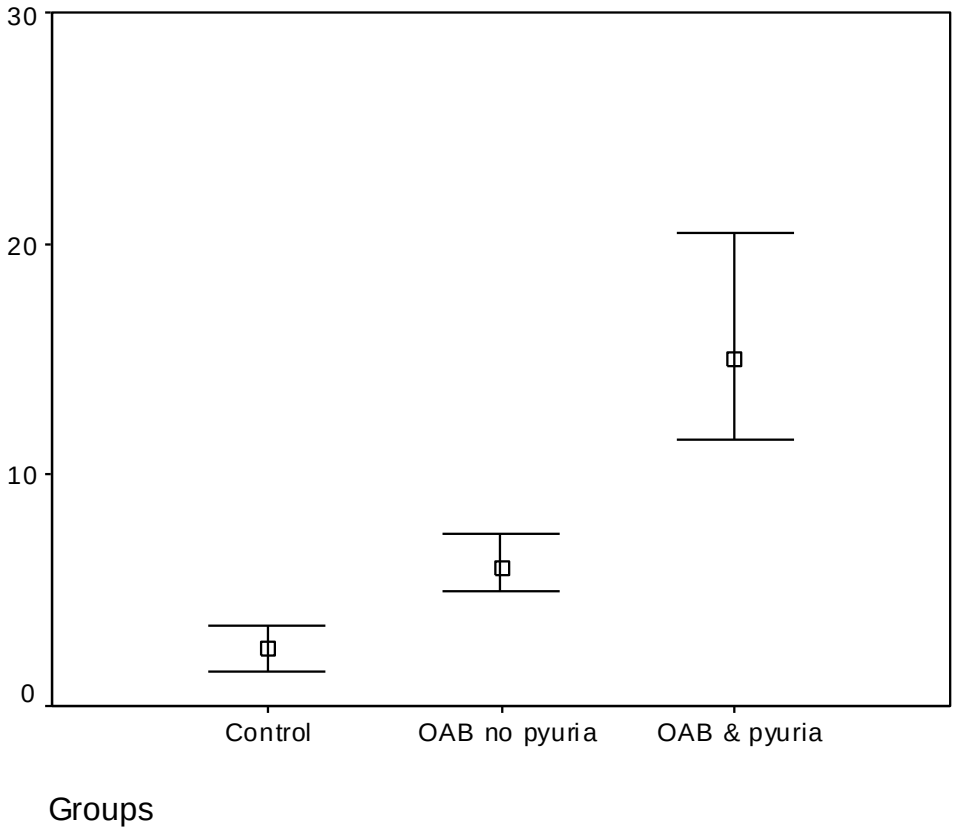
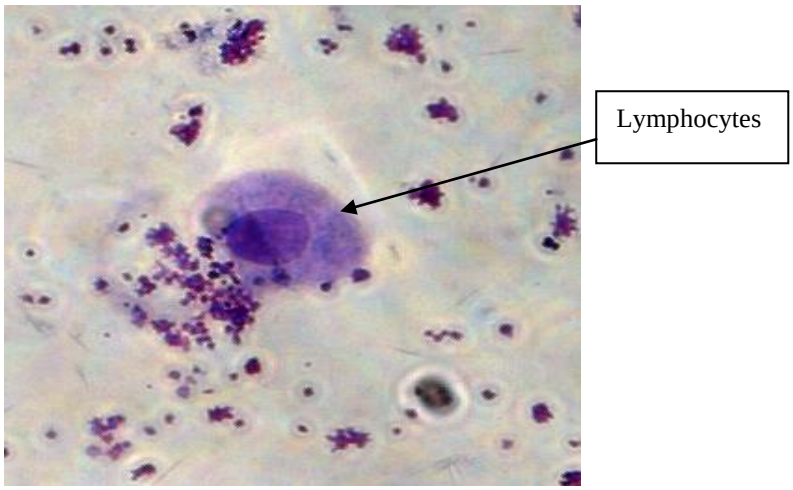



**Figure 6.4a – Picture of Lymphocyte**

**(Olympus light microscope, X20 Magnification, Giemsa stained slide)**



**(Olympus light microscope, X20 Magnification, Giemsa stained slide)**

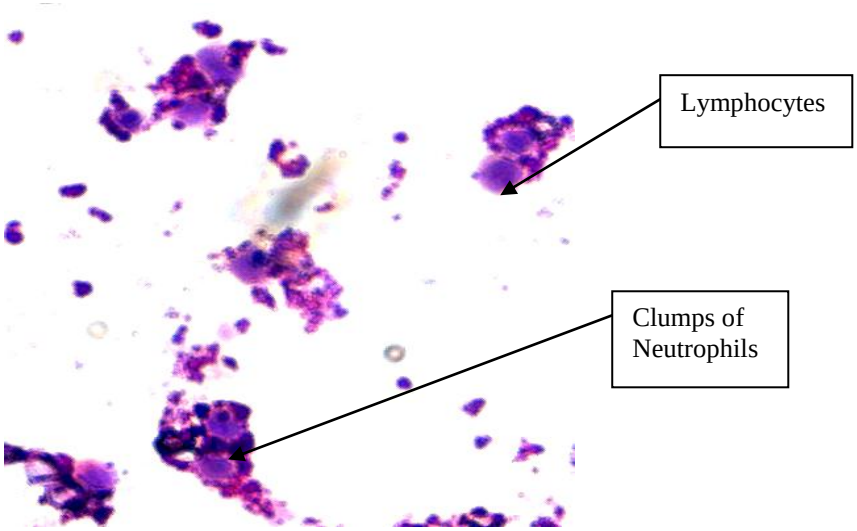
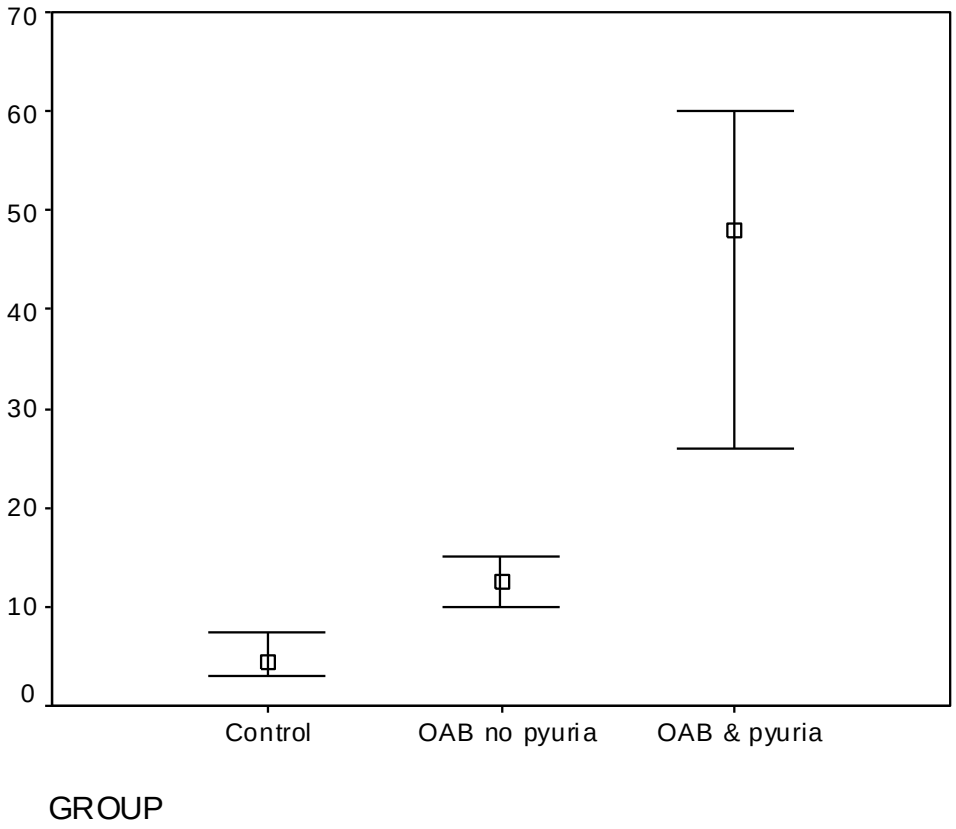



**Figure 6.5 – The median total white cell count and 95% CI between groups**



The results of the Kruskal-Wallis test for these three analyses were as follows:

| | | | |
|---|---|---|---|
| Neutrophils | H=28 | df=2 | p<0.001 |
| Lymphocytes | H=38 | df=2 | p<0.001 |
| Total white cells | H=36 | df=2 | p<0.001 |

An analysis of the ratio of the lymphocyte:neutuophil count was conducted on the 2 OAB symptom groups. It was found that the lymphocyte:neutrophil ratio differed between OAB symptom Groups: 1:1 in OAB symptom zero pyuria but 2:3 in OAB symptom & pyuria ≥1 wbc $\mu l^{-1}$ (H=12, df=1, p=0.001).

A further sub-analysis was carried after removing the patients whose urine culture were positive. A total of 146 patients; 93 OAB with zero pyuria (64 %) and 53 OAB & pyuric ≥1 wbc $\mu l^{-1}$ (36 %) were identified. Their mean age, sex, neutrophil count, lymphocyte count and the Total inflammatory cell count were almost similar to the above statistical calculations, if not were slightly higher. Infact their ratio of lymphocyte:neutrophil were also similar. The OAB & pyuria ≥1 wbc $\mu l^{-1}$ group showed much higher all the counts as compared to OAB with zero pyuria, which in turn showed higher counts than the control group.

The control group data was also similar as only 1 out of original 21 had to be excluded due to positive culture.

It was a conscious decision not to replicate all the statistics, graphical and numerical calculations.

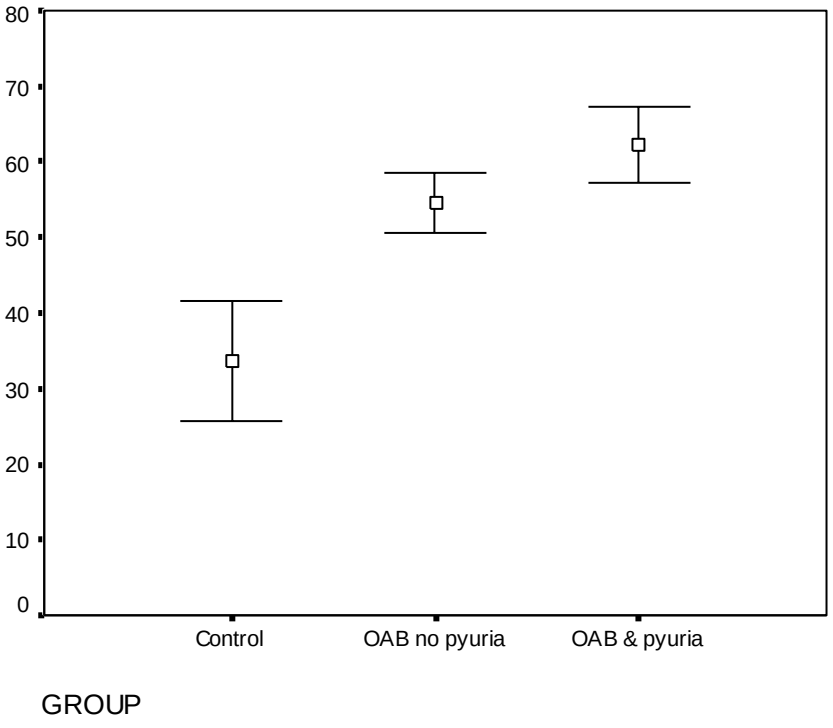
## 6.2 Pap staining Results

The Pap stain experiments were designed to examine the differential urothelial cell counts by enumerating; umbrella cells and deep transitional cells and to seek metaplastic squamous cells. The hypothesis being tested was that chronic urothelial stress and inflammation should be associated with increased urothelial cell turnover and squamous metaplasia. If such changes were not identified then some doubt would be felt about to the proposition that the persistence of pyuria ≥1 wbc $\mu l^{-1}$ in OAB implied significant chronic cystitis. Urine samples were processed through a Shandon Cytospin™ and then stained by the Pap method. Preparations from patients with OAB symptoms were compared with those from normal controls.

The patients with OAB symptoms were further sub-classified on the presence or absence of pyuria ≥1 wbc $\mu l^{-1}$ as OAB symptoms & pyuria or OAB symptoms zero pyuria.

In total 157 patients with OAB symptoms (97 % female) and 29 controls (48 % female) were studied. The controls were younger (mean age 33, 95 % CI 28-38, sd=12) as compared to patients with OAB symptoms (mean age 54, 95 % CI 51-59, sd=19). The age distribution is shown in figure 6.6.

**Figure 6.6 – The mean age with 95 % CI for the experimental groups**




The patients with OAB symptoms & pyuria ≥1 wbc $\mu l^{-1}$ reported an average 24-hour frequency of 10 (sd=5, 95 % CI 8 to 13) and average 24-hour incontinence of 1 (sd=2, 95 % CI 0.5 to 2). Their mean urgency score was 5 (sd=5, 95 % CI 3 to 8), whereas patients with OAB symptoms zero pyuria reported an average 24-hour frequency of 10 (sd=8, 95 % CI 8 to 12) and average 24-hour incontinence of 1 (sd=2, 95 % CI 0 to 1). Their mean urgency score was 4 (sd=3, 95 % CI 3 to 5).

Formal comparisons of the 2 groups, OAB symptoms & pyuria ≥1 wbc $\mu l^{-1}$ and OAB symptoms zero pyuria showed no significant differences in symptoms: 24-hour frequency (df=89, p=0.167, 95 % CI of difference –14 to 2), 24-hour incontinence (df=89, p=0.352, 95 % CI of difference -3 to 1) and urgency score (df=89, p=0.503, 95 % CI of difference 3 to 6).

Some patients could not provide a CSU. A comparison of age and sex between those providing MSU (n=4) against those providing a CSU (n=153), confirmed the predominance of males in the MSU group (100 %) and the fact that the MSU group was younger (mean age = 40, sd= 21) than the CSU group (mean age = 57, sd= 18) although, given the small numbers, this could not be statistically significant. For the same reasons, the symptoms of the MSU group did not differ from those of the CSU group.

CSU group: Average 24-hour frequency = 10 episodes (sd=7); average 24-hour incontinence = 0 episodes (sd=0); mean urgency score = 6 (sd=4)

MSU Group: Average 24-hour frequency = 4 episodes (sd=1); average 24-hour incontinence = 0 episodes, sd=0; mean urgency score = 6 (sd=4)

**Comparisons**

24-hour frequency: p=0.167, 95 % CI of difference -14 to 2

24-hour incontinence p=0.352, 95 % CI of difference -3 to 1

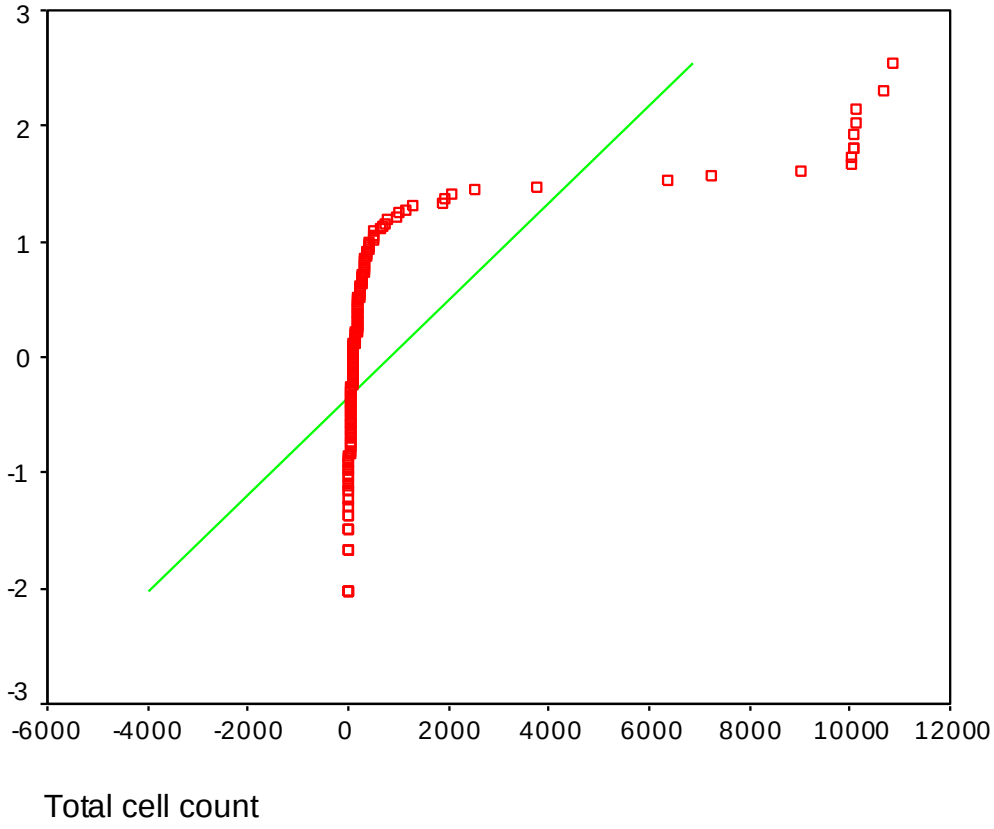
Urgency score p=0.503, 95 % CI of difference -3 to 6

**Analysis of cell counts**

The data describing the total cell counts were first checked for normality using the Quantile-Quantile (QQ) plot available in SPSS. These data were not normally distributed (See figure 6.7). Non-parametric tests were used to analyse these data. Once again there were 3 groups; (1) controls, (2) OAB symptom zero pyuria and (3) OAB symptom & pyuria $\geq$1 wbc $\mu l^{-1}$; so the Kruskal-Wallis test was used and the test statistic H was recorded along with the degrees of freedom (=2) and p value. Similarly, the data distributions were illustrated by plotting the

median count and 95 % CI of the median. These were calculated by using Minitab statistics package.

**Figure 6.7 – Normal QQ plot of the Total cell count**




In the above graph, the X axis shows total urothelial cell count and the green perpendicular line suggest the expected normal lie of the data, and the linearity of points (small red boxes) suggests that the data are not normally distributed.

45 of 157 patients with OAB symptoms (29 %; 95 % CI = 22 % to 36 %) showed pyuria ≥1 wbc $\mu l^{-1}$. 112 of the 157 (71 %; 95 % CI = 64 % to 78 %) patients did not show pyuria. None of the 29 controls had pyuria. The 2 OAB symptom groups were similar in age & sex, as described above.

There were significant differences between controls and OAB symptom groups in the number of transitional and umbrella cells but no significant difference in the number of squamous cells between the groups. However, this finding should be viewed with some caution since 76 % of the control group provided MSU samples and skin cell contamination was a significant risk. This is shown in figures 6.8 to 6.10. Interestingly, there is no difference in transitional cell count between OAB groups, even in presence of pyuria.

**Figure 6.8 – The median umbrella cell count and 95 % CI between groups**

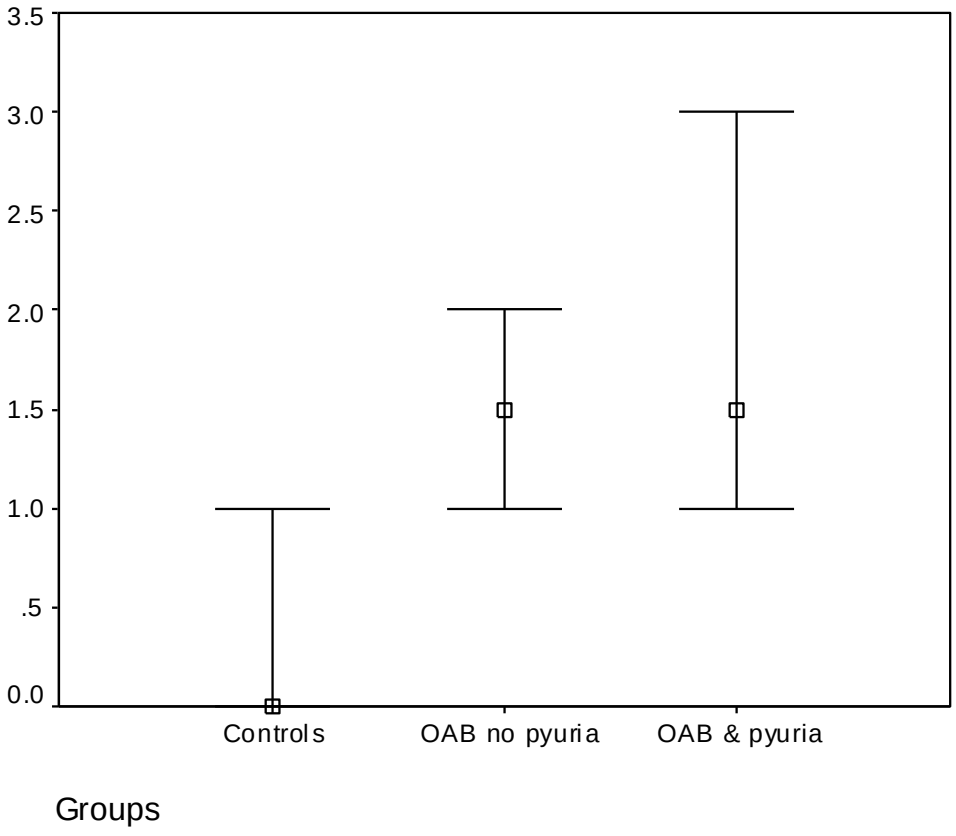


**Figure 6.8a – Picture of Umbrella cell**

**(Olympus light microscope, X20 Magnification, Pap stained slide)**

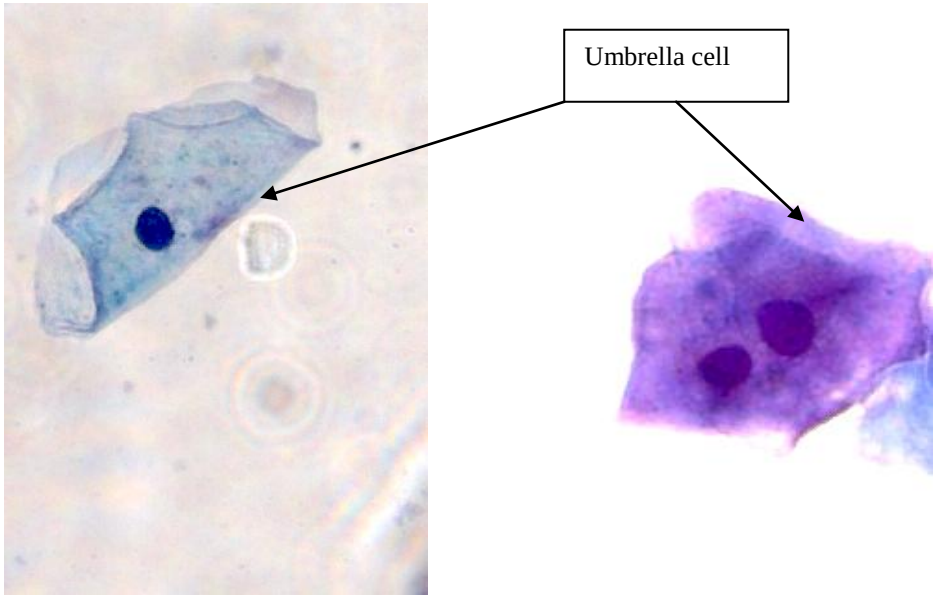



**Figure 6.9 – The median transitional cell count and 95 % CI between groups**

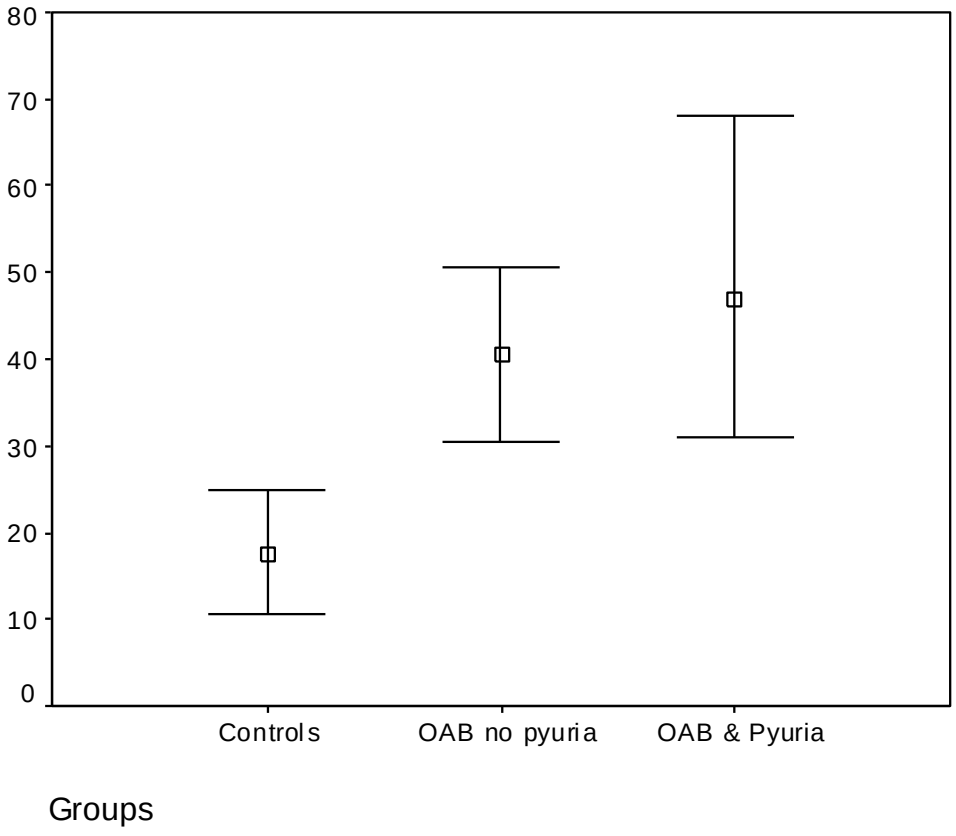


**Figure 6.9a – Picture of Deep Transitional cell**

**(Olympus light microscope, X20 Magnification, Pap stained slide)**

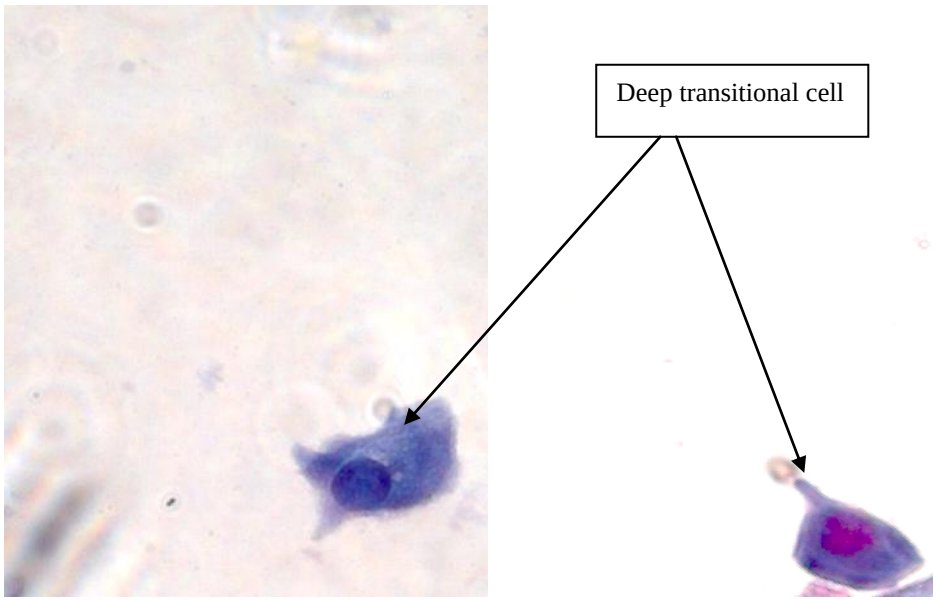



**Figure 6.10 – The median squamous cell count and 95 % CI between groups**

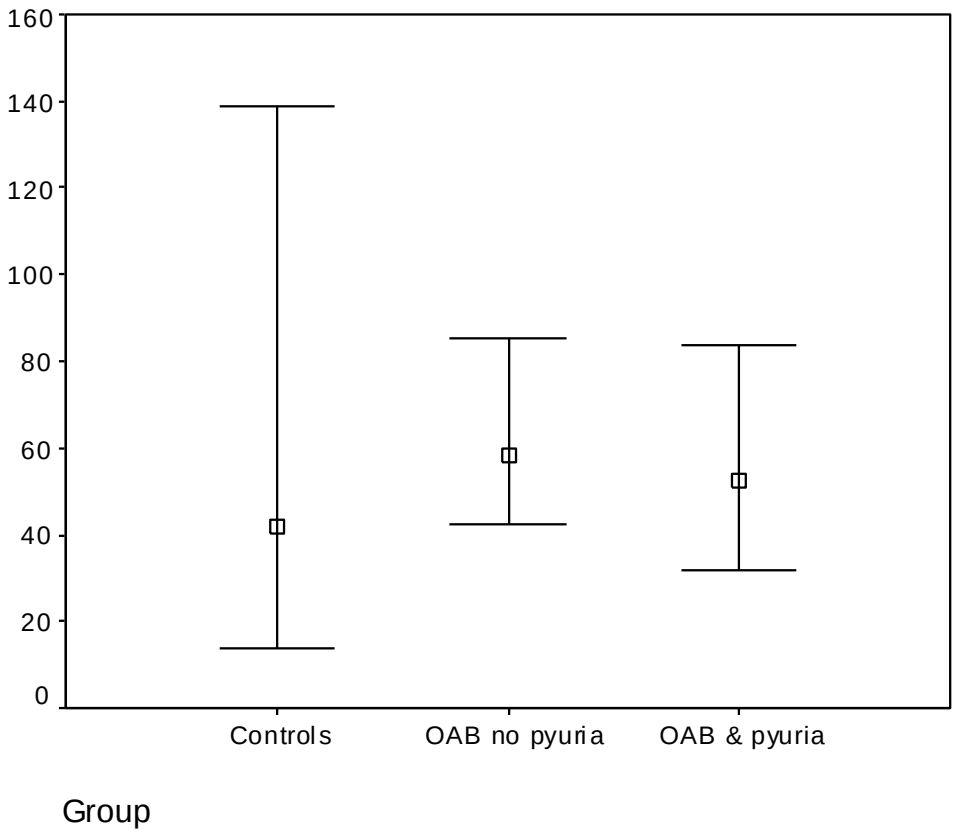
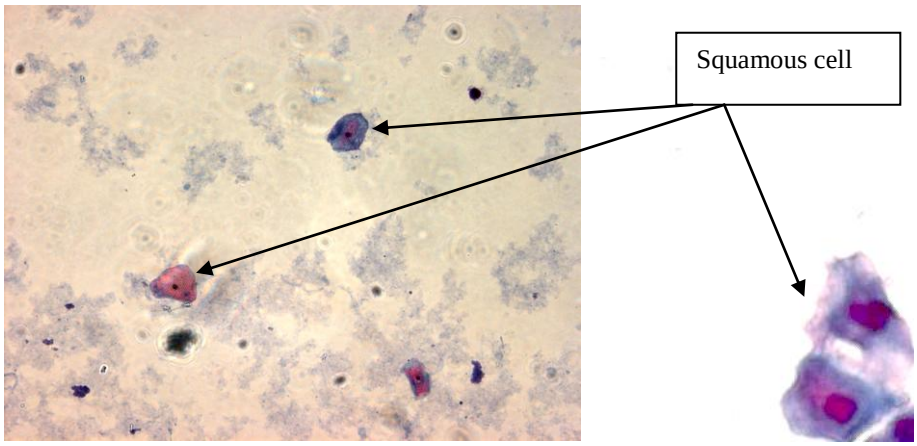


**Figure 6.10a – Picture of Squamous cell**

**(Olympus light microscope, X20 Magnification, Pap stained slide)**




The results of the Kruskal-Wallis test for these three analyses were as follows:

| | | | |
|---|---|---|---|
| Umbrella cell | H=10 | df=2 | p=0.008 |
| Transitional cell | H=11 | df=2 | p=0.004 |
| Squamous cell | H=1 | df=2 | p=0.543 |

## 6.3 Histology Results

The histology experiments were designed to test the hypothesis; that persistent pyuria of ≥1 wbc $\mu l^{-1}$ reflected chronic cystitis in patients with OAB. The test method was direct visualisation of the urothelium under a light microscope. Paraffin sections of urothelium were stained with H&E and scrutinised for the presence of inflammatory changes. In total 79 patients (13 male (16 %) & 66 female (84 %); mean age 56, sd=15) with OAB symptoms, 15 with OAB symptoms & pyuria ≥1 wbc $\mu l^{-1}$, 60 OAB symptoms zero pyuria provided bladder biopsies. 4 patients provided inadequate tissue and could not be included in the analysis. 5 normal asymptomatic controls (all males) provided bladder biopsies. 55 out of 60 OAB zero pyuria patients (92 %; 95 % CI = 82 % to 96 %) and 14 out of 15 OAB & pyuria ≥1 wbc $\mu l^{-1}$ patients (93 %; 95 % CI = 70 % to 99 %) ($X^2$ = 24, df=1, p<0.001) manifest all the features of chronic cystitis viz; urothelial hyperplasia, mixed inflammatory cell infiltrate, oedema/congestion and urothelial denuding.

Only 1 specimen showed the presence of a mast cell. 5 normal controls showed normal urothelium without any features of inflammation. The normal urothelium is 3 to 6 layers in thickness without inflammatory cell infiltrate and without oedema. Figures 6.1 to 6.16 depict this.

**Figure 6.11 – Photomicrograph showing urothelial hyperplasia/metaplasia - Pyuria**

**(Olympus light microscope, X20 Magnification, H & E stained slide)**

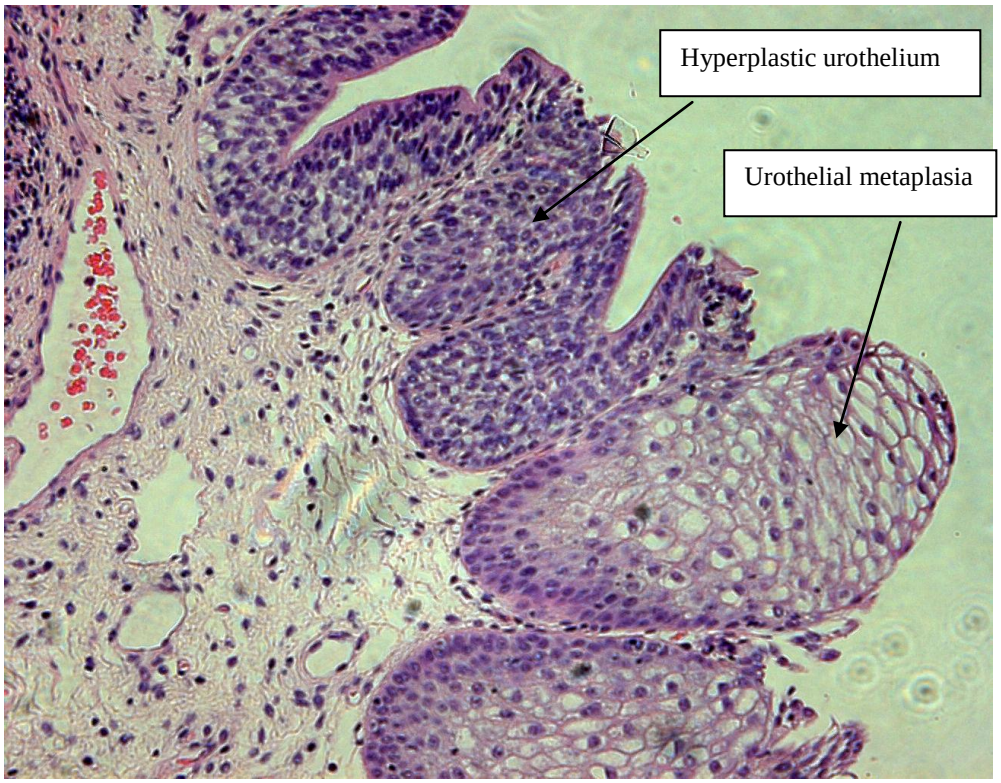



**Figure 6.12 – Photomicrograph showing urothelial hyperplasia/metaplasia – No pyuria**

**(Olympus light microscope, X20 Magnification, H & E stained slide)**

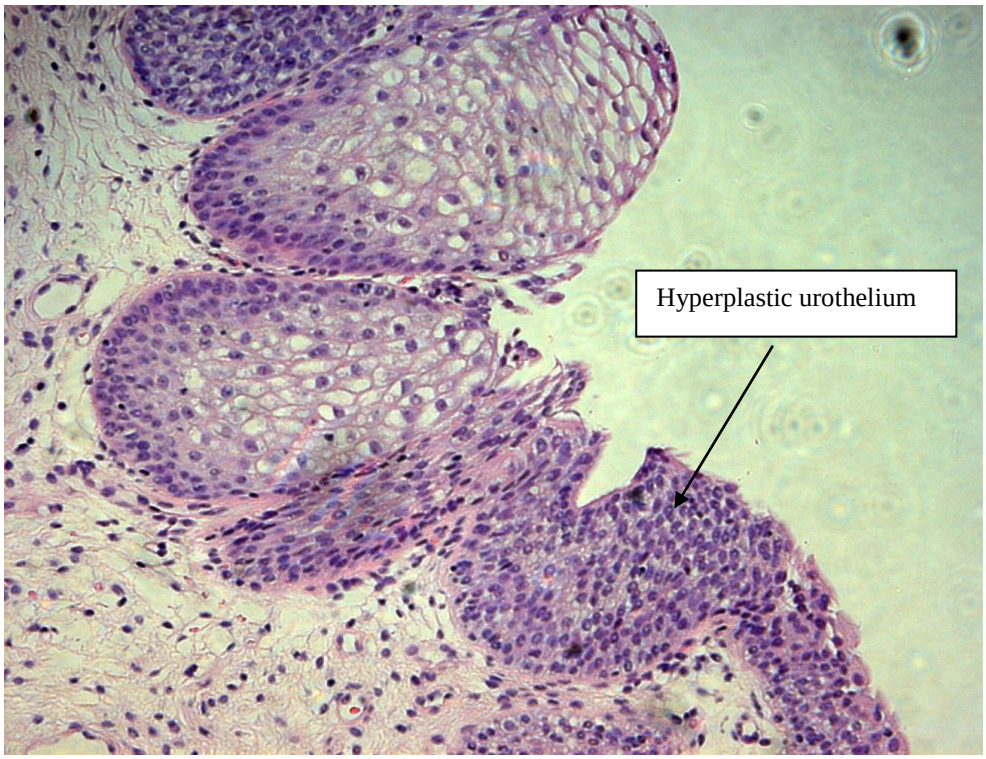


**Figure 6.13 – Photomicrograph showing mixed inflammatory cell infiltrate**

**(Olympus light microscope, X20 Magnification, H & E stained slide)**

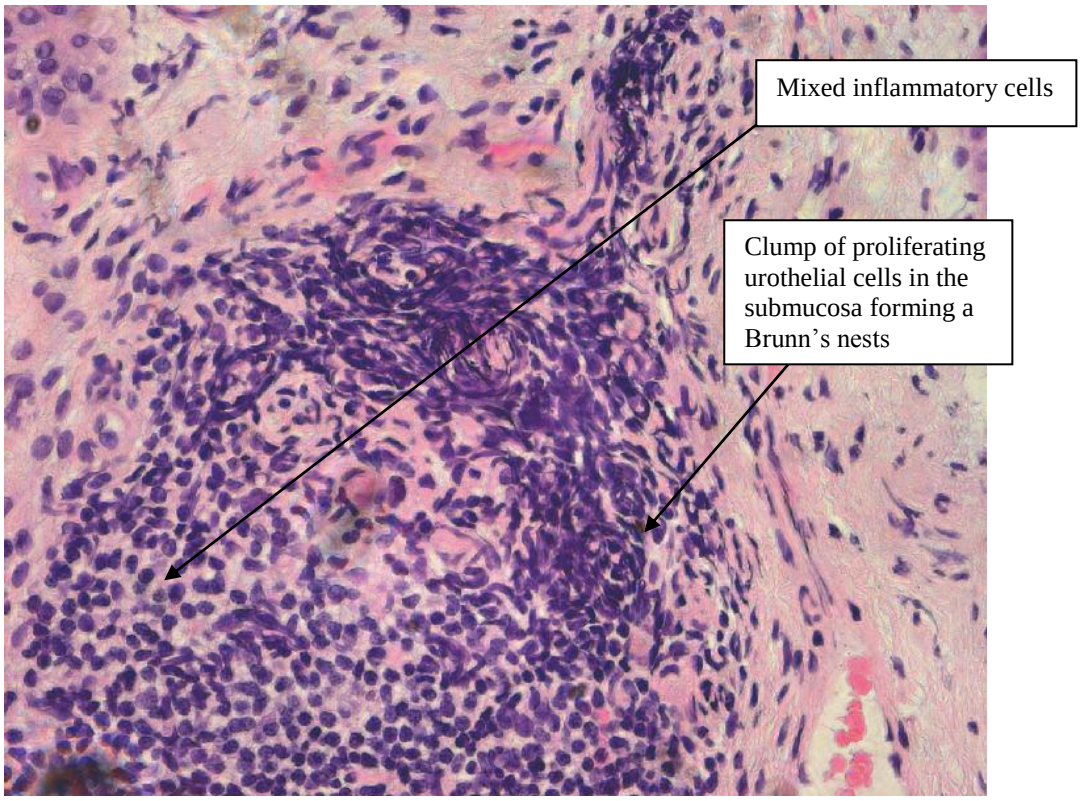



**Figure 6.14 – Photomicrograph showing oedema and congestion**

**(Olympus light microscope, X20 Magnification, H & E stained slide)**

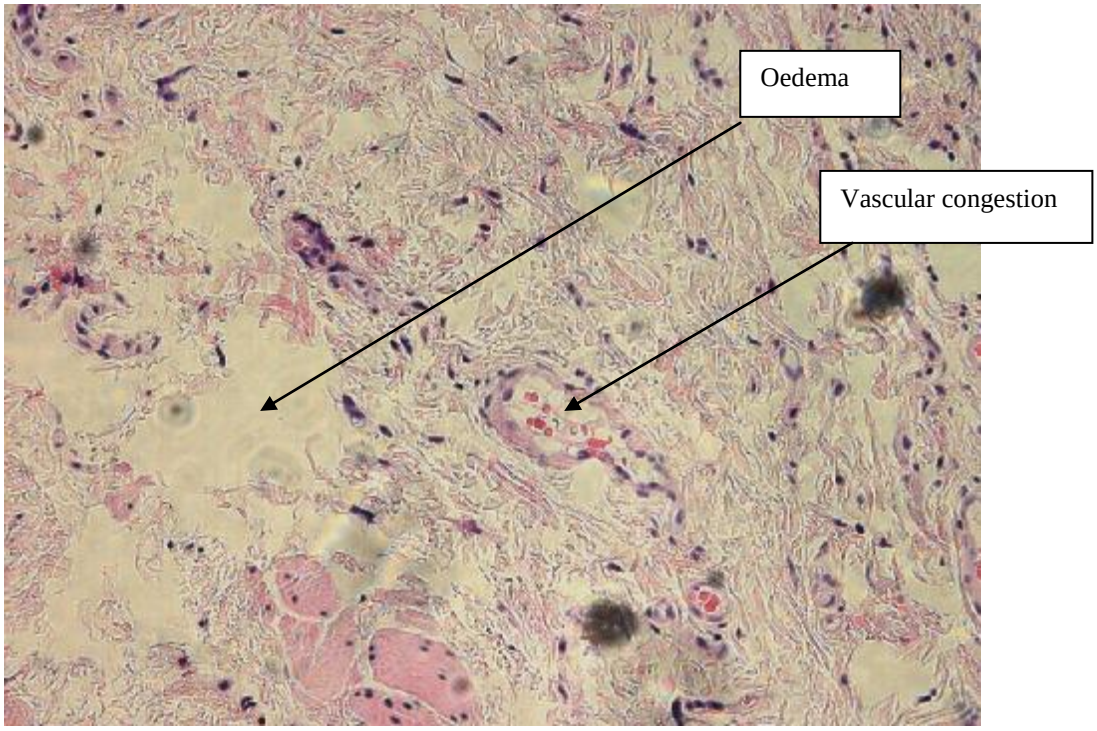


**Figure 6.15 – Photomicrograph showing urothelial denuding**

**(Olympus light microscope , X20 Magnification, H & E stained slide)**

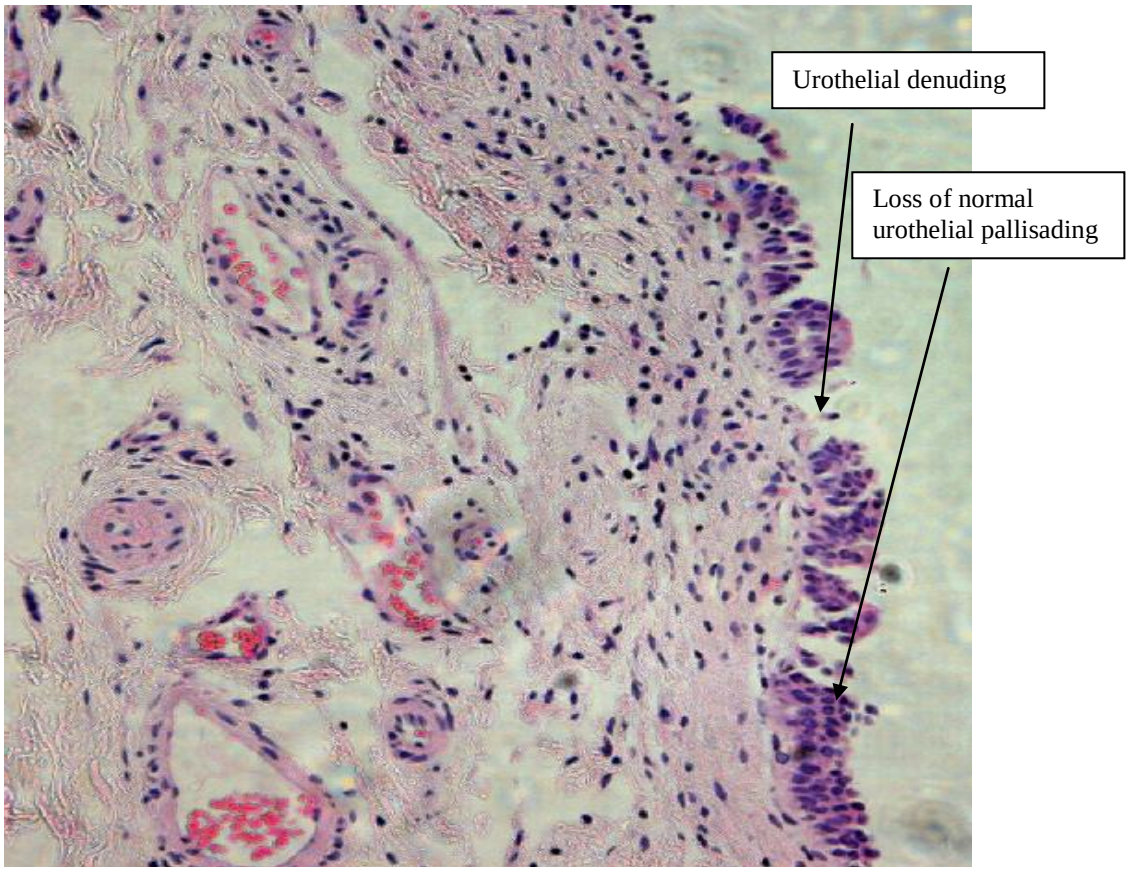
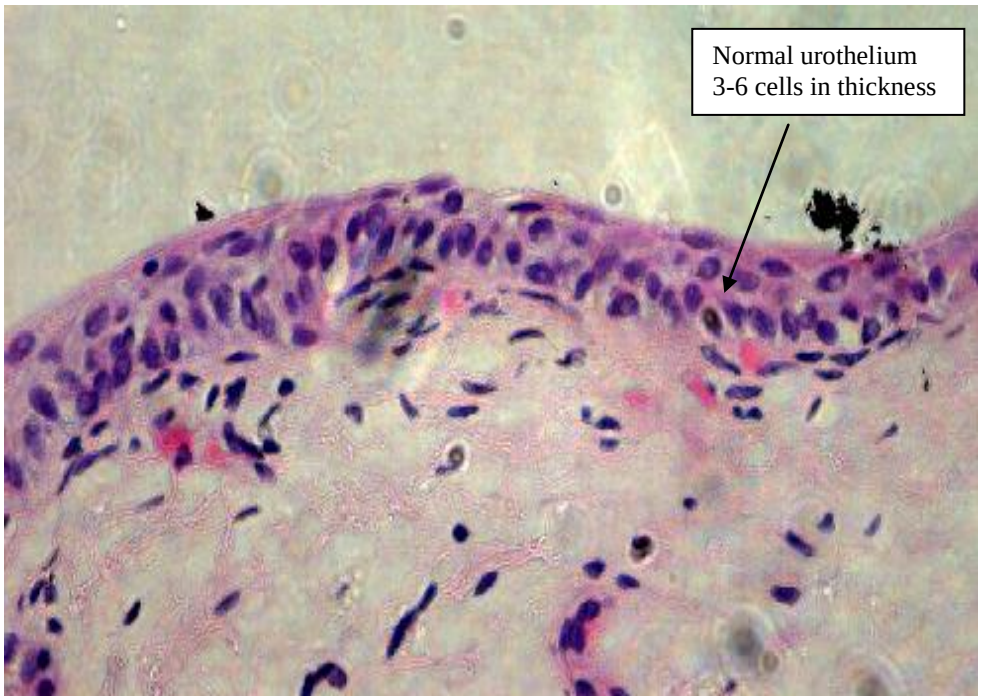



**Figure 6.16 – Photomicrograph showing normal urothelium**

**(Olympus light microscope, X20 Magnification, H & E stained slide)**



## 6.4 Electron Microscopy Results

The aim of this study was to assess the ultrastructure of the urothelium. Once again normal controls were very hard to find and could only achieve specimens from 2 out of 3 biopsies since one produced an inadequate tissue sample. The EM confirmed all the features of chronic cystitis seen on the light microscopy, and already described in the histology results (fig 6.18 - 6.19). In addition, the photomicrographs of the OAB patients, irrespective or pyuria, demonstrated urothelial denuding and a breakdown in the bridging between urothelial cells. There was also a loss of cell cohesion. The same samples showed patches where the cells appeared darker with intact bridging. In areas of shedding the cells appeared lighter. None of these features were exhibited by the controls.

More importantly, it was noted that the basement membrane of the OAB patients thickened in places (fig 6.20). There was a variance in the basement membrane thickness in patients, but less so in the controls. In parts the basement membrane appeared clear and uniform in other parts it was much less uniform with a rather fluffy inferior margin. The measurements were effected by making 100 thickness measurements along the extent of the section at regular intervals (5 microns) between measurements. The mean width was then calculated from these data.

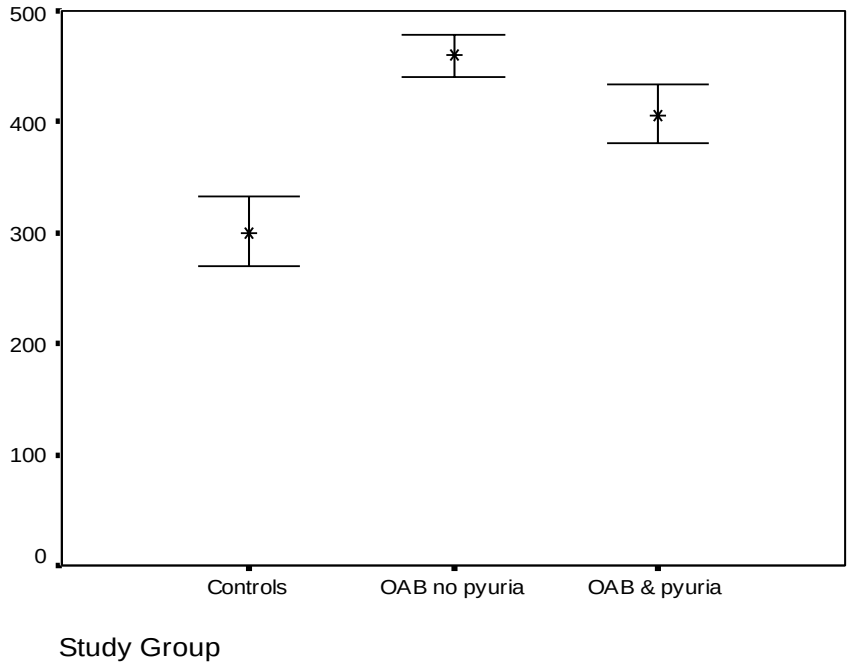
The slides were analysed using Philips CM120 transmission electron microscope (TEM), using a magnification of x8800 and the basement membrane thickness was measured using AMT image capture engine version 600.

22 patients (6 male (27 %) & 16 female (73 %); mean age 50, sd=13) with OAB symptoms (15 OAB symptoms zero pyuria, 7 OAB symptoms & pyuria $\geq$1 wbc $\mu l^{-1}$ and 2 normal

controls provided bladder biopsies for EM analysis. The basement membrane was measured in all of these samples.

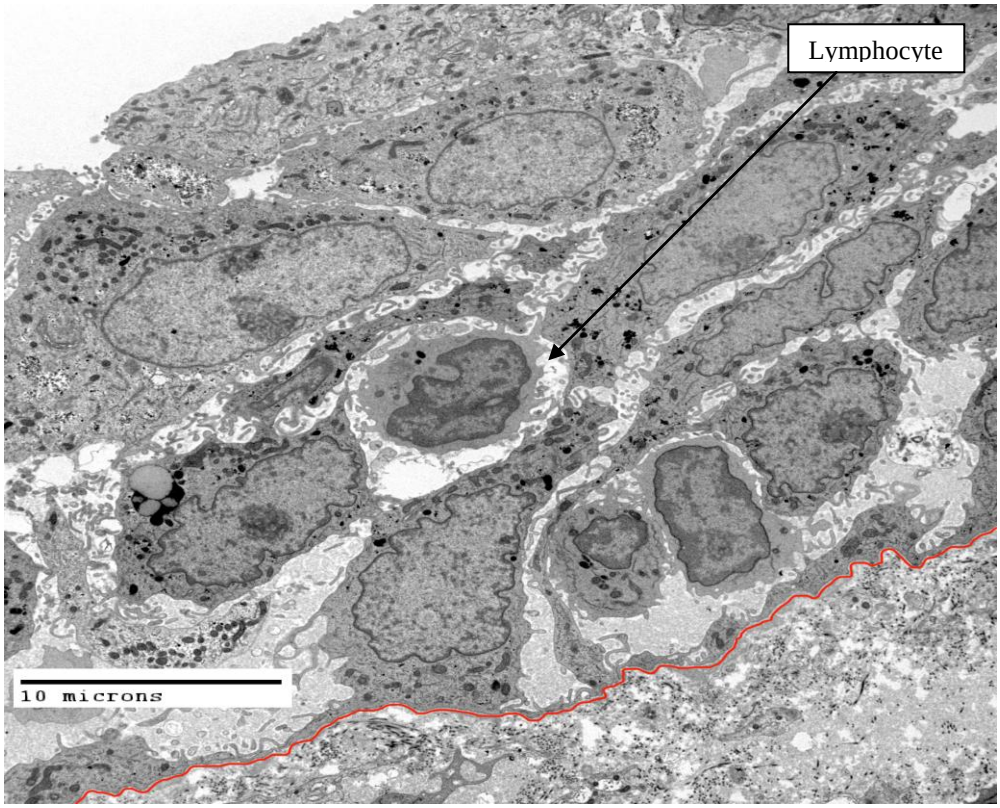
The patients with OAB showed non-uniform thickening of the basement membrane. The mean thickness of the basement membrane, sampled at 100 points along its length, showed a mean thickness of 514 nm, (sd=396) for OAB patients zero pyuria, mean thickness of 459 nm, (sd=354) for OAB patients & pyuria $\geq$1 wbc $\mu l^{-1}$ as compared to controls with a mean thickness of 338 nm, (sd=246) (H=48, df=2, $p<0.001$). The data is illustrated in figures 6.17 with the median and 95 % CI for each group as the comparative statistical analysis was non parametric because of the sample size.

**Figure 6.17 – The median basement membrane width and 95 % CI between groups**



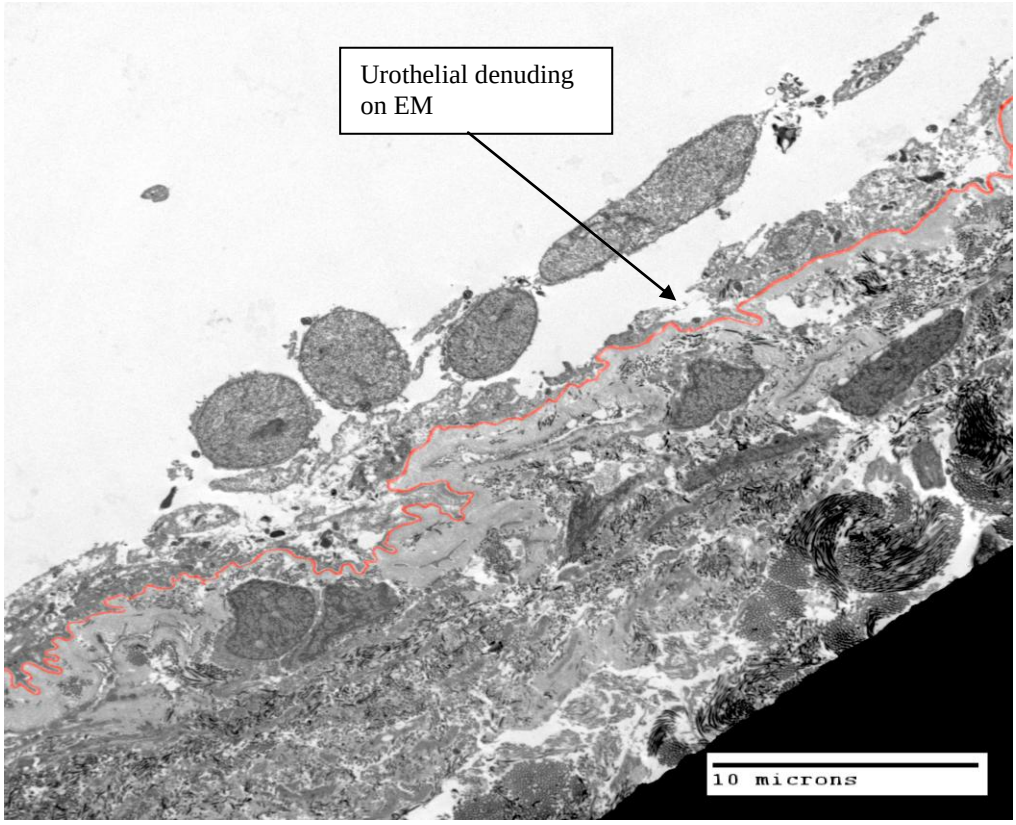
## Figure 6.18 – Electron micrograph showing Lymphocytes

(Philips CM120 transmission electron microscope, X8800 magnification, epoxy resin stained slide)



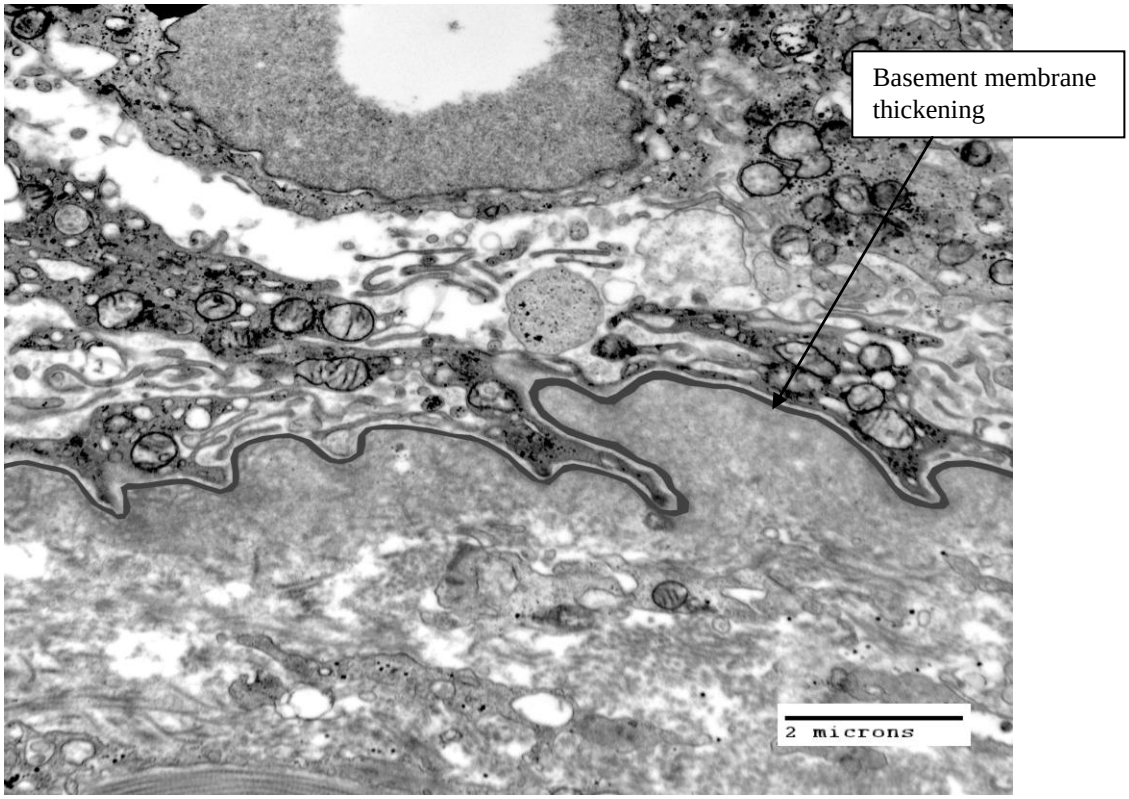
## Figure 6.19 – Electron micrograph showing urothelial denuding

(Philips CM120 transmission electron microscope, X8800 magnification, epoxy resin stained slide)



## Figure 6.20 – Electron micrograph showing basement membrane thickening

(Philips CM120 transmission electron microscope, X8800 magnification, epoxy resin stained slide)



# Chapter 7

# Discussion

# CHAPTER 7

# DISCUSSION

Each result is discussed individually.

## 7.1 Giemsa Stain

A vital step in the sequence leading to the diagnosis of symptoms of overactive bladder (OAB) is the exclusion of urinary tract infection (UTI). A number of recent publications have cast doubt on the veracity of normal midstream urine (MSU) culture and urinary dipstick analysis for accomplishing this step. Since 1968 it has been known that the best surrogate method of diagnosing urine infection, in symptomatic patients, is the identification of pyuria of ≥10 wbc $\mu l^{-1}$, counted by microscopic examination of fresh unspun urine in a haemocytometer. This has been validated in patients with very clear acute urinary tract infection. As mentioned earlier, the threshold of pyuria has not been tested in OAB patients and hence in this study the pyuria was taken as ≥1 wbc $\mu l^{-1}$ because there was no data to justify ≥10 wbc $\mu l^{-1}$ as a threshold in this clinical context and hence it seemed wisest to stick to a clear dichotomy of zero pyuria or some.

Being laborious and requiring certain skills, fresh urine microscopy is not a usual method for screening patients with OAB symptoms. Nevertheless, it has been reported elsewhere that new patients presenting with OAB symptoms manifest pyuria ≥1 wbc $\mu l^{-1}$ 34% of the time (95% CI 32% to 37%) with only 35% of these proving MSU culture positive at $10^5$ colony forming units (cfu) $ml^{-1}$ ( 95% CI 29% to 41%). Asymptomatic controls showed pyuria ≥1 wbc $\mu l^{-1}$ 7% of the time (95% CI 1% to 14%)(178). These data imply that a third of patients with OAB symptoms exhibit microscopic pyuria ≥1 wbc $\mu l^{-1}$ and two thirds of these are routine culture negative.

In focusing on this study; there are several options available for elucidating the nature of this phenomenon. A basic first step would be a morphological description of the inflammatory exudate that is implied by the pus cells in the urine. It would be important to confirm that the pyuria ≥1 wbc $\mu l^{-1}$ does originate from the bladder. Another consideration is the fact that urine output varies and that any inflammatory exudate must be subject to dilution of lesser or greater degree. The morphology of the exudate can be addressed by using stains to achieve differential cell analysis. The bladder origin can be ensured by sampling through a catheter. The effect of dilution can be countered by centrifuging the samples and as all these urine samples were collected in the morning, the effect of variation on urinary dilution was kept to the minimum.

These data used CSU or meticulous MSU so the probability that the urinary cell content originated from the bladder was very high. The data collected for this study reconfirmed the previous identification of pyuria ≥1 wbc $\mu l^{-1}$ in 34% to 48% of OAB patients. None of the controls showed pyuria in this study.

The preparation of spun urinary sediments successfully achieved concentrated samples that nevertheless exhibited some quantitative properties. The Giemsa stain proved successful in permitting a differential analysis of the morphology of white cells in the urinary sediment by discriminating lymphocytes and polymorphonuclear leucocytes. This process has unearthed evidence of a vigorous urothelial inflammatory cell response in patients with OAB symptoms, whether exhibiting pyuria ≥1 wbc $\mu l^{-1}$ or not. Pyuria ≥1 wbc $\mu l^{-1}$ if present was associated with an increased inflammation with numerous acute inflammatory cells with lymphocyte:neutrophil ratio of 2:3 whereas patients with OAB symptoms zero pyuria appears

to be associated with a balanced mixed acute and chronic inflammation manifest by lymphocyte:neutrophil ratio of 1:1.

The use of Giemsa stain for differential counting of urinary sediment has not been previously reported. It is commonly used for differential white cell analysis of blood films. Whilst this was a novel approach it did prove to be most satisfactory in achieving the morphological analysis that was sought.

## 7.2 Histology

In recent years there has been an increased interest in the role of the urothelium in the pathophysiology of overactive bladder (OAB) (182). This has included a focus on afferent nerves and the influence of adenosine triphosphate (ATP) on puringergic receptors. Data from human experiments are limited but increase of stretch activated release of ATP from bladder urothelial cells in patients with interstitial cystitis has been described (183). A human urothelial pathology associated with OAB symptoms has not been rigorously defined. Animal models have shown increased urothelial ATP release in association with inflammation (184) . One mechanism underlying OAB could be a chronic urothelial stress and inflammation resulting in excessive release of ATP. It was reasonable therefore to respond to the inflammatory exudate identified in the urinary sediment by scrutinising the urothelium for evidence of an inflammatory response

Whilst the physiological literature in this arena has been growing there is a striking lack of a histopathological data. This experiment scrutinised urothelial histology in patients with OAB symptoms without pyuria, a sterile urine and who required maximum anticholinergic therapy. Another group of OAB patients with recalcitrant pyuria $\geq$1 wbc $\mu l^{-1}$ were also examined. A very small number of normal controls (total 5 – all males) were achieved because of the difficulty of obtaining biopsies from such persons.

The histological features seen under the light microscope were supportive of the findings in the urinary sediments of patients with OAB whether they had pyuria $\geq$1 wbc $\mu l^{-1}$ or not. The morphological changes implied chronic inflammatory activity that had resulted in urothelial hyperplasia and metaplasia, urothelial denuding, oedema and congestion, and mixed inflammatory cell infiltrate. The normal urothelium is devoid of any of these features

described. The original purpose of this analysis was to test the veracity of the observation of pyuria ≥1 wbc $\mu l^{-1}$ in a significant minority of patients with OAB. The Giemsa staining of spun urinary sediments was to serve a similar purpose. It was a surprise of both experiments to find clear evidence of urothelial inflammation, not only in OAB patients with pyuria ≥1 wbc $\mu l^{-1}$ but those without pyuria. These findings raise the important question as to whether OAB is in fact a manifestation of chronic cystitis that goes undiagnosed by conventional methods. Whether this cystitis is due to urinary tract infection has not been a subject for this thesis. However, it has to be accepted that infection, bacterial or otherwise, must remain a suspect. In the introduction it has been described about the considerable doubts that must affect the routine methods used to exclude urine infection. The data obtained in this project would surely justify a very thorough exploration of urinary infection in this context. The ICS definition of OAB, assumes the exclusion of urinary infection by dipstick or urine culture. The literature indicates that this is included in the definition because excluding infection, amenable to antibiotic treatment, would be a wise precaution. It is not based on a body of evidence that has been gleaned from a careful, critical study of the culpability of bacterial UTI in the generation of the symptoms or pathophysiology of OAB.

## 7.3 Pap Stain

The Pap stain experiment was deployed as a falsification exercise. This implies that the excessive shedding of the urothelial cells doesn't happen in state of chronic stress. We know that epithelia that experience the assault of chronic inflammation will invariably respond by metaplastic changes. The story of pyuria ≥1 wbc $\mu l^{-1}$ in patients with OAB and current evidence pointed to a chronic cystitis as one plausible explanation. If that were to be true it should be expected that evidence of metaplasia should be forthcoming. The urinary spun sediment prepared from a CSU using a Cytospin when stained by the Pap method provides excellent information on the morphology of the urothelial cells. If metaplasia were affecting the urothelium it should be expected to be revealed by an increase in surface cell shedding, increased immature transitional cells and squamous metaplasia. If that was not found it would go a substantial way to refute the hypothesis of chronic cystitis associated with pyuria ≥1 wbc $\mu l^{-1}$ in OAB.

The data demonstrated that OAB with or without pyuria was associated with increased cell shedding with increased numbers of immature transitional cells and superficial umbrella cells. There was no difference in the numbers of squamous cells identified between the groups. There are several explanations for this. The first is the fact that sampling by CSU from normal controls proved very difficult and as a result had to be forced to use MSU, albeit collected with much care. Nevertheless, the probability of contamination by skin cells was high. Regrettably the number of CSU samples from controls was too small to permit a valid comparison. The second explanation could be that the normal controls had squamous metaplasia in their bladders. A macroscopic cystoscopic finding that is commonly described is squamous metaplasia of the trigone which might account for this. This appears as a pearly white carpet at the bladder base which was definitely not seen in the five normal controls who

contributed to this study by cystoscopy and biopsy. The third possibility is that there was some error in the method. This could also be attributed to difference in age, sex and compounding factors but that would act more as a limiting factor that prevented from getting the desired result. This has been mentioned in the limitation of the study.

There was an increased shedding of deep transitional cells in OAB groups when compared with normal controls. This does imply the increased cell turnover of deeper immature cells which would imply chronic urothelial stress. The differences in number of umbrella cells shed were small because these proved to be comparatively rare in the sediments.

The Pap stain result failed to refute the hypothesis of chronic urothelial stress. This experiment lacked some teeth because majority of MSU samples had to be used from the control subjects and that opened the samples to contamination from squamous cells outside the urinary tract. It would be extremely useful for this experiment to be repeated in circumstances where CSU samples could be collected from normal asymptomatic controls. A possible source would be patients undergoing an operative procedure that required bladder catheterisation whilst urinary symptoms did not feature in the clinical story. It may also be wiser to loosen the recruitment criteria for controls as this may have been misguided in setting very stringent criteria for this study.

## 7.4 Electron Microscopy

Electron microscopy is a method for assessing the ultrastructure of the urothelium. At the outset it was not sure what this method was likely to show and had to be approached without preconceptions. The key finding was to be able to identify the inflammatory changes that had been seen on light microscopy. The denuding of the urothelium was well shown and the changes implied that there must be some breakdown in the adhesion molecules that would normally bind the urothelial cells to the basement membrane. In all cases where denudation was apparent the fracture occurred at the basement membrane. An interesting feature was the thickening of the basement membrane. At first site it just seemed to be thicker than comparative images from EM texts and the BM of other epithelia. Proper control comparison was essential but hampered by the paucity of such samples. However, in situations where the membrane was irregular in controls and patients the dimensions of the OAB basement membrane were sufficiently increased to show a significant difference from a very small control sample (male only). It would be wrong to infer too much from this observation and the best option would be to repeat the experiment with some comparative controls, perhaps selected on less rigorous criteria, and mixed gender. The finding of basement membrane thickening is well documented in patients with Interstitial cystitis (IC) (185).

Haferkamp A et al. and Resnick NM et al. have done extensive electron microscopic studies on detrusor muscle in neurogenic bladder dysfunction but it was not focused on urothelium and basement membrane and certainly did not involve patients with OAB symptoms (186-188).

The literature search failed to locate any publications reporting similar studies of the basement membrane in OAB.

## 7.5 Limitations of the Study

There are a number of limitations that must be acknowledged for this study.

The sample selection could not be randomised, had it been done the study would have been unable to achieve the sample sizes that were needed.

There was some overlap between the patients who provided samples for Giemsa and for Pap. Had time and patient numbers permitted it would have been attractive to have studied different samples from the representative groups. In all circumstances it was possible to maintain a blind over the identity and clinical situation as it affected the patients or controls.

The greatest proportion of OAB patients donating bladder biopsies were on anticholinergic therapy and had not responded, to the extent that they required Botox injections. This means that they are a selected group but their symptoms and clinical state re extremely well characterised. It would be a much harder option to seek to effect cystoscopies and biopsies from patients with less troublesome OAB. Let's hope that the development of the methods of scrutinising urinary spun sediments may make this unnecessary.

It was not possible to match the OAB patients with normal controls in terms of age, sex and menopausal status. The controls were mostly men, younger and in the main they provided MSU's. As the screening criteria were very stringent, particularly the requirement for there to be no nocturia, it was very difficult to find matched asymptomatic normal controls. On a brighter note, the controls were extremely normal persons. It was an extremely hard work to recruit asymptomatic normal controls willing to provide CSU samples but that was also very difficult task which was managed in a very few circumstances in the operating theatres.

The greatest difficulty of all was to recruit asymptomatic normal controls for bladder biopsy. This group was recruited from persons undergoing routine cystoscopy so finding individuals without any urinary tract symptoms was a challenge. Such patients were few and far between and all of them were young, unsurprisingly.

For methods involving urine Cytospin™ preparations, staining and reading slides, It would have been useful to have been able to use a standardised counting method but it transpired that such a method had not previously been scribed. At first, the first 100 cells were counted and calculated the relative expression of different cell types. It then became evident that the Cytospin deposit provided a semi-quantitative field and in order to take advantage of this, re-counting of the whole cell set in each preparation was effected.

It would have been preferred to have prepared own tissue samples for histology and electron microscopy. However, It had to accept that it was dealing with material from patients and that the findings could have clinical implications. The researcher did not have the practiced skills of NHS laboratory technicians and the hosting departments felt that it was imperative that such slides be prepared by certified personnel. Nevertheless the researcher certainly learned the methods and could achieve the requisite preparations although at this time they would be of an inferior quality to those produced by experienced laboratory staff.

When it came to analysing the prepared slides for Pap and Giemsa stain, lot of difficulty was encountered before managing to achieve the desired results. It was imperative to be able to identify the cells correctly because it was needed to achieve the quantitative analysis. It all started by comparing the cells under the microscope to follow photomicrographs in a reference Atlas of cytology but this proved far more difficult than at face value as the Atlas

photomicrographs were evidently ideal examples. Therefore a formal training was arranged during five sessions under the supervision of a consultant histopathologist at UCL hospital. She eventually assessed the ability and passed the researcher having sufficiently skilled to assess the samples necessary, given the scope of this project. As audit, cross-checking with her a number of findings during the course of the project were carried out. It should be noted that there is certainly a skill component to this type of work and that is not easy to quantify in arid scientific terms.

There were similarly difficulties during analysing and reading the histology slides. It was found that picking out different features with confidence was indeed difficult. The atlas and textbooks are less helpful than would be expected because of the excellent quality of the illustrations that are shown. The supervisor went through the basics of reading histology slides so as to build the initial confidence. Thereafter, eight sessions were arranged with the consultant histopathologist at the UCL hospital who once again trained and assessed the capacity of the researcher to handle the demands of this project. She cross-checked all of the histopathology slides that were interpreted by the researcher as did a second histopathologist at the Whittington Hospital.

Finally, learning to interpret EM micrographs was similarly challenging but the provision of the excellent support from the electron microscopist at the Royal Free Hospital came into good stead. The basement membrane was measured together by the researcher and the electron microscopist and despite the vast experience of the electron microscopist it was difficult at times to assess and measure the basement membrane thickness. The potential for errors was reduced by the large number of measurements taken.

# Chapter 8

# Conclusion

# CHAPTER 8

# CONCLUSION

This study shows evidence of chronic cystitis and urothelial hyperplasia associated with OAB irrespective of pyuria. This has been scrutinised by three different methods. The very fact that the urine was processed and stained with Giemsa stain and to get the tissue biopsy to undergo the H & E stained histopathological analysis with further unearthing of the ultrastructural changes with the help of epoxy resin stained electron microscopy, and all to show the inflammatory changes it certainly cements the fact that the inflammation/infection lies at the heart of overactive bladder. The routine screening for the exclusion of UTI in patients presenting with symptoms of OAB need to be revisited.

Further studies with large cohort of patients, with matched patients and control, need to be carried out.

# Chapter 9
# Future Work

# CHAPTER 9

# FUTURE WORK

There has been some very excising proposal and ongoing projects being carried out in relation to further the work on inflammation, with regards to OAB. The Interleukin 6 assay on urine sample and comparing it with different markers of inflammation has been going on. The use of Sternheimer-malbin stain and comparing it with various stains to enhance the pickup rate of the leucocytes, along with comparing the decay of leucocytes/pyuria in urine sample subjected to temperature change, delay in processing the urine in laboratory and use of different preservatives, is an ongoing work. There is further work in relation to finding the intracellular bacterial colonies from urine culture and bladder biopsy is being carried out. Further work related to enhance ATP secretion and use of Scanning Electron Microscopy on the Urothelial cells is going on.

The current project on urinary Lactoferrin holds good promise for the future as a surrogate marker of infection.

The role of long term antibiotics and whether or not one shot of intravenous antibiotics in patients with symptoms of OAB, presenting with pyuria but urine culture negative, is to be presented to the Ethical committee to set it up as a pilot project.

In essence, there has been lot of exciting prospect and projects going on to enhance our understanding with regards to inflammation especially in patients with symptoms of OAB.

# Chapter 10
# Study Documents

# CHAPTER 10

# STUDY DOCUMENTS

## 10.1 Appendix 1

## Flexible cystoscopy

• Here, we explain some of the aims, benefits, risks and alternatives (including no treatment) to this procedure. You will also receive a separate clinic information sheet. We want you to be informed about your choices to help you to be fully involved in making any decisions.

• Please ask about anything you do not fully understand or wish to have explained in more detail.

• If you would like this information in another format or language or would like help completing the form, please ask a member of our staff.

**Intended benefits of the procedure**

To find the cause of the bladder symptoms or to check that the bladder is clear of disease.

**Who will perform my procedure?**

A doctor trained in urology, surgery of the bladder, will perform the procedure.

**The procedure:**

A doctor will insert an instrument called a cystoscope (very fine and soft telescopic tube) into your bladder via the water pipe (urethra). Attached to the instrument are a telescopic lens, a light source and some sterile water to fill the bladder so that the lining can be inspected. A local anaesthetic gel is used to numb and lubricate the urethra to make the passage into the bladder as comfortable as possible.

Some patients may experience slight discomfort during the procedure but the majority do not. If you do feel uncomfortable at any time you should inform the doctor performing the examination immediately.

Once the instrument is in place, the examination will take only a few minutes to complete.

Once the inspection is completed, the instrument will be removed, and you will be informed of the findings and the need for any further management.

A nurse will remain with you whilst the treatment is taking place and will explain anything you do not understand.

**Alternative procedures that are available**

The alternative is to have a cystoscopy under a general anaesthetic.

**Serious or frequently occurring risks**

There are potential complications with any procedure. Although these are rare, it is important that you are aware of them and have the opportunity to discuss them with your doctor.

**Common**

Mild burning or bleeding on passing urine for a short period after the Operation.

**Occasional**

Infection of the bladder requiring antibiotics. We shall be giving you a course of antibiotic treatment lasting one day at the time of the procedure

**Rare**

Delayed bleeding requiring removal of clots or further surgery.

Injury to the urethra causing delayed scar formation.

Difficulties in passing urine after the procedure requiring temporary insertion of a catheter.

## 10.2 Appendix 2

## Bladder biopsy via cystoscopy

A study of bladder tissue sample and patient symptoms

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with friends, relatives and your GP if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

Consumers for Ethics in Research (CERES) publish a leaflet entitled 'Medical Research and You'. This leaflet gives you more information about medical research and looks at some questions you may want to ask. A copy may be obtained from CERES, PO Box 1365, London N16 OBW. CERES also has an excellent Website, which contains much advice and information about research (http://www.ceres.org.uk/). You may also obtain information from the hospital Patient Advice and Liaison Service (PALS), Whittington Hospital, Tel 020 7288 5956 or 020 7288 5957

Thank you for taking the time to read this information and for considering helping us with our research project.

**What is the purpose of the project?**

Many people suffer with Overactive Bladder symptoms and urinary incontinence (leaking from the bladder). It is a very unpleasant problem that affects people's lives at home, at work and their general well-being. The aim of this study is to examine urine and samples of the

bladder tissue to better understand how the bladder works and relate these findings to patient symptoms**.**

**Why have I been chosen?**

You have been chosen because you have the symptoms of an overactive bladder. Alternatively you are without lower urinary tract symptoms but are scheduled to have a routine cystoscopy. The cystoscopy procedure is described in the attached leaflet. This test provides an opportunity to take a sample (biopsy) from your bladder. We should like to do this so as to use the sample for research purposes.

**Do I have to take part?**

It is up to you if you decide whether or not to take part. If you do decide to take part you will be given a copy of this information sheet to keep. You will be asked to sign a consent form a copy of which will be given to you. If you decide to take part you are still free to withdraw at any time and without giving a reason. This will not affect the standard of care you receive.

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

If you decide to take part in the project the research clinician will arrange to see you. You will be asked some questions about your bladder and how well it works for you. Your answers will be recorded on a form stored on a computer. This will take about 30-45 minutes. The computer record is closely guarded by the NHS security system so there is no unauthorised access to you record. Blood samples may or may not be taken as part of your normal clinical care. We should like to record the results of these in your research record. A random code will decide whether you will undergo the flexible cystoscopy procedure or not, despite your consent to be a part of the study.

When you have the cystoscopy the doctor will take a very small sample of bladder tissue (about 1-2mm) in addition to any that may be needed as part of your normal clinical care.

**What will be done with the biopsy tissue that is taken for this study?**

The tissue sample taken at biopsy from the bladder will be studied in detail with various staining methods and under a special microscope. The use of your specimen, where it goes, and the results of any experiments that are conducted on it, will be kept on the record and could be made available to you should you so wish. In effect there will be the full story of your specimen.

**What are the alternatives to participating in the study?**

It is entirely your decision if you wish to take part in the study and it will not affect your future care if you do not wish to do so. If you do not wish to take part you will have your assessment in the usual way. If a cystoscopy would be a normal part of your clinical care, this will happen in the usual way.

**What are the possible disadvantages and risks of taking part?**

With each bladder biopsy there is a small amount of bleeding, but this will usually stop after a day or so. This will be manifest as a pink colour to your urine, which rapidly clears.

There is a risk of cystitis but we shall cover you for this by administering a single dose of an antibiotic at the time of the procedure.

**What are the possible benefits of taking part?**

The sample of bladder tissue taken for this research will be considered as a ‘gift’ to the university and after the research has been completed the sample will be destroyed. There are

no benefits directly to you for taking part. But your participation will help us to have a better understanding of how the bladder works and may lead to better treatments being available to people with bladder problems in the future.

**What happens if something goes wrong?**

If taking part in this research project harms you, there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for legal action but you may have to pay for it. Regardless of this, if you wish to complain about any aspect of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms will be available to you.

**What if I want to withdraw from the study?**

You are free to withdraw from the study at any time before and after signing the consent form without needing to give any explanations. If you wish to withdraw after the bladder sample has been taken then you may contact us and we will not use the sample for any experiments.

**Will my taking part in this study be kept confidential?**

All information, which is collected about you, during the course of the research, will be kept strictly confidential. Any information about you which leaves the hospital / surgery will have your name and address removed so that you cannot be recognised from it.

Your GP will be notified of your involvement in the study unless you do not wish us to do so.

**What will happen to the results of the research?**

The results of the experiments made on the samples of bladder tissue will be published in scientific journals so that other researchers working on improving treatments for people with

troublesome bladder symptoms and incontinence will benefit from this knowledge. You will not be identified of any publications.

**Who is organising and funding the research?**

This study is organised by the Department of Medicine at the Whittington Hospital.

**Who has reviewed the study?**

The East London Research Ethics Committee has reviewed this study.

**Contact for further information**

If you have any questions please don't hesitate to contact us.

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## 10.3 Appendix 3

## A study of bladder tissue and patient symptoms

Consent Form

Confidential

Investigator's name: _____

**To be completed by the participant**

| | | |
|---|---|---|
| Have you read the information sheet about this study? | Yes | No |
| Have you had an opportunity to ask questions and discuss this study? | Yes | No |
| Have you received satisfactory answers to all your questions? | Yes | No |
| Have you received satisfactory answers to all your questions? | Yes | No |
| Have you received enough information about this study? | Yes | No |
| Which staff member did you speak to about this study? | Yes | No |
| Do you understand that you are free to withdraw from this study, at any time, without giving a reason for withdrawing and without it affecting your future medical care? | Yes | No |
| Do you understand that you will not benefit financially if the research leads to the development of a new treatment or medical test | Yes | No |
| Do you understand that you will not benefit financially if the research leads to the development of a new treatment or medical test? | Yes | No |
| May we contact your GP to inform him/her that you wish to | Yes | No |

| | |
|---|---|
| take part in this study? | |
| Do you agree to enter this study? | Yes No |

_____ _____ _____

**Signature (Participant)** Print name Date

_____ _____ _____

**Investigator's signature** Print name Date

## 10.4 Appendix 4

## Bladder symptom questionnaire

**Date of birth…….** **Sex** **F / M……**

**Study number**……

**Date**…….

| | None | Some | Much | |
|---|---|---|---|---|
| | ☺ | 😐 | ☹ | |
| Do you experience urgency? That is having to hurry in order to pass urine? →→→→→→→→→ | ☺ | 😐 | ☹ | 1 |
| Do you experience urge incontinence? That is hurrying to pass urine and not making it in time? →→→→→→→→→ | ☺ | 😐 | ☹ | 2 |
| Does cold weather make your bladder symptoms worse? →→→→→→→→→ | ☺ | 😐 | ☹ | 3 |
| Do you find that running water from a tap causes urinary urgency or incontinence? →→→→→→→→→ | ☺ | 😐 | ☹ | 4 |

Do you find that putting a key in the front door when returning home causes urinary urgency or incontinence? →→→→→→→→→ ☺ 😐 ☹ 5

Do you find that on getting up from bed in the morning you experience urgency or urge incontinence? →→→→→→→→→ ☺ 😐 ☹ 6

Is there any pain associated with the urgency? →→→→→→→→→ ☺ 😐 ☹ 7

Do you ever leak urine on coughing, sneezing, running, laughing, lifting? →→→→→→→→→ ☺ 😐 ☹ 8

# Chapter 11

# References

# CHAPTER 11

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