Altered Urothelial ATP Signaling in Major Subset of Human Overactive Bladder Patients with Pyuria

Alberto Contreras-Sanz[1*], Louise Krska[1*], Aswini A. Balachandran[2], Natasha L. Curtiss[2], Rajvinder Khasriya[3], Stephen Kelley[1], Matthew Strutt[4], Hardyal S. Gill[5], Kevin M. Taylor[5], Kylie J. Mansfield[6], Changhao Wu[7], Claire M. Peppiatt-Wildman[1], James Malone-Lee[3], Jonathan Duckett[2], Scott S. Wildman[1]

[1]Medway School of Pharmacy, Universities of Kent and Greenwich, Kent, UK; [2]Obstetrics and Urogynaecology, Medway Maritime Hospital, Kent, UK; [3]Division of Medicine, UCL Medical School, London, UK; [4]Department of Microbiology, East Kent Hospitals University Foundation Trust, Kent, UK; [5]Department of Pharmaceutics, UCL School of Pharmacy, London, UK; [6]School of Medicine, University of Wollongong, NSW, Australia; [7]Faculty of Health and Medical Sciences, University of Surrey, Surrey, UK; *Joint first authors.

Author for correspondence: Dr Scott S. Wildman PhD, Urinary System Physiology Unit, Universities of Greenwich and Kent at Medway, Anson Building, Central Avenue,
Chatham Maritime, Chatham, Kent, ME4 4TB; Tel: +44 (0)1634 202944; Fax: +44 (0)1634 883927; E-mail: s.s.wildman@kent.ac.uk

Abstract (200 words):

Overactive Bladder (OAB) is an idiopathic condition, characterized by urgency, urinary frequency and urgency incontinence, in the absence of routinely traceable urinary infection. We have described microscopic pyuria (≥10 wbc μl^{-1}) in patients suffering from the worst symptoms. It is established that inflammation is associated with increased ATP release from epithelial cells, and extracellular ATP originating from the urothelium following increased hydrostatic pressure, is a mediator of bladder sensation. Here, using bladder-biopsy samples, we have investigated urothelial ATP signaling in OAB patients with microscopic pyuria.

Basal, but not stretch-evoked, release of ATP was significantly greater from urothelium of OAB patients with pyuria than from non-OAB patients or OAB patients without pyuria (<10 wbc μl^{-1}). Basal ATP release from urothelium of OAB patients with pyuria was inhibited by the P2 receptor antagonist suramin and abolished by the hemichannel blocker carbenoxolone, which differed from stretch-activated ATP release. Altered P2 receptor expression was evident in urothelium from pyuric OAB patients. Furthermore, intracellular bacteria were visualized in shed urothelial cells from ~80% of OAB patients with pyuria.

These data suggest that increased ATP release from the urothelium, involving bacterial colonization, may play a role in the heightened symptoms associated with pyuric OAB patients.

Introduction:

Overactive bladder syndrome (OAB) is an idiopathic condition where the bladder detrusor urinae muscle spontaneously contracts before the bladder is full. In the USA, it is ranked in the top 10 of common chronic conditions, competing with both diabetes and depression, with a reported prevalence of up to 31-42% in the adult population (2).

OAB is currently characterized by symptoms of urgency, with or without urgency incontinence, with increased frequency, and nocturia, and in some cases pain, in the absence of urinary tract infection (UTI) or other defined underlying pathology (54). The exclusion of infection is determined by failure to isolate $\geq 10^5$ colony forming units (CFU)/ml of a single species of bacteria from culture of a midstream urine (MSU) specimen (23) and negative leukocyte esterase and/or nitrate urinalysis by dipstick (25). Controversy exists as to whether current methods used to determine UTI are fully accurate (28). It has been established that the bacterial threshold of $\geq 10^5$ CFU/ml, in the presence of symptoms, is not identified in approximately 50% of UTI (31). In addition, we have recently reported the low sensitivity and specificity of routine urinary dipstick tests (20, 51). Interestingly, in recent studies where the threshold has been reduced to $\geq 10^2$ CFU/ml, bacterial cystitis has been identified in approximately one third of patients with refractory OAB, suggesting bacteria may play a significant role in the aetiology of OAB, in at least a subset of patients with OAB (22, 37, 49). It is generally accepted, that the best indicator of UTI is the detection of ≥10 white blood cells (wbc) in 1 µl of fresh, un-spun, urine examined using a haemocytometer (16, 44); however nowadays this is not normal clinical practice. Using this methodology, we have identified a low-grade inflammatory response (pyuria with ≥10 wbc μl^{-1}) in 10-35% of MSU specimens from patients with OAB (*i.e.* symptoms of urgency, with or without urgency incontinence, with frequency and nocturia, in the absence of UTI) (45, and new data not shown). Interestingly, our observation is that these patients showed the worst symptoms of frequency. Most apposite to this finding is that persistent inflammation, caused by infection and thereby accompanied by pyuria, is associated with increased nucleotide (primarily adenosine 5'-triphosphate [ATP]) release from epithelial cells and nucleotide-activated P2 receptor signaling (4, 5, 36, 55).

Extracellular nucleotide signaling via P2 receptor activation is important in the regulation of bladder function (3, 7, 8). Bladder stretch, during filling, induces a cytosolic Ca^{2+} increase via multiple proposed pathways (9) to promote release of ATP through conductive or vesicular pathways (46), and probably other nucleotides from superficial urothelium. Once released, nucleotides bind to P2 receptors on suburothelial sensory afferents (P2X2, 3 and/or 2/3 subtypes) to trigger nerve activation and the sensation of bladder fullness and the urge to urinate (52). Given the rapid breakdown of extracellular nucleotides by nucleotidases, this route should involve intermediate signaling steps involving activation of P2 receptors on other urothelial cells to release additional nucleotides to act in an autocrine/paracrine manner (17, 19, 48, 58). Nucleotide signaling in human urothelium is enhanced in interstitial cystitis (IC) in humans and felines (17, 58), a condition symptomatically similar to OAB yet with the presence of pain and an inflammatory aetiology. In IC, stretch-activated ATP release is significantly increased, P2R expression profiles are altered in the urothelium, and ATP breakdown is purportedly decreased (15, 58). Interestingly, studies on humans with acute cystitis/urinary infection demonstrate that uropathogenic *E. coli* (UPEC) can thrive in the urothelium as intracellular bacterial colonies resisting host immunity (29), that should be accompanied by increased nucleotide release from epithelial cells (see paragraph above).

We hypothesize that in a subset of OAB patients (*i.e.* those with pyuria ≥10 wbc μl^{-1}) there is increased release of ATP, and other nucleotides, from the urothelium caused by low-grade inflammation, which ultimately results in increased sensory nerve excitation and the enhanced symptoms of OAB. Furthermore, in these patients the low-grade inflammation is associated with intracellular bacteria colonization of the urothelium. Here we investigated our hypothesis using human bladder urothelium obtained using flexible cystoscopy.

Methods:

Reagents. Chemical reagents were purchased from Sigma-Aldrich (Poole, UK); with the exception of suramin obtained from Bayer AG (Leverkusen, Germany) and DAPI-containing microscope slide mountant obtained from Vector Labs (Peterborough,

UK). HPLC column and solid phase extraction cartridges were obtained from Phenomenex (Macclesfield, UK).

Human tissue and urine samples. All procedures were performed with consent and approval from the Moorfields and Whittington Hospitals Research Ethics Committee (London, UK) and the NHS Research Authority South East Coast (Kent). Informed written consent was obtained from volunteers involved in the study. Bladder biopsies and urine samples were obtained from either the Whittington Hospital Campus, University College London or Medway Maritime Hospital. Bladder biopsies were obtained using a flexible cystoscope under local (or rarely general) anaesthesia. A catheter specimen of urine (CSU) was obtained from female patients and a MSU from male patients and non-OAB controls. Patients with idiopathic OAB were recruited from Incontinence Clinics and non-OAB controls were recruited from the Haematuria Clinic. The inclusion criteria for OAB, reconfirmed at the time of sample collection, were adults aged ≥18 years of either sex with frequency ≥8 per day, urgency with or without urgency incontinence, and the absence of a UTI or severe concomitant urinary tract pathology (*e.g.* chronic obstruction, catheterisation, neurological disease, prior radiotherapy, anatomical defects, implanted devices, pregnancy, bleeding disorders, and/or anticoagulant therapy). All urine samples obtained were sent for conventional urinalysis and bacteria culture at the time of collection, if a UTI was subsequently identified the sample was retrospectively removed from the study. The inclusion criteria for non-OAB were a single historical episode of microscopic haematuria with no underlying pathology. All OAB and non-OAB patients were additionally investigated for pyuria by trained clinicians as previously described (20); significant pyuria was taken as of ≥10 wbc μl^{-1} of fresh un-spun urine. Thus the following experimental groups were used in this investigation: *i*) **non-OAB controls**, *ii*) **OAB patients without pyuria**, that is patients with no wbc or with <10 wbc μl^{-1}, and *iii*) **OAB patients with pyuria**, that is patients with ≥10 wbc μl^{-1}. Urine was either stored on ice and processed within 1 h (for microscopy) or immediately snap-frozen and kept at -80°C until the time of processing (for HPLC). Bladder biopsies were either immediately transported to the laboratory in ice-cold sterile saline and processed within 1 h of collection or placed in 10% formalin for 48

141 h prior to histological scrutiny.
142
143 *Bioiopsy histology.* Following 48 h in 10% formalin, biopsy tissue was dehydrated
144 with alcohol and xylene in a vacuum infiltration-processing machine for 12 h. The
145 tissue was then impregnated with paraffin wax. A microtome (Microm HM355S;
146 Thermo Fisher Scientific, Loughborough, UK) was used to cut sections of 6 µm
147 thickness. Slices were placed on a glass slide then dried at 60°C for 10 min. The
148 sections were then stained with H&E to evaluate the morphological characteristics of
149 the tissue. A Leica DM4000B upright light microscope (Wetlar, Germany) was used to
150 image the sections.
151
152 *Biopsy ATP release.* A Luciferin Luciferase ATP Bioluminiscence Assay Kit was used to
153 quantify ATP release from intact, live, bladder urothelium according to the
154 manufacturer's protocol. In brief, the urothelial cell layer was manually isolated
155 from the underlying tissue of the bladder biopsy using fine forceps, scalpel and a
156 dissection microscope (final wet tissue weight being 3.4±0.7 mg [n=33]). Two
157 working solutions containing the luciferin luciferase reagents were made up, one in
158 PBS (phosphate buffered saline [1X] containing [mM]: Na_2HPO_4 [10], KH_2PO_4 [1.8]
159 NaCl [137], KCl [2.7], pH 7.4; Solution-A [isotonic solution]) and one with distilled
160 water (Solution-B; hypotonic solution). The intact urothelial cell layer was then
161 incubated for 1 h in Solution-A (100µl) at 37°Cin a 96-well plate (Nunc, Roskilde,
162 Denmark). Basal readings of luminescence were taken after 1 h incubation with the
163 tissue still immersed in Solution-A. Stimulated readings of luminescence (*i.e.*
164 stretch-evoked ATP release) were taken following the addition of Solution-B (100µl)
165 for 1 min. Where stated, stimulated readings of luminescence were taken over a
166 period of 15 min with 3 min interval recordings to observe degradation of ATP with
167 time. Two ATP standard curves, one in Solution-A (100 µl volume), and the other in
168 Solution-A and Solution-B (50:50, 200 µl volume), in combination with blank wells,
169 were prepared alongside each given experiment with concentrations ranging from
170 10^{-10} to 10^{-7} M. ATP-evoked luminescence was quantified using a luminometer
171 (Synergy 2, BioTek, Winooski, USA). ATP concentration from samples was calculated

from the ATP standards using linear regression analysis. All data were normalised as nM ATP per 5 mg of wet tissue, and stimulated readings presented following the subtraction of basal readings. In experiments investigating the effect of drugs, the same protocol was followed, allowing a minimum of 5 min for the drugs to take effect before luminescence was read. Parallel standard curves were also run in the presence of drugs to investigate any possible interactions with the luciferin luciferase reaction.

Biopsy vesicle staining. Following immediate transport to the laboratory in ice-cold sterile saline, biopsy tissue was placed in PBS (1X) and incubated for 1 h at 37 °C to create *resting* conditions, or, to imitate *stretch* conditions (*i.e.* bladder filling), biopsy tissue was then incubated in hypotonic PBS (0.5X) for 1 min. Both the *resting* and *stretch* biopsy tissues were immediately transferred to PFA (4%) for 48 h, before being wax-embedded and sectioned (as above: *Biopsy histology*). The tissue sections were permeated with a 0.1% Triton X100 solution and incubated with quinacrine (100 µM) for 30 min before being washed with PBS (1X). Sections were mounted with DAPI-containing mountant, and visualised under an inverted confocal microscope using the x63 oil immersion objective (Leica SP5; Wetzlar, Germany). Using ImageJ software, mean fluorescence intensity was measured in all images taken from quinacrine-labelled tissue.

Biopsy RNA extraction and RT-PCR. Following immediate transport to the laboratory, in cold sterile saline, the urothelial cell layer was manually isolated from the underlying tissue of the bladder biopsy using fine forceps, scalpel and a dissection microscope. Intact urothelium was homogenised in Tri-reagent and then freeze/thawed in liquid nitrogen followed by chloroform extraction and ethanol precipitation. Precipitated RNA was loaded onto Qiagen RNeasy columns (Qiagen, Crawley, UK) for DNase treatment and further purification. RNA concentration was measured using a spectrometer, Nano N-1000 system (Nanodrop Technologies, Wilmington, USA).

Total RNA (10 ng) from each sample was reverse transcribed in a 20 µl reaction volume using the One-step Quantitect Reverse Transcription Kit (Qiagen, Crawley,

UK) according to manufacturer's instructions. Primers for P2 receptor subtypes were designed using Primer 3 Web-software (Whitehead Institute for Biomedical Research, Cambridge, USA) and manufactured by Eurofins-MWG (Ebersberg, Germany) (see Appendix for primers and mRNA accession numbers used). The real-time PCR, based on SyBR green detection, (Qiagen, Crawley, UK) was performed using a Chromo-4 thermal cycler (Bio-Rad, Hemel Hempstead, UK) with 2 µl total RNA. Using a standard curve, created by duplicate serial dilutions of standard DNA (target sequence of interest) over 12 logarithmic orders and the thermal cycler software the relative concentrations of the target amplicons were determined. In addition, the standard curve was used to verify the linearity of amplification of each transcript; r^2>0.99 in all cases. The relative concentrations of target in each run were expressed as a ratio to the housekeeping gene, GAPDH. All PCR products were checked for specificity and purity from a melting curve profile created after each run by the thermal cycler software. Homology of the PCR products was further checked for size by agarose gel electrophoresis.

Urine sediment immunofluorescence. Fresh urine (50 µl; within 1 h of collection and stored on ice) was spun onto slides using a cytospin (Sandon Cytospin 4, York, UK) at 800 rpm for 5 min. The deposit was stained with acridine orange (0.5% in Gey's solution) for 30 min; previously shown to fluoresce green in the presence of viable organism DNA after excitation by a laser at 436-490 nm (12, 32). In order to differentiate extracellular from intracellular bacteria, a crystal violet counter-stain (0.1% in 150 mM NaCl) was added to quench the fluorescence of extracellular microorganisms. Images were acquired using an upright fluorescence microscope (Leica DM4000B, Wetlar, Germany), samples were excited at 488 nm and emitted light collected with a 505-550 nm band pass filter. On those slides found to have cells containing bacteria, the deposit was fixed with PFA (4%) for 2 min, then further treated with anti-Uroplakin III (Santa Cruz Biotechnology Inc; Santa Cruz, USA) for 12 h, a specific marker of urothelial cells (10). Alexa 555 (Invitrogen, UK) was used as a secondary antibody and incubated for 2 h, after which the slides were mounted with DAPI-containing mountant. Images were acquired with a confocal microscope (Leica SP5; Wetlar, Germany) and Z-series processed using Volocity software (Improvision,

Coventry, UK). Alexa-555 and DAPI were excited at 543 nm and 405 nm, and emittedlight was collected using a 560 nm long-pass filter and 420 nm long pass filter, respectively.

Urine nucleotide and nucleoside quantification. Urine, that had previously been immediately snap-frozen and kept at -80°C, was thawed and sterile filtered through a 0.22 µm membrane prior to HPLC analysis. HPLC analysis was performed as previously described (11). In brief, the samples were then subjected to a solid phase extraction cleaning procedure through Strata-X columns (30 mg/ml; Phenomenex, Macclesfield, UK). Elution was achieved using 25 mM ethanolamine at pH 5.0 and 30% methanol in ethanolamine (pH 5.0). A standard curve was prepared alongside each HPLC experiment consisting of ADP only. Samples (100 µl) were injected into the column (Polar-RP 4 µm 80A 250x4.6 mm, Phenomenex, Macclesfield, UK) and a gradient profile of 2%-30% acetonitrile in phosphate buffer was run for 20 min, with an additional 15 min stabilisation period. Nucleotides and nucleosides were UV-detected at 254 nm and areas measured with the Agilent software (Agilent Technologies, Wokingham, UK). Areas were then adjusted to the SPE cartridge performance and compared against standard curves to obtain the final concentration. Urinary creatinine levels were measured using a commercial test (R&D Systems, Abingdon, UK) as per manufacturer's instructions to ensure data were comparable.

Statistical analysis. All numerical data were assessed for normality using the Kolmogorov-Smirnov test. Significance level was evaluated by two-tailed paired and unpaired *t*-tests, parametric and non-parametric one-way ANOVA with appropriate post-hoc tests. *P* values less than 0.05 were considered statistically significant. All data presented as mean±SEM, and n equals number of patients or samples.

Results:

Bladder biopsies obtained from patients using flexible cystoscopy have full-thickness intact urothelium. H&E staining of wax-embedded and sliced biopsies obtained using flexible cystoscopy routinely demonstrated intact urothelium of full-thickness in samples of all three experimental groups of patients (*i.e.* non-OAB [8 of 8 specimens], OAB without pyuria [11 of 13 specimens], and OAB with pyuria [6 of 6 specimens]) (Figure 1A). Morphologically distinct umbrella cells were evident on the luminal side of the urothelium further demonstrating the integrity of the tissue (Figure 1B).

Basal ATP release is significantly greater from urothelium of OAB patients with pyuria. To measure ATP release from microdissected urothelium, we used a luciferin luciferase assay. ATP levels became detectable after 15 min and stabilized at 30-40 min (data not shown) and consequently recordings were taken at 60 min. The subtle increase in concentration of ATP, which stabilized with time, was taken to represent basal (i.e. unstimulated) release of ATP. Basal release of ATP was significantly greater from urothelium of OAB patients with pyuria (78.1±20.6 nM/5 mg of wet tissue [hereafter referred to as simply 'nM'], n=15, $P<0.05$) than from non-OAB patients (1.9±1.5 nM, n=9) or OAB patients without pyuria (2.2±1.7 nM, n=33) (Figure 2A). Application of a hypotonic solution, to mimic stretch and thus bladder filling, resulted in substantial, and similar increase in ATP release from the urothelium of all three experimental groups (non-OAB patients, 129±48 nM, n=9; OAB patients without pyuria, 38±18 nM, n=33; OAB patients with pyuria, 268±188 nM, n=15) (Figure 2B). Peak stretch-evoked ATP levels (*i.e.* the highest concentration of ATP measured following stimulation) was achieved within 1 min irrespective of experimental group (n=18; data not shown). The concentration of ATP decreased during hypotonic insult suggesting degradation by endogenous tissue ATPases. As expected, increasing hypotonicity caused additional ATP release from urothelium (~2-fold with 25% hypotonic buffer, ~10-fold with 50% hypotonic buffer, and ~20-fold with 75% hypotonic buffer). The concentration of ATP released from urothelium following osmotic insult decreased by 51±8% (n=9) and 52±11% (n=9)

after 3 min for samples from non-OAB patients and OAB patients with pyuria, respectively, and levels returned to basal concentrations within 10-12 min in both cases. However, for samples from OAB patients without pyuria, the decrease in concentration of stimulated-ATP release was significantly less after 3 min (26±7%; n=9; $P<0.05$) and returned to basal concentrations in ~30 min (Figure 2C). The slower rate of stimulated ATP decay seen with urothelium obtained from OAB patients without pyuria was similar to that with urothelium obtained from non-OAB patients in the presence of the ATPase inhibitor ARL 67156 (100 μM; n=5) (Figure 2C); ARL 67156 did not alter the peak concentration of stimulation-evoked ATP release (data not shown). For ATP concentration measurements following stimulation, results obtained using HPLC correlated well with results from the luciferin luciferase assay (data not shown); however it was not possible to use HPLC to measure basal ATP release as, in some cases, concentrations were below the level of accurate quantification using the HPLC technique.

Basal ATP release mechanisms differ from stretch-evoked stimulated release. We pharmacologically investigated the molecular mechanism(s) by which ATP is released (primarily stretch-evoked) from human urothelium obtained from non-OAB patients, OAB patients without pyuria, and OAB patients with pyuria, using a luciferin luciferase assay. Basal ATP release from urothelium of OAB patients with pyuria was significantly inhibited (by 67±9%, n=3) by the P2 receptor antagonist suramin (1 mM) and almost abolished by the hemichannel and gap junction blocker carbenoxolone (CBX, 50μM; n=3), yet, was significantly potentiated (by 74±13%, n=3) by the P2 receptor agonist UTP (1 μM) (Figure 3A). The UTP-evoked potentiation of ATP was subtly inhibited (by ~30 nM, n=3) by co-incubation with CBX (50 μM) and significantly inhibited by co-incubation with suramin (1 mM; $P<0.05$, n=3), suggesting at least two mechanisms of ATP release (*i.e.* hemichannel-mediated and downstream P2 receptor-evoked) (Figure 3A and 7A). Botulinum toxin-A (BTX-A; 20 units/ml) known to inhibit vesicular release of ATP, brefeldin-A (BFA; 20 μM) known to inhibit vesicular trafficking, capsazepine (3 μM) a blocker of stretch-activated TRP channels, and 4,4'-diisothiocyano-2,2'-stilbenedisulfonic acid (DIDS, 100 μM) a

calcium-activated chloride channel blocker, did not significantly alter basal ATP release (all n=3-4) (Figure 3A). Unfortunately, basal ATP release, as opposed to stretch-evoked ATP release, from urothelium of non-OAB patients and OAB patients without pyuria could not be fully investigated due to barely detectable levels of ATP (see Figure 2A). However, the effects of UTP (1 μM, n=3) were investigated in these tissues and found not to significantly increase basal ATP concentration. Stretch-evoked ATP release (*i.e.* release evoked by a hypotonic stimulus) from urothelium of OAB patients with pyuria was significantly inhibited (by 72±14%, n=5) by suramin (1 mM; $P<0.05$) and almost abolished by BTX-A (20 units/ml, n=3) and BFA (20 μM, n=3), but unaffected by capsazepine, CBX, DIDS or UTP (n=3-5) (Figure 3B). Stimulated ATP release from urothelium of non-OAB patients and OAB patients without pyuria, in addition to being abolished by suramin, BTX-A and BFA, was significantly inhibited by capsazepine (by 67±11% [n=3] control and 83±8% [n=4] OAB-pyuria; $P<0.05$).

Given that BTX-A abolished hypotonicity-evoked ATP release from urothelium of OAB patients with pyuria (Figure 3B) we investigated whether vesicles were evident in the urothelium. Quinacrine staining of wax-embedded and sliced biopsies demonstrated ATP-containing vesicular structures throughout the urothelium and in underlying tissue (Figure 4A). Hypotonic challenge of biopsies from OAB patients with pyuria prior to wax embedding and slicing resulted in significantly less dense quinacrine staining (3.9±1.3 arbitary units [AU], n=3, as compared to 0.9±0.3 AU, n=3), suggesting vesicle emptying following hypotonic stimulation (Figure 4B), which was inhibited by the addition of BTX-A (1.3±1.3 AU, n=3).

Altered expression of P2 receptor mRNA in bladder urothelium of OAB patients. To quantify the relative abundance of P2 receptor mRNA in microdissected urothelium of non-OAB patients, OAB patients without pyuria, and OAB patients with pyuria, we calculated a ratio of the P2 receptor gene of interest to a constitutively expressed housekeeping gene (GAPDH) using RT-PCR.

We failed to detect significant levels (*i.e.* >5 arbitrary units) of P2X4 and $P2Y_4$ mRNA in urothelium from any experimental group. In contrast, significant amounts

of mRNA were detected for P2X1, 2, 3, 5, 6 and 7, and $P2Y_{1,\ 2,\ 6,\ 11,\ 12,\ 13,\ 14}$ in urothelium of non-OAB controls; order of expression:$P2Y_{14}$>>P2X1, 3, 5, 6 and 7=$P2Y_{1,\ 6,\ 11,\ 12\ and\ 13}$>P2X2=$P2Y_2$ (Figure 5). Urothelium from OAB patients without pyuria showed a significant increase in abundance of $P2Y_{11\ and\ 13}$ mRNA (by 200-fold and 10-fold, respectively; n=6; $P<0.01$). Whereas, urothelium from OAB patients with pyuria showed a significant increase in abundance of $P2Y_{2\ and\ 11}$ mRNA (100-fold and 50-fold, respectively; n=6; $P<0.01$) (Figure 5B).

Intracellular bacteria in shed urothelial cells of OAB patients with pyuria. To investigate whether intracellular bacteria are responsible for increased basal release of ATP from urothelium from OAB patients with pyuria we stained cytospun fresh urine samples with acridine orange and crystal violet. Biopsy tissue was not used in this part of the investigation given its precious nature and the necessity of its use in mechanistic luciferin luciferase studies. Planktonic bacteria were observed in 1 of 16 samples from non-OAB patients, whereas, intracellular bacteria were not observed in any sedimentary cells (8±2 urothelial cells per sample, n=16) (Figure 6A). Similarly, planktonic bacteria were observed in <10% of samples from OAB patients without pyuria (n=33) and intracellular bacteria were not observed in any sedimentary cells (Figure 6B). The number of sedimentary cells identified as urothelial cells in urine samples from OAB patients without pyuria was 15±3 (n=33). Planktonic bacteria were observed in 9 of 16 samples from OAB patients with pyuria, and intracellular bacteria were observed in sedimentary urothelial cells from 13 samples (19±3 urothelial cells per sample, n=16). In the 13 samples, 52±9% of urothelial cells were found to contain intracellular bacteria (Figure 6C). To confirm that the cells containing intracellular bacteria were urothelial cells, the deposit was fixed with PFA (4%) then further treated with anti-Uroplakin III (UP-III) and DAPI. In all cases, those cells initially identified as urothelial cells by their morphology alone, were confirmed as urothelial cells by positive UP-III immunofluorescence. Z-stack images obtained by confocal microscopy further confirmed the intracellular localization of bacteria (Figure 6D).

Urinary AMP and adenosine levels are elevated in OAB. Given that basal ATP release is significantly greater from urothelium of OAB patients with pyuria we investigated whether this was detectable by HPLC in MSU samples, as well as other nucleotides (ADP, AMP, GTP, GDP, GMP, UTP, UDP and UMP) and nucleosides (adenosine, guanosine and uridine) that may be altered (Table 1). Urinary AMP levels were significantly greater in samples from OAB patients without pyuria (14.4±8.3 μM, n=17, $P<0.05$) and OAB patients with pyuria (8.5±2.3 μM, n=16, $P<0.05$) than non-OAB patients (2.7±0.5 μM, n=11). In addition, urinary adenosine levels were significantly greater in samples from OAB patients without pyuria (228±106 μM, n=17, $P<0.05$) than non-OAB patients (61±58 μM, n=11). Nucleotide and nucleoside release from biopsy tissue was not investigated using HPLC given the necessity of the tissue's use in mechanistic luciferin luciferase studies.

Discussion:

The main findings of this investigation revealed that basal release of ATP from the urothelium is significantly greater for human OAB patients with pyuria ≥10 wbc μl^{-1} than for OAB patients without pyuria (or with pyuria <10 wbc μl^{-1}) or non-OAB patients, which may account for the heightened symptoms seen in these patients (36). More specifically, we present evidence that (*1.*) bacteria reside in some urothelial cells of OAB patients with pyuria ≥10 wbc μl^{-1}, and (*2.*) a sequential signaling mechanism occurs whereby (i) basal ATP release from the urothelium is via hemichannels, (ii) ATP released through hemichannels acts in an autocrine/paracrine manner by activating P2 receptors expressed throughout the urothelium (likely to be the $P2Y_2$ subtype, shown here to be upregulated in OAB patients with pyuria), and (iii) P2 receptor activation causes yet further ATP release from the urothelium via an undetermined mechanism. Taken together, these findings lead to the proposal that in a subset of OAB patients (*i.e*. OAB patients presenting with pyuria ≥10 wbc μl^{-1}), there is heightened basal ATP release from, and increased P2 receptor expression in, the urothelium originating from intracellular bacteria colonization, that culminates in inappropriate sensory nerve excitation and the symptoms of OAB seen in these patients (see Figure 7A).

In addition to reporting altered urothelial ATP release from OAB patients presenting with pyuria, we also describe stretch-evoked ATP signaling in human urothelium (which does not significantly differ between OAB patients without pyuria, OAB patients with pyuria, and non-OAB patients). Specifically we present evidence that a sequential signaling mechanism occurs whereby (i) urothelial cell stretch (as occurs in bladder filling) evokes vesicular ATP release, (ii) ATP released from vesicles acts in an autocrine/paracrine manner by activating P2 receptors (of which a variety and abundance are expressed throughout the urothelium), and (iii) P2 receptor activation causes yet further vesicular ATP release from the urothelium to presumably activate P2X receptors on suburothelial sensory nerves and therefore signal bladder fullness (see Figure 7B).

Mechanisms of urothelial ATP release.

It is well established that extracellular nucleotide signaling arising from the urothelium is important in the regulation of bladder function (3, 8). However, to date little work has been performed on human urothelium. This, in part, is because of the reported fragility of the urothelial cell layer and superficial umbrella cells, and, the logistic difficulty in obtaining tissue samples using the cold-cup biopsy technique (normally performed under general anaesthesia). However, here we demonstrate that full-thickness intact urothelium obtained using flexible cystoscopy, a routinely used procedure using a fibre optic instrument and not requiring general anesthesia of the donor, can be used successfully to study the physiology/pathophysiology of the urothelium *in vitro*.

Using urothelium samples, obtained by flexible cystoscopy, we have presented evidence for an autocrine/paracrine ATP signaling mechanism in human urothelium that presumably culminates in sensory nerve excitation (see Figure 7). Our proposed mechanisms of autocrine/paracrine ATP signaling are broadly in keeping with others. Autocrine/paracrine signaling by ATP, and other nucleotides, in epithelial tissues is a well documented phenomenon, especially in renal tissue where basal ATP release and stretch-evoked ATP release (a function of tubular flow rate) influence ion and water transport mechanisms, and may even influence intrarenal blood flow (21, 35, 39, 60). With respect to the bladder, the group of Birder was the first to propose that the abundance, and variety, of P2 receptors expressed throughout the urothelial cell layer might serve to amplify stretch-evoked ATP signaling (15). Non-neuronal cellular release mechanisms of ATP are not well understood. Proposals to date involve a number of complementary pathways that include transport via ATP-binding-cassette (ABC) proteins, connexin hemichannels, large-diameter anion channels and exocytotic vesicular release (1, 31). Our data using human urothelium demonstrate ATP release via hemichannels and vesicles (see Figure 3 and 4). This investigation extends findings of earlier studies using urothelium by demonstrating multiple, and different, release mechanisms for both basal release (in OAB patients with pyuria) and stretch-evoked release (from all experimental groups). However, disappointingly we report at least one unidentified ATP release mechanism in OAB patients with pyuria that is insensitive to all blockers/antagonists tried (see Figure 7). It is perhaps prudent to mention at this

juncture that the effect of bacteria on urothelial cell permeability has not be investigated, which of course may also account for increased increased basal release of ATP from the urothelium of patients with OAB and pyuria.

Current OAB therapies include antimuscarinic and/or BTX-A treatment. The former is associated with severe side-effects and high rates of withdrawal, and the latter is notoriously expensive. Our study tested the ability of suramin and BTX-A to alter the ATP signaling cascades seen in urothelium from OAB patients. Suramin, successfully inhibited the exaggerated basal ATP release unique to OAB patients with pyuria, whereas BTX-A was without effect (see Figure 3A). However, stimulated ATP release, similar in non-OAB patients and OAB patients alike, was inhibited by both suramin and BTX-A (see Figure 3B). These findings suggest that BTX-A may not be a suitable therapy for those suffering from OAB with pyuria, and that selective targeting of urothelial ATP receptors may be an alternative pharmacological strategy to currently used antimuscarinics in the treatment of OAB (irrespective of pyuria status).

P2 receptor expression in urothelium.

Using real time-PCR, we molecularly characterized the P2 receptor subtypes expressed in urothelium of non-OAB patients, OAB patients without pyuria, and OAB patients with pyuria. We consistently demonstrated mRNA expression of P2X1-3 and 5-7 receptor subunits, but not P2X4, in samples from all 3 experimental groups. The level of expression for each subtype did not significantly differ between experimental groups. Given the ability of P2X receptors to form homomeric and heteromeric ion channels, the possibility exists that up to 10 subtypes of P2X receptor (P2X1, 2, 3, 5, 6, 7, 1/2, 1/5, 2/3 and 2/6) may be functionally expressed in human urothelium. For P2Y receptors, we consistently demonstrated mRNA expression for all subtypes ($P2Y_{1, 2, 6 \text{ and } 11\text{-}14}$) with the exception of $P2Y_4$. However, levels of $P2Y_{2, 11 \text{ and } 13}$ were significantly increased in OAB patients, with an increase in $P2Y_2$ being specific to OAB patients with pyuria and $P2Y_{13}$ being specific to OAB patients without pyuria (see Figure 5).

Our PCR findings demonstrating expression of almost all P2 receptors in human urothelium are broadly in accordance with previous studies that collectively

reported all P2X (P2X1-7) and $P2Y_{1, 2, 4, 6 \text{ and } 11}$ localization/expression in native urothelium of human, rat, mouse, rabbit, guinea-pig and cat and in a human urothelial cell line (UROtsa cells) (4, 6, 13-15, 18, 19, 24, 40, 42, 50, 57, 61). That others have not demonstrated $P2Y_{12\text{-}14}$ expression is perhaps due to the recent discovery and cloning of these subtypes from human tissue. Our PCR investigation also extends findings of earlier studies in which P2 receptor expression in the urothelium is described for both human and feline IC patients, whereby P2X1 and 3 and $P2Y_2$ are decreased (6, 15).

Interestingly, stimulation of some P2 receptors (P2X2, 4 and 7, and $P2Y_6$) results in the release of key proinflammatory cytokines (*e.g.* IL-1ß, IL-6, IL-8 and TNFα) (3, 47). IL-1, IL-6 and IL-8 have been shown to presage UTI symptoms (27, 53). Here we present data suggesting that $P2Y_6$ expression may be increased (not quite significant; Figure 5B) in the urothelium of OAB patients with pyuria, which may be critical to the release of these cytokines, and amplify the cascade of events leading to the heightened symptoms of OAB.

Bacterial colonization of bladder urothelium.

With our findings in mind, the most apposite series of animal experiments have been reported by the Hultgren group (41, 59). Using a murine model of chronic urinary infection, they demonstrated the ability of *E. coli* to colonize the superficial urothelium forming intracellular bacterial colonies (IBCs). These colonies exhibit a reduced susceptibility to antibiotics and host immune mechanisms. Electron microscopy studies showed that colonies formed pod-like protrusions from the cell wall, with the resident bacteria encased in a polysaccharide-rich matrix surrounded by a protective shell. Eventually, bacteria detached from the pod and burst into the bladder lumen where the escaped bacteria then infected fresh cells. However, in this study we failed to identify bacteria residing within the urothelial cells lining the bladder (*i.e.* in biopsy material) but instead bacteria were identified inside shed urothelial cells found in urine samples. The possibility exists that the shed umbrella cells were from the renal pelvis, ureters, urethra and bladder; although unlikely given that the urine was obtained by CSU sampling.

Interestingly, Rosen and colleagues have published data that showed IBCs forming in urothelial cells of patients with acute cystitis (60). UPEC lipopolysaccharide (LPS) is an extremely potent activator of innate immune responses acting via binding to CD14 and Toll like receptors in the bladder urothelium to activate p38 MAP kinase, Ca^{2+} and cAMP signalling which in turn triggers IL-6 and IL-8 production. (34, 56). Furthermore, ATP released from both UPEC and infected cells can situmlate IL-8 production via P2 receptor signalling (4, 43, 55). IL-6 is associated with activation of the acute phase response, which increases production of C-reactive protein from the liver and IL-8 acts as a chemotactic factor for neutrophils (pyuria). The presence of pyuria in the absence of infection (determined by failure to isolate $\geq 10^5$colony forming units) in a subset of OAB patients, which suggests bladder infection and IBC, is a relatively recent discovery (44).

Urinary nucleotides and nucleosides as biomarkers of OAB.

Previous studies have utilized the luciferin luciferase assay to investigate the potential for urinary ATP concentration to serve as a suitable biomarker for UTIs. These studies report ATP concentrations of 5-25 nM in MSU samples collected from patients testing negative for UTIs, and 112-140 nM for patients with positive culture (26, 38), suggesting that ATP could be a biomarker for urinary infection. However, a more recent study looking at ATP concentrations in the urine of OAB patients found similar low levels of ATP in both OAB and non-OAB control groups (1.5 and 1.4 nM [normalized to creatinine concentrations], respectively) (33). Using a novel HPLC technique we found significantly higher levels of AMP in MSU samples from OAB patients (irrespective of pyuric status) than in samples from non-OAB patients. In addition, we detected elevated adenosine in OAB patients without pyuria. Whereas these data are currently too preliminary to propose biomarkers for OAB, it may be useful in determining additional signaling mechanisms responsible for the symptoms of OAB.

Should pyuria inform the diagnosis and treatment of OAB?

Data presented here raise the question as to whether OAB with pyuria should be classified and treated differently to OAB without pyuria. Considerable overlap already exists between OAB and chronic prostatitis or "chronic pelvic pain syndrome" and IC, also called "painful bladder syndrome", and in all cases the diagnosis, rightly or wrongly, relies on exclusion of urinary infection by routine culture methods (54). Our findings suggest that OAB with pyuria has a subclinical UTI component (intracellular bacteria in the urothelium), not dissimilar to recent findings for IC yet without pain and inflammation (29). That significant basal ATP release is novel to OAB with pyuria suggests treatment could be designed accordingly. Further multi-centre investigations are required to fully answer this question; which may need to wait until routine UTI detection methodologies are updated.

***Summary**:*

These data provide a better understanding of OAB aetiology and, nucleotide release and signaling in bladder epithelium (in health and disease). The former may prompt clinical re-classification of OAB, to aid successful diagnosis and treatment in the future. The later may help identify alternative, efficacious, and acceptable therapeutic treatments for the unpleasant symptoms of OAB. Conceivably, efficient therapeutic strategies may be evolved to target nucleotide signaling by intravesicular delivery methods to alleviate OAB symptoms and/or treat intracellular bacteria of OAB with pyuria.

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The author(s) declare to have no competing interests.

592 ***References:***

1 **Abrams P, Cardozo L, Fall M., Griffiths D, Rosier P, Ulmsten U, Van Kerrebroeck P, Victor A, Wein A.** The standardisation of terminology in lower urinary tract function: report from the standardisation sub-committee of the International Continence Society. *Urology* 61:37-49. 2003.

2 **Anderson GG, Palermo JJ, Schilling JD, Roth R, Heuser J, Hultgren SJ.** Intracellular bacterial biofilm-like pods in urinary tract infections. *Science* 301:105-107. 2003.

3 **Appleby PA, Shabir S, Southgate J, and Walker D.** Cell-type-specific modelling of intracellular calcium signalling: a urothelial cell model. *J R Soc Interface* 10:20130487. 2013

4 **Birder L, Andersson KE.** Urothelial signaling. *Physiol Rev* 93(2):653-80. 2013.

5 **Birder LA, Barrick SR, Roppolo JR, Kanai AJ, de Groat WC, Kiss S, Buffington CA**. Feline interstitial cystitis results in mechanical hypersensitivity and altered ATP release from bladder urothelium. *Am J Physiol Renal Physiol* 285:F423-429. 2003.

6 **Birder LA, Ruan HZ, Chopra B, Xiang Z, Barrick S, Buffington CA, Roppolo JR, Ford AP, de Groat WC, Burnstock G.** Alterations in P2X and P2Y purinergic receptor expression in urinary bladder from normal cats and cats with interstitial cystitis. *Am J Renal Physiol* 287:F1084-1091. 2004.

7 **Burnstock G**. Pathophysiology and therapeutic potential of purinergic signaling. *Pharmacol Rev* 58:58-86. 2006.

8 **Burnstock G.** Physiology and pathophysiology of purinergic neurotransmission. *Physiol Rev* 87:659-797. 2007.

9 **Chopra B, Gever J, Barrick SR, Hanna-Mitchell AT, Beckel JM, Ford AP, Birder LA.** Expression and function of rat urothelial P2Y receptors. *Am J Physiol Renal Physiol* 294:F821-829. 2008.

10 **Clare J, Griffiths D, Chet de Groat W.** The neural control of micturition. *Nature Reviews Neuroscience* 9(6):453-66. 2008.

11 **Contreras-Sanz A, Scott-Ward TS, Gill HS, Jacoby JC, Birch RE, Malone-Lee J, Taylor KM, Peppiatt-Wildman CM, Wildman SS.** Simultaneous quantification of

12 different nucleotides and nucleosides released from renal epithelium and in human urine samples using ion-pair reversed-phase HPLC. *Purinergic Signal.* 8(4):741-51. 2012.

12 **Crawford C, Kennedy-Lydon TM, Callaghan H, Sprott C, Simmons RL, Sawbridge L, Syme HM, Unwin RJ, Wildman SSP, Peppiatt-Wildman CM.** Extracellular nucleotides affect pericyte-mediated regulation of rat in situ vasa recta diameter. *Acta Physiologica* 10:1748-1716. 2011.

13 **Davis IC, Sullender WM, Hickman-Davis JM, Lindsey JR, Matalon S.** Nucleotide-mediated inhibition of alveolar fluid clearance in BALB/c mice after respiratory syncytial virus infection. *Am J Physiol Lung Cell Mol Physiol* 286:L112-120. 2004.

14 **Elneil S, Skepper JN, Kidd EJ, Williamson JG, Ferguson DR.** Distribution of P2X(1) and P2X(3) receptors in the rat and human urinary bladder. *Pharmacology* 63:120-128. 2001.

15 **Ferguson DR, Kennedy I, Burton TJ.** ATP is released from rabbit urinary bladder epithelial cells by hydrostatic pressure changes--a possible sensory mechanism? *J Physiol* 505 (Pt 2):503-511. 1997.

16 **Hurlbut TA 3rd, Littenberg B.** The diagnostic accuracy of rapid dipstick tests to predict urinary tract infection. *Am J Clin Pathol* 96:582-588. 1991.

17 **Inscho EW.** ATP, P2 receptors and the renal microcirculation. *Purinergic Signal* 5:447-460. 2009.

18 **John AS, Boyd JC, Lowes AJ, Price CP.** The use of urinary dipstick tests to exclude urinary tract infection. *Am J Clin Path* 126:428-436. 2006.

19 **Justice SS, Hung C, Theriot JA, Fletcher DA, Anderson GG, Footer MJ, Hultgren SJ.** Differentiation and developmental pathways of uropathogenic Escherichia coli in urinary tract pathogenesis. *Proc Natl Acad Sci* U S A 101:1333-1338. 2004.

20 **Kass EH.** Bacteriuria and the diagnosis of infections of the urinary tract; with observations on the use of methionine as a urinary antiseptic. *AMA Arch Intern Med* 100:709-714. 1957.

21 **Kelley SP, Courtneidge HR, Birch R, Contreras-Sanz A, Kelly MC, Durodie J, Peppiatt-Wildman CM, Farmer CK, Delaney MP, Malone-Lee J, Harber MA,**

Wildman SSP. Urinary ATP and visualisation of intracellular bacteria: a superior diagnostic marker for recurrent UTI in renal transplant recipients? *SpringerPlus Med* 3:200 (p1-7). 2014.

22 **Khasriya R, Khan S, Lunawat R, Bishara S, Bignal 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* 183:1843-1847. 2010.

23 **Khasriya R, Sathiananthamoorthy S, Ismail S, Kelsey M, Wilson M, Rohn JL, Malone-Lee J.** Spectrum of bacterial colonization associated with urothelial cells from patients with chronic lower urinary tract symptoms. *J Clin Microbiol* 51(7):2054-62. 2013.

24 **Kim JC, Yoo JS, Park EY, Hong SH, Seo SI, Hwang TK.** Muscarinic and purinergic receptor expression in the urothelium of rats with detrusor overactivity induced by bladder outlet obstruction. *BJU Int* 101:371-375. 2007.

25 **Ko YC, Mukaida N, Ishiyama S, Tokue A, Kawai T, Matsushima K, Kasahara, T.** Elevated interleukin-8 levels in the urine of patients with urinary tract infections. *Infect Immun* 61:1307-1314. 1993.

26 **Kruse R, Demirel I, Save S, Persson K.** IL-8 and global gene expression analysis define a key role of ATP in renal epithelial cell responses induced by uropathogenic bacteria. *P2 Receptor Signal* 10(3):499-508. 2014.

27 **Kruse R, Save S, Persson K.** Adenosine triphosphate induced P2Y2 receptor activation induces proinflammatory cytokine release in uroepithelial cells. *J Urol* 188(6):2419-25. 2012.

28 **Kunzelmann K, Scheidt K, Scharf B, Ousingsawat J, Schreiber R, Wainwright B, McMorran B.** Flagellin of Pseudomonas aeruginosa inhibits Na+ transport in airway epithelia. *FASEB J* 20:545-546. 2006.

29 **Lee HY, Bardini M, Burnstock G.** Distribution of P2X receptors in the urinary bladder and the ureter of the rat. *J Urol* 163:2002-2007. 2000.

30 **Malone-Lee J, Ghei M, Lunawat R, Bisahara S, Kelsey M.** Urinary white cells and the symptoms of the overactive bladder. *Neurourol Urodyn* 26:656-657.

2007.

31 **Mansfield KJ. Hughes JR.** P2Y receptor modulation of ATP release in the urothelium. *Biomed Res Int* 2014:830374. 2014.

32 **Mansfield KJ, Cowan T, Cheng Y, et al.** Predicting refractory detrusor overactivity: are there any clues at diagnosis? *EMJ Urol* 1:87-91. 2013.

33 **Miliotis, MD.** Acridine orange stain for determining intracellular enteropathogens in HeLa cells. *J Clin Microbiol* 29:830-831. 1991.

34 **Moore KH, Malykhina AP.** What is the role of covert infection in detrusor overactivity, and other LUTD? *Neurourol Urodyn* 33(5):606-10. 2014.

35 **Osterberg E, Hallander HO, Kallner A, Lundin A, Aberg H.** Evaluation of the adenosine triphosphate test in the diagnosis of urinary tract infection. *Eur J Clin Microbiol Infect Dis* 10:70-73. 1991.

36 **Praetorius HA, Leipziger J.** ATP release from non-excitable cells. *Purinergic Signal*. 5:433-446. 2009.

37 **Praetorius HA, Leipziger J.** Intrarenal purinergic signaling in the control of renal tubular transport. *Annu Rev Physiol* 72:377-393. 2010.

38 **Rapp DE, Lyon MB, Bales GT, Cook SP.** A role for the P2X receptor in urinary tract physiology and in the pathophysiology of urinary dysfunction. *Eur Urol* 48:303-308. 2005.

39 **Reeves P, Irwin D, Kelleher C, Milsom I, Kopp Z, Calvert N, Lloyd A.** The current and future burden and cost of overactive bladder in five European countries. *Eur Urol* 50(5):1050-1057. 2006.

40 **Rosen DA, Hooton TM, Stamm WE, Humphrey PA, Hultgren SJ.** Detection of intracellular bacterial communities in human urinary tract infection. *PLoS Med* 4:e329. 2007.

41 **Save S, Persson K.** Extracellular ATP and P2Y receptor activation induce a proinflammatory host response in the human urinary tract. *Infect Immun* 78:3609-3615. 2010.

42 **Schwiebert EM, Zsembery A.** Extracellular ATP as a signaling molecule for epithelial cells. *Biochim Biophys Acta* 1615:7-32. 2003.

43 **Song J, Duncan MJ, Li G, Chan C, Grady R, Stapleton A, et al.** A novel TLR4-

mediated signaling pathway leading to IL-6 responses in human bladder epithelial cells. *PLoS Pathog* 3(4):e60. 2007.

44 **Stamm WE.** Measurement of pyuria and its relation to bacteriuria. *Am J Med* 75:53-58. 1983.

45 **Stamm WE.** Quantitative urine cultures revisited. *Eur J Clin Microbiol* 3:279-281. 1984.

46 **Stamm WE, Counts GW, Running KR, Fihn S, Turck M, Holmes KK.** Diagnosis of coliform infection in acutely dysuric women. *N Engl J Med* 307:463-468. 1982.

47 **Studeny S, Torabi A, Vizzard MA.** P2X2 and P2X3 receptor expression in postnatal and adult rat urinary bladder and lumbosacral spinal cord. *Am J Physiol Regul Integr Comp Physiol* 289:R1155-1168. 2005.

48 **Sugaya K, Nishijima S, Kadekawa K, Miyazato M, Mukouyama H.** Relationship between lower urinary tract symptoms and urinary ATP in patients with benign prostatic hyperplasia or overactive bladder. *Biomed Res* 30:287-294. 2009.

49 **Sui G, Fry CH, Montgomery B, Roberts M, Wu R, Wu C.** Purinergic and muscarinic modulation of ATP release from the urothelium and its paracrine actions. *Am J Physiol Renal Physiol* 306:F286-298. 2014.

50 **Sui GP, Wu C, Fry CH.** Characterization of the purinergic receptor subtype on guinea-pig suburothelial myofibroblasts. *BJU Int* 97:1327-1331. 2006.

51 **Sun Y, Chai TC.** Up-regulation of P2X3 receptor during stretch of bladder urothelial cells from patients with interstitial cystitis. *J Urol* 171:448-452. 2004.

52 **Sun Y, Chai TC.** Augmented extracellular ATP signaling in bladder urothelial cells from patients with interstitial cystitis. *Am J Physiol Cell Physiol* 290:C27-34. 2006.

53 **Svanborg C, Agace W, Hedges S, Lindstedt R, Svensson ML.** Bacterial adherence and mucosal cytokine production. *Ann N Y Acad Sci* 730:162-181. 1994.

54 **Tempest HV, Dixon AK, Turner WH, Elneil S, Sellers LA, Ferguson DR.** P2X and P2X receptor expression in human bladder urothelium and changes in interstitial cystitis. *BJU Int* 93:1344-1348. 2004.

55 **Thore A, Ansehn S, Lundin A, Bergman S.** Detection of bacteriuria by luciferase assay of adenosine triphosphate. *J Clin Microbiol* 1:1-8. 1975.

56 **Truschel ST, Ruiz WG, Shulman T, Pilewski J, Sun TT, Zeidel ML, Apodaca G.** Primary uroepithelial cultures. A model system to analyze umbrella cell barrier function. *J Biol Chem* 274:15020-15029. 1999.

57 **Uhlen P, Laestadius A, Jahnukainen T, Soderblom T, Backhed F, Celsi G, et al.** Alpha-haemolysin of uropathogenic E. coli induces Ca^{2+} oscillations in renal epithelial cells. *Nature* 405(6787):694-7. 2000.

58 **Vial C, Evans RJ.** P2X receptor expression in mouse urinary bladder and the requirement of P2X(1) receptors for functional P2X receptor responses in the mouse urinary bladder smooth muscle. *Br J Pharmacol* 131:1489-1495. 2000.

59 **Wang EC, Lee JM, Ruiz WG, Balestreire EM, von Bodungen M, Barrick S, Cockayne DA, Birder LA, Apodaca G.** ATP and purinergic receptor-dependent membrane traffic in bladder umbrella cells. *J Clin Invest* 115:2412-2422. 2005.

60 **Wildman SS, King BF.** P2X receptors: epithelial ion channels and regulators of salt and water transport. *Nephron Physiol* 108:p60-67. 2008.

61 **Wilkinson SM, Uhl JR, Kline BC, Cockerill FR 3rd.** Assessment of invasion frequencies of cultured HEp-2 cells by clinical isolates of Helicobacter pylori using an acridine orange assay. *J Clin Pathol* 51:127-133. 199

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Table 1:

Compound	Non-OAB	OAB without pyuria	OAB with pyuria
ATP	1.4±0.8 nM	4.2±3.6 nM	2.0±0.8 nM
ADP	19.0±8.5μM	11.7±2.5μM	24.2±6.5 μM
AMP	2.7±0.5μM	14.4±8.3 μM*	8.5±2.2 μM*
adenosine	61.0±58.5μM	228±86 μM*	113±42.3 μM
GTP	44.9±13.9 μM	157±92.7μM	89.8±42.3 μM
GDP	128±54.0 μM	83.2±20.0 μM	138±47.3 μM
GMP	5.5±2.0μM	20.7±10.3 μM	15.9±6.7 μM
guanosine	478±174 μM	225±81.5μM	527±176 μM
UTP	9.9±2.4 μM	22.9±14.5 μM	8.6±2.6 μM
UDP	30.6±13.9μM	20.2±6.3μM	46.5±19.3 μM
UMP	1.4±0.6 mM	734±393 μM	667± 261 μM
uridine	7.3±3.4μM	25.0±8.1 μM	10.0±4.8 μM

Urinary AMP levels are elevated in OAB patients presenting with pyuria. HPLC was used to measure the concentration of nucleotides and nucleosides in urine samples from non-OAB patients, OAB patients without pyuria or with pyuria <10 wbc μl^{-1} (OAB without pyuria), or with pyuria≥10 wbc μl^{-1} (OAB with pyuria). Creatinine levels were similar in all 3 patient groups (non-OAB, 119±25 mg/dl; OAB without pyuria, 132±20 mg/dl; OAB with pyuria, 121±14 mg/dl). Data are mean±SEM (n=11 for non-OAB, n=16 for OAB without pyuria, n=17 for OAB with pyuria). * denotes statistical difference from non-OAB patients (P<0.05).

Appendix:

Human P2 receptor primers and mRNA accession numbers. Primers were designed using Primer 3 Web software (Whitehead Institute for Biomedical Research, Cambridge, USA) and manufactured by Eurofins-MWG (Ebersberg, Germany).

Gene	Accession No.	5'to 3' Sequence	Position
P2X1	NM_002558	S: CGCCTTCCTCTTCGAGTATGA	471-491
		AS: AGATAACGCCCACCTTCTTATTAC	538 – 514
P2X2	NM_170682	S: GCCTACGGGATCCGCATT	958 – 975
		AS: TGGTGGGAATCAGGCTGAAC	1024– 1005
P2X3	NM_002559	S: GCTGGACCATCGGGATCA	135 – 152
		AS: GAAAACCCACCCTACAAAGTAGGA	205 – 182
P2X4	NM_002560	S: CCTCTGCTTGCCCAGGTACTC	1108– 1128
		AS: CCAGGAGATACGTTGTGCTCAA	1176– 1155
P2X5	NM_002561	S: CTGCCTGTCGCTGTTCGA	311 – 328
		AS: GCAGGCCCACCTTCTTGTT	378 – 360
P2X6	AF065385	S: AGGCCAGTGTGTGGTGTTCA	488 – 507
		AS: TCTCCACTGGGCACCAACTC	555 – 536
P2X7	NM_002562	S: TCTTCGTGATGACAAACTTTCTCAA	401 – 425
		AS: GTCCTGCGGGTGGGATACT	476 – 458
$P2Y_1$	NM_002563	S: CGTGCTGGTGTGGCTCATT	1352 – 1370
		AS: GGACCCCGGTACCTGAGTAGA	1419 – 1399
$P2Y_2$	NM_176072	S:GAACTGACATGCAGAGGATAGAAGAT	1495 – 1520
		AS: GCCGGCGTGGACTCTGT	1567 – 1551
$P2Y_4$	NM_002565	S: CCGTCCTGTGCCATGACA	725 – 742
		AS: TGACCGCCGAGCTGAAGT	793 – 776
$P2Y_6$	NM_176797	S: GCCGGCGACCACATGA	1171 – 1186
		AS: GACCCTGCCTCTGCCATTT	1227 – 1209
$P2Y_{11}$	NM_002566	S: CTGGAGCGCTTCCTCTTCAC	511 – 530
		AS: GGTAGCGGTTGAGGCTGATG	586 – 567
$P2Y_{12}$	NM_022788	S: AGGTCCTCTTCCCACTGCTCTA	318 – 339
		AS: CATCGCCAGGCCATTTGT	385 – 368
$P2Y_{13}$	NM_023914	S: GAGACACTCGGATAGTACAGCTGGTA	223 – 248
		AS: GCAGGATGCCGGTCAAGA	291 – 274
$P2Y_{14}$	NM_014879	S: TTCCTTTCAAGATCCTTGGTGACT	433 – 456
		AS: GCAGAGACCCTGCACACAAA	505 – 486

Figure legends:

Figure 1. *Bladder biopsies obtained using flexible cystoscopy have full-thickness urothelium*. Human bladder biopsies were obtained using a flexible cystoscope under local or general anaesthetic. Biopsies were placed in 10% formalin for 48 h prior to dehydration with alcohol and xylene, and parrafin wax embedding. Sections (6 µm thick) were stained with H&E to investigate urothelium integrity. (A) Representative microphotograph of a biopsy section from an OAB patient that presented with pyuria of <10 wbc μl^{-1}. Full-thickness urothelium is evident in the sample (between arrowheads). (B) Representative microphotograph of a biopsy section from an OAB patient that presented with pyuria of ≥10 wbc μl^{-1}. Full-thickness urothelium is evident in the sample (as in A); umbrella cells lining the luminal membrane are clearly visible (arrowheads). Scale bars equal 200 µm.

Figure 2. *Greater ATP release from urothelium of OAB patients with pyuria of ≥10 wbc μl^{-1}.* ATP release from microdissected urothelium was measured using a luciferin luciferase asssay. ATP release was first measured at rest (classified here as 'basal' release) and then after addition of a hypotonic solution (to cause cell stretch and mimic bladder filling; classified here as 'stimulated' release). Data for stimulated ATP release is presented following subtraction of basal release values. (A) Basal ATP release from urothelium of OAB patients with pyuria ≥10 wbc μl^{-1} (OAB + pyuria; n=15) was significantly greater than basal ATP release from urothelium of OAB patients without pyuria, or with pyuria <10 wbc μl^{-1} (OAB - pyuria; n=9), or non-OAB patients (P<0.05; n=9). (B) Stimulated ATP release from the urothelium was not significantly different between experimental groups (non-OAB patients, n=9; OAB patients without pyuria, n=9; OAB patients with pyuria, n=15). (C) The rate at which the concentration of ATP decreased, following stimulation and in the continued presence of hypotonic solution, was greatest from urothelium of non-OAB patients (n=9) and OAB patients with pyuria (n=15). The decreased rate of stimulated ATP degradation seen from urothelium of OAB patients without pyuria (n=9), was similar to that from urothelium of non-OAB patients in the presence of the ATPase inhibitor ARL 67156 (100 µM, n=5). Data shown are mean±SEM. * denotes significant difference from non-OAB, where P<0.05. In (A) and (B) data were compared by using one-way ANOVA and Dunnett's post-hoc test against non-OAB (the identified control); in (C) data were compared by using two-way ANOVA (*time after stimulation* being the repeated measures factor, and the between factor being the *patient conditions*) and Dunnett's post-hoc test against non-OAB (identified control).

Figure 3. *Mechanisms of ATP release from urothelium of OAB patients with pyuria.* ATP release from microdissected urothelium of OAB patients with pyuria ≥10 wbc μl^{-1} (OAB + pyuria) was measured using a luciferin luciferase asssay. ATP release was first measured at rest ('basal') or after addition of a hypotonic solution ('stimulated'). Data for stimulated ATP release is represented following subtraction of basal release values. Urothelium was pre-treated

with the stated drug for 5 min prior to measuring ATP release (basal or stimulated). (A) Basal ATP release from urothelium was significantly attenuated by both suramin (1 mM, n=3) and carbenoxolone (CBX, 50 μM, n=3), and significantly potentiated by UTP (1 μM, n=3) (P<0.05 in all cases). The potentiating effects of UTP were inhibited by co-treatment with either CBX or suramin. (B) Stimulated ATP release from the urothelium was significantly attenuated by suramin (n=5), botulinum toxin-A (BTX-A, 20 units/ml, n=3) or brefeldin-A (BFA, 20 μM, n=3) (P<0.05 in all cases). Co-treatment of either BFA or suramin with UTP (n=3 in both cases) had no further effect. Data shown are mean±SEM. * denotes P<0.05. In (A) and (B) data were compared by using one-way ANOVA and Dunnett's post-hoc test against non-OAB (the identified control).

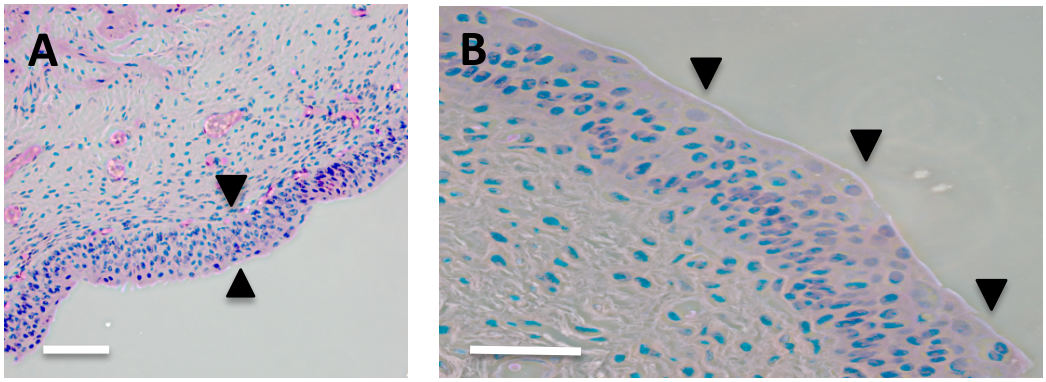
Figure 4. *Urothelial cell stretch evokes vesicular release of ATP*. Human bladder biopsies were obtained using a flexible cystoscope under local anaesthetic. Biopsies were placed in 10% formalin for 48 h prior to dehydration with alcohol and xylene, and paraffin wax embedding. In some cases, biopsies were challenged with a hypotonic solution (to cause cell stretch and mimic bladder filling) prior to fixation. Sections (6 μm thick) were stained with DAPI and quinacrine to investigate localisation of ATP-containing vesicles. (A) Representative microphotograph of a biopsy section from an OAB patient with pyuria ≥10 wbc μl^{-1}; *i*) DAPI staining (blue), *ii*) quinacrine staining (green), *iii*) composite image of DAPI and quinacrine staining, *iv*) bright field image. ATP containing vesicles are evident throughout the urothelium. (B) Representative microphotograph of a biopsy section from an OAB patient with pyuria ≥10 wbc μl^{-1}, challenged with a hypotonic solution prior to fixation; *i*) - *iv*) as above. ATP containing vesicles are less evident throughout the urothelium. Scale bars equal 100 μm.

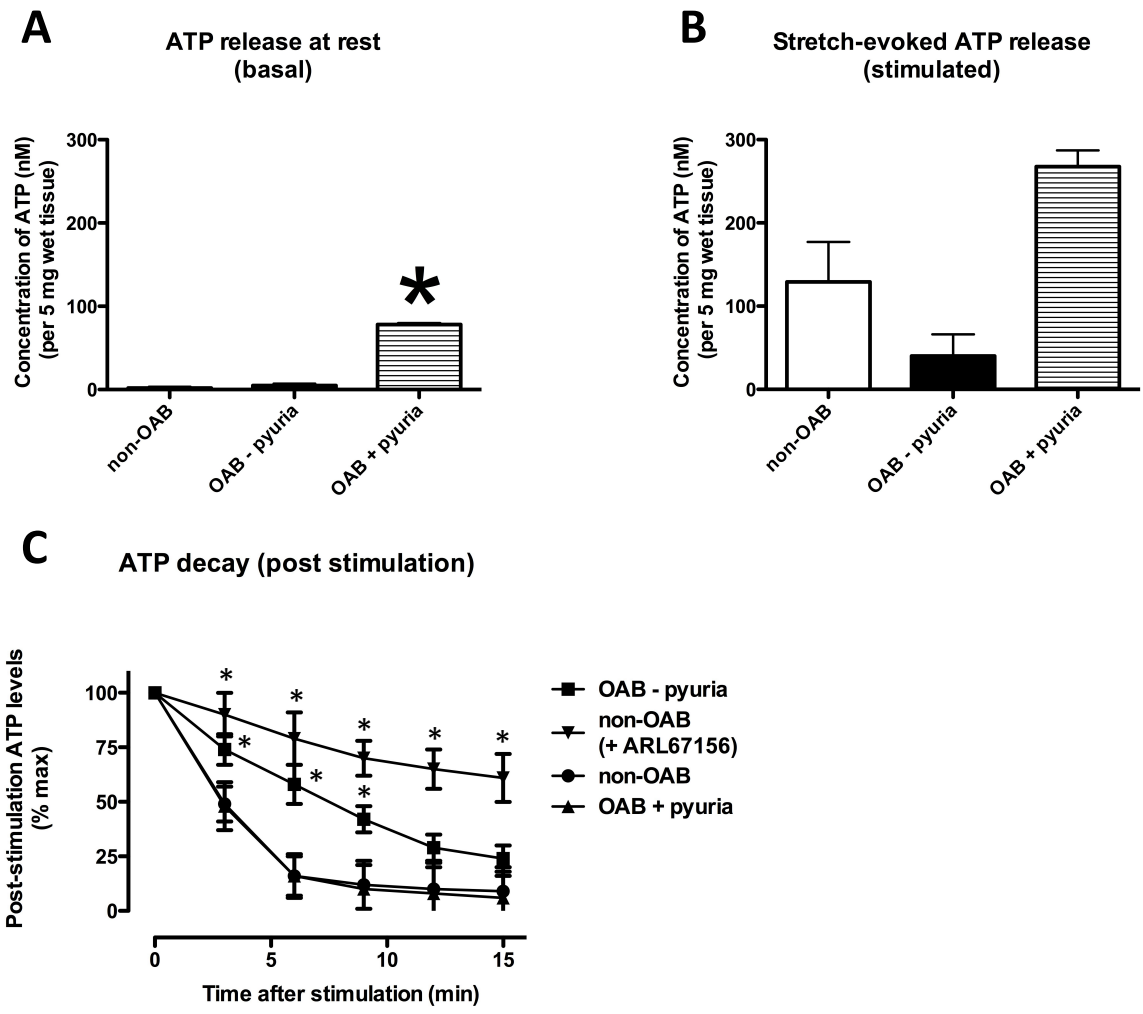
Figure 5. *Altered P2Y receptor expression in urothelium of OAB patients.* RT-PCR was performed on microdissected urothelium of non-OAB patients, OAB patients without pyuria, or with pyuria <10 wbc μl^{-1} (OAB – pyuria), and OAB patients with pyuria ≥10 wbc μl^{-1} (OAB + pyuria). The ratio of the P2 receptor gene of interest to a constitutively expressed housekeeping gene (GAPDH) was calculated. (A) Ionotropic P2X receptor mRNA levels were similar in urothelium from the three experimental groups (n=6). (B) Urothelium from OAB patients without pyuria showed a significant increase in abundance of metabotropic $P2Y_{11\text{ and }13}$ mRNA (n=6; P<0.01). Whereas, urothelium from OAB patients with pyuria showed a significant increase in abundance of metabotropic $P2Y_{2\text{ and }11}$ mRNA (n=6; P<0.01). Data shown are mean±SEM. * denotes significant difference from non-OAB, where P<0.01. In (A) and (B), data were compared by using one-way ANOVA and Dunnett's post-hoc test against basal or stimulated, respectively (the identified controls).

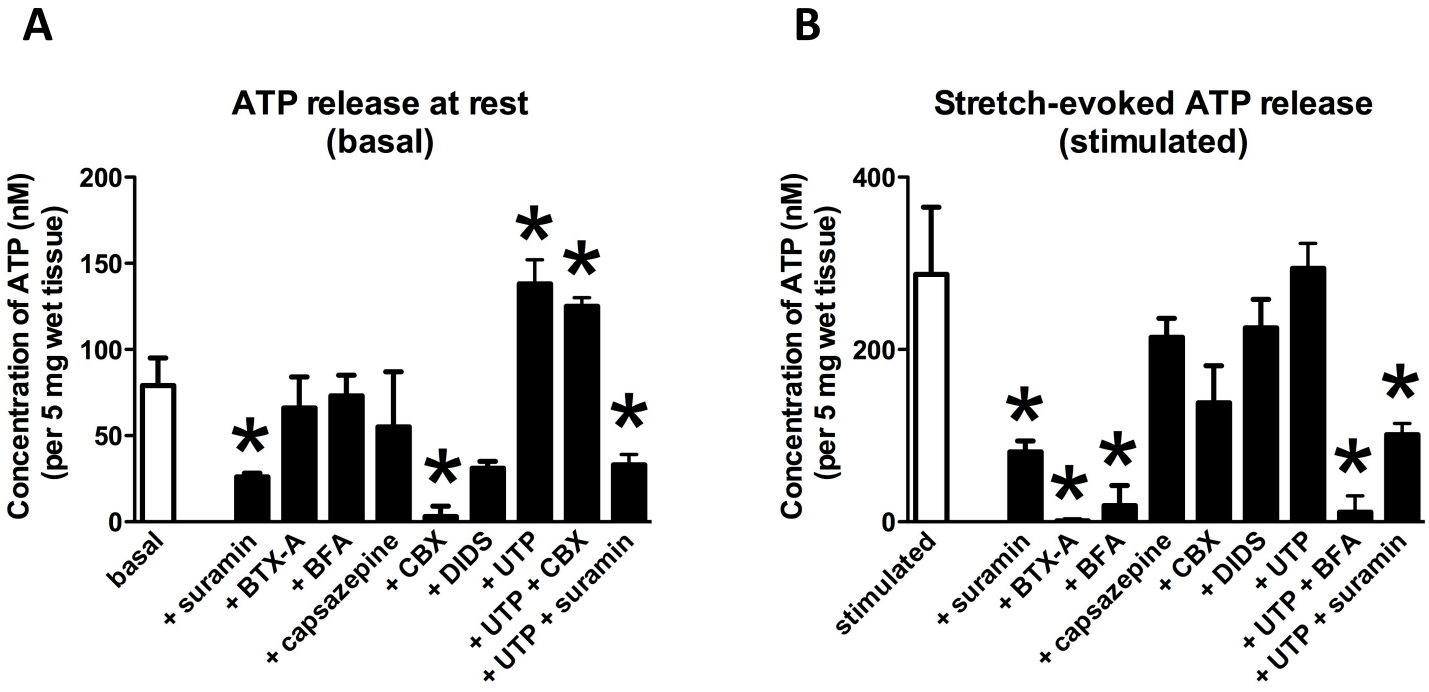
Figure 6. *Intracellular bacteria identified in shed urothelial cells from OAB patients with pyuria ≥10 wbc μl^{-1}.* Fresh urine was spun onto slides using a cytospin and the deposit stained with acridine orange (which fluoresces green in the presence of viable organism DNA). In order to differentiate extracellular from intracellular bacteria, a crystal violet counter-stain was added to quench the fluorescence of extracellular microorganisms. Images were acquired using an upright fluorescence microscope. Intracellular bacteria were not observed in sedimentary cells from the urine of non-OAB patients (see A for a representative image) or OAB patients without pyuria, or with pyuria <10 wbc μl^{-1} (OAB – pyuria; see B for a representative image). (C) Representative microphotograph of shed urothelial cell colonized by intracellular bacteria (arrow) from an OAB patient with pyuria ≥10 wbc μl^{-1} (OAB + pyuria). Bacterial colonization of urothelial cells was seen in 81% of urine samples tested (n=16), and of those, 52±9% of urothelial cells contained bacteria (19±3 urothelial cells per sample). Further confirmation of the intracellular localisation of bacteria and cell type was obtained by treatment with anti-uroplakin III (red; a marker of urothelial cells) and DAPI (blue; marker of DNA). Images were acquired with a confocal microscope and Z-series processed using Volocity software. (D) Representative 2D microphotograph of three shed urothelial cells from urine of an OAB patient with pyuria (Di), whereby the intracellular localisation of bacteria is evident in the side projections (Dii and Diii; dashed line shows origin of side-projection). Scale bars equal 10 μm.

Figure 7. *Proposed mechanisms of ATP release from urothelium of OAB patients with pyuria.* In our investigations, basal release of ATP was ~15-fold greater from urothelium of OAB patients with pyuria, nearing levels normally associated with stretch and bladder fullness. Histology demonstrates that bacteria reside in urothelial cells, and RT-PCR suggest that levels of $P2Y_2$ and $P2Y_{11}$ are increased. (A) When the bladder is at rest there is a substantial release of ATP (basal release) from the urothelium, which in our experiments is abolished by CBX, suggesting release via hemichannels (*1.*). We propose that the released ATP acts in a paracrine/autocrine fashion to activate P2 receptors expressed throughout the urothelium (including the upregulated $P2Y_2$ and $P2Y_{11}$ subtypes) (*2.*). P2 receptor activation in turn evokes further ATP release (in our experiments attenuated by suramin and potentiated by UTP) via an undetermined mechanism (*3.*). Presumably, released ATP reaches levels able to activate P2 receptors expressed on suburothelial sensory nerves, resulting in inappropriate signalling normally associated with bladder fullness (*4.*). We hypothesise that the presence of intracellular bacteria (IB) is responsible for increased P2 receptor expression and hemichannel mediated ATP release. (B) When the urothelium is stretched (*i.e.* when the bladder is full) there is a substantial release of ATP (stimulated release), which in our experiments is abolished by BTX-A or BFA, suggesting release from vesicles (*1.*). We propose that the released ATP acts in a paracrine/autocrine fashion to activate P2 receptors expressed throughout the urothelium *(2.*). P2 receptor activation in turn evokes further ATP release (in our experiments attenuated by suramin) again from vesicles (*3.*). Presumably, released ATP reaches

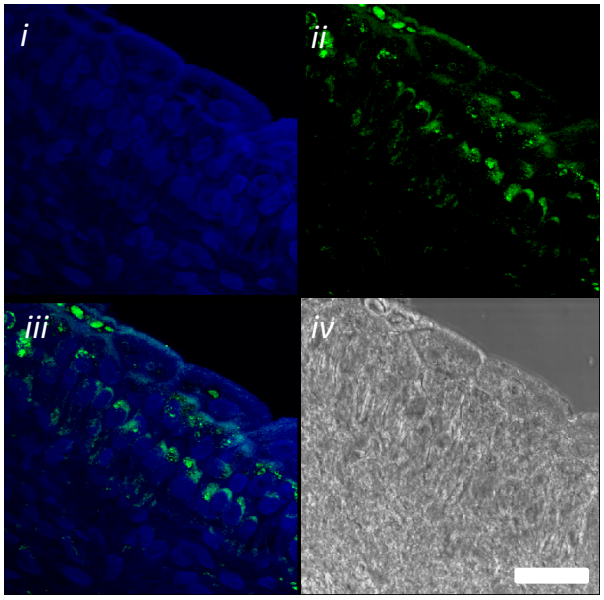
levels able to activate P2 receptors expressed on suburothelial sensory nerves, resulting in signalling of bladder fullness *(4.*). The presence of intracellular bacteria (IB) does not appear to alter stimulated ATP release from urothelium.


A
B

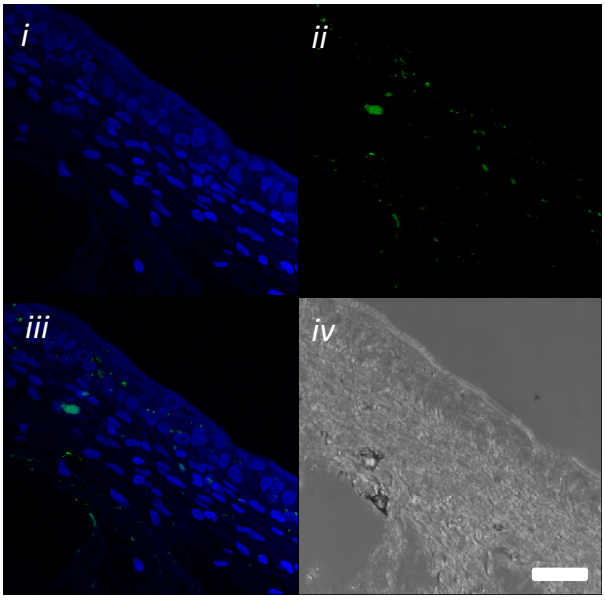

A
ATP release at rest
(basal)
Concentration of ATP (nM)
(per 5 mg wet tissue)
300
200
100
0
*
non-OAB
OAB - pyuria
OAB + pyuria
B
Stretch-evoked ATP release
(stimulated)
Concentration of ATP (nM)
(per 5 mg wet tissue)
300
200
100
0
non-OAB
OAB - pyuria
OAB + pyuria
C
ATP decay (post stimulation)
Post-stimulation ATP levels
(% max)
100
75
50
25
0
0
5
10
15
Time after stimulation (min)
OAB - pyuria
non-OAB
(+ ARL67156)
non-OAB
OAB + pyuria

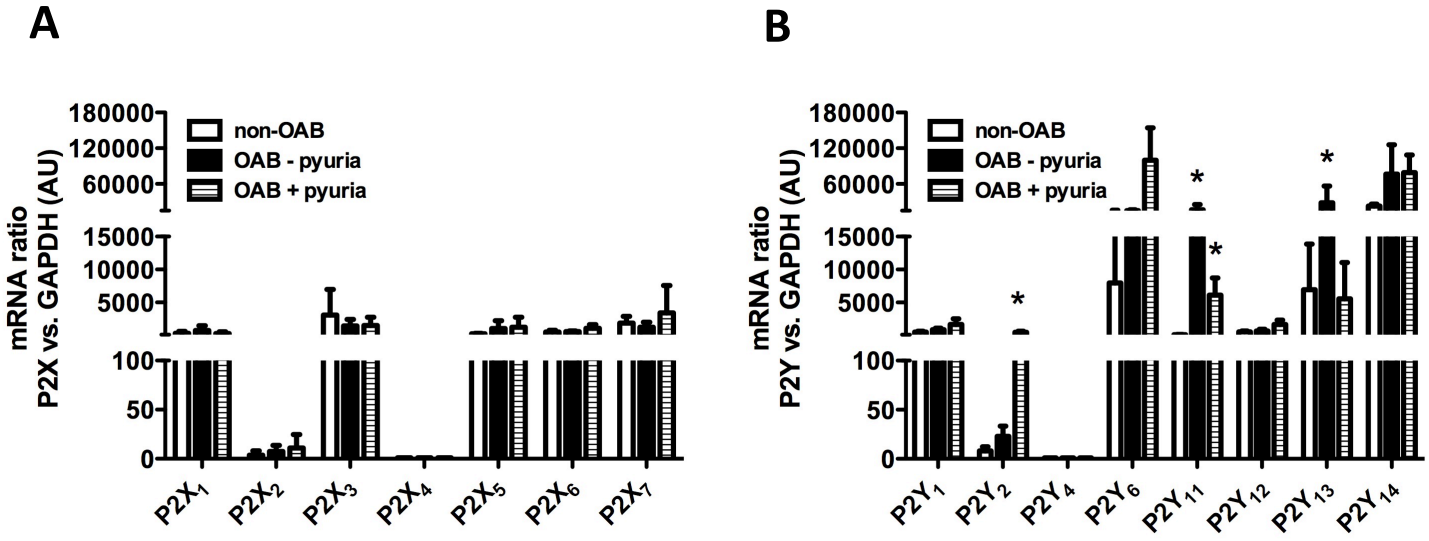

A
ATP release at rest
(basal)
Concentration of ATP (nM)
(per 5 mg wet tissue)
200
150
100
50
0
basal
+ suramin
+ BTX-A
+ BFA
+ capsazepine
+ CBX
+ DIDS
+ UTP
+ UTP + CBX
+ UTP + suramin
B
Stretch-evoked ATP release
(stimulated)
Concentration of ATP (nM)
(per 5 mg wet tissue)
400
200
0
stimulated
+ suramin
+ BTX-A
+ BFA
+ capsazepine
+ CBX
+ DIDS
+ UTP
+ UTP + BFA
+ UTP + suramin

A



B




A
B
non-OAB
OAB - pyuria
OAB + pyuria
mRNA ratio
P2X vs. GAPDH (AU)
P2Y vs. GAPDH (AU)
180000
120000
60000
15000
10000
5000
100
50
0
P2X1
P2X2
P2X3
P2X4
P2X5
P2X6
P2X7
P2Y1
P2Y2
P2Y4
P2Y6
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P2Y14
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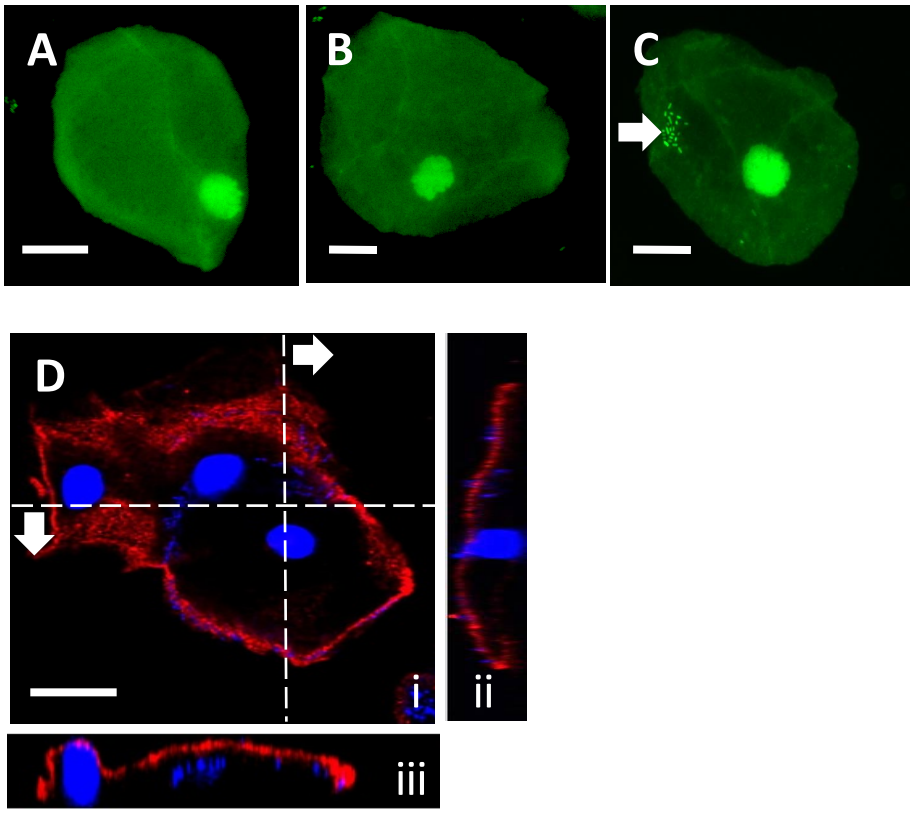
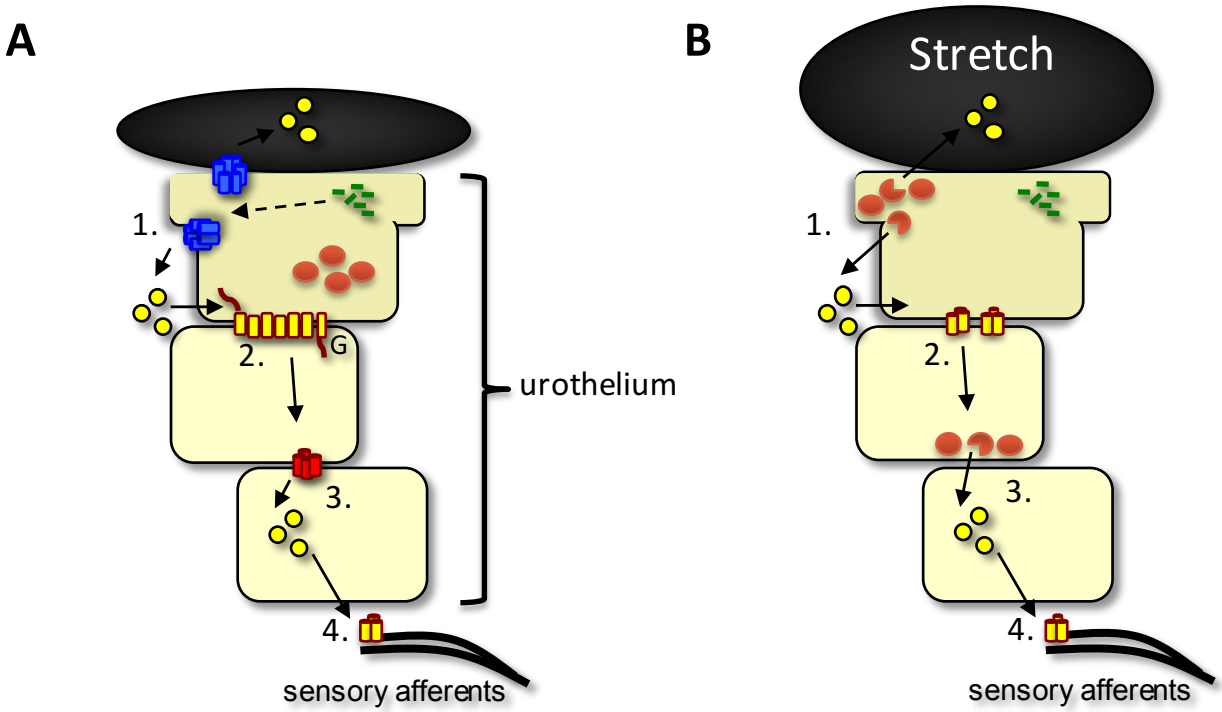

A
B
C
D
i
ii
iii



Key: , intracellular bacteria; , hemichannel; , ATP; , P2Y receptor; , undetermined ATP channel; , P2X receptor; , ATP-containing vesicle.